# THÈSE

**En vue de l'obtention du**

# DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

**Délivré par :**

Institut National Polytechnique de Toulouse (INP Toulouse)

**Discipline ou spécialité :**

Développement des Plantes

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## Présentée et soutenue par :

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le mardi 3 décembre 2013

## Titre :

CHARACTERIZATION OF TOMATO SIARF FAMILY AND SIARF8A VARIANTS REVEALS A SELECTIVE TRANSCRIPTIONAL CONTROL OF ARF8 BY ALTERNATIVE SPLICING AND MIRNA STRESS IN AUXIN-MEDIATED FRUIT SET

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**Ecole doctorale :**

Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

**Unité de recherche :**

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# Comprehensive description of Sl-ARF gene family members in the tomato and the central role of Sl-ARF8A in regulating the flower to fruit transition and in parthenocarpic fruit formation

# Aknowledgments

Formost, I would like to thank Prof. Mondher Bouzayen for gracefully allowing me to be part of their teams. To my two supervisors Prof. Mondher Bouzayen and HDR Mohamed Zouine for their guidance, advice, involvement and support in my work. I would have never made it without the help of Prof. Zhengguo Li and Dr. Alain Latche who was there in all the phases of the project.

A big thank you to Dr. Yingwu Yang, Ji Li, Dr. Isabelle Mila, Dr. Pierre Frasse, Dr. Corinne Audran-Delalande for their important contribution to the project and for sharing their knowledge with me.

I would like to thank Simone Albert, Dominique Saint-Martin and Lydie Tessarotto-Leronnier for doing all the transformation and Olivier Berseille for doing the sequencing. Thank you all the GBF team, for their support, advice and friendship, you all contributed to the accomplishment of the thesis.

All the microscopy was done at the Imagery platforme under the advice of Alain Jauneau, thank you very much.

Thank you our secratery Brigitte Lafforgue who brought me support in all the administrative jungle. And I would like to thank to all my colleagues from Chongqing University for their friendship and support.

My scholarship was granted by the Chinese Embassy in Paris and the Crous who contributed also to the aquision of a good room. Thank you for making my staying in France possible.

And last but not the least I would like to thank to my family and friends for bearing with me all these years and for being there for me all the way.

# Résumé

La formation des fruits charnus est un processus de développement impliquant trois stades principaux : (i) la transition fleur/fruit ou nouaison, (ii) la croissance et enfin (iii) la maturation des fruits. Chacune de ces étapes correspond à une transition développementale associée à d'importants changements physiologiques et structurels. Parmi toutes les hormones, l'auxine est connue pour jouer un rôle important dans l'initiation et la coordination du processus de nouaison et des phases précoces de développement du fruit. La mise en place de la réponse à l'auxine nécessite l'intervention de facteurs de transcription appartenant à la famille des ARF (Axin Response Factor) connus pour réguler l'expression des gènes de réponse précoce à l'hormone en se liant aux Cis-éléments de type AuxRE (Auxin Response Element) possédant le motif conservé de réponse à l'auxine. Les ARF sont de ce fait des candidats forts pour faire partie du mécanisme moléculaire par lequel l'auxine intervient dans le processus de nouaison. Le projet de recherche réalisé au cours de la thèse a permis d'isoler et de caractériser au total 22 gènes Sl-ARF chez la tomate (*Solanum lycopersicum*), la plante modèle pour l'étude du développement et de la maturation des fruits charnus. Les gènes Sl-ARF montrent des profils d'expression distincts selon les tissus et organes considérés, suggérant des fonctions spécifiques pour les membres de cette famille multigénique. Il est de plus montré que certains gènes Sl-ARF sont régulés à la fois par l'auxine et par l'éthylène, suggérant qu'ils participent potentiellement au dialogue entre les voies de signalisation des deux hormones. L'expression transitoire a révélé la capacité des Sl-ARF à agir comme activateur ou répresseur transcriptionnel des gènes de réponse à l'auxine. L'étude des profils d'expression globale, réalisée par RNA-seq à l'échelle du génome entier, a révélé pour la première fois l'existence d'un niveau important de régulation par épissage alternatif des ARFs pendant la transition fleur-fruit. La localisation nucléaire des protéines Sl-ARF8A / B a été déterminée par fusion avec le gène rapporteur GFP puis expression dans un système "signle cell". L'étude d'expression a révélé des profils distinctifs entre ARF8A et ARF8B avec une augmentation notable des transcrits Sl-ARF8A suite à la pollinisation des fleurs. Le rôle physiologique du gène Sl-ARF8A a été par la suite abordé par une approche de génétique inverse fournissant un nouvel éclairage sur les événements moléculaires qui sous-tendent la mise à fruit. La surexpression de Sl-ARF8 dans la tomate engendre des phénotypes pléiotropiques touchant la croissance

végétative (réduction de la taille des plantes, altération du développement racinaire et des tiges latérales) et l'appareil reproducteur avec la formation de fruits parthénocarpiques (absence de graines). L'analyse histologique a révélé une modification notable du placenta et des ovules chez les lignées de sur-expression de Sl-ARF8 et les études par RNA-Seq ont identifié plus de 2632 gènes différentiellement exprimés chez les surexpresseurs par comparaison avec les lignées non transformées. Au total, l'étude réalisée au cours de la thèse fournit une description exhaustive de la famille des ARF chez la tomate et une caractérisation fonctionnelle du gène Sl-ARF8 qui souligne son rôle comme figure centrale du mécanisme de contrôle de la nouaison des fruits.

# Abstract

The making of a fleshy fruit is a developmental process involving three main stages known as (i) fruit set, (ii) fruit growth and (ii) fruit ripening each corresponding to a transition step associated with major physiological and structural changes. Among other hormones, auxin is known to play a dynamic role in triggering and coordinating the changes associated with the process of fruit set and early fruit development. Auxin responses are mediated at the transcriptional level by Auxin Response Factors (ARFs) which regulate early auxin-responsive genes by specific binding to TGTCTC Auxin Response Elements (AuxREs). ARFs are therefore good candidates for being among the components of the molecular mechanism by which auxin mediates the fruit set. In the present study, a total of 22 *Sl-ARF* genes have been isolated and characterized in tomato (*Solanum lycopersicum*), a model plant for the study of fleshy fruit development and ripening. Expression profiling revealed distinctive patterns for *Sl-ARF* genes in different tomato tissues. Hormone treatment indicated that *Sl-ARFs* can be regulated both by auxin and ethylene with *Sl-ARF2B*, *5* and *9* likely to be involved in the cross-talk between the two hormones. Transient expression using a single cell system uncovered the ability of Sl-ARFs to act either as transcriptional activator or repressor in regulating the expression of auxin-responsive genes. Genome-wide expression profiling performed by deep RNA-Sequencing revealed for the first time the importance of the alternative splicing mode of regulation of *ARF* genes during tomato fruit set. The physiological significance of two closely related *Sl-ARFs*, *Sl-ARF8A* and *Sl-ARF8B*, was addressed in the present study via a reverse genetics approach providing new insight on the molecular events underlying tomato fruit set. Fusion to GFP reporter gene indicated that both Sl-ARF8A/B proteins are nuclear localized. Expression analysis by RT-qPCR revealed some distinctive features between *Sl-ARF8A* and *Sl-ARF8B* with a notable increase in *Sl-ARF8A* transcript upon flower pollination. Over-expression of *Sl-ARF8A/B* in tomato resulted in pleiotropic phenotypes, including dwarf plants, altered root and lateral shoot development and parthenocarpic fruits (seedless). Histological analysis revealed altered placenta and ovules development in *SlARF8A-OX* flowers and RNA-Seq profiling identified over 2632 differentially expressed (DE) genes in *SlARF8A-OX* flower buds compared to wild type control plants. Considering the dramatic change in gene expression of genes related to auxin, jasmonate and ethylene displayed in *SlARF8A-OX* lines, these phytohormones are likely to play an active role in coordinating the fruit set process. Altogether, the present

study provided a comphensive description of the tomato *ARF* gene family and a functional characterization of Sl-ARF8 defining this ARF member as a central figure of the control mechanism of the fruit set process.

# 中文摘要

植物浆果的发育包括三个主要的过程，即坐果，发育和成熟，其中，每一个过程都有相应的生理和结构变化。植物激素生长素是公认的在坐果和果实早期发育过程中起着重要的作用。生长素反应是通过生长素响应 因子 (ARF) 特异性的结合到 TGTCTC 生长素响应原件(AuxRE) 调节的. 因此，ARF 是很好的候选基因用于解释生长素调节坐果的分子机理。在本研究中，一共有 22 个 *ARF* 基因从模式植物番茄中被分离和鉴定。表达模型分析揭示了 *Sl-ARF* 在不同的番茄组织有独特的表达模式。激素诱导处理表明 *ARF* 基因对生长素和乙烯有着显著的相应，其中 3 个基因 *Sl-ARF2B*，*Sl-ARF5* 和 *Sl-ARF9* 可能包含在生长素和乙烯之间的通路反应中。单细胞转化表达表明 Sl-ARF 家族成员具有激活或抑制生长素早期相应基因的功能。另外，通过 RNA 测序分析第一次成功揭示了包含 Sl-ARF 家族在坐果过程中的选择性剪接调节机制。为了提高对番茄 ARF 家族的认识，通过反向遗传学的方法进一步分析了 *Sl-ARF8A* 和 *Sl-ARF8B* 两个同源基因的生理功能。GFP 融合基因表达显示 Sl-ARF8A 和 Sl-ARF8B 两个蛋白是定位于细胞核内的。定量 RT-PCR 分析表明，*Sl-ARF8A* 基因在授粉后呈现高水平表达，而 *Sl-ARF8B* 基因在开花授粉前已呈现出高水平表达。超表达 *Sl-ARF8A* 和 *Sl-ARF8B* 基因在番茄中产生了多效性影响，比如矮化植株，侧芽发育，侧根生长和单性结实的果实等。通过组织切片学分析显示，在 *Sl-ARF8A* 超表达的转基因系中发现了异常发育的胎座和胚珠。通过对转基因和野生型花苞进行 RNA 测序，揭示了超过 2632 个差异性表达的功能基因。进一步对生长素，茉莉酸和乙烯信号通路基因表达分析显示这些基因是差异性表达在 *Sl-ARF8A* 超表达系中，表明植物激素在坐果过程中发挥了很重要的作用。总之，该研究全面分析和鉴定了番茄 Sl-ARF 家族，揭示了 Sl-ARF8A / 8B 作为一个中心角色在坐果过程中发挥着关键性的控制性作用。

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# Abbreviations

ABA: Abscisic Acid

ABP1: Auxin Binding Protein 1

ACC: 1-Aminocyclopropane-1-Acide Carboxilique

ACO: 1-AminoCyclopropane-1-Carboxilique Oxydase

ACS: 1-AminoCyclopropane-1-Carboxilique Synthase

AD: Activation Domain

AFB: Auxin signalling F-Box proteins

ARF: Auxin Response Factor

AuxRE: Auxin Response Element

Aux/IAA: Auxin/ Indole-3-Acetic Acid

CBP1: C-Terminal Binding Protein

cDNA: Complementary Desoxyribonucleic Acid

CTD: Carboxy-Terminal Dimerization Domain

CaMV: Cauliflower mosaic virus

CDK: cyclin-dependent kinase

CYP707A: ABA 8′ -hydroxylase

DBD: DNA Binding Domain

DE: Differently Expressed genes

Dgt: Diageotropica

DPA: Days Post Anthesis

DRM: dormancy-associated

DST: downstream element

EIL: EIN3-like

ERD: early response to dehydration

ERF: ethylene response factor

ETR: ethylene resistant

ER: Endoplasmic Reticulum

GFP: Green Fluorescent Protein

GA: Gibberellic Acid

GUS: β-Glucoronidase

GH3: Gretchen Hagen

GUS: ß-glucuronidase

IAA: Indole-3-Acetic Acid

IAR: IAA-alanine resistant

JA: Jasmonate

MTT: 2:5-diphenyl monotetrazolium bromide

NBP: NPA-binding protein

NCED: 9-cis-epoxycarotenoid dioxygenase

NPH: NON-PHOTOTROPHIC HYPOCOTYL

NS: neoxantin synthase

NLS: Nuclear Localization Signal

NAA: α-Naphtalene Acetic Acid

NPA: N-(napthyl) Phthalamic Acid

PCR: Polymerase Chain Reaction

PGP: p-glycoprotein part

PIN: In-Formed Protein

PP2C: protein serine/threonine phosphatases 2C

q RT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

RACE: Rapid Amplification of cDNA-ends by Polymerase Chain Reaction

RT: Reverse Transcription

RD: Repression Domain

RNA-Seq: Deep RNA Sequencing Analysis

SCF: Skp1/Cullin/F-box

SGN: Solanaceae Genomics Network

SAUR: Small Auxin Up-regulated RNA

SKP2A: S-Phase Kinase-Associated Protein 2A

SRDX: Superman Repression Domain X

RT - q PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

Semi - q PCR: Semi-quantitative Reverse Transcription Polymerase Chain Reaction

TIR1: Transport Inhibitor Response 1

X-Gal: 5-bromo-4-cloro-3-indolil- β-D-galactoside

XTH: Xyloglucan endotransglucosylase

YFP: Yellow Fluorescent Protein

2:4-D: 2:4-Dichlorophenoxyacetic Acid

# Publications

## Articles

Mohamed ZouineΨ, Yongyao FuΨ, Anne-Laure Chateigner-Boutin, Isabelle Mila, Hua Wang, Alain Latche, Jean-Claude Pech and Mondher Bouzayen, Characterization of the tomato ARF gene family uncovers a multi-levels post-transcriptional regulation including alternative splicing. Plos one, *In press*, 2013.

Yongyao FuΨ, Mohamed ZouineΨ, Isabel Mila, Zhengguo Li, Yingwu Yang, Ji Li, Pierre Frasse, Jean-Paul Roustan, Alain Latche and Mondher Bouzayen, Characterization of Sl-ARF8A and Sl-ARF8B tomato genes reveals their critical role in auxin-mediated fruit-set. *in preparation*, 2013.

Ψ These authors participated equally in the work.

## Poster presentation

Yongyao Fu, Ji Li, Isabel Mila, Zhengguo Li, Mohamed Zouine and Mondher Bouzayen (2012) Characterization of Sl-ARF8 involved in tomato fruit set and development. November 6th, Grand Auditorium-UPS-Toulouse, France.

Zouine M, Frasse P, Maza E, Semin P, Yongyao F, Bouchez O, Marsaud N and Bouzayen M (2012) Transcriptomic profiling of tomato fruit set by deep sequencing uncovers de novo genes. April 18th-19th, 4th statseq workshop, Verona, Italy.

# Main components of the thesis

Plant development relies on organ and tissues differentiation processes that are triggered and coordinated by a complex interplay between exogenous and endogenous factors among which hormones are the most prominent players. In this regard, fruit development offers a unique example of a series of developmental shifts tightly regulated by intricate signaling pathways that remain largely unknown. Indeed, the making of a fleshy fruit involves three main stages known as fruit set, fruit growth and fruit ripening. Understanding the molecular and genetic basis of the transition leading from flower to fruit may have an important implication on yield and fruit quality traits. Therefore, elucidating the fundamental mechanisms underpinning fruit set is essential for designing new approaches to improve crop yield and fruit quality.

The plant hormone auxin is known to play a key role in triggering the flower-to-fruit transition and recent studies enlarged our understanding of the auxin-dependent mechanism underlying the fruit set process. Transcriptional regulators such as ARFs (Auxin Response Factors) and Aux/IAAs (Auxin/Indole Acetic Acid factors) emerged as main actors in mediating auxin responses during the fruit set process. However, the components of these important mechanisms and their role in driving developmental processes in crop species remain largely unknown.

The aim of the thesis project is to better characterize the auxin signaling pathways in tomato, a model species for fleshy fruit research and an economically important crop, during the flower-to-fruit transition, the so-called fruit set. This developmental process strongly impacts fruit yield and quality. To further improve our understanding on how auxin regulates fruit initiation it is essential to better unveil the functional role of the molecular components involved in auxin responses. Auxin Response Factors are the last known downstream components of the auxin signal transduction pathway. These transcription factors are encoded by a large gene family and as such they are well suited to contribute to the diversity and specificity of auxin responses. Using tomato (*Solanum lycopersicum*) as a model plant, my PhD research project aimed at identifying and characterizing the tomato Sl-ARF family members and using advanced reverse genetics and genomics methodologies, it addresses the specific role of Sl-ARF8 in controlling the flower-to-fruit developmental transition.

The thesis manuscript comprises four main chapters. The first chapter is a general introduction to the topic providing a bibliographic review on auxin. It presents the actual knowledge regarding the biosynthesis, perception and signal transduction of the phytohormone. The main roles of auxin in regulating different plant growth and development

processes and the interaction with other phytohormones are also described. The last section of this chapter is dedicated to emphasizing the advantages of using tomato as plant model for my research project. It also summarizes the actual knowledge of the mechanisms regulating fruit set and early development.

Chapter II is presented in the form of an article recently published in PLoS ONE and deals with the comprehensive identification of the entire members of the ARF family in tomato with structural and functional features of the genes and encoded proteins. The work allows renaming of the tomato ARF family members to provide a consensus nomenclature for all *ARF* genes across plant species. The data brings new insight on the complexity of their expression control at the post-transcriptional level. The data shed new light on the distinctive spatio-temporal pattern of expression of *ARF* genes in the tomato using RT-qPCR and describes their differential responsiveness to auxin and ethylene. Transactivation assays defined tomato ARFs as repressors or activators of auxin-dependent gene transcription and in silico search predicted the putative target site for small interfering RNAs and identified potential small uORF regulatory elements in their 5‟-leader sequences. In addition, RNA-seq revealed that some *Sl-ARF* genes undergo alternative splicing mode of regulation. All together, the data bring new insight on the complexity of the expression control of *Sl-ARF* genes at the transcriptional and post-transcriptional levels.

Chapter III is dedicated to the characterization of Sl-ARF8 and shows that two functional copies of the gene (*Sl-ARF8A* and *Sl-ARF8B*) are present in the tomato genome. The overexpression of both *Sl-ARF* genes via stable genetic transformartion of tomato results in vegetative and reproductive phenotypes including the initiation of the fruit set process independently from pollination/fertilization. Expression studies carried out both by RT- qPCR analysis and through the analysis of *Sl-ARF8A/B* promoter-GUS fusion plants revealed that *Sl-ARF8A* is up-regulated upon pollination/fertilization and that in contrast to *Sl-ARF8B* its expression is negatively regulated by auxin and positively regulated by ethylene. Moreover, analysis of RNA-sequencing revealed over 2632 differently expressed genes in *Sl-ARF8A* overexpressing lines. Altogether, the data bring new insight into the ARF8-mediated control of the fruit set process and point out to the interactive role of Sl-ARF8 and Sl-IAA9 in relaying auxin signalling during the flower to fruit transition.

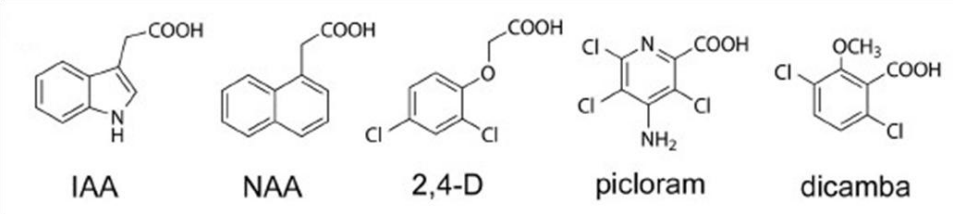
Chapter IV, summarizes in short the main scientific outcome of the thesis project and outline some perspectives open by the work.

# Chapter I

## Bibliographic review and general introduction

### I.Auxin

The study of auxin is one of the oldest fields of plant experimental research. Early in 19th century, *Charles Darwin* carried out an interesting experiment and proposed that the plant growth was regulated by a matter which transmits its effects from one part of the plant to another [1]. After that, the term auxin was firstly found from human urine as calledauxin A and B and then the structural distinct compound was isolated from fungi called heteroauxin, which was finally determined to be indole-3-acetic acid (IAA) [2].




Auxin is a central hormone proved to play critical roles in a number of plant developmental and physiological processes, such as embryogenesis, apical dominance, gravitropism, phototropism, lateral root initiation, leaf elongation, shoot branching, flowering, fruit development and ripening [3]. Indole-3-acetic acid (IAA), which is the predominant naturally occurring auxin, coordinates plant growth and developmental responses through transcriptional regulation of gene expression. Some synthetic auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), picolinic acid-type auxins (picloram) and benzoate-type auxins(dicamba) have been developed and are being widely used in agronomical practices [4].

### II.The auxin biosynthesis pathway in plants

Auxin biosynthesis is fairly complex in plants (Fig. 1) and multiple pathways have been postulated to contribute to *de novo* auxin biosynthesis. As different plant species have specific strategies to regulate their metabolic pathways, it is likely that plants would share evolutionarily conserved core mechanisms for auxin biosynthesis for the reason that IAA is a fundamental substance in the plant life cycle. To date, two major pathways for IAA biosynthesis have been proposed: the tryptophan (Trp)-independent and Trp-dependent

pathways [5]. In Trp-dependent IAA biosynthesis, four pathways have been postulated in plants: (i) the indole-3-acetamide (IAM) pathway; (ii) the indole-3-pyruvic acid (IPA) pathway; (iii) the tryptamine (TAM) pathway; and (iv) the indole-3-acetaldoxime (IAOX) pathway. For the Trp-independent IAA biosynthesis, little is known about the molecular or biochemical pathway to IAA despite that many genes have been found in the Trp-dependent IAA biosynthesis [6]. In addition, among the pathway of Trp-dependent IAA biosynthesis, IAM or IPA is the major route(s) to IAA in flowering plants.

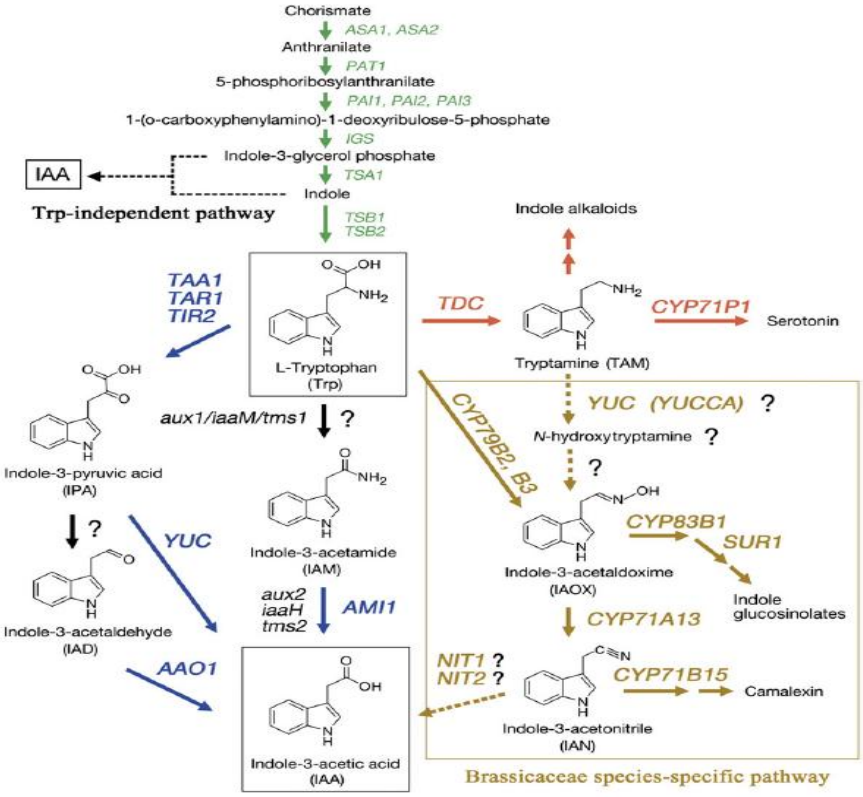



**Fig. 1 Presumptive pathways for IAA biosynthesis in plants. Green arrows indicate the tryptophan synthetic pathway in the chloroplast.A thin dashed black arrow denotes the tryptophan-independent IAA biosynthetic pathway. Blue arrows indicate steps for which the geneand enzymatic function are known in the tryptophan-dependent IAA biosynthetic pathway. Red arrows indicate the indole alkaloid andserotonin biosynthetic pathway. Mustard-coloured arrows indicate the Brassicaceae species-specific pathway. Black arrows indicatesteps for which the gene(s) and enzymatic function(s) are unknown. Dashed mustard-coloured arrows indicate steps for which the geneand enzymatic function(s) remain poorly understood. Letters in italics show genes involved in the conversion process. Lower case lettersin italics indicate bacterial genes [6] .**

### II. 1 The indole-3-acetamide pathway

The IAA biosynthetic pathway via IAM was found to be in a bacteria-specific pathway. In hairy roots, where the plant pathogen *Agrobacterium rhizogenes* harbours, IAA is synthesized from Trp by a two-step reaction as a result of the expression of the integrated genes *aux1* (iaaM/tms1) and *aux2* (iaaH/tms2) of Ri TR-DNA [7]. Based on overexpression of *aux1* gene of the Ri plasmid, the indole-3-acetamide hydrolase gene (GenBank accession

number AB457638) was isolated from *Nicotiana sp.* and named *NtAMI1*[8]. Fusion proteins of AtAMI1 and NtAMI1 have the enzyme activity which converts IAM to IAA, suggesting that *AMI1* genes encode indole-3-acetamide hydrolase. In addition, the fusion protein of AMI1–GFP shows the AMI1 protein was confirmed to be located in the cytoplasm of plant cells [9]. So far, the AMI1 family has been found to be widely distributed in plants, including tobacco, tomato, grape, poplar, *Arabidopsis* , maize, sorghum, rice, and wheat [10].

### II. 2 The indole-3-pyruvic acid pathway

The Tryptophan Aminotransferase of *Arabidopsis* 1 (TAA1) gene, encoding an amino transferase converting Trp to IPA was isolated based on the characterization of mutants that were defective in shade avoidance [11] and in ethylene responses [12]. Mutations in *TAA1* gene result in dramatic reduction in IAA levels, which indicates that IPA-dependent IAA biosynthesis could be a critical pathway for the biosynthesis of free IAA. Furthermore, overexpression of TAA1–YFP and TAA1–GFP fusion proteins shows the TAA1 protein was located in the cytoplasm of plant cells [11,12]. In addition, Yamada et al. revealed that the Transport Inhibitor Response 2 (TIR2) gene was identical to the *TAA1* gene [13]. Overexpression of *TIR2* does not lead to some growth defects and the plants display normal sensitivity to exogenous Trp, which indicates that increasing endogenous IPA levels does not result in the synthesis of more IAA [13].

## III. Auxin transport and the role of PIN proteins

Auxin molecules, like indole-3-acetic acid (IAA), function as mobile signals between cells, tissues and organs and, as such, they are involved in spatial and temporal coordination of plant morphogenesis and in plant responses to their environment. Auxin is actively moved around the plant by a series of transmembrane pumps or pump components [14]. Auxins, both native ones and their synthetic analogues, are weak organic acids. Their molecules undergo reversible dissociation, the equilibrium of which is pH dependent. Auxin molecules are partly dissociated when the pH value is about 5.5 at the extracellular space [15] (Fig 2). As such it can freely diffuse into the cell, where the pH is higher, resulting in ionisation. The auxin ions are then trapped in the cell and can only leave through active transport, energised by the electrochemical gradient across the plasma membrane. A widely discussed example of this is the so-called PAT-„polar transport stream“, in which auxin synthesised in the young expanding leaves at the shoot apex is pumped in cell files associated with the plant vascular

system down the stem, into the roots, and to the root tip.The polar transport stream is a highly stable route for auxin movement down the plant, supplemented by phloem transport, which can be considered to be an expressway that delivers auxin in bulk to the root tip [16,17].

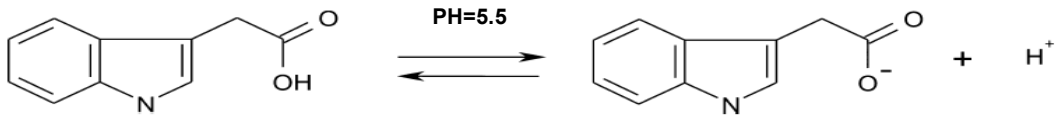



**Fig. 2 Reversible dissociation of the molecule of native auxin – indole-3-acetic acid (IAA) [15].**

As a result, auxin physiology has benefited from the use of drugs that act specifically to inhibit polar auxin transport. Of which naphthylphthalamic acid (NPA) is the most response-specific chemical and has been widely used in agronomy.

### III.1 The efflux carriers: PIN proteins

The PIN-FORMED (PIN) protein family is a group of plant transmembrane proteins with a predicted function as auxin transporters. In *A. thaliana*, there are eight sequences assigned to PIN proteins [18]. PIN are transmembrane proteins that belong to the group of secondary transporters. AtPINs share a limited sequence similarity with some prokaryotic and eukaryotic transporters [19,20]. PINs have a crucial role in the polar auxin efflux machinery: some *pin* mutants were shown to have serious defects in polar auxin transport [21,22]. PIN proteins are localized in the cells in a polar manner corresponding to the direction of the auxin flow [23,24,25]. The polar auxin transport inhibitors can phenocopy loss-of-function *pin* mutations in wild type plants [21,24,26]. Today, the auxin transporting activity of PINs had not been biochemically demonstrated [27]. However, the heterologous expression of AtPIN1 protein with GFP fusion demonstrated its preferential localization at transversal PMs [28]. Moreover, the auxin-specific efflux was shown to be directly proportional to the degree of PIN expression and this AtPIN related auxin efflux was sensitive to polar auxin transport inhibitors NPA [27]. These evidences for the auxin-efflux-catalyzing role of PINs points to their crucial role in polar-auxin-transport-regulated physiological processes.

### III.2 A flexible PIN network

PIN proteins are transporters excreting compounds and functional redundancies between individual members of the PIN family and all PIN proteins seem to be subject to a multi-level regulation. As shown in Fig 3, various transporters, such as auxin influx carrier permease

AUX1; auxin efflux carriers of the PIN and PGP (P-glycoprotein part) type, are depicted together with PIN constitutive cycling. Plasma membrane H+-ATPase, involved in maintenance of the proton gradient across the plasma membrane, is included. The vesicle trafficking itself may serve as an auxin transport pathway as suggested by the question marks accompanying the arrows representing auxin flow into vesicles. NBP (NPA Binding Protein) is a hypothetical protein, which binds 1-naphthylphthalamic acid, the non-competitive inhibitor of auxin efflux of the phytotropin type and possibly also other polar auxin transport inhibitors, PATIs with high affinity.

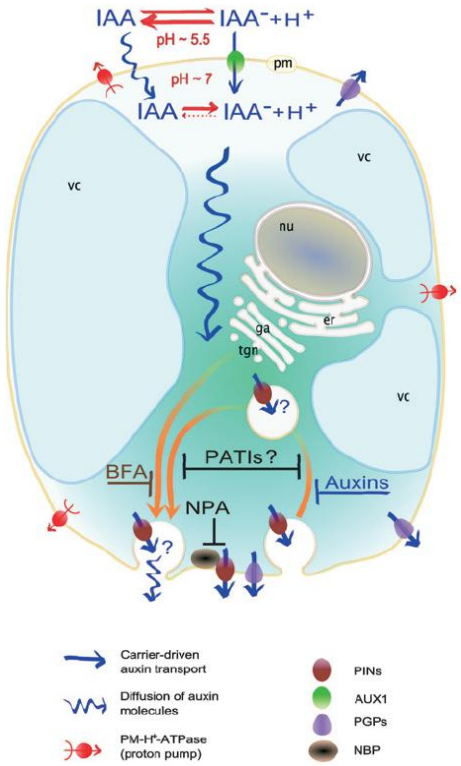



**Fig. 3 Scheme for the role of PINs in the polar auxin transport machinery in plant cells. pm, plasma membrane; vc, vacuole; nu, nucleus; er, endoplasmic reticulum; ga, Golgi apparatus; tgn, trans-Golgi network [15].**

NBP is believed to be connected with actin filaments and its interaction with auxin efflux carriers may be mediated by another, metabolically very unstable component. Protein BIG seems to be a part of the endocytic path of constitutive cycling of PINs. PINs may interact with alternative auxin efflux carriers of the PGP type; however, the mechanism of such interaction is still not clear. Taken together, the PIN network underlies the directional auxin flux (polar auxin transport) providing any cell in the plant, which together with auxin signalling system, coordinates the plant growth and development.

## IV. The auxin perception and signalling pathway in plants

According to the current model, auxin signal is transduced by the SCF class E3 ubiquitin ligase-proteasome pathway. Firstly, auxin binds to TIR1/AFB nuclear receptors, which are F-box subunits of the SCF ubiquitin ligase complex, to promote *Aux/IAA* protein ubiquitination and subsequent degradation. Thereafter, the auxin signal is controlled by the quantitative and qualitative responses between Aux/IAA repressors and Auxin Response Factors (ARFs). ARFs regulate the downstream auxin-responsive genes by specific binding to auxin-response elements (AuxRE), located in their promoters. When auxin concentration is low, the regulatory complex of Aux/IAAs binding with ARFs inhibits the activity of ARF factors. Increased auxin accumulation promotes the degradation of Aux/IAA repressors and consequently activates auxin-responsive gene expression by removing the inhibition of ARF activity (Fig. 4). An alternative hypothesis is that Aux/IAAs prevent ARFs from reaching their AuxRE target sites by dimerizing with ARFs at low auxin concentrations. In the latter hypothesis, Aux/IAA proteins would be degraded when auxin concentrations are increased, allowing the binding of dimerized ARFs to AuxREs, and subsequently the activation of early auxin-response genes [29,30].

In addition, multiple pathways have been postulated that contribute to *de novo* auxin response. Auxin Binding Protein 1 (ABP1), as a candidate protein of auxin receptor [31,32], may control the local auxin distribution by regulating the localization of PIN transporters that are responsible for interdigitated growth. Nevertheless, the downstream components and molecular action of the ABP1 pathway remain poorly understood. Recently, S-Phase Kinase-Associated Protein 2A (SKP2A), has been identified as another candidate for a F-box-type auxin receptor [33], its binding to auxin might regulate the proteolysis of cell cycle transcription factors, such as E2FC and DPB [34] to trigger cell division. Another SKP2A-like protein might also be involved in the auxin signal pathway [33].

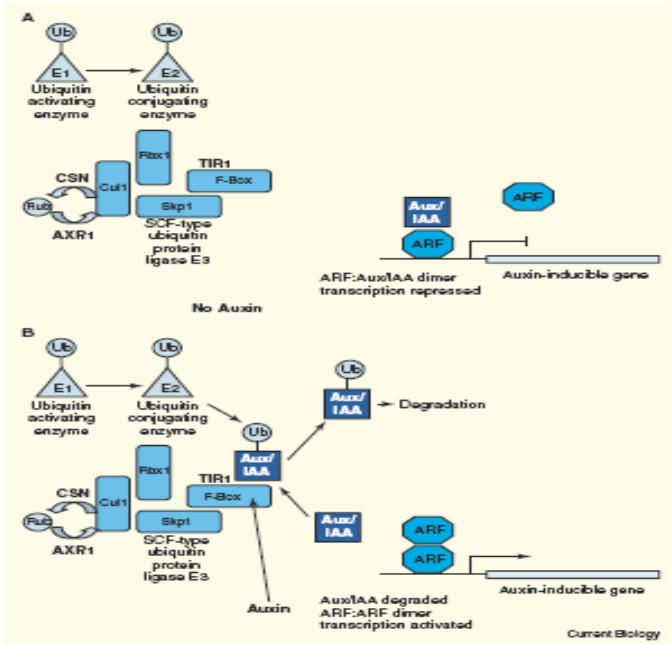



**Fig. 4 The auxin response pathway in plants. Auxin-responsive genes are activated by members of the ARF family with Q-richtranscriptional activation domains. (A) Activationis blocked by dimerisation with members of the Aux/IAA family, which have a powerful transcriptional repression domain. (B) Auxin promotes degradation of the Aux/IAAs by binding directly to the F-box protein (TIR1 or its close relatives), promoting interaction between the F-box protein and the Aux/IAAs, releasing ARFs to form homodimers and promote transcription [17].**

### IV.1 Auxin receptors: TIR1 the heart of auxin signalling

The Transport Inhibitor Response 1 (TIR1) gene was firstly identified in a genetic screen for *Arabidopsis* plants tolerant to NPA, the auxin transport inhibitor [35]. Nevertheless, it was soon indicated that TIR1 functioned in auxin responsiveness, not auxin transport [36]. The TIR1 contained some recognisable motifs, which were an F-box domain and a set of leucine-rich repeats. F-box protein TIR1 contributes to a familiar and important protein degradation pathway, the ubiquitination pathway [37,38]. TIR1 is a component of a cellular protein complex known as $SCF^{TIR1}$ (Skp1/Cullin/F-box) involved in ubiquitin-mediated protein degradation. In auxin-mediated transcriptional control, the F-box protein TIR1 binds to ASK1 to determine the specificity for the protein to be ubiquitinated. Ubiquitins are added from the activated RBX1 and, once the target proteins are polyubiquitinated, they are recruited by the COP9 signalosome and the 26S proteasome where they are degraded [39] (Fig 5). The SCF regulatory proteins, auxin resistant 1 (AXR1), ECR1 and Ras-converting enzyme 1 (RCE1), are involved in the conjugation of CUL1, and mutations in these components confer auxin-resistant phenotypes and lead to defects in auxin-related developmental processes [40,41]. *Tan* et al. (2007) have demonstrated that auxin enhanced the binding of an Aux/IAA protein

to the complex and they obtained crystal structures for the complex alone and for complexes bound to IAA along with an Aux/IAA peptide [42]. The crystal structures showed that the TIR1–ASK1 complex had a mushroom shape, with the leucine-rich-repeat domain of TIR1 forming the cap, and the F-box of TIR1 along with ASK1 forming the stem.

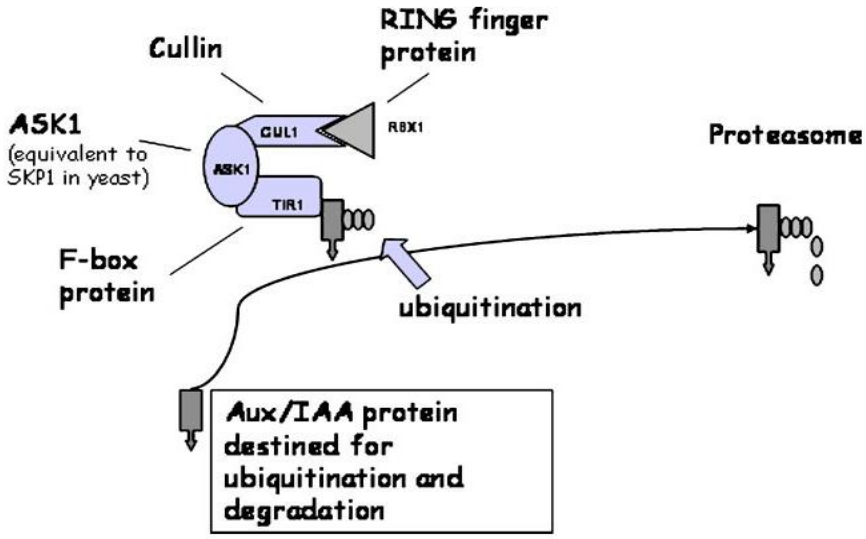



**Fig. 5 The plant $SCF^{TIR1}$ complex ubiquitinates Aux/IAA transcription factors [39].**

Auxin acts like "molecular glue" to stabilize the interaction between TIR1 (gray) and domain II of the Aux/IAA (orange) (Fig 6). Gain-of-function mutations in domain II of several Aux/IAAs result in reduced binding to TIR1 and stabilization of the Aux/IAA. A variety of loss-of-function mutations in TIR1/AFB proteins have been characterized, T-DNA insertions and point mutations, that results in an auxin-resistant phenotype.

A number of genetic and biochemical analyses have revealed that TIR1 itself binds auxin and acts directly as a receptor in this response. Indeed, partial or complete loss of function mutants in TIR1 subunits or its regulators exhibit auxin resistant phenotypes, suggesting that the targets of TIR1 are negative regulators of auxin signalling [43,44].




**Fig. 6 Schematic diagram of auxin functioning as a „molecular glue" to enhance TIR1–substrate interactions. In contrast to an allosteric mechanism, auxin binds to the same TIR1 pocket that docks the Aux/IAA substrate. Without inducing significant conformational changes in its receptor, auxin increases the affinity of two proteins by simultaneously interacting with both in a cavity at the protein interface [42].**

So far, it is possible to say that the $SCF^{TIR1}$ complex (including the bound Aux/IAA substrate) holds the binding site, although Aux/IAA proteins do not bind IAA, they might contribute to a composite binding site. The $SCF^{TIR1}$ receptor system is always beautifully functional. By controlling the free concentration of transcription factors, small errors are buffered, but once $SCF^{TIR1}$ is activated and transcription promoted, amplification of many auxin-regulated genes will be rapid and very specific.

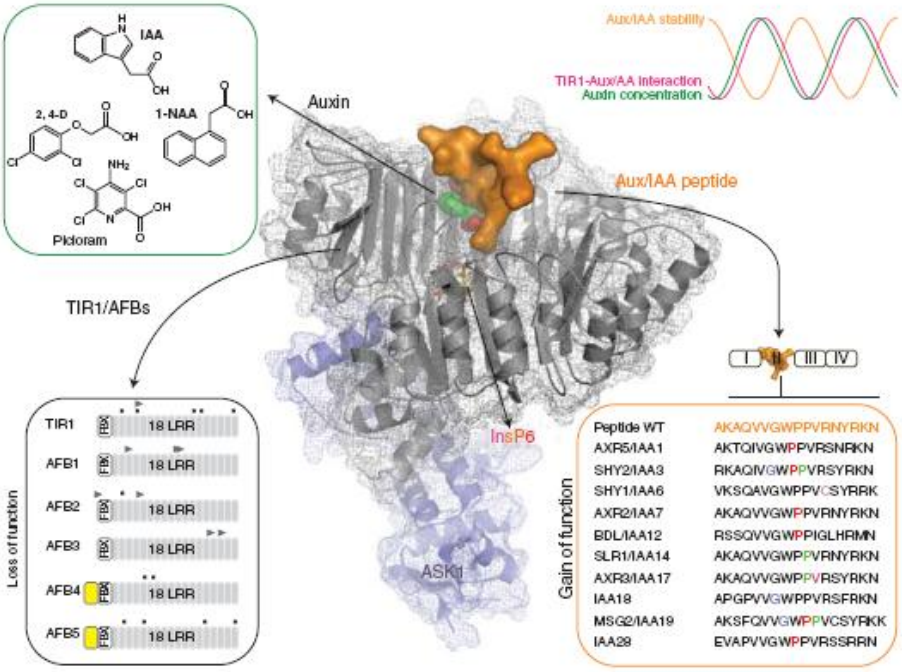



**Fig. 7 Regulation of ASK1-TIR1-Aux/IAA auxin receptor complexes. Indole-3-acetic acid (IAA) is the major natural auxin but other auxinic compounds, including a-Naphthalene aceticacid (1-NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 4-Amino-3,5,6-trichloropicolinic acid (picloram) promote auxin specific responses in the root or shoot of the plant. These compounds and other natural auxins may bind to the promiscuous auxin binding pocket of TIR1 with different affinities [45].**

Response specificity is conferred at several complements, by the receptor"s binding site for active auxins, by TIR1"s specificity for Aux/IAAs and also by the specificity of auxin response elements for ARFs [45] (Fig 7).

**IV.2 Auxin receptors: the role of ABP1**

A huge step in the elucidation of auxin soluble receptors was made in 2005 after discovering of the receptor function of transport inhibitor resistant 1 (TIR1) protein. In addition, another putative auxin receptor, the AUXIN BINDING PROTEIN1 (ABP1) was isolated based on its capacity to bind auxin and is involved in a set of early auxin responses such as rapid activation of ion fluxes at the plasma membrane [46]. The ABP1 is encoded by a small gene family in *Arabidopsis*, and varies in the number of genes and their localization in different plants [47,48]. The protein has two conserved domains, Box A, responsible for auxin binding, and Box B, as well as a C-terminal KDEL tetrapeptide for ER targeting, are present in the ABP1 structure [46,49] as shown in Fig 8.

The ABP1 has long been characterized as an essential component of early auxin action at the plasma membrane but its role in downstream responses had remained elusive until recently. The identification of an *Arabidopsis* ABP1 gene knocked out exhibited developmental arrest at early embryogenesis stage [50]. Developmental map of gene expression in *Arabidopsis* revealed that ABP1 (At4g02980) exhibits a fairly constant expression in almost all tissues throughout vegetative plant development suggesting that its role is not restricted to embryo development [51,52]. It has also been shown that ABP1 is required for post embryonic shoot development and root growth in a context-dependent manner [53,54] as shown in Fig 9. Addition of ABP1 to protoplasts from tobacco leaf cells enhanced the sensitivity to auxin 100- to 1000-fold. It was suggested that an over-expression of ABP1 enhanced the sensitivity of guard cells to auxin [55].

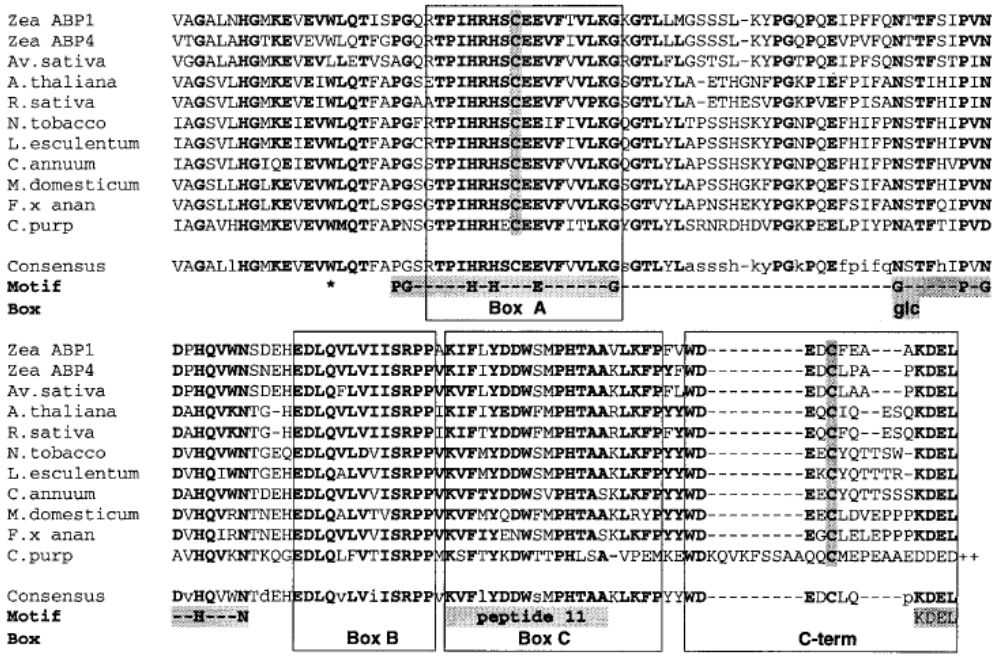



**Fig. 8 Pile-up of ABP1 sequences. ABP1s are listed against their genus names. Conserved residues are shown in bold type. A consensussequence is shown at the foot and lower case letters represent residues not conserved. Two boxes of completely conserved residues are outlined, Boxes A and B, and a third box with high identity is labelled Box C. Several sequence motifs are listed; glc represents N-glycosylationsites, KDEL the ER retention motif, the residues listed in the shaded boxes represent the core cupin motif and the peptide reported to carryphoto-activated IAA [49] is labelled peptide 11 [46].**

The sequence and structure of ABP1 classify the protein as a soluble protein, but so far ABP1 has been exclusively found at the plasma membrane. Accordingly, the protein is localized in the ER, at least in higher plants, but a whole body of biochemical and functional evidence supports that ABP1 is located and acting at the outer face of the plasma membrane [46,56] as shown in Fig10.

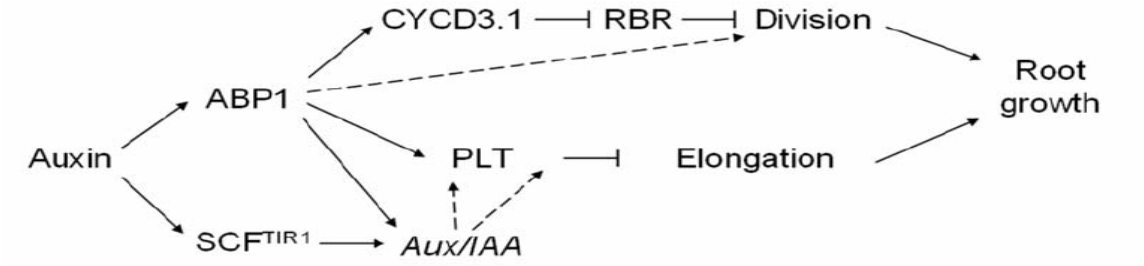



**Fig. 9 A model for ABP1 mediated auxin responses in roots. The permissive effect of auxin on cell division is dependent on ABP1. In root stem cells, the D-type CYCLIN/RETINOBLASTOMA (RBR) pathway acts downstream of ABP1 and controls the G1/S transition. In meristematic cells, ABP1 might also affect the D-type CYCLIN/RBR pathway but other critical regulators of the G1/S transitionphase are dependent on ABP1 activity. ABP1 contributes to the auxin control of cell elongation by modulating a zone of competence for PLETHORA and by acting on the auxin-mediated regulation of Aux/IAA transcriptional repressors. It is worthwhile noticing that expression of PLTs was reported to be regulated downstream of ARF transcription factors (Auxin Response Factors) and consequently of Aux/IAAs. ABP1 might act indirectly on PLT via Aux/IAAs regulation. It is well established that regulation of gene expression by auxin involves the TIR1 receptor which, within the SCFTIR1 E3 ligase, controls the degradation of Aux/IAA repressors. ABP1 and TIR1 might collectively control gene regulation and elongation [54].**

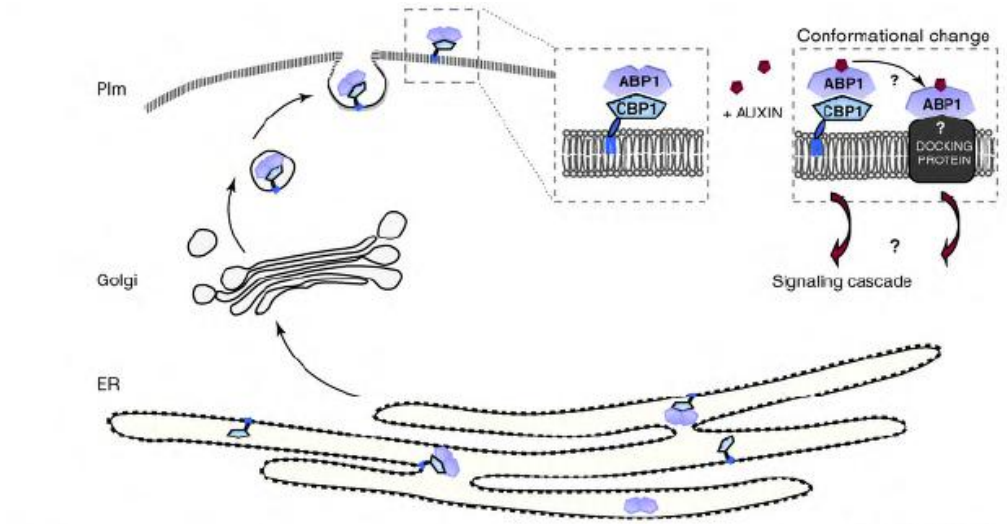



**Fig. 10 Hypothetic model for ABP1 targeting at the plasma membrane. ABP1 is located in the endoplasmic reticulum (ER) and at the outer surface of the plasma membrane. The protein is always associated with membrane fractions despite the absence of an intrinsic hydrophobic domain. The targeting of ABP1 to the Plm has beenhypothesized to result from the interaction of its C-terminal domain with another protein, thus masking the KDEL ER-retention motif. The identification of C-TERMINALBINDING PROTEIN 1(CBP1), as a protein interacting with a synthetic peptide corresponding to the C-terminus of Zm-ABP1, supports this suggestion. Interaction of ABP1 with the GPI-anchored protein CBP1 might promote targeting of ABP1 to the Plm with CBP1 acting as a carrier. Interaction of ABP1 with CBP1 is however not confirmed yet.The involvement of a third component for the transduction of the signal after binding of auxin to ABP1 cannot be ruled out [56].**

ABP1 is important throughout the life of plants, including embryogenesis, and differentially affects the cells depending on the developmental context. All experimental data

support that ABP1 is a crucial component of auxin signaling and can be considered as the auxin binding subunit of a plasma membrane auxin receptor.

So far, there are two clear auxin perception receptors, one involving ABP1 and the other TIR1/AFBs, as shown in Fig 11. The broad variety of auxin effects is likely to require both ABP1 and TIR1 coordinately control the physiological events in plants. In addition, a recent research showed S-Phase Kinase-Associated Protein 2A (SKP2A), as another candidate protein for auxin receptor, containing in the auxin signal pathway [57].

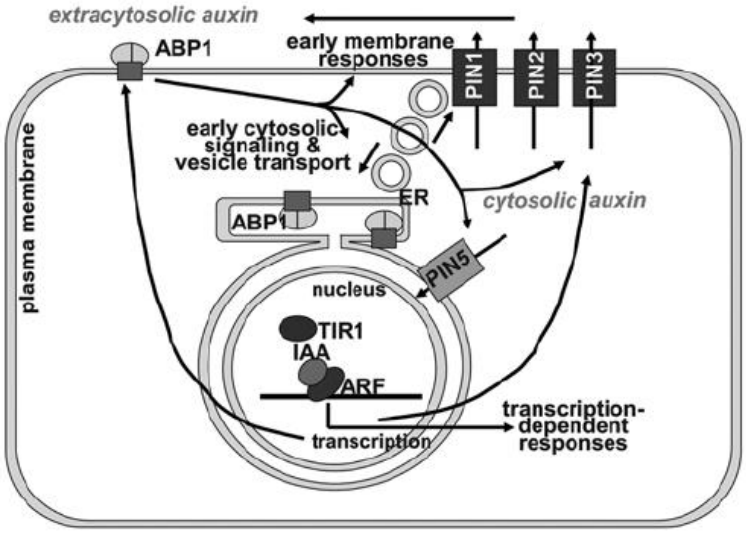



**Fig. 11 Model of auxin signal transduction. The receptor ABP1 is depicted as a dimer in complex with a transmembrane "docking protein." ABP1 triggers a number of typical signaling pathways in the cytosol (Scherer, 2011). These responses include the phosphorylationstatus of PIN proteins (not shown in drawing) and control of endocytosis/exocytosis balance (Kleine-Vehn & Friml, 2008). Several PIN proteins, including PIN 1, PIN 2and PIN 3 are integrated into cell polarity and auxin efflux transport toregulate extracytosolic auxin concentration and, thereby, polar auxintransport and tropisms. Indirectly, they may also regulate cytosolicauxin concentration as is assumed for PIN 5 which is localized to theER. This localization is postulated to increase nuclear auxin concentrationwhere it is sensed by the receptor TIR 1. Formation of the ternary complex [TIR 1 x auxin x IAA] leads to ubiquitination of IAA proteins and their hydrolysis by the proteasome. At least PIN 2 and PIN 3 are regulatedby phosphoryation and rapid transcriptional responses. TIR 1 is alsoassumed to be the relevant receptor for *ABP1* transcriptional regulationso that ABP1, PIN s and TIR 1 are a completely interlocking system oftwo receptors linked by auxin transport. Other systems of two or morereceptors for one signal where one receptor is closely associated toregulation of proteasomal activity are known or likely in plants [32].**

### IV.3 Auxin early response genes and auxin responsive element, AuxRE

Auxin rapidly and transiently induce the expression of hundreds of genes with minutes [58] .The early auxin-responsive genes can be classified into three major families: *SMALL AUXIN-UP RNAs* (*SAURs*), *GRETCHENHAGEN* (*GH3*)-related transcripts and *Auxin/INDOLE-3-ACETIC ACID* family members(*Aux/IAA*).

**SAUR.** Small auxin-up RNAs (SAURs) are the early auxin-responsive genes represented by a large multigene family in plants. SAURs were originally identified in auxin-treated soybean elongating hypocotyl sections [59]. Auxin induction of soybean SAURs was transcriptionally regulated and specific for active auxin [60]. Sequence analysis of three soybean *SAUR* cDNAs and genes revealed that the genes contain no introns [60].

Subsequently, Auxin-inducible *SAURs* have also been identified in mung bean [61], *Arabidopsis* [62], tobacco [63], maize [64] and rice [65]. In *Arabidopsis*, there are over 70 *SAUR* genes which appear to be lack of introns with only one exception *AtSAUR11*. As like in soybean, most of the *SAUR* genes in *Arabidopsis* are found in clusters. In rice, 58 *OsSAUR* gene family members were identified and also the coding sequences of *OsSAURs* do not possess any introns. The *SAURs* encode highly unstable mRNAs with a very high turnover rate due to the presence of a conserved downstream (DST) element in their 3-untranslated regions [66].

The SAUR proteins remain largely uncharacterized. Recently, It has been reported that *ZmSAUR2*, a nuclear-localized *Zea mays* SAUR protein, are shortlived, with a half-life of about 7 min [64]. Expression of *SAUR19* subfamily members in *Arabidopsis* confers numerous auxin-related phenotypes indicative of increased and/or unregulated cell expansion [67]. In addition, the calcium-dependent *in vitro* binding of SAUR proteins with calmodulin has been demonstrated, which provides a link between the Ca2+/calmodulin second messenger system and auxin signalling [64,68].

**GH3.** *Gretchen Hagen 3* (*GH3*) genes were a large multigene family and originally identified in *Glycine max* (soybean) as responsive to the phytohormone auxin [69]. *GH3* mRNA is transcriptionally induced within 5 minutes of auxin treatment, and is specifically induced only by active auxins [70]. By contrast, soybean *GH3* mRNA levels are unaffected by treatment with protein synthesis inhibitors [70,71]. Up to now, *GH3* genes has been identified in many plant species [72], which can be devidied into four different types, Chlorophyta, Bryophyta, Coniferophyta, and Magnoliophyta. In *Arabidopsis thaliana*, several genes were identified in genetic screens for altered phytohormone-mediated responses, including *DFL1* [73] and *JAR1* [74].

All of the *GH3* genes encode proteins with predicted molecular masses of 65–70 kDa. It has been shown plant GH3 proteins can be divided into three major clades, identified as Groups I, II, and III [74,75]. Groups I and II contained genes from many more species than Group III, the latter containing genes from only three species, *Arabidopsis thaliana*, *Brassica napus*, and *Gossypium hirsutum* [72]. In *Arabidopsis,* Group I was composed of two proteins, AtGH3.11(JAR1), that adenylated jasmonic acid (JA) *in vitro* and displayed JA-amino synthetase activity and AtGH3.10 (DFL2) that doesn‟t have these capabilities [76]. Group II contains most of the members, including AtGH3.2 (YDK), AtGH3.5 (AtGH3a), AtGH3.6 (DFL1), and AtGH3.17, which were able to adenylate indolacetic acid (IAA) and to catalyze IAA conjugation to amino acids through amide bounds [74,77]. In addition, AtGH3.6,

AtGH3.5 and AtGH3.17 might be the targets of the auxin response factor ARF8 [78]. There was no adenylation activity on the substrates tested found in group III.

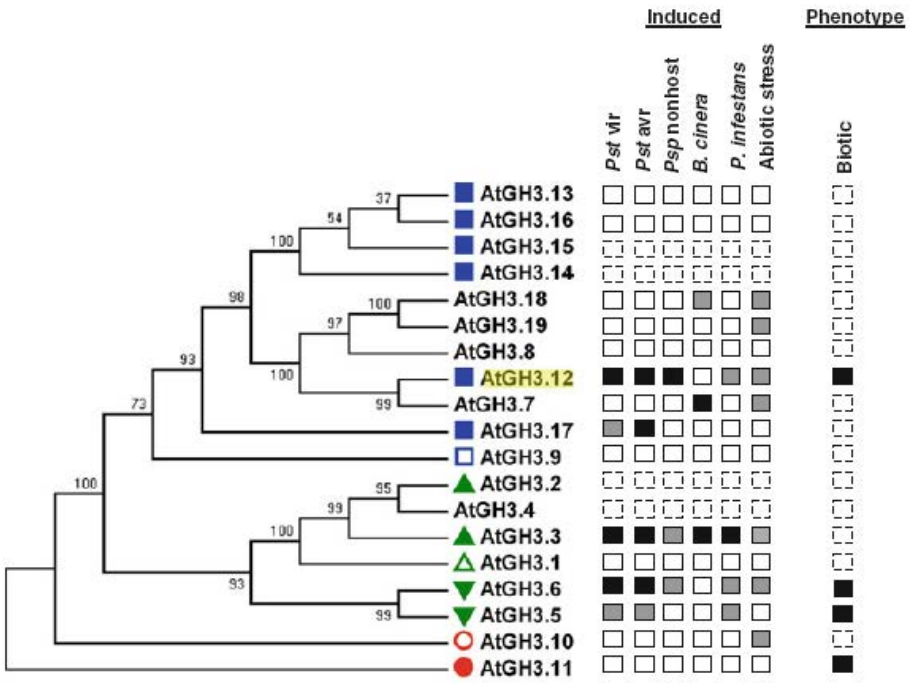



**Fig. 12 Maximum likelihood phylogenetic tree of *Arabidopsis* GH3 proteins and expression in response to biotic and abiotic stress [79].**

Recently, *GH3* genes showed different responses to a variety of stimulus [79] (Fig. 12). The *fin219* (far-red-insensitive 219) mutant was isolated as a suppressor of a mutation in a key regulatory component of photomorphogenesis, COP1 [80]. The *fin219* mutant plants display a long hypocotyl phenotype when grown under continuous far-red light. The dominant *dfl1-D* (dwarf in light 1) mutant was selected in a screen for hypocotyl length mutants from *Arabidopsis* activation-tagged lines [73]. Both hypocotyl cell elongation and lateral root formation are inhibited in the *dfl1-D* mutant in the light. Furthemore, Overexpression of *AtGH3.6*, *AtGH3.10*, and *AtGH3.2* [73,81,82] produced short hypocotyl in light (YDK also in dark) but only adult plants overexpressing *AtGH3.6* and *AtGH3.2* resulted in a dwarf phenotype. Most recently, the *GH3* family encodes the enzyme that catalyzes the conjugation of IAA with an amino acid to yield an inactive storage form of IAA [83,84]. In total, the above evidence suggests *GH3* genes are clearly implicated in the regulation of plant phenotype, an agronomical trait of great relevance.

**Aux/IAA.** *Aux/IAA* gene was first isolated from soybean hypocotyls [85]. Subsequently, many *Aux/IAA* genes have been characterized based on the analysis of gain-of-function

mutants in *Arabidopsis* [86], tomato [87,88] , rice [89] and maize [90]. *Aux/IAA* genes are present as multigene families in plants. *Arabidopsis* contains 29 *Aux/IAA* genes and most of them are induced by auxin and show a range of induction kinetics except *Aux/IAA* 28 [91,92]. In rice, a total of 31 Aux/IAAs distributed on 10 of the 12 chromosomes (except chromosome 4 and 10) have been reported [93] . In tomato, it has been shown that a total of 26 Aux/IAAs distributed on 9 of the 12 chromosomes (except chromosome2, 10 and 11) and IAA4, IAA9, IAA22, IAA29 and IAA32 proteins were located in the nuclus of the tobacco protoplasts fused with –GFP (green fluorescent protein) or –YFP (yellow fluorescent protein), suggesting that Aux/IAA proteins are nuclar localized [94,95]. In maize, a total of 31 *Aux/IAA* genes were also identified, which are distributed in all the 10 chromosomes except chromosome 2 [96]. Until now, 17 IAA genes in potato genome, 35 *Aux/IAA* genesin *Populus* genome and several IAA genes in tobacco have been reported [97,98].

Aux/IAAs proteins generally range in size from 20 to 35 kDa. They are short-lived and localize to the nucleus as previous described. So far all the Aux/IAAs are characterized as repressor of auxin response [99]. It has been found four highly conserved domains (Fig 13). Domain I of Aux/IAA functions as an active repression domain and contains a conserved Leu-rich (LxLxL) motif that is located in the ethylene response factor-associated amphiphilic repression (EAR) motif [99]. Domain II contains a WPPV motif that is responsible for the stability of Aux/IAA proteins and directly binds to the TIR1/AFB auxin receptors in an auxin-dependent manner [100]. Domain III contains a predicted *βαα* fold, which has been characterized in DNA-binding domains of Arc and Met J repressor proteins [101]. Domains III and IV of the Aux/IAA proteins contain a protein-protein interaction domain that shares a homology with the C-terminal domain (CTD) of ARF proteins [102].

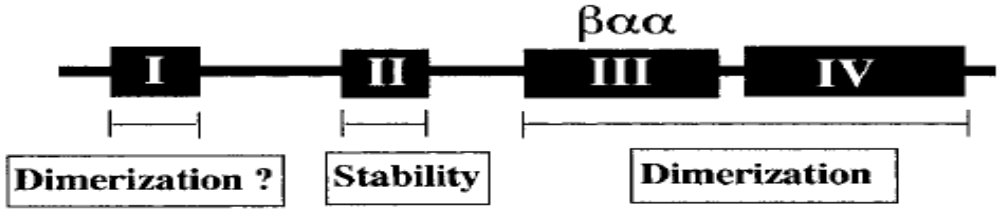



**Fig. 13 Diagram of an Aux/IAA protein with conserved domains I, II, III and IV [111].**

*Aux/IAA* family genes play an important role in many aspects of plant responses to auxin. In *Arabidopsis*, it has been shown that *iaa28-1* may act as transcription repressor in promoting lateral root initiation in response to auxin signals [103]. IAA14 was regarded as a fundamental

regulator in lateral root formation as its mutant completely lacked lateral roots [104]. In tomato, antisense down-regulation of *Sl-IAA3* resulted in the alteration of auxin and ethylene-related phenotypes, such as apical dominance, auxin sensitivity, apical hook and petiole epinasty [87]. While *Sl-IAA9* was down-regulated, the antisense lines exhibited simple leaves instead of compound leaves, and fruit development was triggered before fertilization, giving rise to parthenocarpy [88]. Most recently, it was shown that *Sl-IAA15* was involved in trichome development as IAA15-inhibitedd lines displayed strong reduction of type I, V and VI trichomes[105]. Likewise, suppression of *Sl-IAA27* gene revealed multiple phenotypes related to vegetative and reproductive growth, including higher auxin sensitivity, altered root development, reduced chlorophyll content in leaves, dramatic loss of fertility and enlarged placenta and small fruits [106]. Taken together, these phenotypes uncover specialized roles for Aux/IAAs in plant developmental processes, clearly indicating that *Aux/IAA* gene family members perform both overlapping and specific functions in plant.

**AuxRE.** The promoters of several auxin-responsive genes, like *SAURs*, *GH3* and *Aux/IAAs*, shared a common sequence which had been identified as a six-base pair sequence, TGTCTC or variants [107,108]. This element known as Auxin Responsive Element (AuxRE), was first identified from the promoter region of the pea Ps-IAA4/5 gene[109]. AuxREs were functional in both composite and simple auxin-response elements (Fig. 14). In composite AuxREs, the TGTCTC element is only functional if combined with a coupling or constitutive element [110]. For simple AuxREs, which derived from the alteration of naturally occurring AuxREs, may function in the absence of a coupling element with the TGTCTC elements occurred as direct or palindromic repeats. And usually, the simple AuxREs are 5–10 times more auxin-responsive than natural AuxREs [111].

In the last two decades, natural AuxRE promoter-reporter constructs have often been used to study organ and tissue expression patterns of auxin-responsive genes. Synthetic AuxRE-reporter genes have been used to monitor cell and/or tissue responses to endogenous auxin in wild type and mutant plants carrying the reporter gene [112,113]. More recently, genome-wide profiling experiments have revealed a lot of auxin-induced genes, which contain AuxREs in putative regulatory regions [114,115].

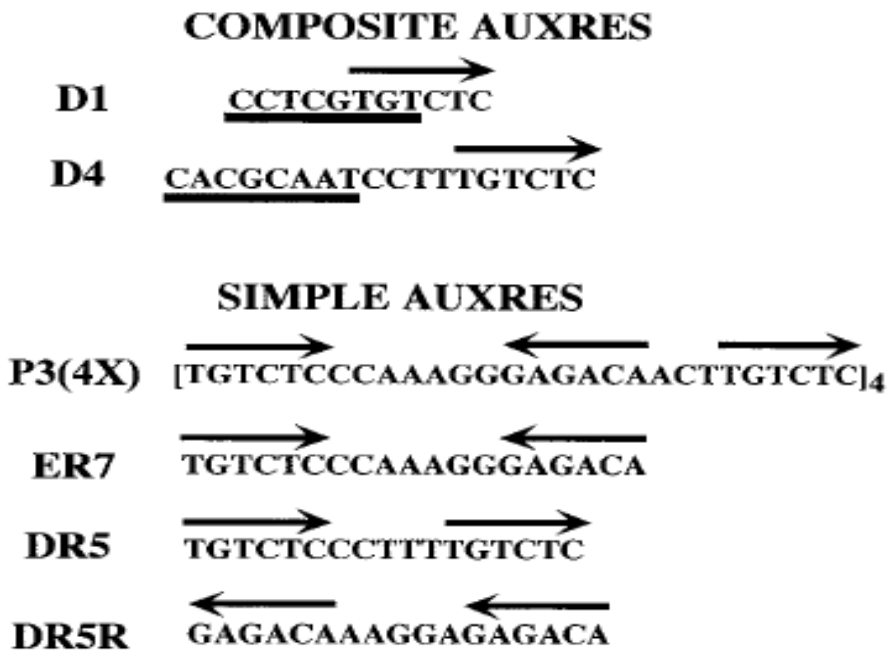



**Fig. 14 Composite and simple AuxREs. The TGTCTC elements or inverted GAGACA elements are indicated by arrows. The coupling or constitutive elements in the D1 and D4 promoter fragments of the *GH3* gene are indicated by the underlines. P3 (4X) contains an everted repeat separated by 7 bp, which is identical to ER7, and an inverted repeat separated by 3 bp. ARF1 protein was shown to bind to the ER in a P3 (4X) probe (Ulmasov et al, 1997a). DR5 is a direct repeat, and DR5R is the inverse of the DR5 repeat; both are active AuxREs, indicating that the orientation of the DR has no effect on activity [111].**

### IV4. Auxin Response Factor (ARF)

Auxin response factors (ARFs) are transcription factors in the auxin signal pathway which bind with specificity to TGTCTC auxin response elements (AuxREs) to regulate the primary/early auxin response genes, such as *GH3s*, *SAURs* and *Aux/IAA* genes [116]. ARF is a large gene family recruiting Aux/IAA repressors to confer an auxin response to functional genes. ARF gene family functions by activating or repressing auxin response genes. Recent advances have revealed that *ARF* family genes play a critical role in a host of developmental process, such as embryo patterning [117], leaf expansion and senescence [118], lateral root growth [119], floral organ abscission and petal growth [120,121], fruit set and development [122,123,124] and apical hook formation[125], as well as various responses to environmental stimuli. In addition, ARF family genes are involved in the interactions between auxin and gibberellins [126], auxin and ethylene [127], auxin and ABA [128] and also brassinosteroid response [129]. Studies employing different species have indicated that a total of 23 *ARF* genes from *Arabidopsis*, 25 *ARF* genes from rice (Oryzasativa) [130], 39 ARF genes from Populus trichocarpa[97], 24 *ARF* genes from sorghum (sorghum vulgare) [131] and 31 *ARF* genes from maize [132] have been identified, respectively. Most recently, 22 ARF genes have

also been isolated from tomato (*Solanum lycopersicum*) genome comparing with *AtARF* genes [133,134].

A typical ARF protein consists of an amino-terminal DNA binding domain (DBD), a middle region (MR) that functions as an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD) (Fig. 15) [135]. The ARF DBD is identified as a plant-specific B3-type, which requires additional amino-terminal and carboxy-terminal amino acids for efficient in vitro binding to TGTCTC AuxREs [136]. The modular nature of ARF DBDs has been checked by carrying out domain swap experiments with the DBDs of an ARF repressor and an ARF activator and the results showed DBDs targeted to AuxREs was independently of ARF MRs and CTDs [137]. The ARF MRs is located just between the DBDs to the CTDs and contains biased amino acids. The ARF ADs are rich in glutamine (Q), serine (S), and leucine (L) residues while ARF RDs are rich in proline (P), serine (S), threonine (T), and glycine (G) residues. The identification of ARF ADs and RDs were demonstrated by fusing the ARF middle regions to yeast GAL4 DBDs and the results indicated that ARF MRs function as repression or activation domains in an auxin-independent manner [138,139].

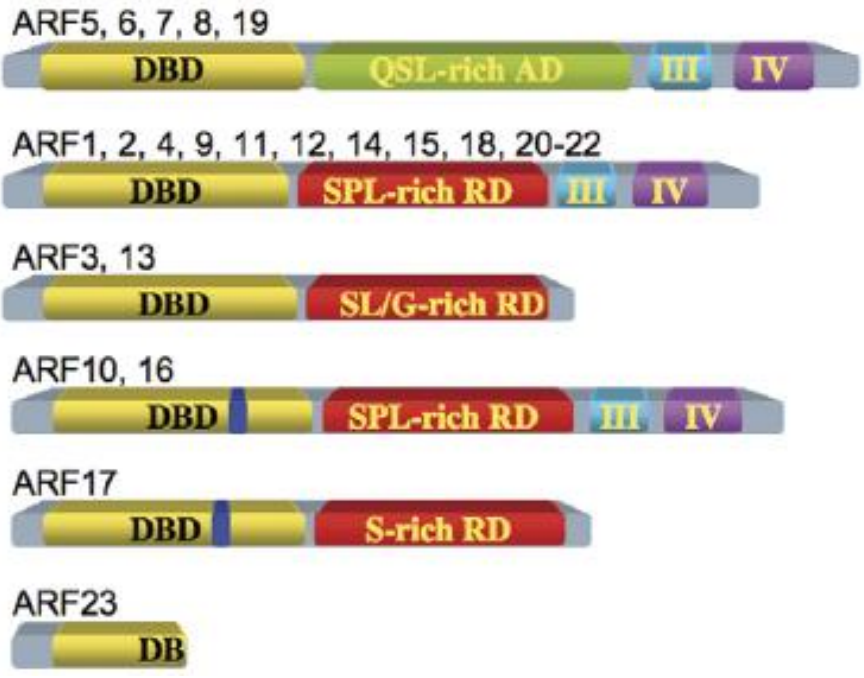



**Fig. 15 The ARF family of transcription factors in *Arabidopsis*. The ARF family consists of five transcriptional activators (ARF5–8 and 19) with an AD that is enriched in glutamine (Q), serine (S), and leucine (L). The remainder of the ARF family is thought to consist of transcriptional repressors with an RD that is usually enriched in serine(S) and in some cases proline (P), leucine (L), and/or glycine (G). All of the ARFs contain a conserved DBD; however, the DBD in ARF10, 16, and 17 contains an additional 32–36 residues (indicated in blue within the DBD), and ARF23 contains only a truncated DBD. ARF3,13, and 17 lack the conserved CTD found in most ARFs [138].**

The ARF CTDs contribute to formation of either ARF/ARF homo- and hetero-dimers or

ARF/Aux-IAA hetero-dimers (Fig. 16), which were based on the results in yeast two-hybrid assays with selected ARF CTDs and Aux/IAA proteins [107,108,140] as well as in protoplast transfection assays [138,139]. ARF activators with a CTD truncation activate auxin response genes constitutively in transfected protoplasts, suggesting that ARF CTDs is required for an auxin response but not ARF DBDs and MRs [137].

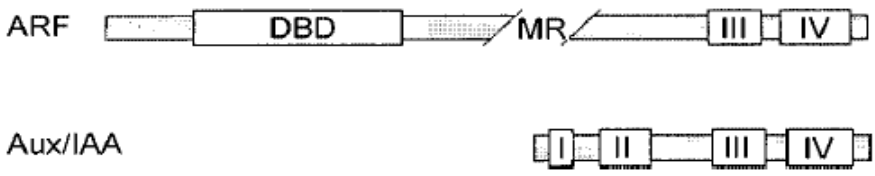



**Fig. 16 Domain properties of auxin response factor (ARF) and auxin/ indoleacetic acid (Aux/IAA) proteins [141].**

Since cloning of the first *AtARF1* gene from *Arabidopsis*, 23 members of this family, distributed over all five chromosomes have been identified [141]. The function and characterization of most *AtARF*genes was revealed by mutant analysis approach (Fig. 17 and 18), for example, *arf1* and *arf2* T-DNA insertion mutation indicated ARF2 regulated leaf senescence and longevity [118] as well as floral organ abscission, and ARF1 acted in a partially redundant manner with ARF2 and increased transcription of Aux/IAA genes in *Arabidopsis* flowers as a transcriptional repressor [120]. The *arf7* and *arf19* mutations were also found to enhance *arf2* mutation phenotypes as transcriptional activators. And the *arf7arf19* double mutant had stronger auxin resistance than the single mutant and displayed phenotypes not seen in the single mutant [127]. The *arf8* mutants were reported to regulate fertilization and fruit development, such as *arf8-1* showed a long-hypocotyl phenotype in either white, blue, red or far-red light conditions [78], *arf8-3* mutant indicated a significantly larger petal than that of the wild type[121], *arf8-4* mutant results in the uncoupling of fruit development from pollination and fertilization and gives rise to big and parthenocarpic fruit [122], and *arf6 arf8* double mutant flowers arrested as infertile closed buds with short petals, short stamen filaments, undehisced anthers and immature gynoecia [142]. Up to now, the roles of most *ARFs* in *Arabidopsis* have been identified, in detail, *ARF1* plays a role in developing flowers [120], *ARF2* in developing floral organs and in light-grown or dark-grown seedlings [120,143,144], *ARF3* and *4* in developing reproductive and vegetative tissues [145,146], *ARF5* in developing embryos and vascular tissues [147,148], *ARF6* in developing flowers [142], *ARF7* in seedlings, roots, and developing embryos [149,150], *ARF8* in seedlings and developing flowers and fruits [78,122], *ARF10* in serrated leaves, curled stems,

contorted flowers and twisted siliques[151], *ARF16* in the basal region of embryos, root caps, vascular tissue of roots, and leaves [152], and *ARF19* in seedlings, leaves and lateral roots[150,153]. Furthermore, *AtARF* genes fall into related sister pairs on the chromosome and may have overlapping functions during different developmental processes, including *ARF1* and *2*, *3* and *4*, *5* and *7*, *6* and *8*, *7* and *19*, or *ARF10*, *16* and *17* [142,154,155].

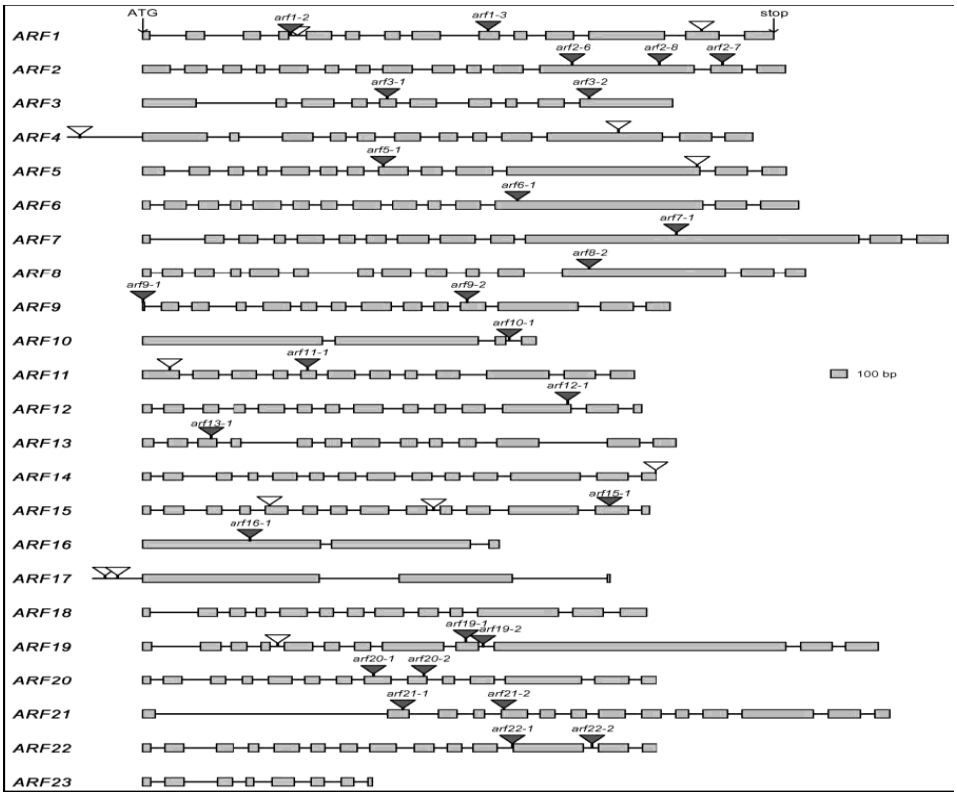



**Fig. 17 Location of T-DNA Insertions in the *ARF* Gene Family Members. Boxes represent exons. T-DNA insertions with gray triangles denote lines whose characterization has been completed. T-DNA insertions with white triangles denote lines not yet characterized [144].**

In tomato, it has been reported that down-regulation of *Sl-ARF2B* resulted in a color change in fruit break stage and partly pathenocarpic fruits (unpresented data). Also, down-regulation of *Sl-ARF4* (DR12) in the tomato resulted in a pleiotropic phenotype including dark green and blotchy ripening fruit [156]. Recently, down-regulation of *Sl-ARF7* gene in tomato produced parthenocarpic fruit, showing different form in contrast to wide type fruit [157]. Moveover, the mutant *mSl-ARF10* (Sl-miR160a-resistant version) plant developed narrow leaflet blades, sepals and petals, and abnormally shaped fruit [158].

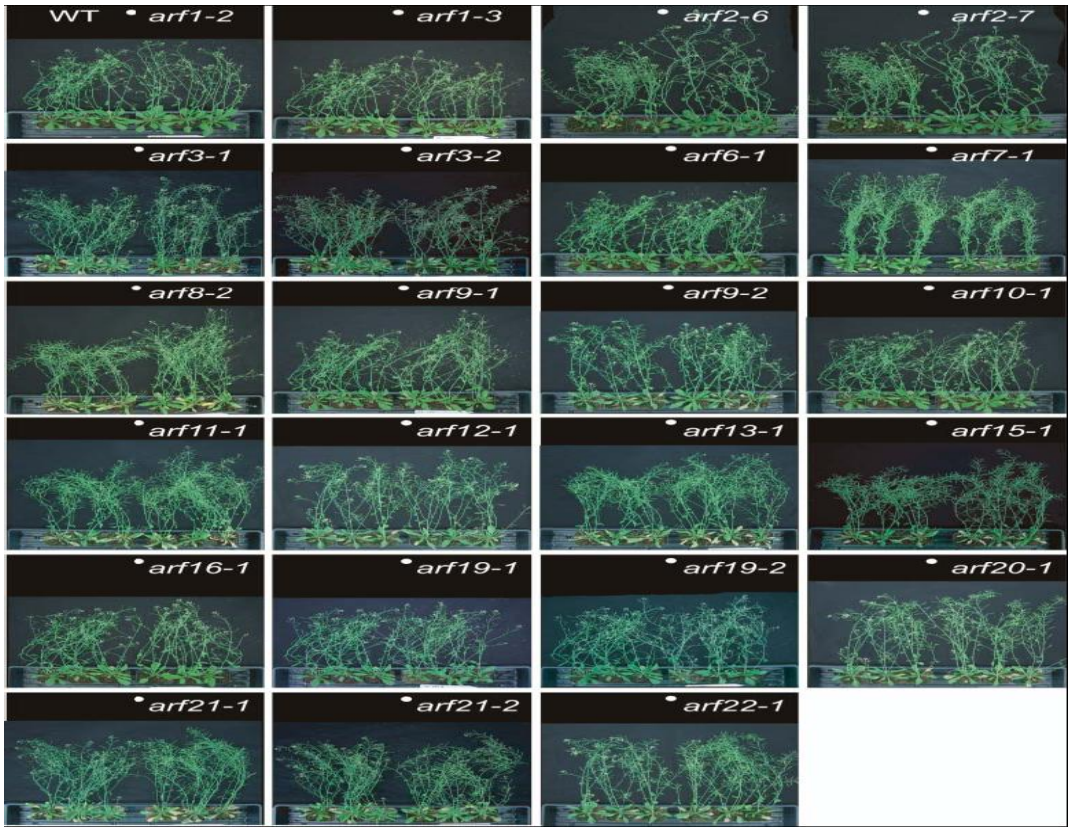



**Fig. 18 Phenotypes of Mature Mutant Plants. Three wild-type (left) and three mutant plants (right) are shown. The plants were grown at the same time. White dots indicate the boundaries between the wild-type and the mutant plants [144].**

Recently, the post-transcriptional regulation of ARF transcript abundance by several microRNAs (miRNAs) has been found to play specific roles in regulating plant growth and development [159]. For example, the miR160 is complementary to *ARF10*, *ARF16*, and *ARF17* and regulates gene expression by directing mRNA cleavage [160]. It has been shown that over-expression of miR160 in *Arabidopsis* reduced root length, increased lateral root number and suppressed root gravitropism [152], while disruption of miR160, acting on ARF17 (*mARF17*), led to severe developmental abnormalities, as like prior description mARF10 in tomato. The miR167 is complementary to *ARF6* and *ARF8* and over-expression of miR167 mimicked the phenotypes of *arf6arf8* double mutant, showing ectopic ovules and abnormal anthers [161,162]. Moreover, the phenotypic plasticity of adventitious rooting in *Arabidopsis*is controlled by complex regulation of *ARF6*, *8* and *17*, correspondence to miR160 and 167 (Fig. 19) [163]. Taken together, the miRNA-based regulation of ARFs in auxin signal pathway is essential for *Arabidopsis* growth and development.

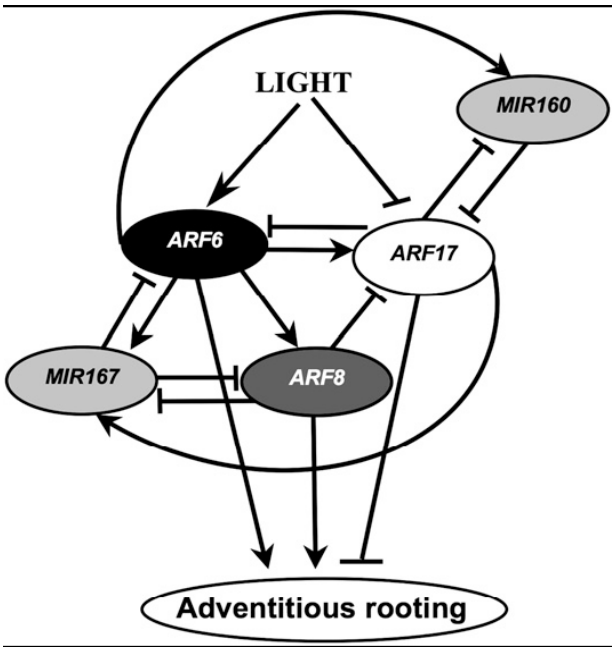



**Fig. 19 A model Integrating the regulatory loops between ARF and miRNAs in the control of adventitious rooting based on results obtained in this study [163].**

To investigate the roles of ARF transcription factors in auxin action, identification of the potential target genes under the direct transcriptional control of ARFs has been to the point. Recently, several methods including differential hybridization, protoplast transfection assays, transcript profiling using microarrays of auxin-induced genes, as well as microarrays from auxin signaling mutants, have been performed [147,164,165,166]. As been known, ARFs can bind specifically to the AuxREs within the promoters of early/primary auxin response genes, thus those genes containing the AuxRE sequences TGTCTC or GAGACA in their promoter regions have been of specific interests. Some reports have shown that *ARF7* plays a major role in regulating a wide variety of early/primary auxin response genes in *Arabidopsis* seedlings [149,167]. *ARF5* and *19* appear to be involved in activating expression of some *Aux/IAA* genes (e.g. *IAA1* and *19*) [164,167]. In floral tissues, *arf6 arf8* double mutants display lower expression of a *SAUR* clade (*SAUR62–SAUR67*) as well as a subset of *Aux/IAA* genes, suggesting that *ARF6* and *8* may activate at least some auxin response genes in reproductive organs [142]. In accordance, some *ARF* genes in *Arabidopsis* might bind to *GH3's* promoters to regulate gene expression. *ARF8* was proved to regulate the expression of three *AtGH3* genes, *AtGH3a*, *DFL1*, and *YDK1* [78]. These genes were down-regulated in *arf8-1* mutant and up-regulated in *ARF8*-sense lines, suggesting that *ARF8* probably maintains auxin homeostasis *in vivo* by positively regulate expression of *GH3*, which leaded to adenylating IAA to form IAA-AA. In addition, an auxin signal pathway containing

*microRNA167-ARF8-GH3-IAA* pathway in rice has been founded recently [168], which further proved that *GH3s* are the target genes of *ARF8* in the downstream of auxin signalling pathway. It is also interesting that a mutant gene *Fer1-1* acted synergistically with the *ARF8* gene to control the development of the anther and filament in *Arabidopsis*[169] and AtARF8 interacts with BPEp to affect petal growth and development. The petals of *bpe-1arf8-3* double mutants are significantly larger than those in wide type [121]. Otherwise, over-expression of a microRNA160-resistent *ARF17* mRNA, showing that *ARF17* could negatively regulate gene expression of *GH3-5*, *DFL1* and positively regulate gene expression of *GH3-2* and *YDK1*[170]. By contrast, ARF7 mutation causes reduced expression of some *GH3* genes including *YDK1*, showing that ARF7 regulates the expression of *YDK1* once again [82,171]. Recently, Okushima et al. described ARF7 binding to the AuxREs in promoters of two *ASL2/LOB* genes *in vitro* activated their expression in an auxin-dependent manner in *arf7arf19* mutant lines [172].

**Conclusion:** Genetic and molecular/biochemical experimental approaches have provided a large part of preliminary evidence that both ARF and Aux/IAA proteins function as transcription factors on primary auxin-response genes. Regulation of gene expression by auxin will surely be interesting and complicated. So far, our understanding remains limited by ARF and Aux/IAA transcription factors involved. Based on the current model, when auxin concentrations are low, auxin response genes are repressed due to the dimerization of Aux/IAAs and ARFs by domains III and IV. These heterodimers might determine whether or not an ARF transcription factor is bound to its TGTCTC target site. When auxin concentrations are increased, transcription is rapidly derepressed or activated due to the dissociation of Aux/IAA repressors from their ARF counterparts, while the Aux/IAA proteins were subsequently degradated by the ubiquitin/proteasome pathway. As of the transcriptional regulation, there are two patterns, one alterlative is, tissue-specific expression of ARF and Aux/IAA proteins could determine which ARF can target an AuxRE and which Aux/IAA protein can interact with an ARF to regulate the transcription of auxin response genes. The other is, transcription activation could be further potentiated by the binding of additional ARF activator to the DNA-bound ARF. However, there are also some questions need to be addressed. How does this model explain what role of ARF repressors, like ARF1 and ARF2? Do Aux/IAA proteins possess intrinsic repressor activity? Is there selectivity in the types of Aux/IAA and ARF homo- and heterodimers (or multimers) that can form? What role, if any, do Aux/IAA homodimers or Aux/IAA heterodimers play in auxin-responsive transcription?

Thus far, our understanding of the signal transduction pathway from auxin perception to gene expression is still rudimentary, progress is being made and interesting challenges remain.

## V. The physiological role of auxin in plant development

The plant hormone auxin as a central hormone is necessory for a number of plant growth and developmental processes as well as in many environmental and stress responses. One of the most profound actions of auxin on plants is the control of cell division. Accordingly, higher auxin concentrations stimulate cell division, and low auxin concentrations stimulate cell elongation and enlargement, and cell differentiation [173]. Regulation of organ patterning is another critial role of auxin, such as phyllotaxis, root initiation and gravitropism. Phyllotaxis is the regular arrangement of leaves on a stem. The accumulation of IAA in cells at the side of the shoot apical meristem startes organogenesis and determines the position of next leaf primordium. Generally, auxin is transported into roots and stimulates the initiation of lateral roots, which the specific PIN proteins, as PIN4 and AUX1 proteins is involved in this action [24]. In addition, auxin controls the apical dominance and shoots branching. It has been known for several years that auxin transported down from shoot apex controls the outgrowth of shoot branches from axillary buds. It also has been shown that auxin gradients, generated by polar auxin transport, have been implicated in tropisms. Adventitious rooting formation is also closely related to auxin induce. Addition of auxin to damaged stems often induces a strong adventitious rooting response, and this has been made good use in horticultural industries. As known, auxin plays a critial role in fruit development and maturation. It has been postulated that auxin is produced by the elongatation of pollen tubes and after by the embryo and endosperm in the developing seeds. Parthenocarpy has been induced in tomato by down-regulated DR12 (ARF4), a transcription factor in auxin singal pathway [156]. Today, the largest commercial exploitation for auxin is the use of synthetic auxins as selective herbicides, like 2,4-D and NAA [174]. The genetic data have showed the target sites for auxinic herbicide seems to be the auxin receptors. As more auxin receptors become known, auxins as agrochemicals are also growing.

## VI. Tomato as a model plant for fleshy fruit development

Tomato (*Solanum lycopersicum*) is the center piece of the Solanaceae family and has worked widely as a model system of fleshy fruit development and ripening due to the following reasons. Firstly, tomato is a short-lived perennial, grown as an annual, 5-6 months. The fruit development usually goes through several remarkable stages, including ovary

development, fruit set, developing fruit (8 DPA), young fruit, green fruit, breaker stage, pink stage and ripening (Fig. 20). Secondly, there are extensive germplasm collections, well characterized mutant stocks, high-density genetic maps, immortalized mapping populations, efficient transient and stable transformation, deep expressed sequence tag (EST) resources, microarrays and an ongoing genome sequencing database (http://solgenomics.net/) and all these efforts contribute to the utility of this experimental system. In addition, well characterized ripening mutations, a long history of biochemical and molecular investigations related to fruit development and maturation and most interest in the species as a major crop, have made considerable effort on the understanding of ripening in tomato [175].

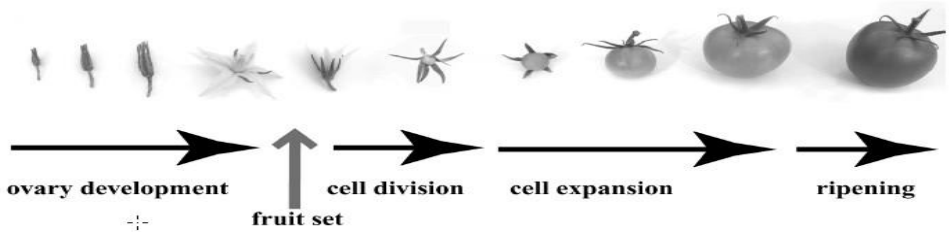



**Fig. 20 Tomato fruit development can be divided in five phases: ovary development, fruit set, cell division phase, cell expansion phase and ripening [188].**

### VI.1 Fruit set and phytohormones

The shift from the static flower ovary to fast-growing young fruit is a phenomenon known as fruit set, and this is a key step in the development of all the sexually reproducing higher plants. Generally, fruit set is triggered after pollination and successful fertilization of the egg cells in the ovules and requires a tight coordination among molecular, biochemical, and structural changes. In the case of tomato (*Solanum lycopersicum*), fruit set is the first phase of fruit development, which contains the initiation of the floral primordial and carpel development up to anthesis, after that, if it is pollinated and fertilized, the flower will resume the process, resulting in fruit set; or the carpel will senesce in the natural situation [176]. As an alternative pathway of fruit production, parthenocarpy promotes the ovary growth into the fruit without fertilization and seed formation, under the guidance of exogenous hormone treatment or endogenous, genetic stimuli.

Phytohormones are considered to be important mediators of fruit set signalling after pollination and fertilization. In 1936, *Gustafson* was the first to demonstrate that the application of substances closely related to auxins onto the tomato stigmas stimulates the ovary into seedless fruits [177]. Shortly thereafter, a second type of growth substance, gibberellins (GAs) can also stimulate parthenocarpic fruits and gibberellin-like hormones

were identified in different families of flowering plants [178], suggesting that gibberellins are also involved in the fruit set programme. In tomato, pollination signals and senescence leaded to an increase in ethylene synthesis followed by a decrease at 72 h after anthesis [179] and the decrease in ethylene biosynthesis and signaling genes in the ovaries of tomato flowers at 3DPA in pollinated or GA3 treated ovaries [180]. Recent years, many studies have contributed to the role of phytohormones in tomato (*Solanum lycopersicum*) fruit set relying on the transcriptome analysis of ovaries by several approaches, such as microarray, cDNA-amplified fragment length polymorphism (AFLP), Real-Time qRT-PCR, GC-MS and isolation cDNA clones of genes encoding enzymes [181,182,183]. A comparative analysis between developing fruit and other plant organs showed that most genes active in the fruit are not exclusively expressed there, indicating fruit development depends on the regulation of gene activity both in time and intensity [184]. Another transcriptomic analysis of tomato carpel development revealed the differentially expression of hormone genes in parthenocarpic fruit set with respect to normal fruit set, including auxin related gene, as *ARF* family, *IAA*2, *IAA*10 and *GH3-like*; gebberellin related gene, as *GASA5-like*, *DWARF3*, *GA2-oxidase*, *GA20-oxidase2* and *3*; ethylene related gene, *KNAT3*, *ACO5* and *3-keto-acyl-thiolase 2*; abcisic acid related gene, as *MOSC domain*, *ABA I zeaxanthin epoxidase* and ABA-induced protein so on [181]. The similar analysis underlying pollination-dependent and -independent tomato fruit set showed that many genes involved in hormone response with different percentages (Fig. 21) [185]. In addition, Vriezen et al. [182] compared the transcriptomes from pollinated ovaries and gibberellin-treated ovaries, as expected, pollination triggered genes that were not all triggered after the application of GA3 and vice versa. Pollination appeared to have significant effects on the expression of the auxin signalling genes, such as *Aux/IAA* and *ARF* family genes [186]. In contrast to the previous model of fruit set, in which gibberellins might induce an increase in auxin content within the ovary [187], these data showed that auxin may act prior to gibberellin in the onset of tomato fruit development. Moveover, several genes involved in ethylene biosynthesis and ethylene signalling pathway decreased after pollination. As well as the expression of genes involved in ABA biosynthesis seemed to decrease while the mRNA levels in ABA degradation increases [188]. These data implied that the function of ABA and ethylene in fruit set may be antagonistic to that of auxin and gibberellin to keep the ovary in a temporally dormant state before pollination and fertilization (Fig. 22).

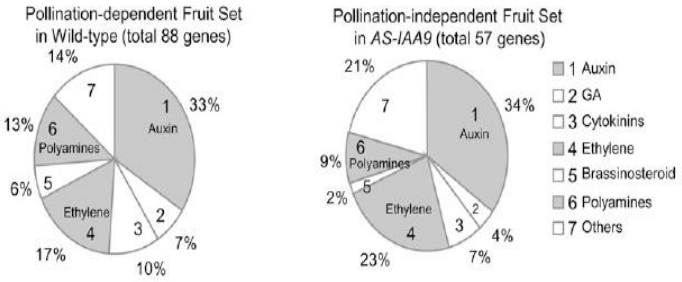



**Fig. 21 Expression of hormone-related genes during fruit set [185].**

## VI.2 Parthenocarpy and functional genes

As previous description, parthenocarpy is the fruit set in the absence of pollination and fertilization and the ovary develops into a fruit without seed formation. Parthenocarpy is thought to be a desirable trait for many commercially grown fruits if undesirable changes to structure, flavour, or nutrition can be avoided. For example, the mutation of parthenocarpic fruit (*pat*) is of particular interest because of its strong expressivity and because it confers earlier ripening, higher fruit set and enhanced fruit quality [189]. Parthenocarpy has been currently extensively studied in tomato due to its application in unfavourable environmental conditions that reduce pollen production, anther dehiscence and lead to fruit set, such as in South European Countries when tomatoes are grown in winter or in unheated greenhouses or tunnels. Parthenocarpy in tomato can be induced by exogenous hormone treatments, such as auxins and gibberellins. However, such methods sometimes cause malformed fruit and vegetative organs, inhibit further flowering, and usually yield poor quality fruit [190].

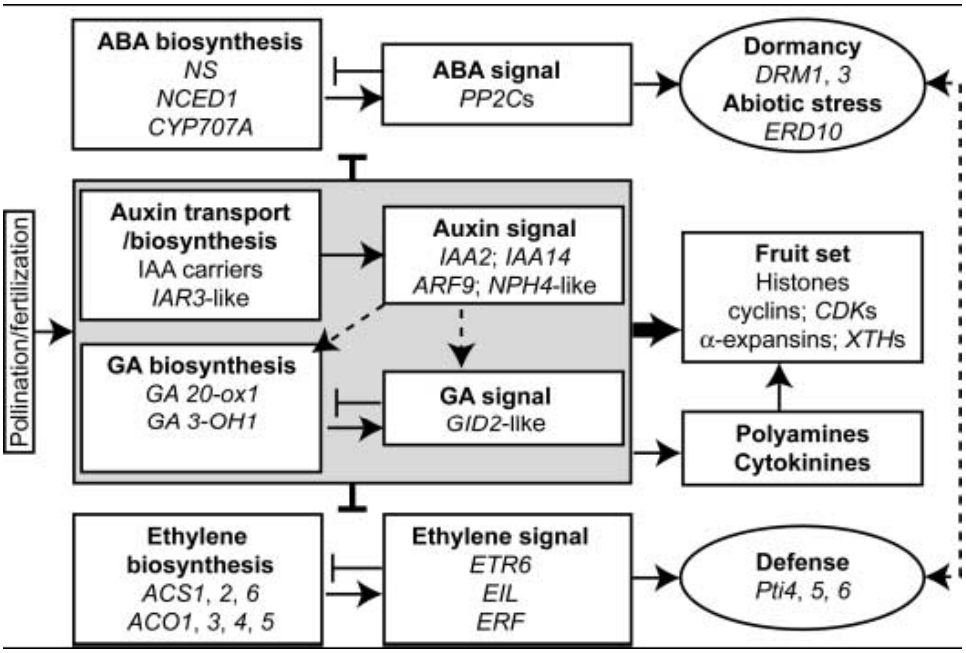



**Fig. 22 Model for hormonal signaling in the tomato ovary after pollination/fertilization. The central shaded squareshows auxin and gibberellin acting together to inhibit abscisic acid (ABA) and ethylene biosynthesis and signaling in order to stimulate fruit set,either directly or in cooperation with other factors such as polyamines and cytokinins. Dashed arrows show suggested interactions; auxin might regulate gibberellin biosynthesis and signaling, and ABA and ethylene might act together in a mechanism to prevent fruit set without fertilization [180].**

Generally, parthenocarpy is correlated with plant hormones in the ovaries, particularly auxins and gibberellins, which regulated the expression of fruit initiation genes during the critical period of anthesis and promoted the ovary growth independent of the pollination and fertilization. So far, genetic strategies offer effective approaches involving specific mutations or introduction of specific genes. In auxin signaling components, diageotropica (*DGT*) gene, which encodes for a cyclophyllin catalysing cis-trans isomerization of proline residues in peptides, was the first reported in tomato fruit set. The *dgt* mutants showed that fruit set, fruit weight, numbers of locules and seeds are all reduced [191]. Another recently identified gene *AUCSIA*, which is reduced by by RNA interference led to a pleiotropic phenotype, especially when the flower petals were emasculated before pollination [192]. In our lab, Jones demonstrated for the first time that down-regulation of *Sl-ARF4* in tomato, resulted in a pleiotropic fruit phenotype including dark green and blotchy ripening fruit [156]. Parthenocarpic fruits have also been identified with the silencing of *Sl-IAA9* [88], *SlIAA27* [106], *Sl-ARF2B* (unpublished data) and *Sl-ARF7* [157], and mutations in the *AtARF8* gene have been shown to induce parthenocarpy phenotype in tomato [123]. In contrast to *Sl-IAA9*, overexpression of *Sl-TIR1* homologue resulted in a pleiotropic phenotype including parthenocarpic fruit formation and leaf morphology [193]. Nevertheless, most of the

parthenocarpic fruits were heart shaped and had a rather thick pericarp compared to the fruits in wild type (Fig. 23).



**Fig. 23 Phenotypic characteristics of the tomato fruit in wild-type and SlARF7-RNAi [157].**

Several genes functioning in gibberellin biosynthesis and signaling cascade have been identified during fruit initiation recently. For example, the expression of a GA 20-oxidase gene was induced by pollination in tomato [194]. Reduction of *Sl-DELLA* mRNA levels induced facultative parthenocarpy in tomato fruit [195], in which, the pericarp contained fewer but bigger cells than wild type similar to the gibberellin-induced parthenocarpic fruit [196]. In addition, two knox genes, *LeT6* and *LeT12*, may have a role to play in formative events in ovule and embryo morphogenesis in tomato. [197]. Recently, it has been shown *Sl-ARF7* may mediate the cross-talk between auxin and gibberellins signalling during tomato fruit set [126].

Notably, in ABA pathway, several genes such as *ABA RESPONSE ELEMENT BINDING PROTEIN 1*, *ABA INSENSITIVE1*-like genes, and dehydrin genes, were found to be highly expressed in mature ovaries while their expression rapidly decreased after pollination [182]. In addition, *LeNCED1* gene, encodes for an important regulator of ABA biosynthesis in tomato, and *Sl-CYP707A1* gene, which was expressed specifcally in ovules and placenta, were found to regulate the abscisic acid levels in tomato ovaries [188]. Accordingly, ABA-deficient mutants, *notabilis* and *flacca* (*not/flc*) double mutant showed reduced fruit set and small fruit compared with wide type, suggesting that these two genes mingt act synergistically to control the fruit development. The *Sl-PP2C1* gene, a homologue of AtABI1, which encode a negative regulator of ABA response, was differentially expressed in tomato ovaries during fruit set. Over-expression and co-suppression of *Sl-PP2C1* in tomato leaded to ABA-insensitive and hypersensitive plants. Additionally, Sl-DRM1, a dormancy gene, is strongly regulated by

pollination, auxin and gibberellins through its 3‟-UTR and the mRNA is stongly localized in the ovules.

As well known, ethylene plays a critical role throughout fruit ripening of tomato fruit. In fact, 1-aminocyclopropane-1-carboxylic acid oxidase 5 (*ACO5*) gene was clearly activated at anthesis in the parthenocarpic line with respect to the control in emasculated ovaries and 1-Aminocyclopropane-1-Carboxilique Synthase (*ACS*) family genes were also found in the early stages of fruit development in tomato [198]. A recent research showed that over-expression of Sl-TPR1, which was a tomato tetratricopeptide repeat protein interacting with the ethylene receptors NR and LeETR1, modulated ethylene and auxin responses to stimulate the parthenocarpic fruits in tomato (Fig. 24) and *Arabidopsis* [199]. Accordingly, it has been also shown that Ethylene Response Factor 4 (*ERF4*) was involved in the process of fruit development. The *ERF4-SRDX* lines showed a reduced size of tomato fruit and seed number (unpublished data). In brief, the plant hormone ethylene may activate with auxin and gibberellin to regulate tomato fruit set and development.

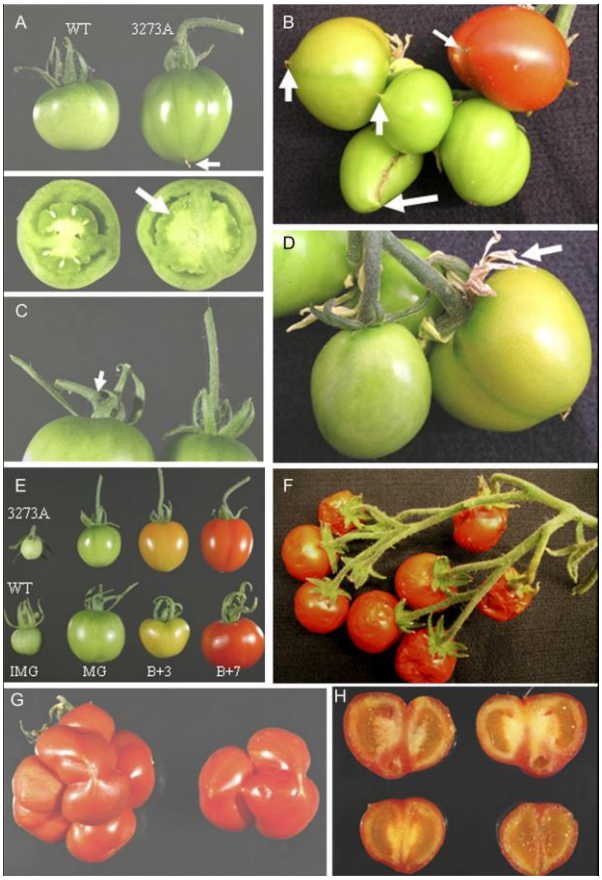



**Fig. 24 Altered fruit morphology in line 3273A overexpressing SlTPR1. (A) Control and transgenic fruit with enlarged columella (arrow) and no seeds. (B) Transgenic fruits with a beak-like structure and an attached style (arrows). (C) Elongated pedicel without the knuckle (abscission zone) in the transgenic fruit compared with the wild-type fruit which has a shorter pedicel with an obvious knuckle (arrowed). (D) Senescing petals attachedto the mature fruits. (E) Comparison of the transgenic fruits with the wild type at different developmental stages. (F) Transgenic fruits on line 3273Afirmly attached to the pedicel 80 d after ripening. (G, H) Fused multiple parthenocarpic fruits produced by plants developed from cuttings of line3273A [199].**

Otherwise, parthenocarpic fruits could be generated through ovule-specific expression of the *iaaM* or *iaaH* genes from *Agrobacterium tumefacians,* which affects the auxin

biosynthesis, and the *rolB* gene from *Agrobacterium rhizogenes*, which affects auxin responses [200,201]. As well known, flower development is largely under the control of transcription factors that belong to specific classes of MADS box homeotic genes, in which *DEFICIENS* (*Sl-DEF*) and *TM29*, were found to play roles in control of ovary growth in tomato [202]. It has also been domonstrated that down-regulation of *TM29* gene resulted in a parthenocarpic fruit and an aberrant flower morphology, which the petals and stamens are partially converted to a sepaloid identity [203].

# Objectives of the study

The research carried out at the laboratory of Genomics and Biotechnology of Fruit deals with the regulation of fleshy fruit development and mainly focus on two important developmental shifts both leading to a change of the organ fate. The first transition leads from flower to fruit, the so-called fruit set, and the second one from unripe to mature fruit. The role of phytohormones in regulating fruit development is addressed mainly through the characterization of transcription factors known to mediate hormone responses. Within this framework, my thesis project focus on the mechanisms underlying the auxin-dependent regulation of the fruit set process. Previous studies performed by the GBF group and based on global gene profiling of the shift from the static flower ovary to a fast-growing young fruit indicated that among all genes related to hormone metabolism and signaling that show significant change in their expression, about one third are related to auxin [185]. In addition, an increase in auxin concentration occurs at the end of the fruit growth phase just before fruit ripening. Taken together, these data strongly suggest that auxin plays an important role in the fruit set and early development steps. To further decipher the mechanisms by which auxin mediates fruit initiation, the GBF lab set up a large program aiming at functional characterization of transcription factors known to regulate the expression of auxin responsive genes. ARFs and Aux/IAAs are two multigene families required for controlling the expression of auxin response genes. Previous work [156] demonstrated for the first time that down-regulation of *Sl-ARF4* in tomato, resulted in a pleiotropic fruit phenotype including dark green and blotchy ripening fruit. Few years later, it was reported [122] that *arf8-4* mutant in Arabidopsis resulted in the uncoupling of fruit development from pollination/fertilization and giving rise to parthenocarpic fruits. More recently, it was shown that silencing of *Sl-IAA9* [88] and *Sl-ARF7* [157] in tomato resulted in a fertilization-independent fruit set leading to parthenocarpic fruits. Nevertheless, it is obvious that there is more to learn about the molecular mechanism underlying the auxin-mediated fruit set. My research project aimed at uncovering the role of members of the ARF family in the flower to fruit transition and the tomato was used as a model system to carry out my project for all the reasons stressed in Chapter I.

To date, the functional significance of members of the tomato *ARF* gene family remains poorly understood and the identification of the ARF members involved in fruit setting has not been undertaken. Moreover, at the beginning of my thesis, unlike the situation prevailing in

Arabidopsis where all members of the *At-ARF* gene family were knownin, the annotation of the tomato *Sl-ARF* gene family was only partial in the absence of the complete genome sequence information for this plant species. The first part of my thesis was then devoted to the identification and isolation of all members of the *ARF* gene family members in the tomato. Overall, 22 *Sl-ARF*genes were found in the tomato genome by BLAST search using 23 predicted AtARF protein sequences as query. Functional characterization of the isolated Sl-ARF genes included the investigation of their ability to work as transcriptional activators or repressors on auxin-responsive promoters. Subsequently, specific expression patterns in different tomato organs and tissues were established for each *Sl-ARF* family members, as well as their regulation by auxin or ethylene treatment at the transcriptional level. In addition, deep RNA-Sequencing revealed that most *Sl-ARF* genes undergo alternative splicing which may represent an import level of regulation of their expression during fruit initiation. This study provided a better insight on distinctive structural and functional features among tomato ARF proteins and set the foundation for more targeted studies on functional characterization of selected ARF genes.

Building on the outcome of the first part of my thesis research project, the second part was dedicated to the functional analysis of Sl-ARF8 using reverse genetics approaches. A dedicated plant material was generated consisting of transgenic lines altered in the expression of this gene. Through expression studies were carried out both by RT-qPCR and via analysis of *Sl-ARF8A/B* promoter-GUS fusion to establish a clear picture for transcript accumulation of *Sl-ARF8A* and *Sl-ARF8B* in the ovary before and after flower pollination/fertilization. The investigation of the hormone regulation of the two ARFs indicated that the expression of *Sl-ARF8A* was sharply inhibited by auxin and up-regulated by ethylene while that of *Sl-ARF8B* escaped any regulation by none of the two hormones. The physiological significance of the two *Sl-ARF8* genes was then addressed by ectopic expression of a sense construct of these genes in the tomato. This resulted in pleiotropic phenotypes, including dwarf plants, altered root and lateral shoot development and seedless fruits. These *Sl-ARF8* over-expressing lines were used for histological analysis of flower buds revealing the putative involvement of this gene in the growth of reproductive structures, especially placenta and ovules. The implementation of RNA-sequencing analyses on this transgenic tomato lines revealed the set of genes that show altered expression in *Sl-ARF8A*-Overexpressing lines. Altogether, the study provided a comphensive description of the functional characterization of Sl-ARF8 allowing to define this tomato ARF member as a central figure of the control mechanism underlying the fruit set process.

# Chapter II

## Characterization of the tomato ARF gene family uncovers a multi-levels post-transcriptional regulation including alternative splicing.

*PLoS ONE - 2013 (in press)*

# Chapter II:

## Characterization of the tomato ARF gene family uncovers a multi-levels post-transcriptional regulation including alternative splicing.

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# Abstract

Background

The phytohormone auxin is involved in a wide range of developmental processes and auxin signaling is known to modulate the expression of target genes via two types of transcriptional regulators, namely, Aux/IAA and Auxin Response Factors (ARF). ARFs play a major role in transcriptional activation or repression through direct binding to the promoter of auxin-responsive genes. The present study aims at gaining better insight on distinctive structural and functional features among ARF proteins.

Results

Building on the most updated tomato (*Solanum lycopersicon*) reference genome sequence, a comprehensive set of *ARF* genes was identified, extending the total number of family members to 22. Upon correction of structural annotation inconsistencies, renaming the tomato ARF family members provided a consensus nomenclature for all *ARF* genes across plant species. *In silico* search predicted the presence of putative target site for small interfering RNAs within twelve *Sl-ARFs* while sequence analysis of the 5‟-leader sequences revealed the presence of potential small uORF regulatory elements. Functional characterization carried out by transactivation assay partitioned tomato ARFs into repressors and activators of auxin-dependent gene transcription. Expression studies identified tomato *ARFs* potentially involved in the fruit set process. Genome-wide expression profiling using RNA-seq revealed that at least one third of the gene family members display alternative splicing mode of regulation during the flower to fruit transition. Moreover, the regulation of several tomato ARF genes by both ethylene and auxin, suggests their potential contribution to the convergence mechanism between the signaling pathways of these two hormones.

Conclusion

All together, the data bring new insight on the complexity of the expression control of *Sl-ARF* genes at the transcriptional and post-transcriptional levels supporting the hypothesis that these transcriptional mediators might represent one of the main components that enable auxin to regulate a wide range of physiological processes in a highly specific and coordinated manner.

# Introduction

The plant hormone auxin, indole-3-acetic acid (IAA), is a simple signaling molecule that plays a critical role in plant development and growth. This phytohormone regulates cell division and cell elongation and exerts pleiotropic effects on a wide range of developmental processes including organ differentiation, embryogenesis, lateral root initiation, apical dominance, gravitropism and phototropism, leaf elongation, shoot architecture and fruit development [1,2,3,4,5]. A critical move towards understanding the mechanisms underlying auxin action [6] happened when it was shown that the hormone coordinates plant development essentially through transcriptional regulation of genes such as *Aux/IAA*, *Gretchen Hagen3* (*GH3*), *Small Auxin Up RNA* (*SAUR*) and *Auxin Response Factor* (*ARF*). It was subsequently found that these so-called early auxin-responsive genes contain in their promoter one or more copies of a conserved motif, TGTCTC or its variants, known as the auxin-responsive element (AuxRE) [7]. Experimental evidences were then provided showing that transcription factors from the ARF type specifically bind to this AuxRE to mediate the transcription of auxin responsive genes [8]. The components of the pathway linking auxin perception to gene expression are now well established indicating that ubiquitination of Aux/IAA proteins by the TIR1/AFB subunit of the $SCF^{TIR1/AFB}$ ubiquitin ligase leads to their degradation by the 26S proteasome thus releasing the Aux/IAA-mediated inhibition of ARFs which allows these transcription factors to modulate the expression of their target genes [9].

Three types of transcriptional regulators are required for the control of auxin-responsive genes, Auxin Response Factors (ARFs), Aux/IAAs and Topless (TPLs) [10,11]. Members of the Aux/IAA and TPL families have been reported to function as repressors of auxin-induced gene expression [10,12,13,14]. An increasing number of studies demonstrate the critical role of ARFs in a variety of developmental processes, such as embryo patterning [15,16], leaf expansion and senescence [17,18,19], lateral root growth [18,20,21], floral organ abscission and petal growth [19,22], fruit set and development [23,24,25,26], apical hook formation [27], and various responses to environmental stimuli [28]. In addition, *ARF* genes are involved in the cross-talk between auxin and other hormones like gibberellins [29], ethylene [30], ABA [31] and brassinosteroid signaling [32]. A typical ARF protein consists of a conserved N-terminal B3-type DNA Binding Domain (DBD) that regulates the expression of early auxin response genes, a variable middle region (MR) that function as a transcriptional activation or repression domain (AD or RD), and a conserved C-terminal dimerization domain (CTD) that contributes to the formation of either ARF/ARF homo- and hetero-dimers or ARF/Aux-IAA

hetero-dimers [8,33,34]. The amino acid composition of MRs, located between the DBD and CTD, showed that AD types are rich in glutamine(Q), serine (S), and leucine (L) residues while RD types are rich in proline (P), serine (S), threonine (T), and glycine (G) residues [33,35].

Since the cloning of the first *AtARF1* from Arabidopsis, 22 members of this family, distributed over all five chromosomes, have been identified [33]. The functional characterization of *AtARF* genes was revealed by mutant analysis approach. For instance, *arf1* and *arf2* T-DNA insertion mutations indicated that ARF2 regulates leaf senescence [17] and floral organ abscission [19]. The *arf7/arf19* double mutant had stronger auxin resistance than the single mutant and displayed phenotypes not seen in the single mutant [30]. ARF8 was reported to regulate fertilization and fruit development, and *arf8-4* mutation results in the uncoupling of fruit development from pollination and fertilization giving rise to parthenocarpic fruit [23], while flowers in *arf6/arf8* double mutant are arrested as infertile closed buds with short petals, short stamen filaments, undehiscent anthers and immature gynoecia [36]. In tomato, recent studies have shown the involvement of *ARF* genes in fruit set, development, ripening and fruit quality [3,4,5,24,25,26,37]. Because of these findings, members of this gene family are becoming one of the main targets towards improving fruit traits in tomato and more broadly in fleshy fruits.

Studies using different species have indicated a total of 25 ARF genes in rice (*Oryza sativa*), 39 ARF genes in *Populus trichocarpa*, 24 ARF genes in sorghum (*Sorghum vulgare*) and 31 ARF genes in maize [38,39,40,41]. Though 21 *ARF* genes have been previously identified in the tomato (*Solanum lycopersicum*), yet, the list was incomplete and some the family members were either misannotated or suffered structural inconsistency due to the lack at that time of a high quality assembled tomato genome sequence [42,43]. The present study, while comprehensively revising the entire *ARF* gene family in tomato, brings new insight on the complexity of their expression control at the post-transcriptional level. The distinctive spatio-temporal pattern of expression of tomato *ARF* genes and their differential responsiveness to auxin and ethylene lay the foundation for a deeper functional characterization of these transcriptional mediators.

## Results

*Genome-wide search for tomato ARF genes*

Comprehensive identification of the *ARF* gene family members in the tomato was achieved using all ARF proteins previously reported from Arabidopsis and other plant species in BLAST queries on the recently published tomato genome sequence (SL2.40 genome sequence and iTAG2.30 whole protein sequences). Twenty four significant hits corresponding to non-redundant putative *Sl-ARF* genes were identified. PCR amplification of full length coding sequences (CDS) revealed two structural annotation inconsistencies reducing the total number of ARFs in the tomato genome to 22 (Table 1). Indeed, four sequences previously annotated as distinct *ARF* genes in iTAG2.30 corresponded to C-terminal or N-terminal parts of two ARF proteins (Solyc12g006340/Solyc00g196060; Solyc11g013480/Solyc11g013470). The mapping of tomato RNA-Seq data allowed to further improve the annotation of tomato *ARFs* by identifying the 3‟ and/or 5‟ UTR regions for 13 *Sl-ARF* genes (Table 1). All Sl-ARF proteins were found to contain a typical DBD domain (Figure 1A) as revealed by the Pfam analysis tool (http://pfam.sanger.ac.uk/). The molecular weight of the deduced Sl-ARF proteins showed a large variation ranging from 68 to 126 kDa (Table1). Of particular note, *Sl-ARF6B* contains a premature stop codon in the region corresponding to the DBD domain and is therefore likely to be a pseudo-gene whose expression at the protein level is not expected (data not shown). Using cNLS Mapper, nuclear localisation signals (NLS) were also identified in all of Sl-ARFs (data not shown).

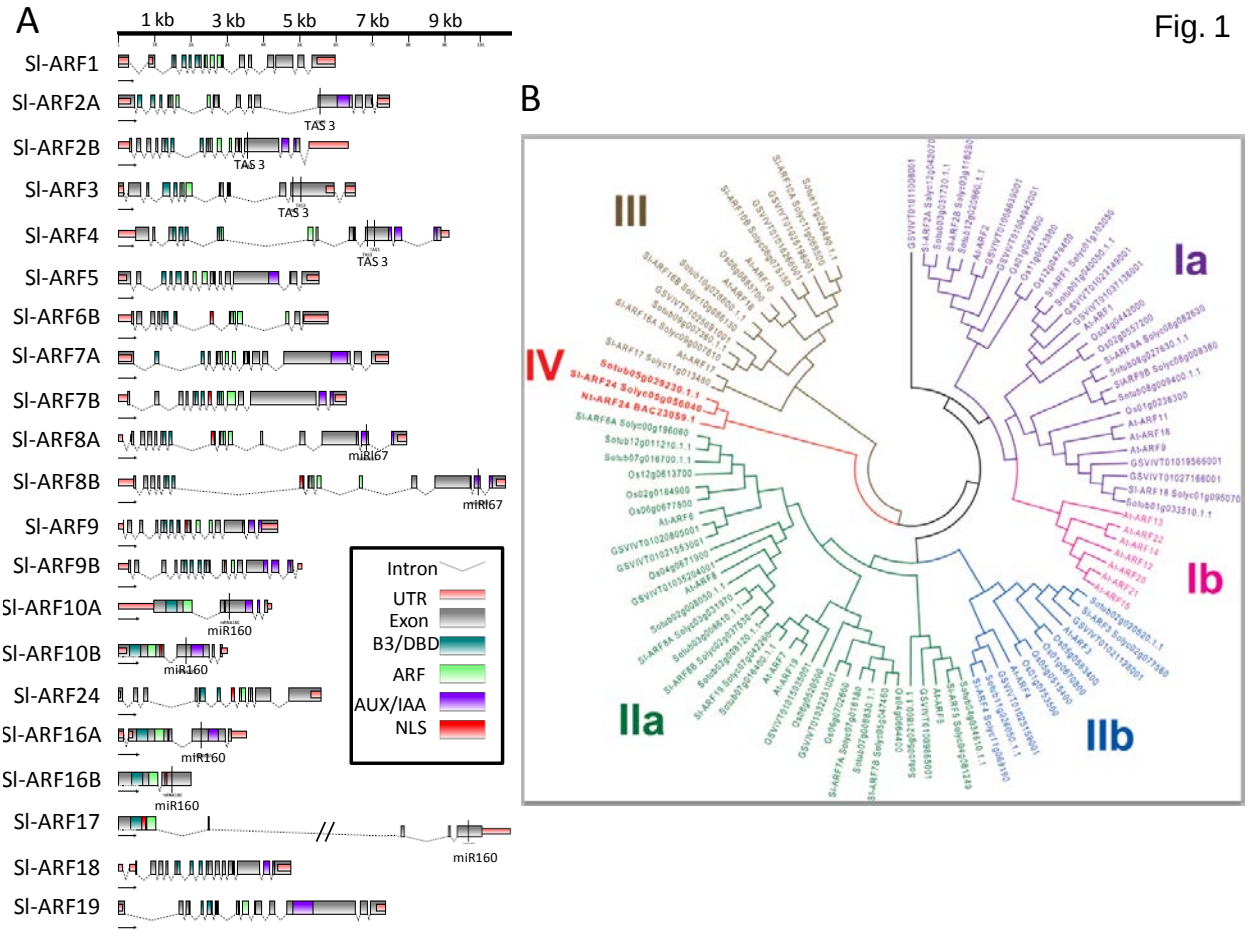



**Figure 1**. **The ARF family structures in tomato and phylogenetic relationship between rice, potato, tomato, grape and Arabidopsis.**(A) The generic structures of Sl-ARF family except *Sl-ARF6A*. The gene size (kb) is indicated in the upper panel. The domain of Sl-ARF gene is indicated by different colours. The marker in Sl-ARF family shows*Sl-ARF2A, 2B, 3* and *4*genes are spliced by TAS 3, *Sl-ARF8A* and *8B* spliced by miRl67, and *Sl-ARF10A, 10B, 16A, 16B* and *17* spliced by miR160.(B) The unrooted tree was generated using MEGA4 program by neighbor-joining method. Bootstrap values (above 50%) from 1000 replicates are indicated at each branch. All Sl-ARFs contain a DBD (brown). Most of the Sl-ARF proteins except Sl-ARF3, 10, 24, 16 and 17 contain a carboxy-terminal domain related to the domains III and IV found in the Aux/IAA proteins (blue).Sl-ARF5, 6A, 7, 8A, 8B, 19 contains a middle region that corresponds to the predicted activation domain (green) found in some AtARFs. The remaining Sl-ARFs contains a predicted repression domain (red). Sl-ARF-6B and AtARF23 contain only a truncated DBD (B3 domain).

Building on the available tomato genome assembly sequence, the mapping of *Sl-ARF* genes revealed that *Sl-ARF* family members are distributed among the 12 tomato chromosomes. Chromosome 7 and 11 are found to harbor three *ARF*genes each; chromosome 1, 2, 3, 5, 8 and 12 bear two *ARFs*, while each of chromosome 4, 6, 9 and 10 contains only a single *ARF*

gene (Supplementary Figure S1). Unlike the situation prevailing in Arabidopsis, there is no evidence for tandem or segmental duplication events involving members of the tomato ARF family.

*Phylogenetic relationship and consensus nomenclature for Sl-ARFs*

To explore phylogenetic relationship among ARF proteins in largely distributed land plant species, a phylogenetic tree (Figure 1B) was constructed that included ARF family members from tomato, *Arabidopsis,* potato, grape and rice. The phylogenetic distribution revealed that *ARF* genes group into four major classes named Class I, II, III and IV. ARFs predicted to function as transcriptional activators, based on the presence the Q-rich activation domain in their middle region, belong to sub-class IIa (Sl-ARF5, Sl-ARF6A, Sl-ARF7A, Sl-ARF7B, Sl-ARF8A, Sl-ARF8B and Sl-ARF19) while ARFs from the remaining classes (Ia, IIb, III and IV) all harbor a repression domain in the middle region and are consequently predicted to function as transcriptional repressors.

Compared to Arabidopsis which contains 23 members, the size of the tomato *Sl-ARF* gene family is slightly contracted to 22 members. In order to reach a consensual nomenclature for *ARF* genes across species, the tomato members of this gene family were renamed, based on phylogenetic relationship and according to the numbering of the closest Arabidopsis homolog. While complying with the most complete classification available in Arabidopsis [33], the proposed nomenclature better clarifies the correspondence between ARF subclasses in plant species. Noteworthy, sub-class Ib which has no representative in the tomato, contains 7 members in Arabidopsis that are likely to derive from multiple duplications of At-ARF13 which has no ortholog in any of the plant species tested in the present study. A distinctive feature of the tomato ARF family is the expanded size of the activators‟ sub-class (IIa) which represents 36.5% of the *ARF* genes whereas the activators only account for 21.7% of Arabidopsis ARFs. Another specific feature of the tomato ARF family is the presence of *Sl-ARF24* (sub-class IV) that is not found out of the *Solanaceae* family. Interestingly, this presumably *Solanaceae*-specific gene encodes an ARF protein that lacks domain III and IV involved in protein/protein interactions and required for the binding to Aux/IAA proteins. Likewise, Sl-ARF3, Sl-ARF16B and Sl-ARF17 are also deprived of domain III and IV necessary for interaction with Aux/IAAs (Figure 1A and Supplementary Figure S2). It is

therefore likely that these Sl-ARFs escape the classical mechanism underlying auxin signaling which implies the sequestration of ARF proteins through interaction with Aux/IAAs.

*Predicted siRNA-mediated degradation and multiple upstream ORFs in the 5' leader sequences of tomato ARF transcripts*

ARF genes have been already reported to undergo post-transcriptional regulation involving small interfering RNAs. *In silico* analysis at the RNA level predicted that 12 out of the 22 tomato Sl-ARFs have a putative target site for small interfering RNAs (Figure 1A). That is, Sl-ARF2A, Sl-ARF2B, Sl-ARF3 and Sl-ARF4 are predicted to be potentially targeted by TAS3; Sl-ARF6A, Sl-ARF8A and Sl-ARF8B by miR167; and Sl-ARF10A, Sl-ARF10B, Sl-ARF16A, Sl-ARF16B and Sl-ARF17 by miR160.

The uORFs are elements found in the 5‟-leader sequences of specific mRNAs that modulate the translation of downstream ORFs by ribosomal stalling and inefficient re-initiation or by affecting transcript accumulation through nonsense-mediated mRNA decay pathway. Among the 19 *Sl-ARFs* for which the 5‟ leader sequences are available in iTAG2.30 (8 members) or identified in this study (11 members), uORFs were predicted for 17 genes, ranging from 1 to 52 amino acids in size with four genes (Sl-ARF2A, Sl-ARF5, Sl-ARF10A and Sl-ARF16A) having five or more uORFs (Supplementary Table S1).The average number of uORF per Sl-ARF gene is similar in tomato (2.8/leader) and Arabidopsis (3.3/leader), indicating that tomato ARFs are suitable candidates to be regulated through translational uORFs depending mechanism

*Transcriptional activation and repression activities of tomato ARFs*

To characterize the capacity of tomato ARF proteins to *in vivo* activate or repress gene transcription, tobacco cells were co-transfected with an effector construct expressing the full-length coding sequence of Sl-ARFs and a reporter construct carrying the auxin-responsive *DR5* promoter fused to *GFP* coding sequence [44]. DR5 is a synthetic auxin-responsive promoter made of 9 inverted repeats of the conserved Auxin-Responsive Element, the so-called TGTCTC box, fused to a *CaMV35S* minimal promoter. The *DR5*-driven GFP chimeric gene showed low basal activity which was induced up to 5-fold by exogenous auxin treatment (Figure 2). Co-transfection of tobacco protoplasts with the *DR5::GFP* reporter construct and effector plasmids expressing either *Sl-ARF1, Sl-ARF2A, Sl-ARF2B, Sl-ARF3, Sl-ARF4, Sl-ARF9A, Sl-ARF10A* or *Sl-ARF17* coding sequences, resulted in strong repression of the auxin-

induced expression of the reporter gene (Figure 2). By contrast, co-transfection with effector constructs expressing *Sl-ARF5, Sl-ARF6A, Sl-ARF7, Sl-ARF8B* or *Sl-ARF19* enhanced the auxin-induced expression of the reporter gene. Noteworthy, with the exception of *Sl-ARF6A* and *Sl-ARF7*, these activator ARFs were unable to enhance the basal activity of the DR5 promoter in the absence of auxin treatment (Figure 2) suggesting that most ARFs require the input of an active auxin signalling for transcriptional activation of target genes.

Fig. 2

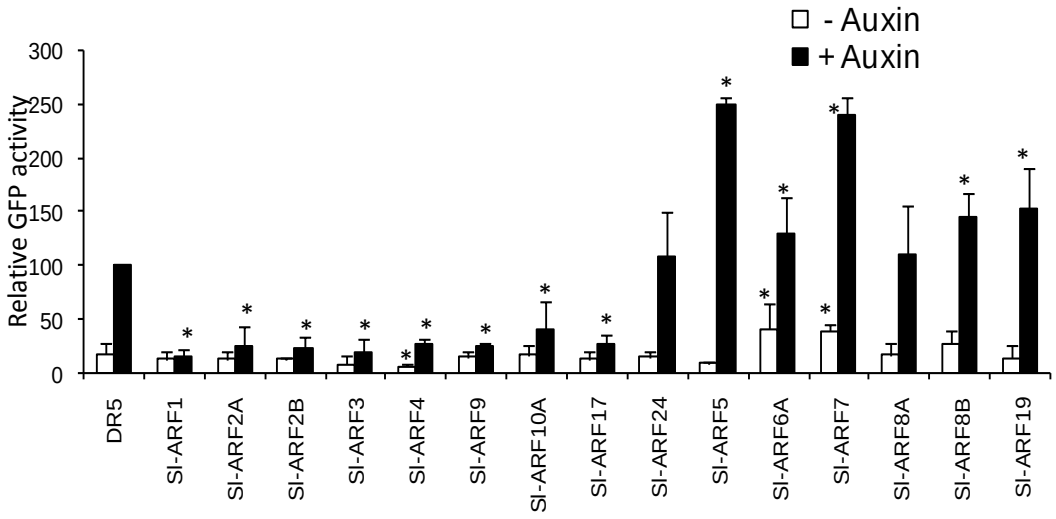



**Figure 2. Sl-ARF factors differentially regulate the expression of reporter genes driven by synthetic and native auxin-responsive promoters.** Sl-ARF factors were challenged with a synthetic auxin-responsive promoter called *DR5*, consisting of seven tandem copies of the AuxREtgtctc element. A transient expression using a single cell system was performed to measure the reporter gene activity. The fluorescence was measured by flux cytometry. Because of the very low basal activity of the DR5 promoter without auxin treatment, the auxin inducible fluorescence obtained by co-transformation with the promoter fused to the reporter gene and with the empty vector is consider as reference. The results shown are the average of 3 independent biological repetitions. Values are means standard deviation (SD) of three replicates. Stars show significant difference using Student‟s t-test at $P<0.05$. Fluorescence Values obtained by co-transformation with plasmids harbouring each Sl-ARF gene were compared to values obtained with basal activity of the DR5 promoter without auxin. Fluorescence Values obtained by co-transformation with plasmids harbouring each Sl-ARF gene and

treated with auxin were compared to values obtained with the activity of the DR5 promoter treated with auxin.

*Expression of Sl-ARF genes in different tomato tissues*

To gain clues on the physiological function of tomato ARFs, the spatio-temporal expression of individual members of the gene family was examined at the transcriptional level using qRT-PCR. Transcript accumulation could be assessed for 15 *ARF* genes in different tissues including root, stem, leaves, flower and fruit at various developmental stages. For the remaining 7 tomato *ARF* genes, transcript detection was unsuccessful in any of the samples tested suggesting their extremely low expression in these tissues. The data indicate that the expression of *ARF* genes is ubiquitous in all tissues with most genes being expressed in reproductive tissues suggesting their putative role in flower and fruit development (Figure 3).

Fig. 3

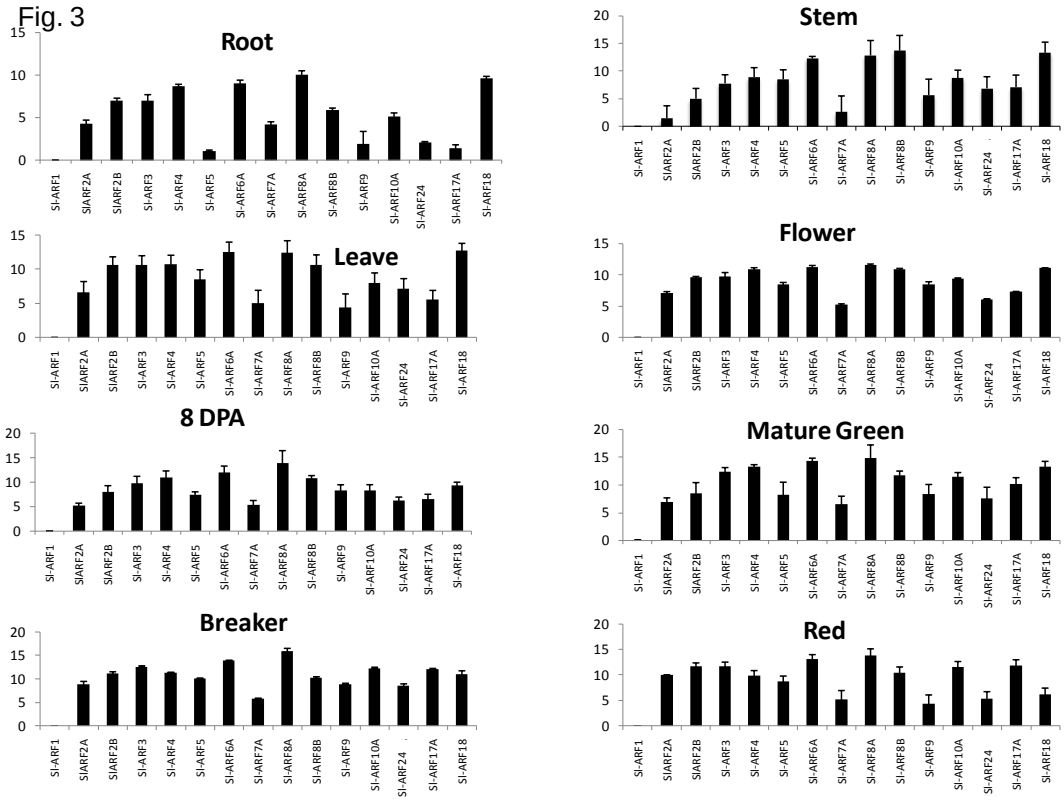



**Figure 3. Real-time PCR expression profiles of individual *Sl-ARF* genes.** Total of 15 *Sl-ARF* genes were performed in different tomato organs (root, stem, leaf, flower, 8DPA, Mature Green, Breaker and Red). X-axis represents different *Sl-ARF* genes, while Y-axis represents three relative expressions of those genes. 8DPA: 8 days after pollination, Mature Green, Breaker and Red represent different stage of the fruit development.

Heatmap representation (Figure 4) allowed the clustering of tomato *ARFs* into two main groups based on their expression pattern: group I (*Sl-ARF1, Sl-ARF2A, Sl-ARF2B, Sl-ARF4, Sl-ARF7A, Sl-ARF6B and Sl-ARF18*) are genes preferentially expressed in roots while group II *Sl-ARFs* are lower expressed in the roots.

Fig. 4

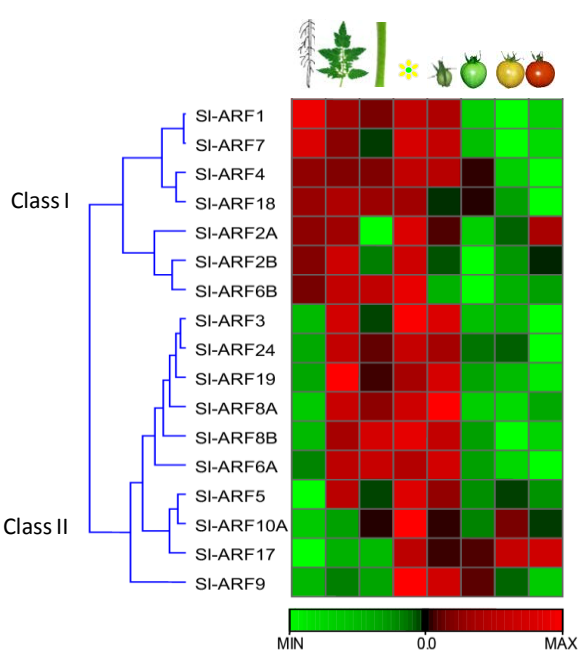



**Figure 4. Heatmap showing *Sl-ARF* gene expression in different tomato tissues.**

Changes in RNA accumulation in different tomato tissues (Roots, Leaves, Stems, Flowers, Early Immature Green (8 DPA), Mature Green, Breaker, Red (Breaker + 7 days) as schematically depicted above the displayed array data, are shown relative to the RNA accumulation levels in roots. Levels of down expression (green) or up expression (red) are shown on a log2 scale from the high to the low expression of each *Sl-ARF* gene.

*Auxin and ethylene regulation of Sl-ARF* genes

Screening for *cis*-acting elements corresponding to Auxin Response Elements (AuxRE) within the promoter regions using the Place database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) identified conserved (TGTCTC) and degenerate (TGTCCC) motifs in most tomato ARF promoters. In addition to these AuxRE, *Sl-ARF* promoters contain conserved Ethylene-Response motifs, the so-called ERELEE4 motif found in the promoter of tomato *E4* gene (AWTTCAAA) (Supplementary Table S2). The presence of these *cis*-regulatory elements suggests a potential regulation of *ARF* genes by both auxin and ethylene. To test the responsiveness of tomato *ARF* genes to both hormones, transcript accumulation was assessed by qRT-PCR in seedlings treated with auxin or

ethylene. All *Sl-ARFs* were found to be auxin-responsive after 2 hour treatment (Figure 5A), with *Sl-ARF4*, *Sl-ARF5* and *Sl-ARF2A* showing the highest up-regulation whereas *Sl-ARF1*, *Sl-ARF7 Sl-ARF10* displayed the most significant down-regulation. On the other hand, the expression of Sl-ARF2B, Sl-ARF5 and Sl-ARF9A showed strong up-regulation (more than four folds increase) when treated 5 hours with ethylene (Figure 5B). Of particular interest, Sl-ARF5 is strongly up-regulated by both hormones and may therefore be involved in mediating responses to both hormones.

Fig. 5

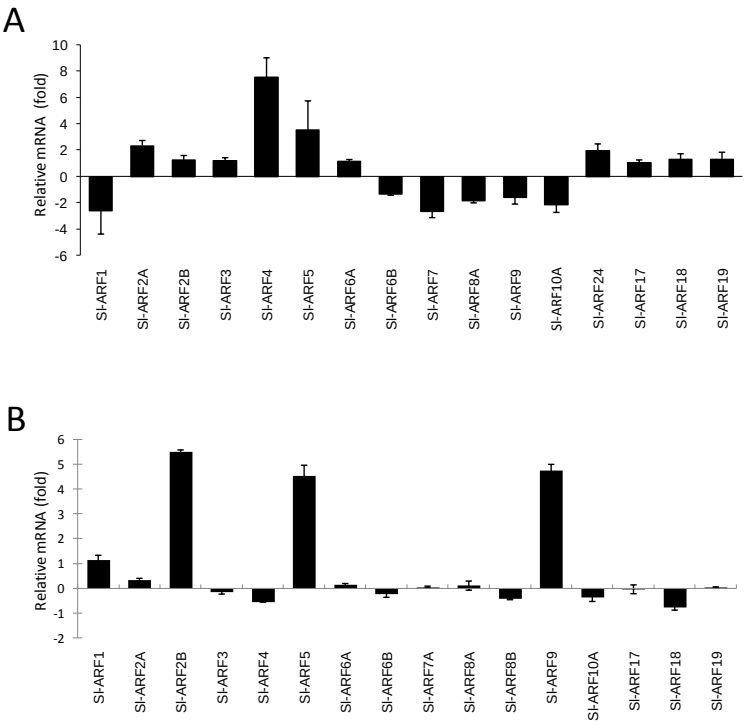



**Figure 5. The expression of *Sl-ARF* family genes in response to auxin and ethylene.** (A) Auxin induction of *Sl-ARF* genes on light grown seedlings. Quantitative RT-PCR of *Sl-ARF* transcripts in RNA samples extracted from 12-day-old tomato seedlings soaked in liquid MS medium with 10µM IAA for 2 hours.ΔΔCT refers to the fold of difference in *Sl-ARF* expression to the untreated seedlings. The *SAUR* gene was used as control to validate the auxin treatment. (B) Ethylene regulation of *Sl-ARF* genes on dark grown seedlings. Quantitative RT-PCR of *Sl-ARF* transcripts in RNA samples extracted from5-days dark-grown tomato seedlings treated 5 hours with ethylene (50µL/L). ΔΔCT refers to fold differences in *Sl-ARF* expression relative to untreated seedlings. The *E4* gene was used as control for efficient ethylene treatment.

*Expression of Sl-ARF genes during tomato fruit set*

The expression of a high number of *Sl-ARFs* in reproductive tissues (Figure 3 and 4) along with the previously reported role of auxin in controlling the fruit set process, prompted us to

investigate the expression of *Sl-ARF* genes during the flower-to-fruit transition. To determine the expression dynamics throughout the fruit set process, transcript accumulation of tomato *ARFs* was monitored by RNA-seq approach at flower buds, anthesis and pos-anthesis stages (young fruit at 4 DPA). For each stage, RNA libraries were generated from three independent biological replicates and subjected to Illumina mRNA-Seq technology sequencing (Data desposited at NCBI SRA database under accession number SRP029978). Reads were then mapped on the tomato genome sequence and read counts were determined as described in Maza et al. 2013 [45]. The data indicate that most *Sl-ARFs* undergo a strong change in their expression associated with the flower-to-fruit transition (Figure 6). Three groups could be discriminated based on RNA counts distribution during the fruit set process. Group 1 corresponds to *Sl-ARFs* whose expression increased following pollination, Group 2 to *ARFs* with unchanged expression and Group 3 to *Sl-ARFs* displaying decreased expression following pollination (Figure 6).

Fig. 6

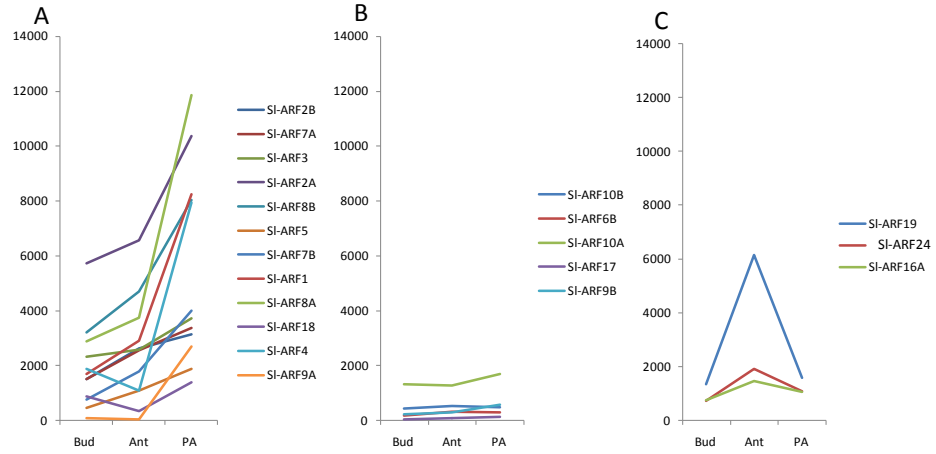



**Figure 6. The expression profile of *Sl-ARF* family genes in tomato fruit set.** (A)12 *Sl-ARF* genes are over-expressed after pollination and fertilization (4DPA), which are Sl-ARF9A, 4, 18, 8A, 1, 7B, 5, 8B, 2A, 3, 7A and 2B genes in turn according to the log change of P/A (Post-anthesis/ Anthesis). (B) 5 *Sl-ARF* genes keep stable expression from flower bud to post-anthesis, includingSl-ARF10A, 10B, 6B, 9B, 17 genes.(C) 3 *Sl-ARF* genes are up-regulated from flower bud to anthesis and down-regulated after pollination and fertilization (4DPA), including Sl-ARF24, 19, and 16A genes. The expression values are taken from RNA-sequencing data and the colors represent different *Sl-ARF* genes.

*Sl-ARF transcripts undergo intense alternative splicing during tomato fruit set*

Closer analysis of the mapping of RNA-seq data on the gene models revealed possible alternative splicing regulation during fruit set for 30% of *Sl-ARF* genes. *Sl-ARF2B* and *ARF19* shows one possible alternative splicing occurring at intron 11 and intron 1, respectively (Figure 6A and Supplementary Figures S3.1). *Sl-ARF3* and *Sl-ARF4* could putatively give rise to two alternative splicing events at introns 7 and 9, and at introns 6 and 10, respectively. Three possible alternative splicings were found at introns 3, 6 and 10 in *Sl-ARF8A* and at introns 9, 11 and 13 in *Sl-ARF8B*. Finally, *Sl-ARF24* offers up to four alternative splicing possibilities at introns 1, 3, 6 and 10 (Supplementary Figures S3.1-6). In all cases, the detected Sl-ARF splice variants resulted in a frame shift within the coding region that generates a premature stop codon. To further validate the occurrence of the alternative splicing forms and assess the relative levels of the various splice variants, a semi quantitative PCR approach was conducted. To this purpose, two pairs of primers were designed, one aiming to specifically amplify the retained intron fragment while the second pair was designed in the margins of the two exons framing the retained intron. A PCR product with the expect size was detected for all genes confirming the presence of the splice variant in each RNA extraction (Figure 7B). Interestingly, the data indicate that the abundance of the *Sl-ARF8B_int11* transcript variant decreases dramatically in young fruits whereas the global expression of the corresponding *Sl-ARF8B* gene increases significantly. This finding suggests that the down-regulation of the *Sl-ARF8B_int11* transcript variant may potentially play a role in the regulation of the flower to fruit transition. By contrast, increased accumulation of the *Sl-ARF19_int1* was observed concomitant to the transition from flower to fruit. Taking together, these data uncover a potential role for alternative splicing in regulating the expression of tomato *ARFs* during the fruit set process.

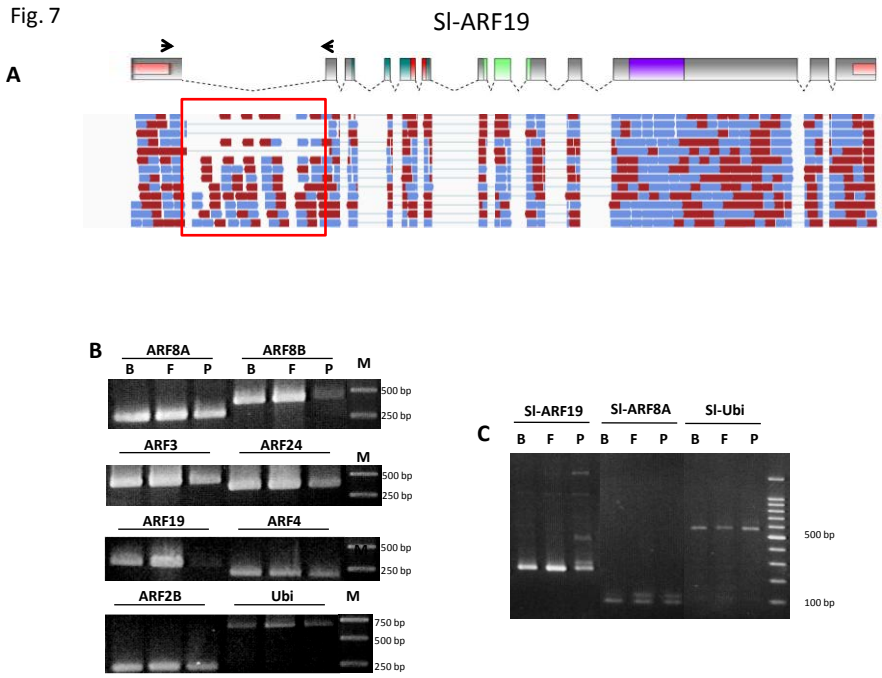



**Figure 7. The ARF family genes showed alternative spilcing mode of regulation in tomato fruit set.** (A) RNA-seq reads generated during the fruit-set and mapped on *Sl-ARF19* gene structure showing one alternative spicing that can be generated in the Intron 1. Reads are represented by red and blue rod arrows (B) The RT-PCR was carried out using pairs of primers designed within the introns of 7 *Sl-ARF* genes highlighted in supplementary Figures S3.1 to S3.6 , such as Sl-ARF8A_Intron 6, Sl-ARF8B_Intron 11, Sl-ARF3_Intron 9, Sl-ARF24_Intron 3, Sl-ARF19_Intron 1, Sl-ARF4_Intron 6 and Sl-ARF2B_Intron 11. The ubiquitin gene was used as the reference. (C) The RT-PCR was performed using pairs of primers nested in the two exons encompassing the intron of target Sl-ARF genes, such as Exon1-Exon2 in Sl-ARF19 and Exon6-Exon7 in Sl-ARF8A. The cDNAs generated from flower bud (B), flower at anthesis (F) and young fruit 4 days post-pollination (P) tissues were used as the template. The ubiquitin was used as the reference.

# Discussion

Being down-stream components of auxin signalling pathway, ARFs likely contribute to the specificity of the hormone responses. Hence, the functional characterization of these transcriptional mediators is essential towards understanding the mechanisms by which auxin triggers appropriate growth and developmental responses in a timely and tissue-specific manner. To better define the role of ARFs in mediating specific auxin responses, the present study brings a complete picture on the main structural features of the tomato *ARF* gene family. Identification of tomato *ARFs* has been already described but this attempt built on a draft tomato genome sequence and ESTs and could therefore not be comprehensive [42,43].

The present work takes advantage of the most updated tomato reference genome sequence [46] to isolate the complete ARF family members and perform functional analysis and expression profiling of these transcriptional regulators. Using these extended resources, the list of tomato *ARFs* has been enlarged to 22 members and manual annotation based on deep RNA-Seq data, allowed the curation of some structural annotation inconsistencies as well as the identification of the 3‟ and 5‟ UTR regions for more than 50% of the *Sl-ARF* gene family. The tomato members of the ARF family were renamed according to the numbering of the closest Arabidopsis homolog, which provides a consensus nomenclature for *ARF* genes across plant species. In this way, the proposed nomenclature better clarifies the correspondence between ARF subclasses in various plant species. The phyllogenetic approach applied on a well distributed set of plant ARFs allowed to identify a specific sub-class (sub-class IV) that is absent out of the *Solanaceae* family. Interestingly, this sub-class contains a specific gene, *Sl-ARF24*, encoding a putative ARF protein that lacks the two protein/protein interaction domains, known as domain III and IV and required for the binding to Aux/IAA proteins. It is therefore likely that Sl-ARF24 escapes the classical mechanism underlying auxin signaling which implies the sequestration of ARF proteins through interaction with Aux/IAAs.

As a preliminary approach towards functional characterization of members of the tomato ARF family, the present study describes their expression pattern, their post-transcriptional regulation and their ability to activate or repress transcriptional activity on synthetic or native auxin-responsive promoters. Transactivation assays revealed that 36% of tomato ARFs are strong repressors of transcriptional activity while only 22% are transcriptional activators. The repressor/activator ratio among ARFs is more than twice higher in tomato (3.6) compared to Arabidopsis (1.7), yet, it remains to be elucidated whether this feature may account for differences in developmental and growth behaviour between the two species. In contrast to repressor ARFs , most activator Sl-ARFs promote transcription of target genes only upon exogenous auxin treatment thus suggesting that activator ARFs require some input from a highly activated auxin signalling pathway in order to potentiate transcriptional activity. It is conceivable that when the auxin level is low, the amount of Aux/IAA proteins available is sufficient to block ARFs at the protein level thus preventing these latter from activating the transcription of the target genes. In this perspective, it has to be postulated that Aux/IAAs are present in excess in the cell when the tissue is not subjected to auxin treatment.

The spatio-temporal pattern of expression indicated that all *Sl-ARF* genes are expressed in flower and fruit suggesting a putative important role in reproductive tissue development. The shift from the static flower ovary to fast-growing young fruit is a phenomenon known as fruit

set and auxin has been shown to play a crucial role in controlling this developmental process [47,48] representing an important step in the development of all sexually reproducing higher plants. Adding to the primary role of Aux/IAAs in triggering the fruit set process previously reported [3,49], the present study reveals the potential active role of a number of Sl-ARFs during this process based on genome-wide transcriptomic profiling of the flower to fruit transition. The expression of 12 members of the gene family sharply increases upon pollination/fertilization, while the expression of a fewer number of Sl-ARF genes peaks at anthesis and then dramatically declines at post-pollination stage. Given the role of auxin signaling in the fruit set process [48,50], the dynamics of the expression pattern of these Sl-ARFs is indicative of their putative involvement in mediating auxin responses during the flower-to-fruit transition. This is consistent with the prominent role reported for *At-ARF8* and *Sl-ARF7* during fruit set and parthenocarpy in Arabidopsis and tomato, respectively [24,26]. Of particular interest, *Sl-ARF8A* shows the most dramatic rise in expression at post-anthesis stage which may designate this ARF among all family members as the main actor of the fruit set process.

The data indicate that tomato ARFs are subject to multi-levels post-transcriptional regulation of their expression. In line with Arabidopsis ARFs [51,52,53], it is shown here that 12 out of the 22 tomato *ARF* genes are potentially regulated by siRNAs. Moreover, the direct evidence for active alternative splicing described here uncover a new layer of complexity in the post-transcriptional regulation of ARF genes in the tomato. This mode of regulation may account for a significant part of the control of ARF expression in developmental processes such as fruit set in the tomato as indicated by the abundance of some transcript splice variants concomitant to the flower to fruit transition. An additional mean towards controlling ARF expression in the tomato may also take place at the translational level via upstream ORFs (uORFs) that have been predicted in most members of *ARF* genes. This mode of regulation has been first suggested in Arabidopsis where *in silico* search revealed an enrichment of uORFs in the ARF 5‟-leader sequences that is not seen in other auxin-related genes such *Aux/IAA*, *YUCCA*, *TIR1* auxin receptors homologs and *PIN* family of auxin transporters [54]. Subsequently, translational control of *AtARFs* by upstream ORF (uORFs) has been proposed as a regulatory mechanism required in modulating auxin responses during plant development [55]. Though direct experimental evidence is still lacking, tomato ARFs may also undergo the same mode of regulation.

In addition of being auxin-responsive, the expression of some *Sl-ARFs* was found to be regulated by ethylene. The presence of auxin and ethylene *cis*-regulatory elements in the

promoter region of a number of *Sl-ARFs*, supports the potential regulation of *ARF* genes by both auxin and ethylene and suggests that these transcription factors have the ability to mediate both auxin and ethylene responses. In support to this hypothesis, Arabidopsis *ARF19* has been shown to be inducible by ethylene and has been reported to contribute to ethylene sensitivity through a cross-talk between auxin and ethylene signalling [27,30]. Also, *ARF2* has been shown to regulate the hook curvature of etiolated Arabidopsis seedlings, a typical ethylene response [27]. Taking together, these data suggest that ARFs may act at the crossroads of auxin and ethylene signaling.

Altogether, the data provide molecular clues on how ARFs can contribute to the specificity and selectivity of auxin responses through (i) structural features, (ii) differential expression of family members at the tissue and organ levels and, (iii) ability to negatively or positively impact transcriptional activity of target genes. The auxin and ethylene regulation of some ARF members suggest their specific role in the multi-hormonal cross-talks. The regulation of the expression of *ARFs* by alternative splicing during fruit set provides new insight into the complexity of regulation of these genes at the post-transcriptional level.

# Materials and Methods

### Plant material and growth conditions

Tomato seeds (*Solanumlycopersicum* cv MicroTom or Ailsa Craig) were sterilized, rinsed in sterile water and sown in recipient Magenta vessels containing 50 mL of 50% Murashige and Skoog (MS) culture medium added with R3 vitamin (0.5 mg $L^{-1}$ thiamine, 0.25 mg $L^{-1}$ nicotinic acid and 0.5 mg $L^{-1}$pyridoxine), 1.5% (w/v) sucrose and 0.8% (w/v) agar, pH 5.9. Plants were grown under standard greenhouse conditions. The culture chamber rooms are set as follows: 14-h-day/10-h-night cycle, 25/20°C day/night temperature, 80% hygrometry, 250 µmol $m^{-2}s^{-1}$ intense luminosity.

### *In silico* Identification of the tomato ARFs

All the ARF gene sequences (ITAG2.3_gene_models.gff3) are download from the Sol Genomics Network (http://solgenomics.net/), and analyzed in Notepad++ software. The NLS location was searched using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). All the obtained sequences were sorted for the unique sequences and these were further used for B3, AUX_RESP, and Aux/IAA domain search using InterProScan Sequence Search (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The UTR of Sl-ARFs were found by two steps, first, the whole tomato genome and Sl-ARF gene structures

(ITAG2.3_gene_models.gff3) were loaded into the Java, and then, the complete cDNA sequences from RNA-Seq data including three stages (flower bud, anthesis and post-anthesis) were blast with Sl-ARF gene structures to identify the final 5‟ or 3‟ UTRs in Sl-ARFs. The miRNA location on the Sl-ARFs were searched depend on the GBF data (http://tata.toulouse.inra.fr/gbf/blast/blast.html) and SGN Blast tools. Taken together, all of the Sl-ARF family structures were drawn by Fancy Gene v1.4 (http://host13.bioinfo3.ifom-ieo-campus.it/fancygene/) with manual correction.

**Transient Expression Using a Single Cell System**

Protoplasts were obtained from suspension-cultured tobacco (*Nicotianatabacum*) BY-2 cells and transfected by a modified polyethylene glycol method as described by Abel and Theologis [56]. For nuclear localization of the selected ARF fusion proteins, the coding sequence of genes were cloned as a C-terminal fusion in frame with GFP under the control of the 35S CaMV, a cauliflower mosaic virus promoter. Transfected protoplasts were incubated for 16 h at 25°C and analysed for GFP fluorescence by confocal microscopy. For co-transfection assays, aliquots of protoplasts ($0.5 \times 10^6$) were transformed either with 10 µg of the reporter vector alone containing the promoter fused to the GFP reporter gene or in combination with 10 µg of ARF contructs as the effector plasmid. Transformation assays were performed in three independent replicates. After 16 h, GFP expression was analyzed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA) on the flow cytometry platform, IRF31, Inserm, Toulouse and and cell sorting platform, INSERM UPS UMR 1048, Toulouse RIO imaging platform. Data were analyzed using Cell Quest software. For each sample, 100 to 1000 protoplasts were gated on forward light scatter and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the population of cells after subtraction of autofluorescence determined with non transformed BY-2 protoplasts. The data are normalised using an experiment, in presence of 50 µM 2.4 D, with protoplasts transformed with the reporter vector in combination with the vector used as the effector plasmid but lacking Sl-ARF coding region.

**RNA isolation and Quantitative RT-PCR**

Total RNA from fruit was extracted according to the method of Hamilton *et al.* [57]. Total RNA from leaves and seedlings was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer‟s instruction. Total RNA was treated by DNase I to remove any genomic DNA contamination. First strand cDNA was reverse transcribed from 2 µg of total RNA using Omniscript kit (Qiagen) according to the manufacturer's instruction.The qRT-PCR analysis was performed as previously described [3]. The sequences of primers are

listed in supplementary Table S3. Relative fold differences were calculated based on the comparative Ct method using the Sl-Actin as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value of genes was normalized to the Ct value for Sl-Actin-51 (accession number Q96483/Solyc11g005330) and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. At least two to three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to real-time PCR analysis in triplicate. Heat map representation was performed using centring and normalized ΔCt value, with Cluster 3.0 software and Java Tree view to visualize dendogram**.**

**Hormone treatment**

For auxin treatment on light grown seedlings, 12-day-old tomato seedlings (30 seedlings) were soaked in liquid MS medium with or without (mock treatment) 10 µM IAA for 2 hours. The efficiency of the treatment was checked by measuring the induction of the tomato early auxin-responsive SAUR gene. For ethylene treatment on dark grown seedlings, 5-days-old MicroTom seedlings (100 seedlings) were treated with air or ethylene gas (50 µL/L) for 5 hours. The efficiency of the treatment was checked by measuring the induction of the tomato ethylene-responsive E4 gene. Experiment was repeated for 3 biological times.

**RNA-Sequencing and RT-PCR**

Total RNA was extracted from bud, flower and post-flower (4DPA) for three biological repeats using a TRIZOL Reagent (invitrogen) according to the manufacturer"s instruction. Total RNA was treated by DNase I to remove any genomic DNA contamination and checked by RNA gel and Agilent RNA 6000 Nano Assay, which the RIN value above 7 was determined to be qualified. After that, the best RNA were sent out for deep RNA sequencing using Illumina Hiseq2000 and the reads generated were mapped to the tomato genome sequence SL2.40. The data are desposited at NCBI SRA database under the accession number SRP029978. The gene expression was calculated for each annotated tomato gene (iTAG2.30). For continuous validation, first strand cDNA was synthesized as previously described and PCR was performed using primers designed from the intron and exon of 7 *Sl-ARF* genes. The primer sequences are listed in Supplementary Table S4. An aliquot of 1ul of the product was used as a template. The PCR amplification cycle was as follows: 95°C for 30 s, 56–60°C for 40 s, 72°C for 30s-2.5min. Samples were taken after 25, 30 or 35 cycles and 10ul of the PCR product was visualized on a 2-2.5% agarose gel. All PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). DNA was stained with ethidium bromide in the gel. Sl-Ubi3 expression was used as an internal control.

## Acknowledgements

This work was supported by the Laboratoire d'Excellence (LABEX, TULIP ANR-10-LABX-41). This work benefited from the networking activities within the European funded COST ACTION FA1106 "Qualityfruit". The authors thank C. Pecher and A. Zakaroff-Girard for their technical assistance and expertise in flow cytometry (Cytometry and cell sorting platform, INSERM UPS UMR 1048, Toulouse RIO imaging platform). The authors also thank O. Boucher and N. Marsaud for their technical assistance and expertise in RNA-seq data production (plateforme genotoul, GeT).

## Authors' contributions

Mohamed Zouine : *In silico* identification and manual annotation of Sl-ARFs, Phyllogenetic characterization, expression analysis by RNA-Seq during fruit set, alternative splicing discovery and analysis.

Yongyao Fu: Manual annotation of Sl-ARFs, expression analysis by RNA-Seq during fruit set, alternative splicing validation by semi-quantitative PCR

Anne-Laure Chateigner-Boutin: PCR validation and Isolation of some Sl-ARFs.

Isabelle Mila: Use of the single Cell approach to uncover the ability of Sl-ARFs to activate or to repress the DR5 promoter, isolation of Sl-ARFs.

Pierre Frasse: Library for RNA-seq and isolation of ARF genes

Hua Wang: Quantitative PCR expression analysis of Sl-ARFs.

Corinne Audran: Identification and analysis of the small uORFs in Sl-ARF transcripts:

Jean-Paul Roustan: Quantitative PCR expression analysis of Sl-ARFs.

Mondher Bouzayen: Management of the project, help of the analysis of all the data generated in this work.

### Tables

Table 1. *Sl-ARF* gene family in tomato.

[a] Sl-ARF gene names

[b] the alias of each *ARF* gene in iTAG2.30 genome annotation

[c] Length of the corresponding Coding Sequence (CDS) in base pairs.

[d] Conserved Domains found in PFAM database: B3 means DNA binding domain, ARF means Auxin response Factor conserved domain, AUX/IAA means AUX/IAA dimerization domain, AD means transcriptional activation domain, RD means transcriptional repression domain.

[e] Corresponding names in Wu et al.; accession numbers are in the parentheses.

[f] Gene Model modification type: UTR means that the UTR sequence have been identified and annotated, CDS means that the the coding sequence have been corrected.

[g] New locations in the tomato genome version Sl2.40 taking into account the manual curation of the previous gene annotation in iTAG2.30

| Generic name[a] | Alias[b] | CDS length[c] | Str | Chr | Domains[d] | Name in Wu et al.[e] | improvement[f] | New location[g] |
|---|---|---|---|---|---|---|---|---|
| Sl-ARF1 | Solyc01g103050 | 1965 | + | 1 | B3,ARF,AUX/IAA,SPL-Rich RD | SlARF1(HM061154.1) | - | |
| Sl-ARF2A | Solyc03g118290 | 2541 | + | 3 | B3,ARF,AUX/IAA,SPL-Rich RD | SlARF2(DQ340255.1) | - | |
| Sl-ARF2B | Solyc12g042070 | 2490 | - | 12 | B3,ARF,AUX/IAA,SPL-Rich RD | SlARF11(HM143940.1) | 5', 3' UTR | 42538600..42544937 |
| Sl-ARF3 | Solyc02g077560 | 2244 | + | 2 | B3,ARF,SL/G-Rich RD | SlARF3(DQ340254.1) | - | |
| Sl-ARF4 | Solyc11g069190 | 2436 | - | 11 | B3,ARF,AUX/IAA,SPL-Rich RD | SlARF4(DQ340259.1) | 5', 3' UTR | 50900912..50910023 |
| Sl-ARF5 | Solyc04g081240 | 2793 | - | 4 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF5(HM195248.1) | - | |
| Sl-ARF6A | Solyc12g006340(Nter); Solyc00g196060(Cter) | 2643 | -/+ | 12/0 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF6(HM594684.1) | 5,, UTR, CDS | 857256..859656(Nter) |
| Sl-ARF6B | Solyc07g043620 | 2673 | - | 7 | B3,ARF,QSL-Rich AD | SlARF6-1(NM_001247611.1) | 5'UTR | 54884781..54890560 |
| Sl-ARF7A | Solyc07g016180 | 3339 | - | 7 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF19(HM130544.1) | - | |
| Sl-ARF7B | Solyc05g047460 | 3294 | - | 5 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF19-1(HM565130.1) | 5'UTR | 58050744..58057040 |
| Sl-ARF8A | Solyc03g031970 | 2535 | + | 3 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF8-1(HM560979.1) | 5'UTR | 8739535..8747501 |
| Sl-ARF8B | Solyc02g037530 | 2529 | + | 2 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF8(EF66734F2.1) | 5'UTR | 21756022..21766699 |
| Sl-ARF9A | Solyc08g082630 | 1977 | + | 8 | B3,ARF,AUX/IAA,SPL-Rich RD | SlARF9(HM037250.1) | 5'UTR | 62527409..62531812 |
| Sl-ARF9B | Solyc08g008380 | 2052 | + | 8 | B3,ARF,AUX/IAA,SPL-Rich RD | SlARF12(HM565127.1) | 5'UTR | 2807931..2812983 |
| Sl-ARF10A | Solyc11g069500 | 2100 | - | 11 | B3,ARF,AUX/IAA,SL/G-Rich RD | SlARF10(HM143941.1) | 5', 3' UTR | 51188434..51192539 |
| Sl-ARF10B | Solyc06g075150 | 2016 | + | 6 | B3,ARF,AUX/IAA,SL/G-Rich RD | SlARF16(HM195247.1) | 3' UTR | 43020594..43023604 |
| Sl-ARF24 | Solyc05g056040 | 1953 | - | 5 | B3,ARF,SPL-Rich RD | SlARF13(HM565128.1); SlARF13-1(HM565129.1) | - | |
| Sl-ARF16A | Solyc09g007810 | 2085 | - | 9 | B3,ARF,AUX/IAA,SL/G-Rich RD | No | 3' UTR | 1332230..1335760 |
| Sl-ARF16B | Solyc10g086130 | 1896 | - | 10 | B3,ARF,SL/G-Rich RD | SlARF16(NM_001247861.1) | - | |
| Sl-ARF17 | Solyc11g013480(Nter); Solyc11g013470(Cter) | 1869 | - | 11 | B3,ARF,SL/G-Rich RD | SlARF17(HQ456923) | 3' UTR, CDS | 6495469..6511349 |
| Sl-ARF18 | Solyc01g096070 | 2058 | + | 1 | ARF,AUX/IAA,SPL-Rich RD | No | 5'UTR | 78941268..78946012 |
| Sl-ARF19 | Solyc07g042260 | 3357 | - | 7 | B3,ARF,AUX/IAA,QSL-Rich AD | SlARF7(EF121545.1) | - | |

**Supplementary Data**

**Supplementary Table S1:** uORF prediction in the 5'UTR leader sequences of Sl-ARFs.

[a]Gene name,

[b]iTAG release 2.30 name,

[c]predicted uORF number,

[d]size of the corresponding uORFs

[e]sequence of the uORFs

| Gene name[a] | ITAG number[b] | uORF number[c] | size uORF (AA) [d] | uORF sequence[e] |
|---|---|---|---|---|
| Sl-ARF1 | Solyc01g103050.2 | 2 | 11 | mnviyvdydei |
| | | | 11 | mmryseirvnt |
| Sl-ARF2A | Solyc03g118290.2 | 5 | 12 | mkkllllsiedr |
| | | | 13 | mnthihtvfrern |
| | | | 4 | mlls |
| | | | 2 | mn |
| | | | 5 | mrdlc |
| Sl-ARF2B | Solyc12g042070.2 | 3 | 52 | mvkslfssfsvfspynhspnslkiyrdkvaisadsefvlqfacldclskgfa |
| | | | 6 | mfglfk |
| | | | 5 | mrdlr |
| Sl-ARF3 | Solyc02g077560.2 | 3 | 1 | m |
| | | | 1 | m |
| | | | 2 | mr |
| Sl-ARF4 | Solyc11g069190.2 | 4 | 29 | mssassvpssfnskmgvemesyfeflvii |
| | | | 16 | mtflffsveflflfll |
| | | | 4 | milt |
| | | | 3 | mln |
| Sl-ARF5 | Solyc04g081240.2 | 6 | 12 | mlscsvvcfhss |
| | | | 16 | mfcsmfsfllriiplt |
| | | | 10 | mwvgsyvsln |

| | | | | |
|---|---|---|---|---|
| | | | 3 | mlv |
| | | | 4 | mssc |
| | | | 11 | mgslvlvlglm |
| Sl-ARF6A | Solyc12g006340 | 3 | 1 | m |
| | | | 16 | mkeltlvvfscgsgvg |
| | | | 9 | mfacflvfv |
| Sl-ARF6B | Solyc07g043620.3 | 2 | 44 | msknmvscflvfawgggvgvggctskvlifleflsgvalcwln |
| | | | 16 | mkqllmvvfscgtrfg |
| Sl-ARF7A | Solyc07g016180.2 | 2 | 8 | mccvlcvw |
| | | | 4 | mnlr |
| Sl-ARF7B | Solyc05g047460.3 | 2 | 1 | m |
| | | | 4 | mnlr |
| Sl-ARF8A | Solyc03g031970.3 | 2 | 9 | mevlekekc |
| | | | 4 | mlgs |
| Sl-ARF8B | Solyc02g037530.3 | 4 | 34 | mekrwgilkigfqlifgvgiegflqfsgllqrrv |
| | | | 27 | mwgdhihngekvgyledrvsvdfwswn |
| | | | 7 | mevleke |
| | | | 8 | mhvgrsys |
| Sl-ARF9A | Solyc08g082630.3 | 1 | 3 | mff |
| Sl-ARF9B | Solyc08g008380.3 | 3 | 23 | mqdleflilklflificdkdtnf |
| | | | 4 | mipi |
| | | | 4 | mkll |
| Sl-ARF10A | Solyc11g069500.2 | 7 | 18 | mlltsltcndpsfrccfv |
| | | | 4 | mhlc |
| | | | 6 | mlniyr |
| | | | 9 | mcflklksl |
| | | | 24 | misfffqflrfnisfhnrkfsvsf |
| | | | 10 | mcftisfsvl |
| | | | 7 | mdllccl |
| Sl-ARF10B | Solyc06g075150.3 | 1 | 1 | m |
| Sl-ARF16A | Solyc09g007810.3 | 5 | 2 | mf |
| | | | 12 | mrsfglnlvklf |

| | | | | |
|---|---|---|---|---|
| | | | 3 | myl |
| | | | 3 | mgk |
| | | | 2 | mr |
| Sl-ARF16B | Solyc10g086130.1 | not expressed | | |
| Sl-ARF17 | Solyc11g013470.2 | not expressed | | |
| Sl-ARF18 | Solyc01g096070.3 | 1 | 36 | mlkcvntengfffklccyrpffvcfgflvkfwvisf |
| Sl-ARF19 | Solyc07g042260.2 | 0 | | |
| Sl-ARF24 | Solyc05g056040.2 | 0 | | |

**Supplementary Table S2**: *In silico* analysis of *Sl-ARF* gene promoters.

| *Gene* | *Code* | *Number of the AuxRE (TGTCTC)* | *Number of the ERE* |
|---|---|---|---|
| Sl-ARF1 | Solyc01g103050 | 1 | 4 |
| Sl-ARF2A | Solyc03g118290 | 3 | 4 |
| Sl-ARF2B | Solyc12g042070 | 1 | 3 |
| Sl-ARF3 | Solyc02g077560 | 4 | 1 |
| Sl-ARF4 | Solyc11g069190 | 5 | 6 |
| Sl-ARF5 | Solyc04g081240 | 0 | 4 |
| Sl-ARF6A | Solyc12g006340(Nter); Solyc00g196060(Cter) | 4 | 1 |
| Sl-ARF6B | Solyc07g043620 | 1 | 1 |
| Sl-ARF7A | Solyc07g016180 | 0 | 5 |
| Sl-ARF7B | Solyc05g047460 | 4 | 2 |
| Sl-ARF8A | Solyc03g031970 | 2 | 1 |
| Sl-ARF8B | Solyc02g037530 | 0 | 4 |
| Sl-ARF9A | Solyc08g082630 | 1 | 1 |
| Sl-ARF9B | Solyc08g008380 | 2 | 2 |
| Sl-ARF10A | Solyc11g069500 | 1 | 3 |
| Sl-ARF10B | Solyc06g075150 | 1 | 0 |
| Sl-ARF16A | Solyc09g007810 | 3 | 2 |
| Sl-ARF16B | Solyc10g086130 | 0 | 4 |
| Sl-ARF17 | Solyc11g013480(Nter); Solyc11g013470(Cter) | 2 | 1 |
| Sl-ARF18 | Solyc01g096070 | 0 | 0 |
| Sl-ARF19 | Solyc07g042260 | 0 | 2 |
| Sl-ARF24 | Solyc05g056040 | 3 | 2 |

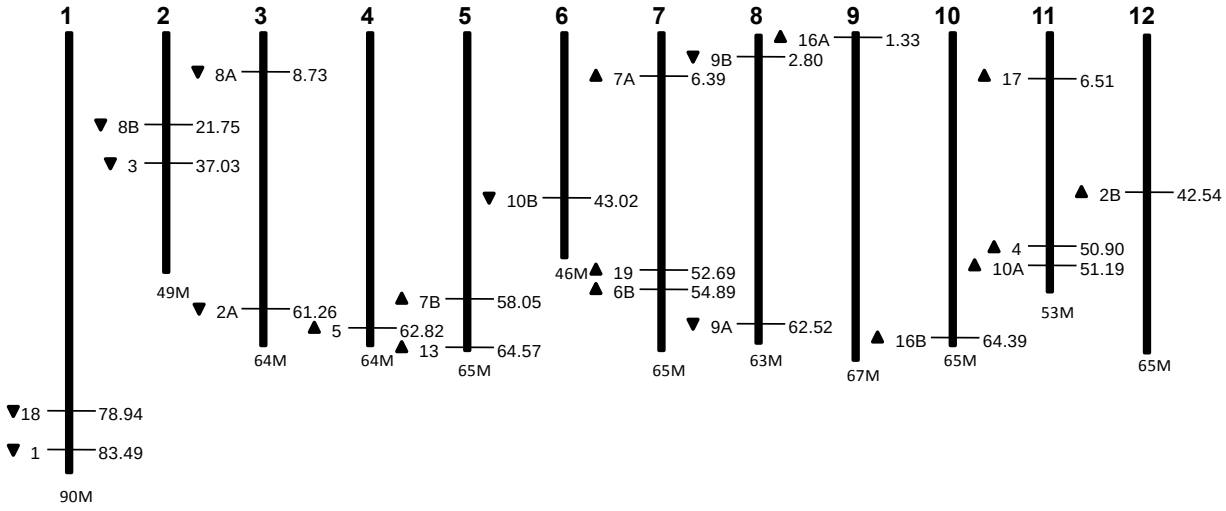
**Supplementary Table S3:** Quantitative RT-PCR primers of *Sl-ARF* genes.

| Gene name | Name in iTAG release 2.30 | Forward primer | Reverse primer |
|---|---|---|---|
| *Sl-ARF1* | Solyc01g103050 | TCTCCTTCATCATTCTCATACTG | GAACCATTCTCACCATAACC |
| *Sl- ARF 2A* | Solyc03g118290 | GCAAGGTCAAGAGTTATCGA | CATTGGTTTCTCAGACAAGTC |
| *Sl- ARF 2B* | Solyc12g042070 | CACTTAATCCACTTCCAATACC | TACAACTACTTTGGATGAACCT |
| *Sl- ARF 3* | Solyc02g077560 | AATTGCAGTATCAGACTTTGG | TCTAGATATCCCAGAACTAGGA |
| *Sl- ARF 4* | Solyc11g069190 | CATTATTGTTGGTGACTTTGTG | GACCTTTGGAAACCTATTGG |
| *Sl- ARF 5* | Solyc04g081240 | CCTTCAGAGTTTGTCATTCCT | AACATCATTCCAAATCTCATACC |
| *Sl- ARF 6A* | Solyc12g006340(Nter); Solyc00g196060(Cter) | CCAACATATCCCTAGTACTTCAG | GTGCCTGAGATATTAGTTGGT |
| *Sl- ARF 6B* | Solyc07g043620 | ACCCTCTAGTATCTTCATCCT | TCCGAGACCTTTGTATTGTG |
| *Sl- ARF 7* | Solyc07g016180 | TCAACTCCTCAAACATACCT | TGAACTATCCAAATAATCCATCTG |
| *Sl- ARF 8A* | Solyc05g047460 | TGACATCGAATGGAAATTCAG | GTCTCTTAGCACTAACAAACAC |
| *Sl- ARF 8B* | Solyc03g031970 | GTCAGTCCGTGATCATAGAG | GGAATCCAAGCTACAATTTCC |
| *Sl- ARF 9* | Solyc02g037530 | ATCATTCAATCTCAAATCAAAGGT | CCTCATCATTGTCTTCTTCAG |
| *Sl- ARF 10* | Solyc11g069500 | ATTCTCTGTGCCTAGATACTG | CTATAAATGTGCCTAAACTTCCA |
| *Sl- ARF 17* | Solyc11g013480(Nter); Solyc11g013470(Cter) | TGAAGTTGATGAAGTTACTATGAG | TCCTCCATTATTCGCATCTG |
| *Sl- ARF 18* | Solyc01g096070 | AATCTACACTCGGCATTGTC | AAGCTTCCTATCTTATCATTGGA |
| *Sl- ARF 19* | Solyc07g042260 | TGGTGGATGAATCTGTTGTC | TACTTAGACAGCTCTGAACCT |
| *Sl- ARF 24* | Solyc05g056040 | TCATTGTTGGATGTTTCAAAGG | GAAGTCTTGGAAAGTAGTATACTC |
| *Sl-Actin* | Solyc11g005330 | TGTCCCTATCTACGAGGGTTATGC | AGTTAAATCACGACCAGCAAGAT |

**Supplementary Table S4**. PCR primers for identifying the alternative splicing expressed forms in *Sl-ARF* genes.

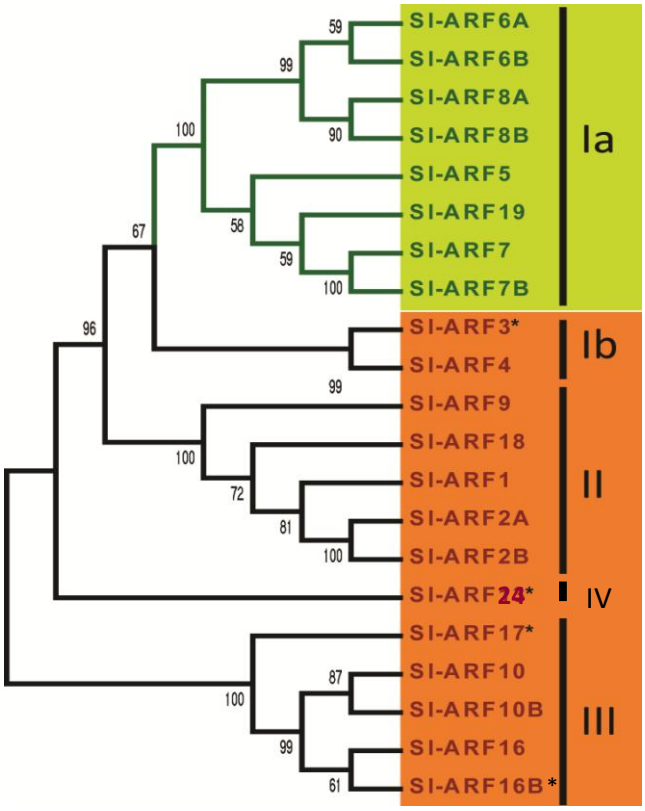
| | | Primers sequence 5'-3' | |
|---|---|---|---|
| **Gene name** | **Details** | **Forward** | **Reverse** |
| *Sl-ARF2B* | Intron 11 | AACCTTAAGAAAGTGCCAAAAGTAC | ATAACTTGCCCTTATATTTGAATTCC |
| *Sl-ARF3* | Intron 9 | AATTCTGAAATACTCCCTCCGT | AGCATATCCAGACCTAGTCTCCA |
| *Sl-ARF4* | Intron 6 | GCACAGAGTGAAAGATTTGGG | GCCAAACCATCAATTATCTTCC |
| *Sl-ARF8A* | Intron 6 | AAGTTGTTATACAGTGGGTCAAGG | GCAAATCTCACTCACAATGTCAG |
| *Sl-ARF8B* | Intron 11 | CGCTGAATGGTCATGTAATAAGAG | ATTCCTTCTATCATGGCTATCTGG |
| *Sl-ARF19* | Intron 1 | ATAAGGAGTTCTGCAAGCCA | GTAGGTTGTGCTATGCTGAC |
| *Sl-ARF24* | Intron 3 | GGACAATATGTAGCAATTAGGGAC | AGACCATGTGATTTGAGTACCA |
| *Sl-ARF2B* | Ex 11-Int 11-Ex 12 | AACATCAGCCTTCTCGTCATCC | GCACTTAATCCACTTCCAATACCA |
| *Sl-ARF3* | Ex 8-Int 9-Ex 9 | TAGATCCAGTTCGATGGCCAG | CTCAATCTCCCATGGTGAAACC |
| *Sl-ARF4* | Ex 6-Int 6-Ex 7 | CTTGTCCCAACAGGAATCCGA | GGAATAAGAAGAGCTGCAAGACCT |
| *Sl-ARF8A* | Ex 6-Int 6-Ex 7 | TGACATCGAATGGAAATTCAGG | CGGCAACAAGTCTCTTAGCA |
| *Sl-ARF8B* | Ex 11-Int 11-Ex 12 | GCCTTTCTATCAAGGAACCTC | GAGAATTGTTGAATCGACTCTC |
| *Sl-ARF19* | Ex 3-Int 3-Ex 4 | GCTCACTGTGACCTTGAGGA | AGGTGAAAGTAGCTTTGTGTTGG |
| *Sl-ARF24* | Ex 1-Int 1-Ex 2 | GGTCTCAGCATAAACCTCTTCC | GGAGCAATCATCCAACCAGGA |
| *Sl-Ubiquitin* | | CTAACGGGGAAGACGATCACCC | TCCCAAGGGTTGTCACATACATC |

Supplementary Figure S1




**Supplementary Figure S1. *Sl-ARF* genes genomic distribution on the tomato chromosomes.** The arrows next to gene names show the direction of transcription. The number near to each *Sl-ARF* designates the position megabases (Mb) of the first ATG in the tomato chromosome pseudomolecules (tomato genome version SL2.40). The chromosome numbers and their corresponding size are indicated at the top and bottom of each bar.

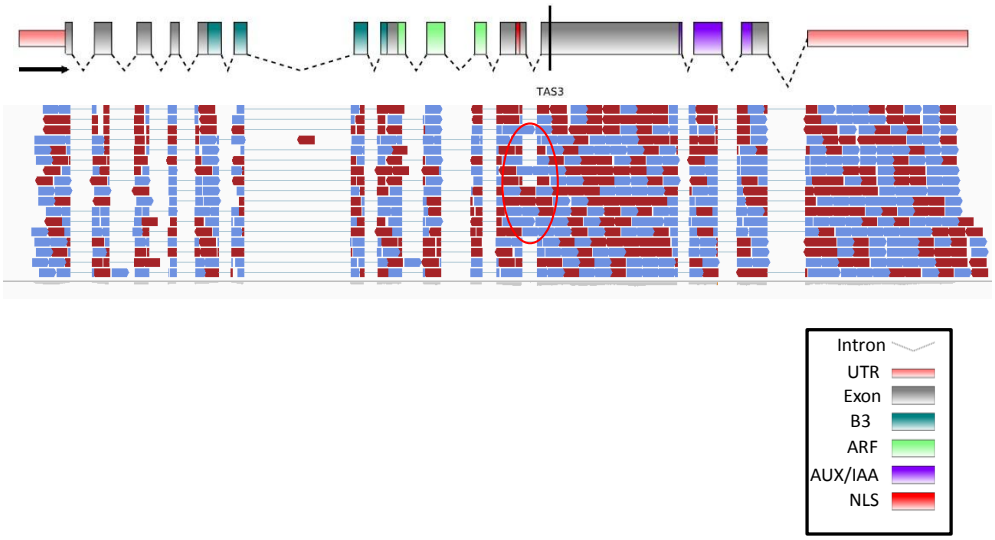
Supplementary Figure S2




**Supplementary Figure S2**. **Phylogenetic relationship between tomato Sl-ARF genes.** The unrooted tree was generated using MEGA4 program by neighbor-joining method. Bootstrap values (above 50%) from 1000 replicates are indicated at each branch. Sl-ARFs with a star (*) are deprived of domain III and IV necessary for interaction with Aux/IAAs.

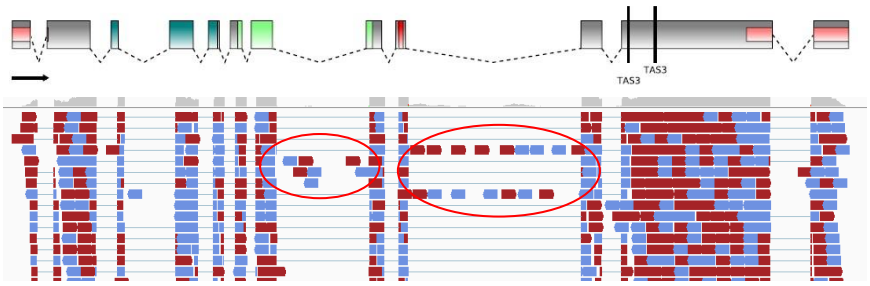
Supplementary Figure S3.1

# Sl-ARF2B



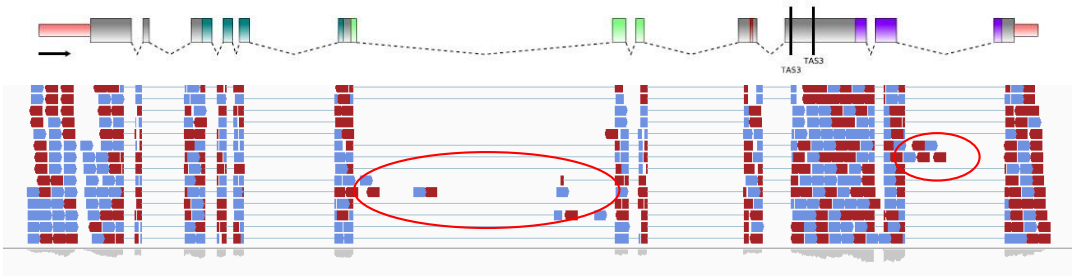

Supplementary Figure S3.2

# Sl-ARF3



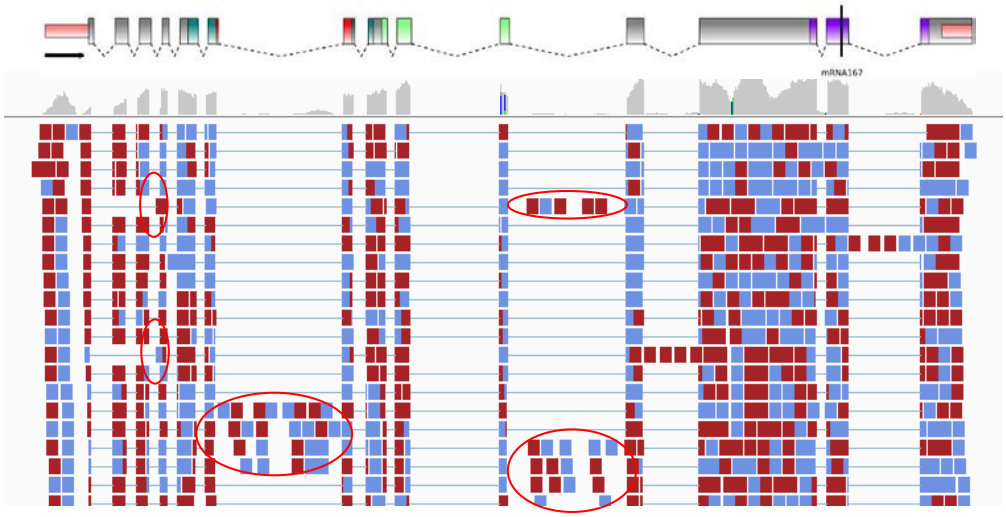
Supplementary Figure S3.3

# Sl-ARF4



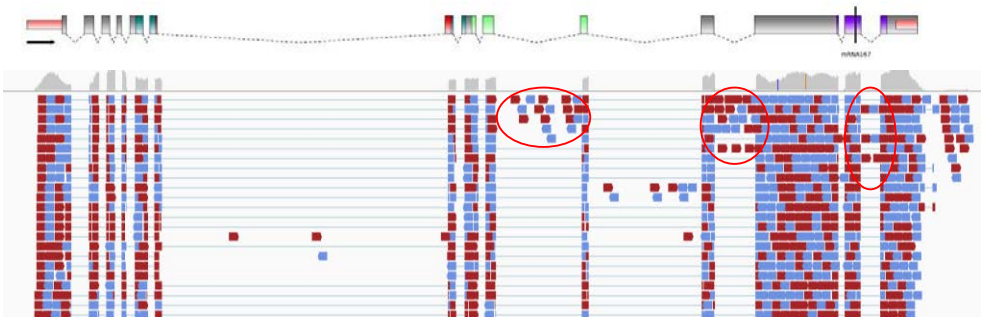
Supplementary Figure S3.4

# Sl-ARF8A



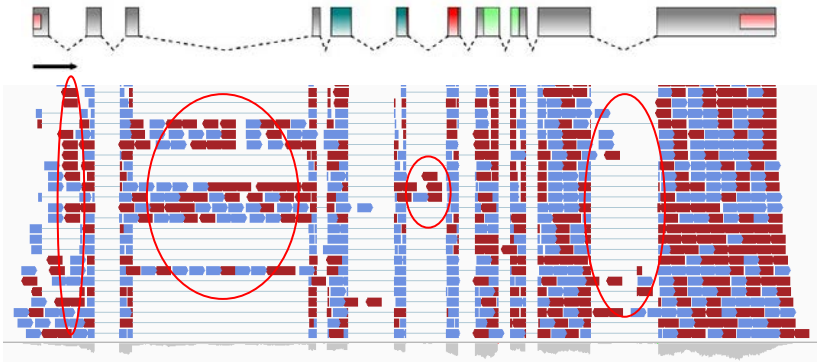
Supplementary Figure S3.5

Sl-ARF8B



Supplementary Figure S3.6

Sl-ARF24



**Supplementary Figure S3.1-6. Predicted alternative splicing in six Sl-ARFs (Supplementary Figure S3.1 to Supplementary Figure S3.6).**

RNA-seq reads generated during the fruit-set and mapped on the corresponding *Sl-ARF* gene sequence (*Sl-ARF2B, 3, 4, 8A, 8B,* and *24*) showing predicted alternative splicing events. RNA-seq reads are represented by red and blue rod arrows.

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# Chapter III

## Characterization of *Sl-ARF8A* and *Sl-ARF8B* tomato genes reveals their critical role in auxin-mediated fruit-set

# Chapter III:

## Characterization of *Sl-ARF8A* and *Sl-ARF8B* tomato genes reveals their critical role in auxin-mediated fruit-set.

**Abstract**

Auxin Response Factor (ARF) is an important regulator involved in auxin-mediated plant growth and development processes. To date, the characteristics of *Solanum Lycopersicum* ARF family genes (*Sl-ARFs*) has remained poorly understood. In the present study, the structural and functional characterization of *Sl-ARF8A/B* was identified and their over-expression in tomato resulted in pleiotropic phenotypes, including dwarf plants, lateral shoots and parthenocarpic (seedless) fruits. The single-cell experiment allowed to showing that the encoded protein, Sl-ARF8A or B was exclusively targeted to the nucleus based on the use of GFP fusion. Expression analysis by RT- qPCR revealed a notable increase in *Sl-ARF8A* transcript level in ovary after pollination/fertilization, and *Sl-ARF8B* transcript picked at anthesis, further done through analysis of *Sl-ARF8A/B* promoter-GUS fusion. The hormone treatment showed *Sl-ARF8A* was sharply inhibited by auxin and up-regulated by ethylene. The histological analysis in flower buds strongly indicated *Sl-ARF8A* regulated the development of the ovary, the placenta and ovules. Moreover, genes whose expression is altered in *SlARF8-OX* lines were identified using a genome-wide transcriptomic profiling by RNA-Seq approach. Almost 2600 differently expressed (DE) genes were identified. Considering the dramatic change in gene expression of genes related to auxin, jasmonate and ethylene displayed in *SlARF8A-OX* lines, these phytohormones are likely to play an active role in coordinating the fruit set process.

**Key words**

Auxin, Fruit set, *Sl-ARF8A/B*, Parthenocarpy

**Introduction**

Fruit set is a complex event requiring a tight coordination among molecular, biochemical, and structural changes. Depending on the progress of the fruit set, the temporal and spatial organization of these changes is mediated by plant hormones such as auxin, gibberellin (GA) and ethylene [1]. It has been shown that fertilization-independent fruit initiation can be triggered by exogenous application of auxin and gibberellin on tomato ovaries but also by increasing auxin sensitivity, resulting in the formation of parthenocarpic (seedless) fruits [2,3]. In normal fruit set, it was hypothesized that successful pollination and fertilization induce an increase of the auxin and GA contents within the ovary [3]. In accordance, both auxin and GA response genes were found to be up-regulated within 48 h after pollination [4,5]. It has also been shown that the effect of auxin on fruit-set was mediated in part by GA, placing some GA biosynthesis genes downstream of auxin signaling pathway [6]. So far, the molecular mechanism of auxin action during fruit set is still largely unclear. Aux/IAAs and ARFs are two type of important auxin responsive gene families, which are involved in leaf morphogenesis [7], lateral root growth [8], floral organ abscission and petal growth [9,10] in Arabidopsis. *Aux/IAAs* are early auxin responsive genes, and by far all the Aux/IAAs are characterized as the repressor of the auxin response [11]. ARF transcription factors regulate the expression of early auxin response genes, such as *Aux/IAA*, *GH3* and *SAUR* genes that share a conserved motif, TGTCTC, which are called auxin-responsive element (AuxRE) [12]. ARFs are specifically binding to the AuxREs in their promoters to mediate the auxin response.

Since cloning of the first *AtARF1* gene from Arabidopsis, 23 members of this family, distributed over all five chromosomes have been identified [13]. Recently, taking advantage of the release of the tomato genome sequence, *in silico* analysis made by our laboratory identified 22 *ARF* genes (Unpublished data). Most of these putative *ARF*s have an N-terminal B3-derived DNA binding domain (DBD) as described previously as typical conserved motifs of this family [14]. The middle region (MR) of these proteins functions as a transcriptional activation or repression domain, depending on its amino acid composition. In general, *ARF*s with a glutamine-rich MR act as transcriptional activators, while ARFs with an MR rich in proline and serine act as transcriptional repressors [15]. The C-terminal domain (CTD) contains two signatures related to motifs III and IV of Aux/IAA proteins. These motifs serve as dimerization sites, facilitating *ARF*s repression by hetero-dimerization with Aux/IAA proteins [16,17].

Up to now, the roles of most *ARFs* in *Arabidopsis* have been revealed by mutant analysis approach. For example, *arf1* and *arf2* T-DNA insertion mutation indicated ARF2 regulated leaf longevity [18] and floral organ abscission, and ARF1 acted in a partially redundant manner with ARF2 and increased transcription of *Aux/IAA* genes in *Arabidopsis* flowers as a transcriptional repressor [10]. And the *arf7* and *arf19* mutations were found to regulate the leaf expansion [19] and lateral root formation via direct activation of *LBD/ASL* genes in *Arabidopsis* [20]. In tomato, down-regulation of *Sl-ARF4* (DR12) resulted in a pleiotropic phenotype including dark green and blotchy ripening fruits [21] and also the mutant *mSl-ARF10* (*Sl-miR160*-resistant version) plant displayed narrow leaflet blades, sepals and petals, as well as abnormal shaped fruit [22].

Most interestingly, recent advance shows three genes involving in auxin signaling pathway play an important role in fruit-set via a reverse genetics approach. Down-regulation of tomato *Sl-IAA9* exhibited an advance fruit set independent of pollinations and fertilizations to lead to parthenocarpic fruits [23]. Thereafter, down-regulated *Sl-ARF7* gene in tomato produced parthenocarpic fruits, which are heart-shaped and showed a rather thick pericap [24]. Several T-DNA insertions of *At-ARF8* in *Arabidopsis* also resulted in the production of parthenocarpic siliques after flower emasculation [25]. All these results pointed out the role of the *Aux/IAA* and *ARF* families in regulating fruit set. In addition, several reports have already focused on the role of *AtARF8* in *Arabidopsis*. *Tian et al.*[26] reported the T-DNA mutant of *arf8-1* showed a long-hypocotyl phenotype in white, blue and red light conditions, in contrast to *AtARF8-OX* displayed short hypocotyls in the light. *Nagpal et al.* [27] found that *arf6arf8* double-null mutant flowers arrested as infertile closed buds with short petals, short stamen filaments, immature gynoecia and undehisced anthers that did not release pollen. More recently, *Varaud et al.* [9] demonstrated that *At-ARF8* interacted with *BPEp* factors to affect the petal growth in mutant *Arabidopsis*, in which the petal was significantly larger than that in the wide-type due to the increased cell number and cell expansion. Thus far, tomato as a model system has been widely researched for fruit development and ripening. It will be pivotal to study the functions of *ARF* family genes during fruit set to improve any fruit-related agronomic trait in tomato.

Considering the number of the *ARF* family members, the extent of functional redundancy for the encoded proteins is still poorly known. In order to provide further insights on the physiological significance and the diversity associated with Sl-ARFs in tomato, we report here upon the functional characterization of *Sl-ARF8A/B* genes *in*

*planta.* The data shows *Sl-ARF8A* and *8B* genes are repressed by auxin and *Sl-ARF8A* is up-regulated by ethylene but not *Sl-ARF8B*. *O*ver-expression of *Sl-ARF8A* or *8B* in tomato resulted in a dwarf phenotype and fertilization-independent fruit set to generate seedless fruits. Comparison of flower buds by histological analysis suggested the implication of *Sl-ARF8A* in ovary development and especially the placenta and ovule growth. Further analysis by RNA-Seq uncovered the possible target genes in *SlARF8A-OX* lines. Although *Sl-ARF8A/B* gene is closely related to *Sl-ARF4* and *Sl-ARF7*, these genes seem to play distinct roles in plant developmental processes and especially fruit growth thus revealing functional diversity within the same gene family.

**Results**

**Structural features, phylogenetic characterization and expression pattern of tomato *Sl-ARF8A* and *Sl-ARF8B* genes**

The ARF8 transcription factor, known to mediate auxin responses, has been previously shown to be encoded by two genes in the tomato *Sl-ARF8A* and *Sl-ARF8B* [28] unlike the case in *Arabidopsis* where only one copy is present in the genome. We show here that *Sl-ARF8A and Sl-ARF8B* display well conserved genomic structure with regard to the number and position of introns and exons, with the exception of an extra intron located in the 5‟-UTR of *Sl-ARF8A* (Figure 1A). The two genes were mapped into different chromosomes with *Sl-ARF8A* being located at the top of chromosome 3 and *Sl-ARF8B* at the middle of chromosome 2 (Figure 1B). The genomic regions and full-length cDNAs corresponding to these genes were isolated showing that *Sl-ARF8A* and *Sl-ARF8* genes encode proteins of 845 and 843 amino acid residues, sharing 75.2% and 75.5% homology with *Arabidopsis* AtARF8, respectively. The derived proteins contain the three conserved domains (B3, ARF, Aux/IAA) characteristics of ARF proteins. In addition, protein domain analysis indicated that both Sl-ARF8A and 8B are predicted to be transcriptional activators, due to the identification of the Q-rich activation domain in their middle region. Based on phylogenetic study, Sl-ARF8A and 8B fell into the activator subclade of the ARF family wich in addition to ARF8 also includes ARF6, ARF5, ARF7 and ARF19; with however ARF6 being the closest paralog (Figure 1C). *In silico* analysis of *Sl-ARF8A/B* at the RNA level predicted the presence of a conserved target site for sly-miR167 and further analysis by *cNLS* Mapper allowed the identification of a putative nuclear location signal (NLS) in Sl-ARF8A/B encoded proteins (Figure 1A).

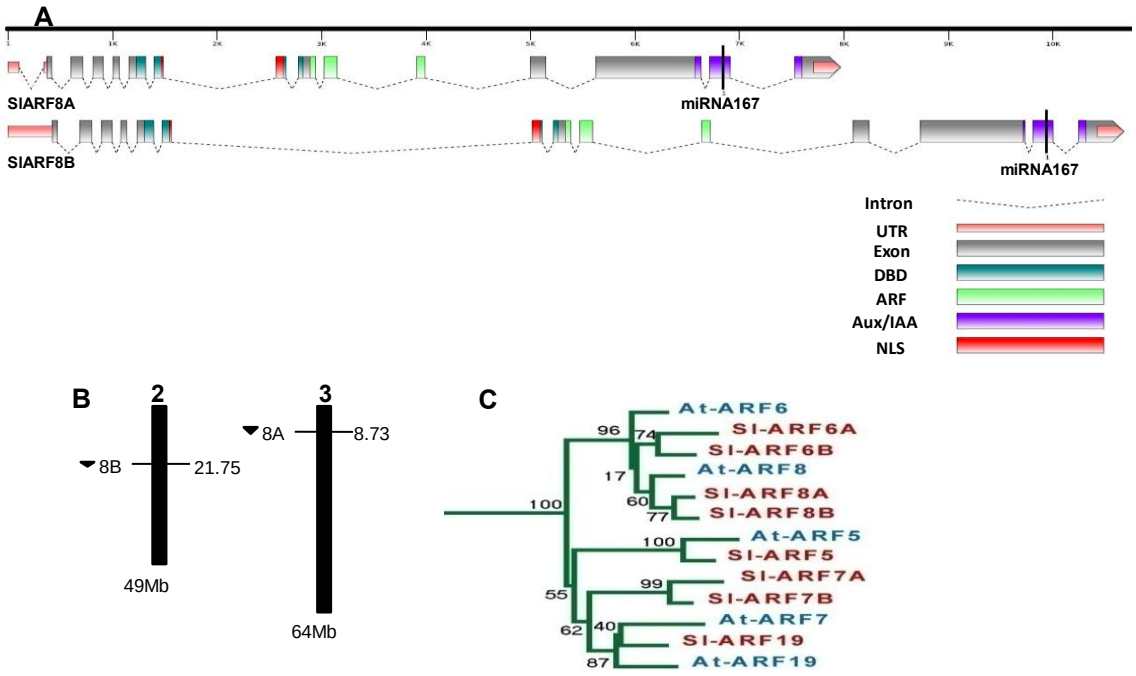


**Figure 1 Transcription Unit of *Sl-ARF8A* and *Sl-ARF8B* in the tomato Genome.**
**(A)** The genomic sequences of *Sl-ARF8A* and *Sl-ARF8B* were extract from SGN database and performed bioinformatic annalysis by using Fancy gene V1.4. These two genes show the same number of exons and introns and are spliced by miRNA167.The colors represent different functional domains.
**(B)** *Sl-ARF8A* and *Sl-ARF8B* are located at two different chromosomes. The arrow next to gene names indicate the direction of transcription. The number in contrast to *Sl-ARF8A* or *Sl-ARF8B* designates the position of the ATG in megabases (Mb) on tomato chromosome pseudomolecules (genome version V5.2). The chromosome numbers are indicated at the top of the bar and the length of each chromosome shown at the bottom of each bar.
**(C)** Phylogenetic relationship of transcription activating class Ia of ARF proteins in Arabidopsis and Tomato. The unrooted tree was generated using MEGA4 program by neighbor-joining method. Bootstrap values from 100 replicates are indicated at each branch.

Since nuclear import of transcription factors is instrumental to their transcriptional activity and in order to validate the functionality of the *NLS in silico* prediction, we investigated the *in vivo* subcellular localization of Sl-ARF8A/B by generating N-terminal fusions with the GFP reporter protein expressed under the 35S promoter (35S::GFP-Sl-ARF8A/B). Transient expression in tobacco BY-2 protoplasts coupled to confocal microscopy analysis clearly indicated that both proteins are exclusively targeted to the nuclear compartment (Figure 2A).

To gain insight into the spatial pattern of expression of the Sl-ARF8A/B genes, accumulation of their transcript was assessed in different plant tissues and organs by quantitative reverse transcription–PCR (qRT-PCR). Sl-ARF8A and Sl-ARF8B transcripts

were detected in all organs tested, with the highest levels for Sl-ARF8A found in young fruit at 8 days post-anthesis whereas the levels of Sl-ARF8B were highest in flowers, stem tissues and young fruit (Figure 2B). As general feature, transcripts of both genes are lowest in fruits and particularely at the ripening stages (Figure 2B).

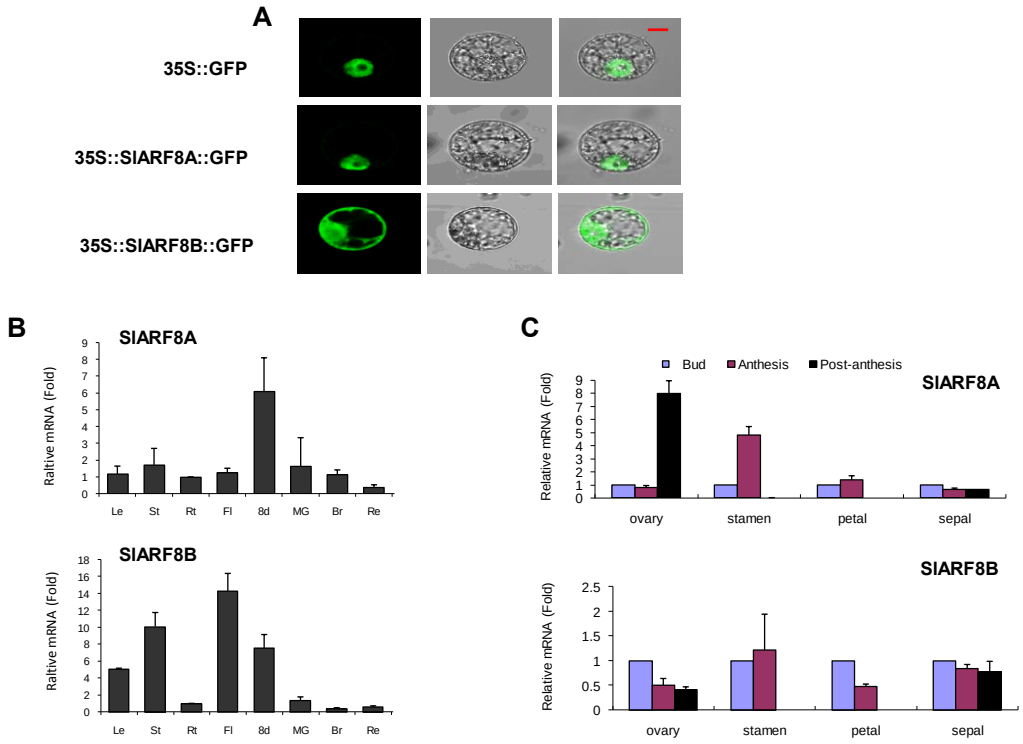


**Figure 2 Nuclear localization of Sl-ARF8A and Sl-ARF8B proteins and comparison of expression patterns of *Sl-ARF8A* and *Sl-ARF8B* genes.**
**(A)** Nuclear localization of Sl-ARF8A and Sl-ARF8B proteins fused to a GFP tag. Pro35S::Sl-ARF8A/B-GFP or pro35S::GFP fusion proteins were transiently expressed in BY-2 tobacco protoplasts and 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.
**(B)** Expression patterns of *Sl-ARF8A* and *Sl-ARF8B* in different tomato tissues. Total RNA was extracted from leaf (Le), stem (St), root (Rt), flower (Fl), fruit at 8 day after anthesis (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 considered. The error bars represent +SE of three independent trials.
**(C)** Expression patterns of *Sl-ARF8A* and *Sl-ARF8B* in different parts of tomato flowers during fruit set. Total RNA was extracted from ovary, stamen, petal and sepal from flower bud to post-flower independently. Data are expressed as relative values, based on the reference ovary at bud stage set to 1.0 at each stage considered. The SE+ represent three biological replicates.

The expression pattern of the two genes was also investigated *in planta* using transgenic lines expressing the β-glucuronidase (GUS) reporter gene driven by either Sl-ARF8A or Sl-ARF8B promoter. Overall, the GUS reported expression confirmed the ubiquitous

expression of the two genes in vegetative and reproductive tissues (Figure 3 and Figure 4). In flowers, Sl-ARF8 and B were expressed in all parts of the reproductive organ (Figure 2C and Figure 3). Notably, Sl-ARF8A shows an increase in transcript levels in ovaries and in young fruits upon flower pollination/fertilization (Figure 3) and in mature green fruit, the expression of Sl-ARF8/B was mainly observed in vascular tissue (figure 3). A clear expression of *Sl-ARF8B* in the root tips were observed where the expression of *Sl-ARF8A* was not detected (Figure 5). In leaves, *Sl-ARF8A and B*are mainly expressed in leaf veins but not in leaflets (Figure 4). Taken together, *Sl-ARF8A/B* genes are typically expressed in reproductive structures and show in some cases distinct spatio-temporal expression.

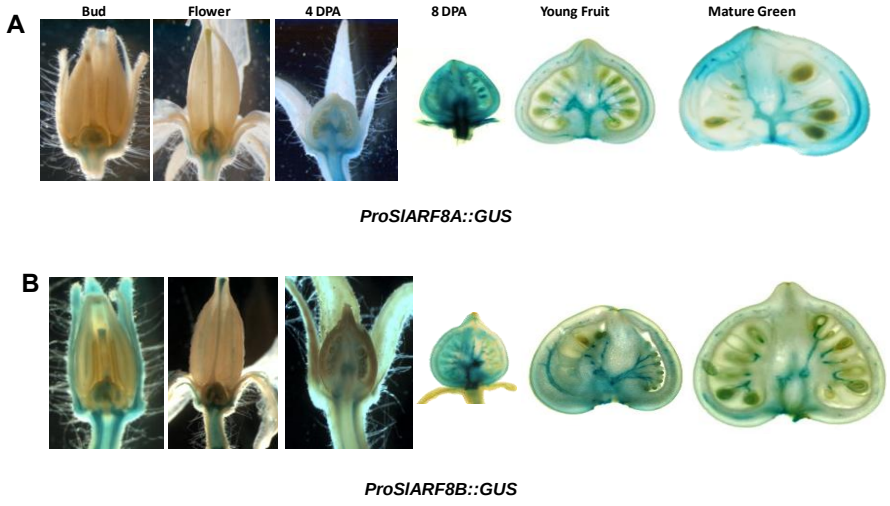


**Figure 3 Expression patterns of *Sl-ARF8A* and *Sl-ARF8B* during the fruit development process assessed by GUS reporter gene driven by their promoters.**
The longitudinal sections of Bud, Flower, 4/8 day post-anthesis (4DPA and 8 DPA), Young fruit and Mature green were carried out through *ProARF8A::GUS* **(A)** and *ProARF8B::GUS* **(B)** displaying a notable increase of *Sl-ARF8A* transcripts after pollination /fertilization (4 DPA and 8 DPA) , and a high expression of *Sl-ARF8B* from flower to fruit transition.

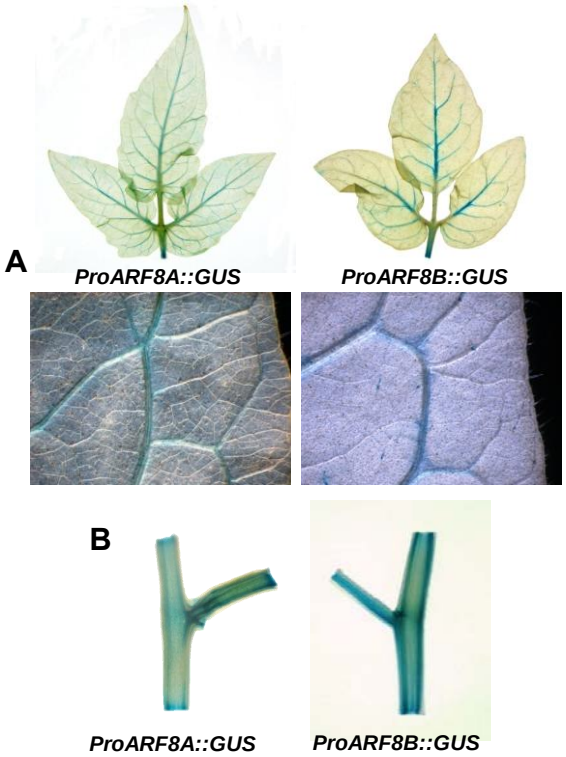


**Figure 4 Expression pattern of *Sl-ARF8A* and *Sl-ARF8B* in the leaves and stems assessed by GUS reporter gene driven by their promoters (*ProARF8A::GUS* and *ProARF8B::GUS*).**
**(A)** The *ProARF8A::GUS* and *ProARF8B::GUS* fusion in the leaves displayed *Sl-ARF8A* and *Sl-ARF8B* were mainly expressed in leaf veins, ex. midveins and lateral veins, but no expression in leaf blades and petiolules.
**(B)** The *ProARF8A::GUS* and *ProARF8B::GUS* fusion in the stems displayed that *Sl-ARF8A* and *Sl-ARF8B* were mainly expressed in Phloem and Xylem.

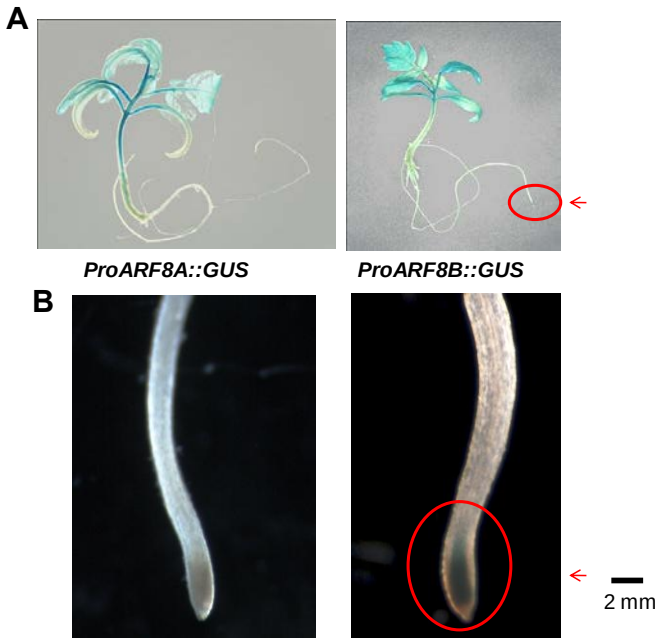


**Figure 5 Expression patterns of *Sl-ARF8A* and *Sl-ARF8B* in 20-day seedlings assessed by GUS reporter gene driven by their promoters (*ProARF8A::GUS* and *ProARF8B::GUS*).**
**(A)** The *ProARF8A::GUS* and *ProARF8B::GUS* fusion in the seedlings displayed a outstanding expression of *Sl-ARF8A* and *Sl-ARF8B* in the vascular tissues.
**(B)** The *ProARF8A::GUS* and *ProARF8B::GUS* fusion in the roots displayed an expression of *Sl-ARF8B* in the root tips compared to *Sl-ARF8A* . These pictures were analysed by microscopy with 2.5 times of maginification. The scale bar indicates 2 mm.

To better uncover their potential role of *Sl-ARF8A and B* in mediating hormone responses, their responsiveness to auxin and ethylene was investigated 12-day wild-type seedlings teated with both hormones. Assessing transcript accumulation of both genes by RT-qPCR revealed a contrasted behaviour with the expression of *Sl-ARF8A* being repressed by auxin and strongly induced by ethylene while the expression of *Sl-ARF8B* displayed weak or no responsiveness to any of the two hormones (Figure 6A, B). The regulation of *Sl-ARF8A* by auxin and ethylene was further validated *in planta* using GUS reporter gene driven by each of Sl-ARF8 promoter in 21-day transgenic tomato seedlings, confirming the repression and stimulation of *Sl-ARF8A* by auxin (Figure 6C) and ethylene (Figure 6D), respectively.

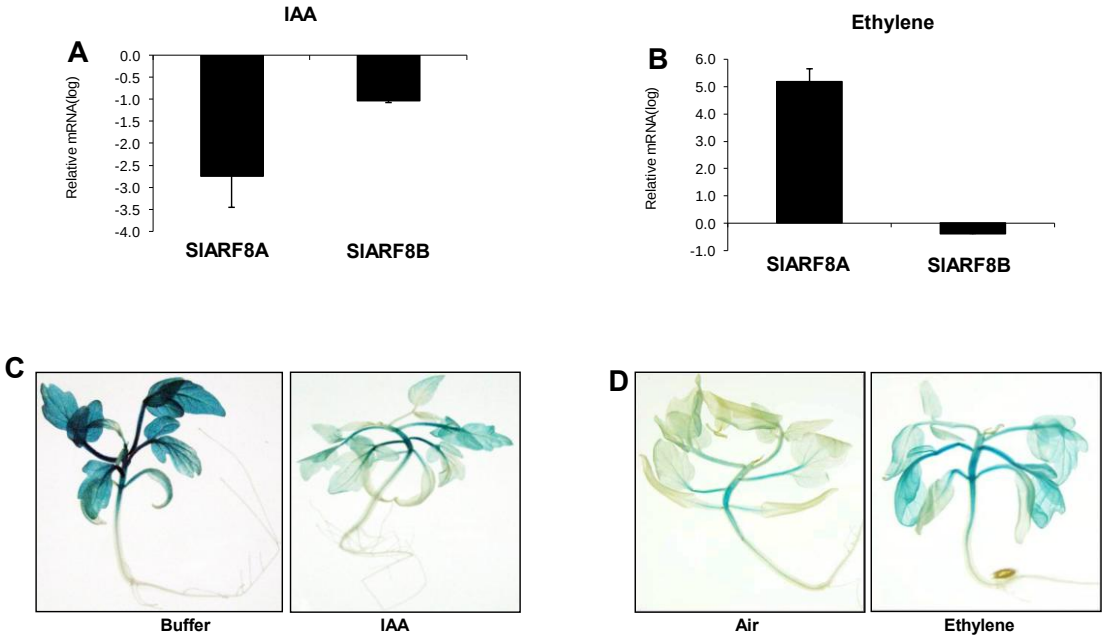


**Figure 6 Expression of *Sl-ARF8A* and *Sl-ARF8B* in seedlings induced by auxin and ethylene.**
**(A)** Quantitative RT-PCR analysis of *Sl-ARF8A* and *Sl-ARF8A* after auxin treatment. The wide-type seedlings were treated with 20 mM IAA for 2h with control and kept in -80℃ for RNA preparation. The SE+ represent three biological replicates.
**(B)** Quantitative RT-PCR analysis of *Sl-ARF8A* and *Sl-ARF8B* after ethylene treatment. The wide-type seedlings were treated with 50 ppm ethylene for 5h with control and kept in -80℃ for RNA preparation. The SE+ represent three biological replicates.
**(C)** Expression pattern of *ProARF8A::GUS* in seedlings treated with auxin. The seedling was treated with 40 mM IAA for 2h (right) and then transfer to 37$^{o}$C for 6h with control (left) displaying *Sl-ARF8A* was inhibited by auxin.
**(D)** Expression pattern of *ProARF8A::GUS* in seedlings treated with ethylene. The seedling was treated with 50 ppm ethylene for 5h (right) and then transfer to 37$^{o}$C for 9h with control (left) displaying *Sl-ARF8A* was up-regulated by ethylene.

### Sl-ARF8A/B over-expression leads to pleiotropic vegetative phenotypes

To gain insight on the physiological significance of the Sl-ARF8A/B encoded proteins, transgenic lines expressing sense constructs of the *Sl-ARF8A or Sl-ARF8B* genes (*Sl-ARF8A-OX* and *Sl-ARF8B-OX)* were generated using MicroTom tomato genotype. Several transgenic lines corresponding to independent transformation events were obtained showing substantial increase in Sl-ARF8A and Sl-ARF8B transcript levels as assessed by RT-qPCR (Figure 5C and D). These overexpressing lines typically displayed

dwarf phenotype (Figure 7A and B) and increased number of lateral roots when compared to wild-type (Figure 8A-E).

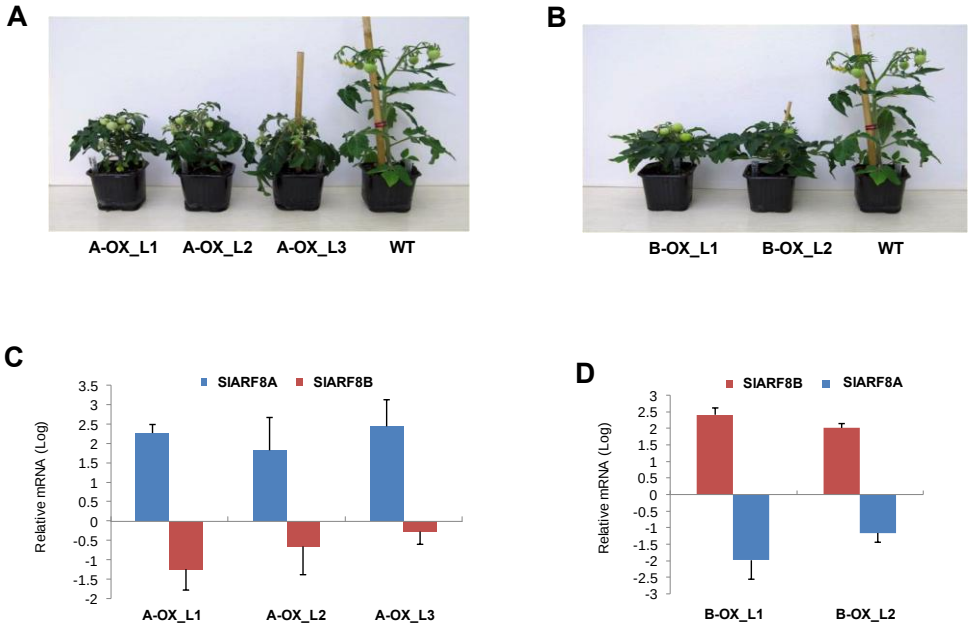


**Figure 7 Phenotype features of *SlARF8A-OX* and *SlARF8B-OX* lines.**
**(A,B)** Over-expression of *Sl-ARF8A* and *Sl-ARF8B* genes displayed the dwarf and parthenocarpic (seedless) phenotypes in three and two independent lines, respectively.
**(C,D)** The transcript levels of *Sl-ARF8A* and *Sl-ARF8B* were assessed by RT- q PCR in three independent *SlARF8A-OX* and two independent *SlARF8B-OX* lines. Relative expression level refers to the Log value and the error bar represents three biological replicates.

Moreover, the apical dominance was strongly reduced in the transgenic plants with a dramatic increase in the number of auxiliary shoot development. Notably, lateral shoots development in wild type plants display a basipetal growth pattern where the first lateral shoot arises from the last leaf node just below the first inflorescence. By contrast, the *SlARF8A/B-OX* plants displayed an acropetal growth pattern where the first lateral shoot arises from the first leaf node just above the cotyledon (Figue 8C). In conclusion, over-expression of *Sl-ARF8A/B* displayed pleiotropic auxin related phenotypes including altered apical dominance and lateral roots development, suggesting an important role for *Sl-ARF8A/B* in plant vegetative growth.

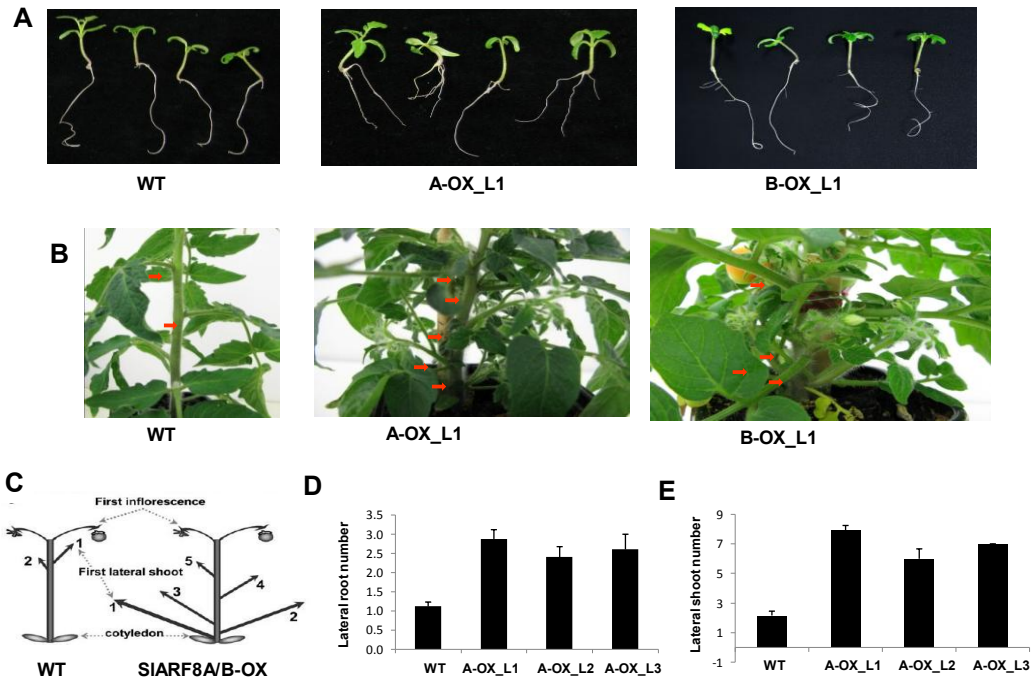


**Figure 8 Analysis of the pleiotropic phenotypes in *SlARF8A-OX* and *SlARF8B-OX* lines.**
**(A)** Increased lateral roots in *SlARF8A-OX* and *SlARF8B-OX* lines accessed in 20-day seedlings compared with the wild type.
**(B)** Increased lateral shoots in *SlARF8A-OX* and *SlARF8B-OX* plants at fruit mature green stage in contrast to the wild-type.
**(C)** Diagram depicting the inverted pattern of shoot branching and reduced apical dominance in *SlARF8A/B-OX* compared with the wild-type. Numbers indicate the emergence order of lateral shoots.
**(D)** Data analysis of the lateral roots in 20-d seedlings of *SlARF8A-OX* and WT. The error bar is representative of three independent statistics.
**(E)** Data analysis of the lateral shoots in *SlARF8A-OX* and WT at fruit mature green stage. The error bar is representative of three independent statistics.

## Over-expression of Sl-ARF8A/B induces fruit set prior to pollination resulting in parthenocarpy

Overexpression of *Sl-ARF8A/B* resulted in a dramatic alteration of early fruit growth, with all *SlARF8A/B-OX* lines exhibiting precocious fruit set prior to anthesis stage, resulting in premature fruit development which proceeds in parallel to flower development (Figure 9). The percent of parthenocarpic fruits was much higher in homozygous lines (90%) than in hemizygous ones (30%) showing a dose effect phenotype (Table 1).

**Table 1 Fruit characteristics in *SlARF8A-OX* and *SlARF8B-OX* Lines**

| Average data | Maximal Diameter(mm)[a] | Maximal Length(mm)[b] | Weight(g)[c] | Flower no.[d] | Fruit no.[e] | Fruit set rate (%) | Parthenocarpic rate (%) [f] |
|---|---|---|---|---|---|---|---|
| SlARF8A-OX-Homo L1 | 9.28 ±0.18 | 8.33 ±0.26 | 0.29 ±0.02 | 99±2 | 69± 9 | 68-78 | 99.00 |
| SlARF8A-OX-Homo L2 | 9.54±0.17 | 10.53 ±0.47 | 0.32 ±0.02 | 78±11 | 41 ±7 | 38-61 | 99.00 |
| SlARF8A-OX-Homo L3 | 9.15 ±0.23 | 10.67 ±0.22 | 0.29 ±0.02 | 87±5 | 55 ±5 | 54-68 | 99.00 |
| SlARF8A-OX-Hemi L1 | 17.04 ±0.40 | 15.71 ±0.32 | 2.37 ±0.16 | N | N | N | 64,72±10.61 |
| SlARF8A-OX-Hemi L2 | 13.19 ±0.34 | 13.76 ±0.31 | 1.23 ±0.09 | N | N | N | 40,18±8.97 |
| SlARF8A-OX-Hemi L3 | 14.07 ±0.38 | 14.19 ±0.35 | 1.31 ±0.09 | N | N | N | 43,35±1.88 |
| SlARF8B-OX-Homo L1 | 15.61±0.35 | 14.96±0.38 | 1.76±0.14 | N | N | N | 76,27 |
| SlARF8B-OX-Homo L2 | 13.98±0.37 | 13.60±0.37 | 1.11±0.09 | N | N | N | 94,52 |
| WT | 22.18 ±0.46 | 20.56 ±0.49 | 5.12 ±0.28 | 18±1 | 10±1 | 47-64 | 13.15±1.97 |

**a,b,c** Check for 3 plants randomly and over 60 fruits in each line of *SlARF8A-OX-Homo* or *-Hemi* and *SlARF8B-OX-Homo*. Check for 20 fruits in wide type.

**d, e** Check for 4 plants randomly in each line of *SlARF8A-OX-Homo* and check for total 20 plants in wide type.

**f** Check for 3 plants randomly and over 150 fruits in each line of *SlARF8A-OX-Homo* or *-Hemi*. Check for 6 plants randomly and over 180 fruits in each line of *SlARF8B-OX-Homo*. Check for 20 plants and over 200 fruits in wide type.

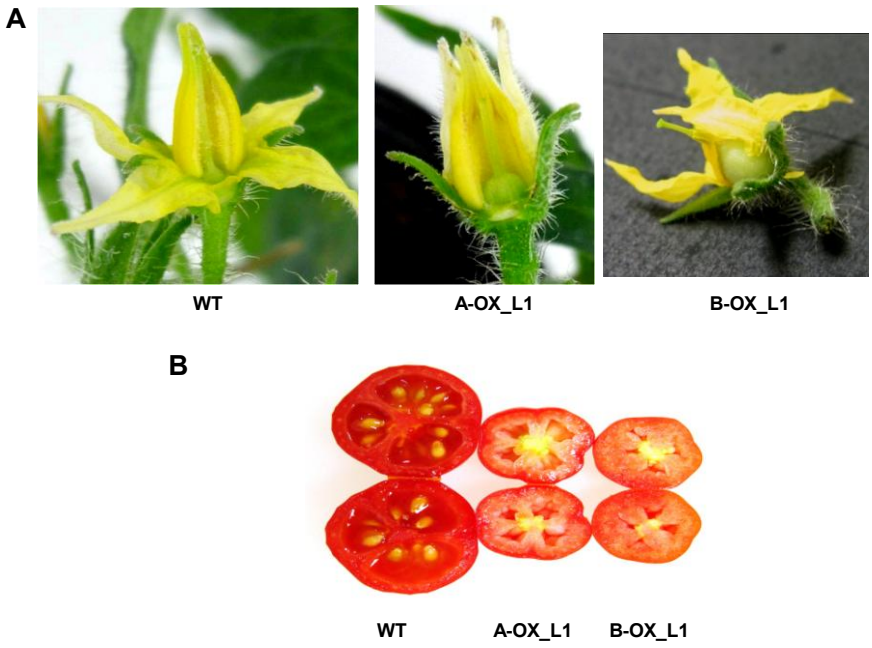


**Figure 9 Analysis of the flower and ripening fruit in *SlARF8A-OX* and *SlARF8B-OX* lines.**
**(A)** The ovary at the flowering stage becomes swollen in *SlARF8A-OX* and *SlARF8B-OX* lines in contrast to the wide-type.
**(B)** The cross section through representive fruits displayed parthenocarpic (seedless) fruits in *SlARF8A-OX* and *SlARF8B-OX* lines.

To get better understanding of the fruit set process in *Sl-ARF8A/B*, emasculation assay was performed on *SlARF8A-OX* lines to assess their ability to set fruit in the absence of pollination and fertilization. As expected, fruit set in control plants never occurs in emasculated wild-type flowers due to the absence of pollination/ferilization, while in *SlARF8A-OX* plants one third of the emasculated unfertilized flowers set fruit leading to the development of seedless fruits (Table 2).

**Table 2 Emasculation assay to assess the ability to set fruit in the absence of pollination**

| Lines | Fruit Set (%) | Fruit Set/Emasculated Flower | Seed Number/Fruit |
|---|---|---|---|
| WT | 0 | 0/16 | 0 |
| SlARF8A-OX L1 | 25 | 24/95 | 0 |
| SlARF8A-OX L2 | 39 | 14/36 | 0 |
| SlARF8A-OX L3 | 30 | 8/27 | 0 |
| WT Pollination | 100 | 8/8 | 25 |

Wild-type and *SlARF8A-OX* flowers were emasculated 1 to 4 d before anthesis. WT Pollination used as a control in emasculation assay. The results represent two independent trials.

To check whether parthenocarpy in *SlARF8A-OX* fruits may also arise from non-functional/fertile pollen, *in vitro* pollen viability test was carried out but no significant differences were observed between wild type and *SlARF8A-OX* pollen thus ruling out any loss of viability for *SlARF8A-OX* pollen (Figure 10 ).

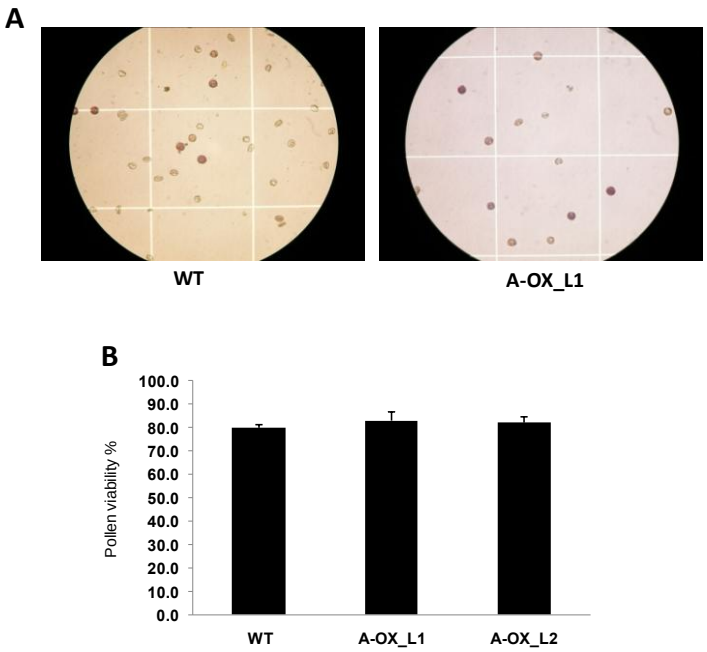


**Figure 10 *In vitro* analysis of pollen viability in *SlARF8A-OX* lines.**

**(A)** The pollens from WT and SlARF8A-OX flowers were treated with 2,5-diphenyl monotetrazolium bromide (MTT) in 5% sucrose solution at room temperature for 15 min. The viable pollen was dyed in red or light red-pink and the dead pollen was not dyed at all.

**(B)** Statistical analysis of pollen viability in WT and two independent SlARF8A-OX lines indicated pollen viability was maintained in *SlARF8A-OX* lines. The error bar represents the mean +SE of three independent tests.

Given that fruit organogenesis is initiated from the floral primordia, light microscopy analysis was therefore performed to investigate flower buds development and early fruit organogenesis in wild type and *ARF8-OX* lines. Observations of a series of longitudinal sections made on resin fixed flower buds from wild-type and *ARF8-OX* lines revealed several major changes in transgenic lines among which a dramatic decrease in the placenta size and a reduction in ovule number. Indeed, in over-expressing lines, the average placenta size was 23% of that of the loggia, while in wild-type it reaches 43%

(Figure 11). In the same way, the average number of ovules was 7.3 in *ARF8-OX* lines and 10.7 in wild-type lines (Figure 11).

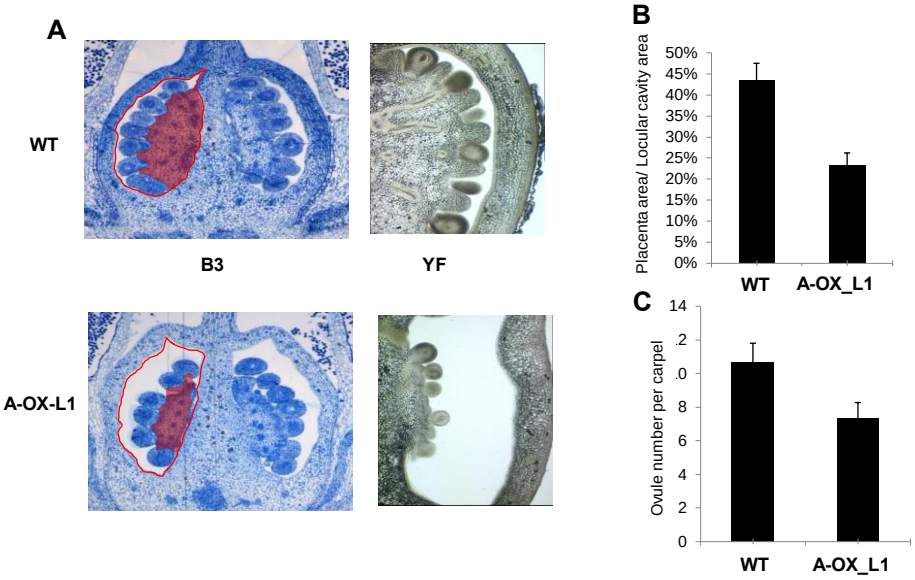


**Figure 11 Histological analysis of the flower bud and post-anthesis fruit in the wild-type and *SlARF8A-OX* line 1.**
**(A)** Comparison of the ovary development in flower bud stage 3 (B3 = 6-8 mm) indicates smaller plencenta and less number of ovules in *SlARF8A-OX* line 1 in contrast to the wide-type. Loggia is delimitated in red and placenta is highlighted. In additon to compare young fruits in the wide-type and *SlARF8A-OX* line 1 displaying the abnormal development of plancenta and ovules.
**(B)** Analysis of area (placenta/locular cavity) for each carpel in the wild-type and *SlARF8A-OX* line 1. Percentage of average placenta size compared to loggia size was lower in *SlARF8A-OX* line 1. The data are measured by at least 10 sections. The error bars are representative of the mean SE+.
**(C)** Analysis of the ovule number for each carpel in the wild- type and *SlARF8A-OX* line 1. The average ovule number for each carpel was reduced in *SlARF8A-OX* line 1. The data are measured by at least 10 sections. The error bars are representative of the mean SE+.

### Identification of the differentially expressed (DE) genes in SlARF8A-OX

In an attempt to identify genes whose expression is altered in *SlARF8-OX* lines, we performed a genome-wide transcriptomic profiling using deep sequencing RNA-Seq approach to compare gobal gene expression patterns in pre-anthesis flower buds (6-8 mm length) of wild type and *SlARF8A-OX*. This developmental srage was selected for the transcriptomic analysis because anthesis and post-anthesis stages are difficult to determine the in *SlARF8-OX*. Three biological replicates were prepared from *SlARF8A-OX* line1 and WT flower buds. Total RNA samples were prepared from each replicate and subjected to

deep sequencing using Illumina Hiseq2000. Almost 30 million reads have been generated for each repeat and after mapping the reads on the latest tomato genome sequence version Sl2.40, the level of gene expression was calculated for each annotated tomato gene (iTAG2.30). A total of 2632 genes differentially expressed (DE) in *SlARF8A-OX* plants were identified using DegSEQ R package (adjusted p-value <0,001 and 1< log(fold change) <-1). All Gene Ontology (GO) functional categories were represented among these genes (data not shown).

Interestingly, many early auxin responsive genes like *GH3*, *SAUR* and *Aux/IAA* were represented in the list of DE genes. It is noteworthy that all auxin biosynthesis genes (*AMI*, *TAA*, *TSB* and *YUC* homologs) except *YUC5* were repressed while all twelve Sl-ARFs genes were induced in the *SlARF8A-OX* lines. Auxin transport genes were all induced with the exception of *Sl-PIN5* whose expression is repressed (Figure 12). Unlike other auxin transporter known to regulate extracellular auxin transport, *Sl-PIN5* encodes an auxin transporter predicted to be ER-localized and involved in modulating the intracellular auxin homeostasis through the regulation of auxin flow from cytosol to ER [29].

The *COL1* gene encoding the Jasmonate receptor was induced in *SlARF8A-OX* lines unlike the *JAZ* genes which encode proteins interacting with the receptor but showing a decrease in transcript accumulation. The *MYC* transcription factor genes whose encoded proteins are negatively regulated by *JAZ* genes are all induced. These findings clearly support the idea that the jasmonate pathway is in active state during the flower to fruit transition. Finally, the situation regarding ethylene is more composite with all ethylene response factor genes displaying differential expression in *SlARF8A-OX* were also induced with the exception of *Sl-ERF.A2* (Figure 12). By contrast ethylene biosynthesis genes *SAMS* and *LeACS8* were down regulated. Considering the dramatic change in gene expression of genes related to auxin, jasmonate and ethylene displayed in *SlARF8A-OX* lines, these phytohormones are likely to play an active role in coordinating the fruit set process.

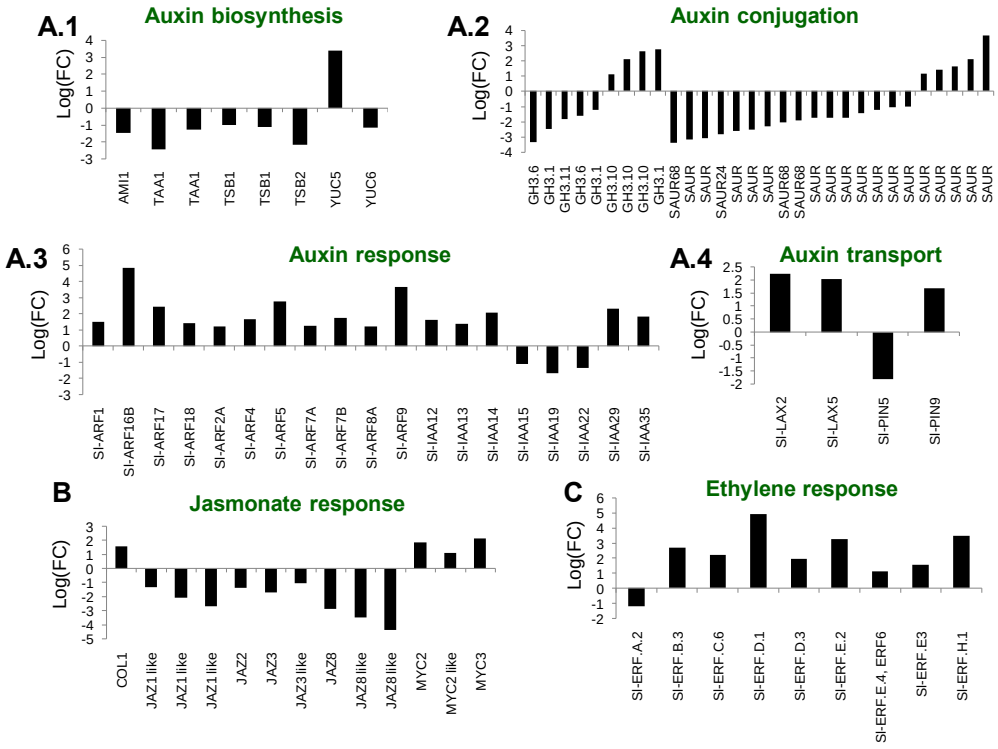


**Figure 12 Analysis of differently expressed (DE) genes involved in Auxin, Jasmonate and Ethylene response from the RNA-Sequencing data.**

(A.1) The DE genes involved in Auxin biosynthesis indicates most of them are down-regulated except YUC5 gene,

(A.2) that in Auxin conjugation indicates the deregulation of GH3 and Saur genes, and (A.3) that in Auxin response indicates up-regulation of 11 Sl-ARF genes but deregulation of 8 Aux/IAA genes. In addition, (A.4) 3 genes in Auxin transport are up-regulated except PIN5 gene.

(B) The DE genes in Jasmonate response indicates up-regulation of COL1 and MYC genes and down-regulation of JAZ genes which interacted with these two genes.

(C) The DE genes in Ethylene response indicates up-regulation of most ERF genes except ERF.A.2 gene. Relative expression level refers to the Log value are indicated in SlARF8A-OX flowers using WT as a control.

## Discussion

The phytohormone auxin plays a critical role in plant growth and development via the control of processes as diverse as lateral root initiation, leaf elongation, shoot architecture, apical dominance, embryogenesis and fruit initiation, development and ripening[3]. The perception and signaling of auxin depends on the cooperative action of several components among which ARF factors which specificallybind to TGTCTC auxin response elements (AuxRE) in the *promoters* of early auxin-responsive genes. Previous work has demonstrated that ARFs and Aux/IAAs significantly contribute to the regulation of the auxin regulated fruit initiation in the plant model *Arabidopsis* and also in tomato (*Solanum lycopersicum*) a model species for fleshy fruit research [5,24,30]. However, the molecular mechanism by which auxin mediates the flower-to-fruit transition, the so-called fruit-set, remains poorly understood.

Following isolation and characterization of all memebers of the ARF gene family in tomato [28], the present study provides new insight on the role of Auxin Response Factor 8 (Sl-ARF8) in tomato (*Solanum lycopersicum*) during fruit-set. Even though the fruit-set developmental process has been investigated by global transcriptome analysis in tomato [28], our understanding of the molecular mechanisms by which auxin mediates this process is still in its primitive steps. Therefore, the functional analysis of Sl-ARF8A/B, carried out here through combined reverse genetics and transcriptomic approaches aims at unlocking some of the key mechanisms underlying the fruit-set in tomato. Expression analysis, characterization of ARF8 over-expressing lines and protein-interaction studies yielded both corroborative and new data on the role of Sl-ARF8 thus allowing to partially decipher the auxin mode of action during fruit initiation and early development. The main conclusion on the important role of Sl-ARF8 came from the data showing that deregulation of Sl-ARF8 expression triggers fertilization-independent fruit-set in tomato. This is consistent with the study on its homolog in Arabidopsis, *At-ARF8,* whose mutation also resulted in fertilization-independent fruit-set [25].

Whereas in Arabidopsis ARF8 is encoded by a single gene, two genes, *Sl-ARF8A* and *Sl-ARF8B*, are present in the tomato genome. The two Sl-ARF8 paralogs share similar genomic structure suggesting that may have resulted from a recent duplication. Both genes seem to encode functional proteins harboring the three conserved domains (B3, ARF, Aux/IAA) characteristics of ARF proteins. Phylogenetic study indicated that Sl-ARF8A and B belong to a subclade gathering ARF6, ARF5, ARF7 and ARF19, with

ARF6 being however the closest paralog. *In silico* analysis of *Sl-ARF8A/B* at the RNA level predicted the presence of a conserved target site for sly-miR167 suggesting that both genes are potentially regulated at the post-transcriptional level by this microRNA. Subcellular localization studies indicated that both proteins are targeted to the nucleus consistent with these DNA-binding proteins being transcriptional regulators as validated by transactivation assays [28].

Spatio-temporal expression profiling indicated that these genes were preferentially expressed at the very early stages of fruit development right after anthesis, which is consistent with the hypothesis upon which Sl-ARF8A/B are probably involved in the flower-to-fruit transition. In order to address the functional significance of *Sl-ARF8A* and *Sl-ARF8B*, a reverse genetic approach was used to alter the expression of these genes in transgenic tomato plants.

Overexpression of Sl-ARF8A/B results in uncoupling fruit set from pollination and fertilization, thus giving rise to parthenocarpic (seedless) fruits.Further characterization of the transformed plants were performed including histological analysis to specifically study the flower bud development in wild type and SlARF8A-OX, with the aim to better decipher the very early stages of fruit-set in the transgenic lines. The data indicated that the ovary is the most affected organ with major modifications of the ovary shape. Abnormal development of placenta and ovules were also observed in the SlARF8A-OX which displayed a limited placenta development and reduced ovule numbers. These results are consistent with the phenotypes of SlARF8A-OX mature fruits, which are parthenocarpic with a lack of locular tissues and the presence of underdeveloped seed structure probably arising from unfertilized ovules.

Parthenocarpic phenotype in SlARF8-OX lines is a result of a fruit-set triggered before any fertilization. However, it is also likely that even in the situation whare ovules are successfully fertilized, seed development would probably have been impaired by the poor development of the placenta. To test this hypothesis, manual pollination of WT ovaries was undertaken to test the viability of these pollen and the results show clearely that SLARF8A-OX pollen are active.

Comparative transcriptomic profiling of wild type and SlARF8A-OX lines identified more than 2600 differentially expressed genes. Functional categorization of the differentially expressed genes revealed that the major groups of genes affected are related to stress, transcriptional regulation and hormones response and metabolism. These data suggest that the fertilization-independent fruit set induced by Sl-ARF8A overexpression

requires complex regulatory control and possibly involves multiple hormonal cross-talks. In this mechanisms, Sl-ARF8A appears to be a key player, which deregulation triggers a cascade of regulatory events leading to desynchronized fruit set.

Of particular interest, a group of nine ERF (Ethylene Response Factor) transcription factors that were differentially expressed, strongly suggesting the involvement of ethylene signaling in fruit set. This is in line with previous transcriptomic data reporting that the fruit set process is associated with the activation of a number of ethylene response genes [5]. More expected, a total of 31 auxin-related genes belonging to auxin biosynthesis, transport and response were found to be deregulated. To further validate the involvement of these genes in the process of fruit set, it will be interesting to analyze their expression pattern during normal fruit set upon fertilization. To uncover which genes among those showing altered expression in the transgenic lines are direct target for SlARF8A, a ChIP-Seq strategy is now considered. Such an approach becomes now possible given the availability of a high quality tomato genome sequence.

The generation of stably transformed tomato lines expressing *ProARF8A::GUS* provided a useful material to unveil the tissue specific expression of *Sl-ARF8A in planta*. The analysis of these plants indicated that *Sl-ARF8A* was highly expressed in the vascular tissues in most tomato organs. Interestingly, although no expression of *ProARF8A::GUS* was detectible in the roots of seedlings, the lateral root development was observed in *SlARF8A-OX* lines, suggesting that up-regulation of *Sl-ARF8A* regulates the auxin homeostasis to induce the lateral roots. The high expression of *Sl-ARF8A* in the axils, where the axillary shoots are generated, is to pout together with the enhanced shoot branching in *SlARF8A-OX* lines. In addition, the high expression of *Sl-ARF8A* found in the stigma is to bring together with the phenotype observed in *SlARF8A-OX* lines, and suggests that *Sl-ARF8A* might be directly involved in the style development.

The identity of the Sl-Aux/IAA(s) putatively interacting with Sl-ARF8 is currently unknown. Noteworthy, a bunch of phenotypes displayed by *SlARF8A-OX* plants are reminiscent of those reported for *Sl-IAA9* down-regulated tomato lines [23], suggesting that Sl-ARF8 might interact with Sl-IAA9 to mediate auxin-dependent developmental processes. In addition of pollination-independent fruit set, the acropetal growth pattern is also common to *SlARF8A-OX* and *Sl-IAA9* suppressed lines. Altogether, these data support the hypothesis that both proteins are components of the same complex involved in auxin signaling, and lowering the abundance of Sl-IAA9 protein is equivalent to increasing the level of Sl-ARF8 protein.

## Materials and methods

### Plant materials and growth conditions

Tomato seeds (*Solanum lycopersicum* cv MicroTom or Ailsa Craig) were sterilized, rinsed in sterile water and sown in recipient Magenta vessels containing 50 mL of 50% Murashige and Skoog (MS) culture medium added with R3 vitamin (0.5 mg $L^{-1}$ thiamine, 0.25 mg $L^{-1}$ nicotinic acid and 0.5 mg $L^{-1}$pyridoxine), 1.5% (w/v) sucrose and 0.8% (w/v) agar, pH 5.9. Plants were grown under standard greenhouse conditions. The culture chamber rooms are set as follows: 14-h-day/10-h-night cycle, 25/20°C day/night temperature, 80% hygrometry, 250 µmol $m^{-2}s^{-1}$ intense luminosity.

### Cloning the full-length Sl-ARF8A/B cDNAs by 5', 3'-Race PCR

BLAST analysis of expressed sequence tag (EST) sequences in the SOL GenomicsNetwork (SGN) (http://sgn.cornell.edu/tools/blast/) revealed that 4 EST sequences (no. BE353344, BF097763, TC161089, AW223741 or BE434602) was highly similar to the previously identified *AtARF8*. This EST sequence was then used as a basis to retrieve the full-length cDNA of *Sl-ARF8A* from „Micro-Tom" Tomato. First, 1 µL RNA from tomato (Kemer) flower, BD Smart IIA primer and ARF8-R2 primer (AATACAAGCTGCCAGCCTGATCT) were used for synthesis of 5' Race cDNA. Then, GSP1 primer (CCGCTTTAGCCTAAGAGGGAACAA) and GSP2 primer (CAGCGAACTGGATCTAAGTCACCA) were used to amplify the 5" cDNA end of this gene according to the manufacturer's instructions of SMART RACE cDNA Amplification Kit (Clontech). For 3" Race PCR, 2 µl RNA (Mix from WT Kemer tomato IG fruit + plantlets+flowers) and 3"-CDS primer A were used for synthesis of 3' Race cDNA. Then, the specific GSP1 primer (AGATCAGGCTGGCAGCTTGTAT) and GSP2 primer(GAATGATGTCCTTCTCCTTGG) were used to amplify the 3" cDNA end of this gene according to the manufacturer's instructions. In addition, a fragment of 1503 bp has been amplified by PCR on tomato Kemer cDNA. Next, the full length of *ARF8A* CDS was amplified from tomato flower cDNA (Kemer) with forward primer (ATGAAGCTTTCAACATCAGGAATG) and reverse primer (TCAGTAATCAAGTGA TCCTATAG) at Tm 62°C, using Isis polymerase and then clone into pGEM-T Easy vector (Promega) for sequence. For *Sl-ARF8B*, a fragment SGN-U328487 with stop cordon of *Sl-ARF8B* CDS has been found in SGN database, then 5' Race PCR was performed as follows. 3 µL RNA Mix from µTom tomato (flower, root, leaves, stems,

IMG, MG, breaker, 3 µg/µL) , 5‟CDS and BD Smart IIA primer were used for synthesis of 5‟Race cDNA. Primers ARF8B_STOP (TCAGTACTCCAGCGATCCAA GAGA) or ARF8B_5‟GSP2 (ATATCCAGTGACCTCCCAACACATCC) were used for first PCR, then ARF8B_5‟GSP3 (GGGAGAATTGTTGAATCGACTCTCTG) or 5‟GSP4 (ATTGGACAGGCTGCTGAAACTGCAT) were used to amplify the 5‟ cDNA ATG of this gene according to the manufacturer's instructions of the smart kit. In the end, the full length of *ARF8B* CDS was amplified with forward primer (ATGAAGCTTTCAACATCAGGAATGGGTCAG) and reverse ARF8B_STOP as before.

**Plant transformation**

To generate *SlARF8A-OX* and *SlARF8B-OX* transgenic plants, the full-length of *Sl-ARF8A* or *Sl-ARF8B* CDS was cloned into pGEMN-T (Promega) vector and then connected to binary vector pGreen in sense orientation under the transcriptional control of the cauliflower mosaic virus 35S promoter and the Nos terminator. Transgenic plants were generated by *Agrobacterium tumefaciens* mediated transformation according to the protocol in the GBF laboratory, and transgenic lines were selected on kanamycin (75 mg/L) and then analyzed by genomic DNA-PCR to check the presence in the various transgenic lines obtained.

**RNA isolation and quantitative RT-PCR**

Total RNA was extracted from fruit according to Hamilton et al. (1991). Total RNA from leaves, stems, roots, flowers, and seedlings was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer‟s instructions. Total RNA from ovary, stamen, petal and sepal from bud, flower and post-flower (4 DPA) was extracted from flower for three biological repeats using a TRIZOL Reagent (invitrogen) according to the manufacturer‟s instructions. Total RNA was treated by DNase I to remove any genomic DNA contamination. First strand cDNA was reverse transcribed from 2 µg of total RNA using Omniscript kit (Qiagen) according to the manufacturer's instructions. q RT-PCR analysis was performed as previously described [31]. The primer sequences are listed in Table 3. Relative fold differences were calculated based on the comparative Ct method using the Sl-Actin as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value of genes was normalized to the Ct value for Sl-Actin-51 (accession number Q96483) and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. At least two to three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to real-time PCR analysis in triplicate.

**Subcellular localization**

The *Sl-ARF8A_GFP* and *Sl-ARF8B_GFP* fusion were cloned into the Gateway vectors by using the primers in Table 3. Protoplasts were obtained from suspension cultured tobacco (Nicotiana tabacum) BY-2 cells and transfected according to the method described previously (Leclercq et al., 2005). Transfected protoplasts were incubated for 16 h at 25 °C and analysed for GFP fluorescence by confocal microscopy. Confocal fluorescent images were obtained on a confocal laser scanning microscope (Leica TCS SP2, Leica DM IRBE ; Leica Microsystems, Wetzlar, Germany). For GFP, the samples were illuminated with a 488 nm ray line of an argon laser and the emission light collected in the 500–525 nm spectral range.

**Hormone treatment**

For auxin treatment, 21-d-old tomato seedlings were harvested and incubated in 1/2 MS buffer containing 20 mM IAA or not (mock treatment) for 3 h. Thereafter, WT tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction, or *ProARF8A::GUS* seedlings were used for GUS analysis. For ethylene treatment, 21-d-old tomato seedlings were treated at 50 ppm ethylene and air as a control for 5 h. After that, WT tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction, or *ProARF8A::GUS* seedlings were used for GUS analysis.

**Histochemical GUS analysis**

Transgenic lines bearing the *ARF8A/B* promoter-GUS fusion constructs (*ProARF8A/B*::GUS) were incubated at 37°C for calculated time with GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton, and 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid) to reveal GUS activity. In fact, to keep 6 h for the flower and 4 DPA, and 3 h for fruit in different stages. Following GUS staining, samples were washed several times to extract chlorophyll using a graded ethanol series.

**Pollen viability analysis**

The pollen from the flower of *SlARF8A-OX* lines and wide type was treated with 2,5-diphenyl monotetrazolium bromide (MTT) in 5% sucrose solution at room temperature for 15 min, and then checked under the microscope with a magnification of 10-40 times.

**Flower emasculation and cross assay**

Flower buds of wild-type or transgenic plants were emasculated before dehiscence of anthers (closed flowers) to avoid accidental self-pollination. Cross-pollination was performed on emasculated flowers one day prior to anthesis. For emasculation and cross-

fertilization experiments, 8 to 10 flowers were kept per plant to ensure equivalent growth conditions for all fruit.

**Histological section analysis**

For histological analysis, flower buds of 2-8 mm in length were fixed in a glutaraldehyde solution (sodium cacodylate 50 mM pH 7.1, 2.5% glutaraldehyde), and dehydrated using graded ethanol series before being fixed in graded LR White Resin series. 99 nm-thick sections were stained with a toluidine blue solution (0.5% in 2.5% sodium carbonate) and viewed under a Leica MZFLIII stereo microscope. Pictures were taken with a Leica DFC 420C camera, while applying shading correction with the Leica Application Suite software (Leica Microsystems). Images analysis and area measures were performed with the Image-Pro plus v4.5 software. In total, 3 flower buds by line have been analyzed.

**RNA isolation and RNA-sequencing**

Total RNA was extracted from flower bud in *SlARF8A-OX* and WT for three biological repeats using a TRIZOL Reagent (invitrogen) according to the manufacturer‟s instructions. Total RNA was treated by DNase I to remove any genomic DNA contamination and checked by RNA gel. The good ones were further analyzed by Agilent RNA 6000 Nano Assay, which the RIN value above 7 was determined to be qualified. After that, the best RNA were sent out for deep RNA sequencing using Illumine Hiseq2000 and the reads generated were mapped to the tomato genome sequence Sl2.40. The gene expression was calculated for each annotated tomato gene (iTAG2.30).

**Table 3 The primers used for the analysis of RT-q PCR and Gateway cloning**

| Genes | Forward Primer | Reverse Primer |
|---|---|---|
| Sl-ARF8A | TGACATCGAATGGAAATTCAG | GTCTCTTAGCACTAACAAACAC |
| Sl-ARF8B | GTCAGTCCGTGATCATAGAG | GGAATCCAAGCTACAATTTCC |
| Sl-Actin-51 | TGTCCCTATTTACGAGGGTTATGC | CAGTTAAATCACGACCAGCAAGAT |
| ProARF8A | AAAAAGCAGGCTTCTTTACCATGTCCCTACCCTCT | AAGAAAGCTGGGTCCTTTCTCCAAGACCTCCATT |
| ProARF8B | AAAAAGCAGGCTTCTTAACTAACAAACCAACACAGCCAAC | AAGAAAGCTGGGTCTTTATTTAAAAACCCACTTCCTCTTGA |
| Sl-ARF8A_GFP Fus | AAAAAGCAGGCTTCATGAAGCTTTCAACATCAGGAATG | AAGAAAGCTGGGTCGTAATCAAGTGATCCTATAG |
| Sl-ARF8B_GFP Fus | AAAAAGCAGGCTTCATGAAGCTTTCAACATCAGGAATGGGTCAG | AAGAAAGCTGGGTCGACGAAAGCTAAAGAAGCCAGG |

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# Chapter IV

# Main conclusions and perspectives

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In fleshy fruits, fruit setting corresponds to a transition step that change the fate of the flower organ to a fruit. This process can be described as a very complex developmental event that is associated with dramatic physiological, structural and molecular changes. The plant hormone auxin is known to play a critical role in the fruit set process, but, so far, the molecular mechanisms underpinning auxin action during the flower to fruit transition remain largely unknown. Auxin Response Factor (ARF), as an important transcriptional factor involved in auxin signaling, regulates early auxin-responsive genes, such as Aux/IAAs, GH3s and SAURs by binding to auxin-responsive elements (AuxREs) present in their promoters. The present study identified and comprehensively analyzed the whole *ARF* gene family in the tomato (*Solanum lycopersicum*), a reference species for Solanaceae plants and the model plant for fleshy fruit development. The main part of my Ph.D. research project was dealing with the structural and functional characterization of two ARF homological members, Sl-ARF8A and Sl-ARF8B.

**The ARF multigene family in tomato**

It is well accepted now that ARF regulators play a key role in various auxin-mediated plant developmental processes by specifically binding to the auxin-responsive elements (AuxREs) found in the promoter of early auxin responsive gene. In the present study, a total of 22 *Sl-ARF* genes have been identified from tomato (*Solanum lycopersicum*) genome using 23 predicted *Arabidopsis thaliana ARF* protein sequences as query. Analysis by Fancy Genev1.4 revealed the genomic structure of each *Sl-ARF* gene and the location of each *Sl-ARF* on tomato chromosomes. Expression analysis by qRT-PCR unveiled the diversified expression patterns of *Sl-ARF* genes in different tomato tissues and organs and revealed their regulation by auxin and ethylene suggesting that these transcriptional regulators may be essential components of the cross-talk between auxin and ethylene signaling pathways. RNA-Seq and RT-PCR analysis provided new insight into the expression profile of each *Sl-ARF* member in fruit set and uncovered a major alternative splicing mode of regulation for 7 *Sl-*

*ARF*s. Overall, the data produced provide important leads towards addressing the role of a specific Sl-ARF in a particular developmental process.

**Structural and functinal features of Sl-ARF8A/B**

Previous studies have showed that *AtARF8* gene played key roles on hypocotyl growth, flower petal development and fruit initiation in *Arabidopsis*. On the other hand, down regulation of *Sl-ARF7* gene in tomato resulted in parthenocarpic phenotypes and a thickened pericarp. The present study uncovers the essential role of *Sl-ARF8A* and *Sl-ARF8B* in controlling fruit set process. Expression analysis carried by by RT-qPCR revealed an increase in *Sl-ARF8A* transcript levels in ovary within 4 days after pollination and fertilization. The hormonal regulation of Sl-ARF8A/B addressed either by qRT-PCR or using transgenic lines expressing the ProARF8::GUS fusion, showed that *Sl-ARF8A* is repressed by auxin and activated by ethylene whereas the expression of *Sl-ARF8B* failed to display regulation by any of the two phytohormones. Over-expression of Sl-ARF8A/B resulted in a fertilization-independent fruit set and parthenocarpic fruit formation in tomato strongly suggesting at least a partial functional redundancy between the two genes. The histological analysis revealed an altered placenta and ovules development in *Sl-ARF8A* over-expressing flowers suggesting that this is involved in the development of these reproductive structures. Taken together, the data uncovers the involvement of *Sl-ARF8* in fruit set and suggest that *Sl-ARF8A* may be essential in relaying both auxin and ethylene responses. In contrast to *Sl-ARF7* gene, previously showed to be up-regulated in non-fertilized flowers and down-regulated after pollination, *Sl-ARF8A* displayed an opposite pattern of expression being up-regulated after flower pollination and fertilization. However, up-regulation of *Sl-ARF8A* and down-regulation of *Sl-ARF7* both resulted in the parthenocarpic fruits in tomato plants, suggesting that these two ARF family members may play an opposite role with regard to fruit initiation. The identity of the Sl-Aux/IAA(s) putatively interacting with Sl-ARF8 is currently unknown. Noteworthy, the parthenocarpic phenotype displayed by *SlARF8A-OX* plants is reminiscent of *Sl-IAA9* antisense tomato lines [83], suggesting that Sl-ARF8 might interact with Sl-IAA9 to mediate auxin-dependent developmental processes. In addition of pollination independent fruit set, the acropetal growth pattern is also common to *SlARF8A-OX* and *Sl-IAA9* suppressed lines. Altogether, these data sustain the idea that both Sl-IAA9 and Sl-ARF8 proteins are part of the same complex involved in auxin signalling involved in fruit set. Considering that Aux/IAA can potentially interact with ARF proteins leading to their sequestration/inactivation, therefore lowering the abundance of Sl-IAA9 protein would increase the level of free/active Sl-ARF8

proteins. In this context, it would be important to know the Aux/IAA partners of Sl-ARF7 in order to determine whether the two ARFs mediate fruit set through a shared mechanism.

**In conclusion,** the data obtained within the thesis research project represent the first description of phenotypes associated with over-expression of an ARF family gene in tomato. The data strongly show that:
(1) Sl-ARFs display distinct structural and functional features and can be divided into activators and repressors of transcriptional activities;
(2) a number of Sl-ARFs undergo a major alternative splicing mode of regulation that is likely important for the control of fruit set;
(3) unlike Arabidopsis, ARF8 is encoded by two genes in the tomato, named *Sl-ARF8A* and *Sl-ARF8B* displaying distinct expression patterns with only *Sl-ARF8A* being regulated by both auxin and ethylene;
(4) fruit set is actively regulated by phytohormone auxin, and Sl-ARF8 plays an important role in auxin-mediated fruit set process;
(5) Over-expression of Sl-ARF8A/B both resulted in common reproductive and vegetative phenotypes indicating a large functional redundancy between these two Sl-ARF8 paralogs.

The research project is now being continued towards seeking for the Aux/IAA partners of Sl-ARF8A and B but also Sl-ARF7. A dominant repression version of Sl-ARF8 obtained by fusing the protein to the EAR repressor motif (ARF8A-SRDX) has been generated and tomato plants expressing this construct are now being generated. However, so far, it has been proven difficult to produce such transgenic plants suggesting that the expression of the repression version of Sl-ARF8 might be detrimental to developmental process essential for plant regeneration. In order to identify Sl-ARF8 direct target genes, tomato lines expressing tagged Sl-ARF8 genes (3*5S::Sl-ARF8A-3HA*, *35S::3HA-Sl-ARF8A*, *35S::Sl-ARF8A-GFP* and *35S::Sl-ARF8B-GFP)* have been generated. This plant material is being used to carry out a ChIP-seq approach. Furthermore, the posttranscriptional regulation of Sl-ARF8 genes by miR167 has been addressed showing that down-regulation of this microRNA gene leads to parthenocarpic (seedless) fruits similar to the over-expression of Sl-ARF8A/B (a paralle project carried out by another Ph.D. student in the GBF lab). Collectively, the data gathered bring a detailed picture of the components of the control mechanism underlying the the molecular events involved in auxin-mediated fruit set.

From the applied side, the data provide multiple new targets for the control of fruit set and parthenocarpy via molecular breeding or genetic transformation strategies.

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