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

Présentée et soutenue par :

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le vendredi 14 novembre 2014

Titre :

AUXIN-MEDIATED FRUIT DEVELOPMENT AND RIPENING: NEW INSIGHT ON THE ROLE OF ARFs AND THEIR ACTION MECHANISM IN TOMATO (S. LYCOPERSICUM)

Ecole doctorale :

Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

Unité de recherche :

Laboratoire Génomique et biotechnologie des fruits (G.B.F.)

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Auxin-mediated fruit development and ripening: new insight on the role of ARFs and their action mechanism in tomato (*S. lycopersicum*)

Acknowledgment

It's a very complicated moment to me. On the one hand, I'm excited about getting the PhD degree but on the other hand, I'm sad since I'm going to say goodbye to the peoples who accompany me these four years. Even though words can't express my appreciation; I think I should say thanks to them.

Foremost, I would like to thank my supervisors Dr. Mondher Bouzayen and Mohamed Zouine. They teach me how to propose and solve questions, how to organize and design expriments, and they try their best to help me in the thesis. Owing to their help, I can be here for my thesis defense. Thanks to Dr. Rothan Christophe and Dr. Benhamed Moussa who accept to report on my PhD thesis and also thanks to Jacqueline Grima-Pettenati who agreed to be examiner of my PhD thesis.

I should say a lot of thanks to Benoit Van-Der-Rest, Isabelle Mila, Xinyu Wang and Guojian Hu for their discussions and important contribution to the project and for sharing their knowledge with me.

I would like to thank Professor Zhengguo Li, who supervised my master degree and suggested me to study in GBF.

I also would like to thank Brigitte Lafforgue for her kind help in the daily life.

I owe many thanks to all members of the GBF team and all the friends studying and living around me during these 4 years. Beacasue of their help in the lab and in the daily life, I can finish my theis on time.

I would like to thank my country, China for offering me the CSC (China Scholarship Council) scholarship supporting my study in France.

And last but not the least; I would like to thank my husband Huailong Qi for his understaning, sacrifice and endless love.

Résumé

L'auxine est une hormone végétale qui coordonne plusieurs processus de développement des plantes à travers la régulation d'un ensemble spécifique de gènes. Les Auxin Response Factors (ARF) sont des régulateurs transcriptionnels qui modulent l'expression de gènes de réponse à l'auxine. Des données récentes montrent que les membres de la famille des ARF sont impliqués dans la régulation du développement des fruits de la nouaison à la maturation. Alors qu'il est établi que les ARF agissent de concert avec les Aux/IAA pour contrôler l'activité transcriptionnelle dépendant de l'auxine, notre connaissances des mécanismes et des partenaires des ARF demeurent très icomplètes. L'objectif principal de la thèse est d'étudier la part qui revient aux ARF dans le contrôle du développement et de la maturation des fruits et d'en comprendre les mécanismes d'action. L'analyse des données d'expression disponibles dans les bases de données a révélé que, parmi tous les ARF de tomates, SlARF2 affiche le plu haut niveau d'expression dans le fruit avec un profil distinctif d'expression associé à la maturation. Nous avons alors entrepris la caractérisation fonctionnelle de SlARF2 afin d'explorer son rôle dans le développement et la maturation des fruits. Deux paralogues, SlARF2A et SlARF2B, ont été identifiés dans le génome de la tomate et des expériences de transactivation ont montré que les deux protéines SlARF2 sont localisées dans le noyau où elles agissent comme des répresseurs transcriptionnels des gènes de réponse à l'auxine. De plus, l'expression de SlARF2A dans le fruit est régulée par l'éthylène tandis que celle de SlARF2B est induite par l'auxine. La sous-expression de SlARF2A, comme celle de SlARF2B, entraine un retard de maturation alors que l'inhibition simultanée des deux paralogues conduit à une inhibition plus sévère de la maturation suggérant une redondance fonctionnelle entre les deux paralogues lors de la maturation des fruits. Les fruits présentant une sous-expression des gènes SlARF2 produisent de faibles quantités d'éthylène, montrent une faible accumulation de pigments et une plus grande fermeté. Le traitement avec de l'éthylène exogène ne peut pas inverser les phénotypes de défaut de maturation suggérant que SlARF2 pourrait agir en aval de la voie de

signalisation de l'éthylène. L'expression des gènes clés de biosynthèse et de signalisation de l'éthylène est fortement perturbée dans les lignées sous-exprimant SlARF2 et les gènes majeurs qui contrôlent le processus de maturation (*RIN*, *CNR*, *NOR*, *TAGL1*) sont sensiblement sous-régulés. Les données suggèrent que SlARF2 est essentiel pour la maturation des fruits et qu'il pourrait agir au croisement des voies de signalisation de l'auxine et de l'éthylène. Alors que l'éthylène est reconnu comme l'hormone clé de la maturation des fruits climactériques, les phénotypes de défaut de maturation chez les lignées sou-exprimant le gène SlARF2 apportent des preuves tangibles soutenant le rôle de l'auxine dans le contrôle du processus de maturation. Dans le but de mieux comprendre les mécanismes moléculaires par lesquels les ARF régulent l'expression des gènes de réponse à l'auxine, nous avons étudié l'interaction des SlARFs avec des partenaires protéiques ciblés, principalement les co-répresseurs de type Aux/IAA et Topless (TPL) décrits comme les acteurs clés dans la répression des gènes dépendant de la signalisation auxinique. Une fois les gènes codant pour les membres de la famille TPL de tomate isolés, une approche double hybride dans la levure a permis d'établir des cartes exhaustives d'interactions protéine-protéine entre les membres des ARFs et des Aux/IAA d'une part et les ARFs et les TPL d'autre part. L'étude a révélé que les Aux/IAA interagissent préférentiellement avec les SlARF activateurs et qu'à l'inverse les Sl-TPL interagissent uniquement avec les SlARF répresseurs. Les données favorisent l'hypothèse que les ARF activateurs recrutent les Sl-TPL via leur interaction avec les Aux/IAA, tandis que les ARF répresseurs peuvent interagir directement avec les Sl-TPL. Les études d'interactions ont permis également d'identifier de nouveaux partenaires comme les protéines VRN5 et LHP1, composantes des complexes Polycomb PRC impliqués dans la repression par voie épigénétique de la transcription par modification de l'état de méthylation des histones. Ces données établissent un lien potentiel entre les ARFs et la régulation épigénétique et ouvrent de ce fait de perspectives nouvelles quant à la compréhension du mode d'action des ARFs. Au total, le travail de thèse apporte un nouvel éclairage sur le rôle et les mécanismes d'action des ARF et identifie SlARF2 comme un nouvel élément du réseau de régulation contrôlant le processus de maturation des fruits chez la tomate.

Abstract

The plant hormone auxin coordinates plant development through the regulation of a specific set of auxin-regulated genes and Auxin Response Factors (ARFs) are transcriptional regulators modulating the expression of auxin-response genes. Recent data demonstrated that members of this gene family are able to regulate fruit set and fruit ripening. ARFs are known to act in concert with Aux/IAA to control auxin-dependent transcriptional activity of target genes. However, little is known about other partners of ARFs. The main objective of the thesis research project was to gain more insight on the involvement of ARFs in fruit development and ripening and to uncover their interaction with other protein partners beside Aux/IAAs. Mining the tomato expression databases publicly available revealed that among all tomato ARFs, *SlARF2* displays the highest expression levels in fruit with a marked ripening-associated pattern of expression. This prompted us to uncover the physiological significance of SlARF2 and in particular to investigate its role in fruit development and ripening. Two paralogs, *SlARF2A* and *SlARF2B*, were identified in the tomato genome and transactivation assay in a single cell system revealed that the two SlARF2 proteins are nuclear localized and act as repressors of auxin-responsive genes. In fruit tissues, *SlARF2A* is ethylene-regulated while *SlARF2B* is auxin-induced. Knock-down of *SlARF2A* or *SlARF2B* results in altered ripening with spiky fruit phenotype, whereas simultaneous down-regulation of *SlARF2A* and *SlARF2B* leads to more severe ripening inhibition suggesting a functional redundancy among the two *SlARF2* paralogs during fruit ripening. Double knock-down fruits produce less climacteric ethylene and show delayed pigment accumulation and higher firmness. Exogenous ethylene treatment cannot reverse the ripening defect phenotypes suggesting that SlARF2 may act downstream of ethylene signaling. The expression of key ethylene biosynthesis and signaling genes is dramatically disturbed in *SlARF2* down-regulated fruit and major regulators of the ripening process, like RIN, CNR,

NOR, TAGL1, are under-expressed. The data support the notion that SlARF2 is instrumental to fruit ripening and may act at the crossroads of auxin and ethylene signaling. Altogether, while ethylene is known as a key hormone of climacteric fruit ripening, the ripening phenotypes associated with *SlARF2* down-regulation bring unprecedented evidence supporting the role of auxin in the control of this developmental process. To further extend our knowledge of the molecular mechanism by which ARFs regulate the expression of auxin-responsive genes we sought to investigate interactions SlARF and putative partners, mainly Aux/IAAs and Topless co-reppressors (TPLs) reported to be key players in gene repression dependent on auxin signaling. To this end, genes encoding all members of the tomato TPL family were isolated and using a yeast-two-hybrid approach comprehensive protein-protein interaction maps were constructed. The study revealed that Aux/IAA interact preferentially with activator SlARFs while Sl-TPLs interact only with repressor SlARFs. The data support the hypothesis that activator ARFs recruit Sl-TPLs co-repressors via Aux/IAAs as intermediates, while repressor ARFs can physically interact with Sl-TPLs. Further investigation indicated that SlARFs and Sl-TPLs can interact with polycomb complex PRC1&PRC2 components, VRN5 and LHP1, known to be essential players of epigenetic repression of gene transcription through the modification of histones methylation status. These data establish a potential link between ARFs and epigenetic regulation and thereby open new and original perspectives in understanding the mode of action of ARFs. Altogether, the thesis work provides new insight on the role of ARFs and their underlying action mechanisms, and defines SlARF2 as a new component of the regulatory network controlling the ripening process in tomato.

Publications

Articles Published

1. **Yanwei Hao*,** Xinyu Wang*, Xian Li, Carole Bassa, Isabelle Mila, Corinne Audran, Elie Maza, Zhengguo Li, Mondher Bouzayen, Benoit van der Rest and Mohamed Zouine. **Genome-wide Identification, phylogenetic analysis, expression profiling and protein-protein interaction properties of the TOPLESS gene family members in tomato.** J. Exp. Bot. 2014 Jan. Vol 65, No.4, pp. 1013-1023.

2. Maha Sagar, Christian Chervin, Isabelle Mila, **Yanwei Hao**, Jean-Paul Roustan, Mohamed Benichou, Yves Gibon, Benoît Biais, Pierre Maury, Alain Latche, Jean-Claude Pech, MondherBouzayenand Mohamed Zouine. **Sl-ARF4, an Auxin Response Factor in volved in the control of sugar metabolism during tomato fruit development.** Pant Physiology, 2013 March, Vol.161, pp. 1362-1374.

Articles In preparation

1. **Yanwei Hao**, Guojian Hu, Mingchun Liu, Isabel Mila, Mondher Bouzayen, Mohamed Zouine, (2014) **Auxin Response Factor SlARF2, a new component of the regulatory mechanism controlling fruit ripening in tomato**. (In preparation)

2. **Yanwei Hao**, Mondher Bouzayen, Eile Maza, Mohamed Zouine (2014) **Auxin signaling during fruit development and ripening.** (In preparation)

Poster presentation

1. **Yanwei Hao**, Isabel Mila, Mondher Bouzayen, Mohamed Zouine. **Characterization of SlARF2 involved in tomato fruit ripening.** 12/11/2013. Journee de l'ecole doctorale SEVAB.

2. Xinyu Wang, **Yanwei Hao**, Isabelle Mila, Carole Bassa, Corinne Audran-Delalande, Mohamed Zouine, Benoit Van DER REST and Mondher Bouzayen. **Identification and Characterization of the TOPLESS Gene Family in Tomato**. 06/11/2012. Grand Auditorium-UPS-Toulouse, France.

3. **Yanwei Hao**, Guojian Hu, Isabel Mila, Mingchun Liu, Eile Maza, Mondher Bouzayen, Mohamed Zouine, **Auxin Response Factor SlARF2, a new component of the regulatory mechanism controlling fruit ripening in tomato.** QUALITYFRUIT 2014, 21st-24th of September 2014, Chania, Crete, Greece.

Content

Abreviations

MCP: 1-Methyl Cyclopropane
ACC: Acide 1-AminoCyclopropane-1-Carboxilique
ACO: Acide 1-AminoCyclopropane-1-Carboxilique Oxydase
ACS: Acide 1-AminoCyclopropane-1-Carboxilique Synthase
AP2: Apetala2
A. thaliana: Arabidopsis thaliana
AA: Amino acid
ABA: Abscisic acid
ABP1: Auxin binding protein 1
AD: Activation domain
AFB: AUXIN RECEPTOR F-BOX
ARF: Auxin response factors
ASP: Asparaginases
At-: Arabidopsis thalianaopless
AuxREs: Auxin response elements
Aux/IAA : Auxin/Indole-3-Acetic Acid
BD: Binding domain
BIFC: Bimolecular fluorescence complementation
bp: Base pair
B. rapa: Brassica rapa
CaMV: Cauliflower Mosaic Virus
cDNA: Complementary deoxyribonucleic acid
CDS: Coding sequence
CNR: Colorless Non Ripening
CTD: C-terminal dimerization domain
CTLH: C-terminal to Lyssencephaly homology domain
DBD: DNA binding domains
dNTP: Deoxyribonucletides
EAR: Ethylene-responsive element binding factor-associated Amphiphilic Repression
EIN: Ethylene insensitive
EDTA: Ethylenediamine tetraacetic acid
ER: Endoplasmic Reticulum
ERF: Ethylene response factor
GH3: Gretchen Hagen
GUS: ß-glucuronidase
GA: Gibberellic acid or gibberellin

GFP: Green Fluorescent Protein
G. max: Glycine max
HDAC: Histone deacetylase
IAA: Indole-3-acetic acid
IFs: Transcriptional factors
JA: Jasmonic acid
LOX: Lipoxigenase
LisH: Lyssencephaly homology domain
LUG/LUH: LEUNIG/LEUNIG HOMOLOG
M. guttatus: Mimulus guttatus
MR: Middle region
NAA: α-Naphthalene acetic acid
NR: Never Ripe
NOR: Non-Ripening
N. benthamiana: Nicotiana benthamiana
NLS: Nuclear localization signal
ORF: Open reading Frame
O. sativa: Oryza sativa
PG: Polygalacturonase
PCR: Polymerase chain reaction
P. patens: Physcomitrella patens
PPIs: Protein-protein interactions
P. trichocarpa: Populus trichocarpa
qRT-PCR: Quantitative reverse transcription Polymerase chain reaction
RIN: Ripening Inhibitor
RD: Repression domain
S. bicolor: Sorghum bicolor
SGN: Solanaceae Genomics Network
SAUR: Small Auxin Up-regulated RNA
SKP2A: S-Phase Kinase-Associated Protein 2A
SRDX: Superman Repression Domain X
SCF: SKP1-Cullin-F-box
Sl-IAA: Solanum lycopersicum Auxin/Indole-3-Acetic Acid
S. lycopersicum: Solanum lycopersicum
S. moellendorffii: Selaginella moellendorffii
S. tuberosum: Solanum tuberosum
SA: Salicylic Acid
TIR1: Transport Inhibitor Resistant 1
TL: Yeast selection medium without Trp and Leu

TLH: Yeast selection medium without Trp, Leu and His
TLHA: Yeast selection medium without Trp, Leu, His and Ade
TPL: Topless
TPR: Topless-related
WT: Wild-type
Y2H: Yeast two-hybrid
YFP: Yellow Fluorescent Protein
Z. mays: Zea mays

General introduction to the thesis

Objectives of the study

Auxin regulates many aspects of developmental processes including fruit set, growth and ripening. In the last decades, important progress has been made to understand how auxin is synthesized, transported and perceived. Similarly, substantial progress has been achieved regarding the auxin-related downstream transcription factors that modulate the expression of auxin-responsive genes through the binding to Auxin Response Elements (AUXRE) present in the promoter region of these gene targets. Yet, the intricate mechanisms by which these transcription factors activate or repress the transcriptional activity of auxin responsive genes in a coordinated manner remain largely unclear.

Auxin response mediators play a primary role in controlling plant developmental processes. Three gene families encoding AuxIAAs, Topless (TPLs) and Auxin Response Factors (ARFs) have been so far identified as the main players involved in auxin-dependent transcriptional regulation (Hagen and Guilfoyle, 2002; Guilfoyle et al., 1998; Causier et al., 2012a). While most of our knowledge on these auxin response regulators came from the plant model *Arabidopsis thaliana*, the Genomics and Biotechnology of Fruits (GBF) group performed a pioneering work towards genome-wide identification and subsequent isolation all members of *ARF* and *Aux/IAA* genes in the tomato, the reference species for fleshy fruit research (Zouine et al., 2014; Audran-Delalande et al., 2012).

In the last period, an increasing number of studies provided molecular clues on how Aux/IAAs and ARFs contribute to the control of specific biological processes and especially fruit development and ripening (Sagar et al., 2013; Jones et al., 2002; De Jong et al., 2011; de Jong et al., 2009; Hendelman et al., 2012; Bassa et al., 2012; Wang et al., 2005, 2009; Deng et al., 2012, 2012). In this regard, the GBF group made a substantial contribution in deciphering the role of *Sl-IAA9* in fruit set (Wang et al., 2005a, 2009). The GBF group also reported recently that down-regulation of an ARF gene member, *SlARF4*, leads to dark green and blotchy ripening in tomato indicating

that this gene plays a role in fruit development and ripening (Sagar et al., 2013). On the other hand, down-regulation of *SlARF7* or over-expression of *SlARF8* result in parthenocarpic fruit development indicating that these two *ARFs* are involved in fruit set (De Jong et al., 2011; De Jong et al., 2009; YongYao 2013 Thesis manuscript). However, functional characterization of most *ARF* genes in the tomato is still lacking and the mechanisms by which they control gene expression remain poorly understood.

The thesis research project builds on the achievement made by the GBF group on tomato *ARF* genes to better uncover their role in fruit development and to elucidate the molecular mechanism underlying their action using the most advanced genomic, proteomic and reverse genetics methodologies.

The study targets *SlARF2* based on its high expression during fruit development and ripening. The first part of the thesis project is to decipher the physiological significance of *SlARF2* in fruit development and ripening using reverse genetics approaches. The second part deals with the identification of the main protein partners of ARFs in the tomato in order to gain new insight on their mode of action.

Overall, the work addresses the putative role of auxin signaling in fruit ripening and the involvement of ARFs in this process. Within this context, the thesis study focus on the following main questions:

- Do SlARF2 regulates fruit development and ripening in the tomato and if so by which mechanism?

- What are the protein partners beside Aux/IAAs that are required for the ARF-mediated tuning of gene expression? In particular, considering that ARFs can function either as repressors or activators of gene transcription, the aim is also to uncover whether these two ARF types interact with the same partners.

The outcome of the work is expected to bring new contribution regarding our knowledge of the involvement of auxin signaling in fruit development and to provide clues on the mechanisms by which ARFs mediate auxin responses.

Main components of the thesis

Fleshy fruit share common steps for development and ripening including fruit set, fruit growth, maturation, and ripening/senescence. Fruit set normally initiates the fruit development and is dependent on the successful pollination and fertilization of the ovary. After fruit set, the fruit undergoes the growth phase via cell division and cell expansion. When the fruit reaches the final size and is mature, it is ready to ripen/senesce. The plant hormone auxin is thought to regulate to various extend these steps of fruit development even though its most prominent role has been demonstrated unambigously only in fruit setting and early growth. Auxin coordinates developmental processes through the regulation of a specific set of auxin-regulated genes. In a widely accepted scheme, auxin is first perceived by the TIR1/AFB receptors and then converted into a signal resulting in the transcriptional control of auxin-responsive genes. The auxin response is mediated by three main players in the auxin signaling pathway: the repressors (Aux/IAAs); the transcriptional factors (ARFs) and the co-repressors (TPLs). The objective of the thesis project is, (i) to investigate the role of auxin in fleshy fruit development and ripening, (ii) to uncover the components that mediate auxin response, and (iii) to uncover the mechanisms by which these components mediate the auxin-dependent regulation of gene expression.

The thesis manuscript comprises four main chapters. The first section (Chapter I) is dedicated to bibliographic reviews providing the state of the art on the role of phytohormones in driving fruit development and ripening. An important part is devoted to ethylene due to its primary role in triggering and coordinating climacteric fruit ripening. An important part of this section deals with transcription factors (RIN, NOR, CNR) shown to function as master regulators of fleshy fruit ripening like the tomato. The introduction provides a description of the tomato as reference species for fleshy fruit research and explains why it was chosen in our study as model species. Given the main focus of the thesis research project on auxin, the last part of the general introduction section is devoted to the components of auxin signaling and their

known role in fruit development. It also addresses the interactions between auxin other hormone signaling. Because the role of auxin in fleshy fruit development is rather poorly covered in the literature, it was decided to differenciate this part of the introduction into a manuscript that will be submitted for publication. This manuscript review describes components of auxin signaling and response mechanisms that are the main material of the thesis research project.

The second section (Chapter II) is dedicated to the functional characterization of SlARF2 and addresses its particular role in fruit ripening using reverse genetics approaches. This part deals with phenotypic, physiological and molecular charactrization of the tomato lines altered in the expression of Sl-ARF2. It proposes a new regulation mechanism model for climacteric fruit ripening that includes Sl-ARF2 in the loop.

The third section (Chapter III) addresses the mechanisms by which tomato ARFs modulate auxin-dependent gene expression. It comprises the search for the main protein partners of ARFs. The work mainly focuses on the tomato TOPLESS (TPL) family members known to be recruited by Aux/IAAs, the main partners of ARFs. This section first describes the isolation of all TPL family members in the tomato and the generation of a comprehensive interactome map between Aux/IAAs and TPLs established via the use of yeast two-hybrid approaches. This section is presented under the form of a published paper.

The fourth section (Chapter IV), summarizes the main scientific outcome of the thesis work and outlines the new prospects and avenues open by the findings. It mainly stresses the potential link between auxin signaling and components of the epigenetic regulation of gene expression.

The thesis manuscript also comprises additional sections dealing with the following items: (i) the list of References cited, (ii) supplemental data, (iii) a published paper describing the physiological significance of Sl-ARF4 to which I made a significant contribution.

Chapter I

Bibliographic review

Fruit development and ripening: the prominent role of ethylene

Tomato, the reference species for fleshy fruit ripening

Tomato anatomy

The tomato fruit is a berry, which develops from ovary. Upon fertilization, the ovary wall is transformed into pericarp, which consists of three distinct layers: exocarp, mesocarp, and endocarp (Fig 1). The external exocarp consists of a cuticle layer which includes an epidermal cell layer and three to four layer of a collenchymatous tissue where starch accumulates and few plastids are retained. The cuticle becomes thicker as the fruit develops (Joubès et al., 2000; Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008). The mesocarp, the intermediate layer, is a parenchymatous tissue formed by big cells with large vacuoles (Joubès et al., 2000; Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008). Finally, the endocarp, the innermost structure, consists of a single cell layer adjacent to the locular region (De Jong et al., 2009; Xiao et al., 2009; Mintz-Oron et al., 2008). The ovary is divided into two or more locules by the septa of the carpels, so the fruit can be bi- or multilocular. The placenta is a parenchymatous tissue, where the seeds are developed. The placenta will become gelatious and fill the locular cavities duiring fruit development and ripening.

Tomato Fruit development and ripening

Tomato fruit development can be divided into four stages sequencially corresponding to fruit set, cell division, cell expansion and ripening/senescence (Fig 1). At stage 1, fruit setting normally initiates the development the fruit organ. Fruit set is dependent on successful fertilization which initiates from the pollen germination, pollen tube penetration and growth in the stylar tissue towards the ovule. When the

pollen reaches the embryo sac, the pollen tube fuses with the egg cell and fertilization occurs which triggers the fruit set (Picken, 1984; Gillaspy et al., 1993). Stage 2, starting immediately after fertilization, is characterized by the activation of cell division. The high cell division activity is first found in the outer and inner pericarp, columellar and placental tissue and peripheral integument layers of the developing seeds (Gillaspy et al., 1993), then is confined to the vascular tissues, outer layer of the pericarp and in the cell layers peripheral to the seeds, at last, is restricted to the cells in the outer pericarp, the outer placenta, the vascular tissue and aslo the developing embryo (Gillaspy et al., 1993). Around two weeks after pollination, when the fruit is about 0.8-1.0 cm in diameter, the sharp fall in the rate of cell division indicates the end of this development step (Harborne, 1971; Nitsch et al., 1960). During stage 3, fruit growth relies maily on cell expansion and leads to a significant increase in weight (Bergervoet et al., 1996). Although the number and timing of cell divisions contributes to the determination of final fruit size, cell expansion makes the greatest contribution to this trait. Cells comprising the placenta, locular tissue, and mesocarp can increase by more than ten-fold during this stage (Gillaspy et al., 1993) and by the end of this step fruits have a diameter of around 2 cm (Giovannoni, 2004; Czerednik et al., 2012). Once cell expansion is complete, fruit reaches stage 4, at the beginning of which fruit enters the maturity phase leading to the mature green (MG) stage and attains its final size (Giovannoni, 2004; Czerednik et al., 2012). About two days after reaching the MG stage, and depending on the genotype, the tomato is ready to undergo the dramatic developmental process associated with ripening (http://link.springer.com/chapter/10.1007%2F978-94-009-3137-4_5). The ripening process can be distinguished into two mian phases: the breaking (BR) and the ripening (RR) stages (Fig 1). At the beginning of the breaking stage, chloroplasts convert into chromoplasts and subsequently the green color changes into yellow-orange, as a result of the carotenoid accumulation and chlorophyII degradation (Gray et al., 1992). In addition to the events described above, tomato fruit ripening is also accompanied by the accumulation of the monomeric sugars, glucose and fructose, organic acids in the vacuoles, and the production of aroma volatiles (Harborne, 1971). Finally, due to the

changes in the cell wall constituents: cellulose, hemicellulose and pectin, there are also substantial changes in the texture of the fruit (Brady, 1987). At the end of the ripening process, the abscission zone (AZ) is formed in the pedicel (Szymkowiak and Irish, 1999; Mao et al., 2000) to allow fruit to fall when it is fully mature. AZs differentiate at predetermined positions and contain a group of small cells lacking large vacuoles (Szymkowiak and Irish, 1999; Mao et al., 2000).

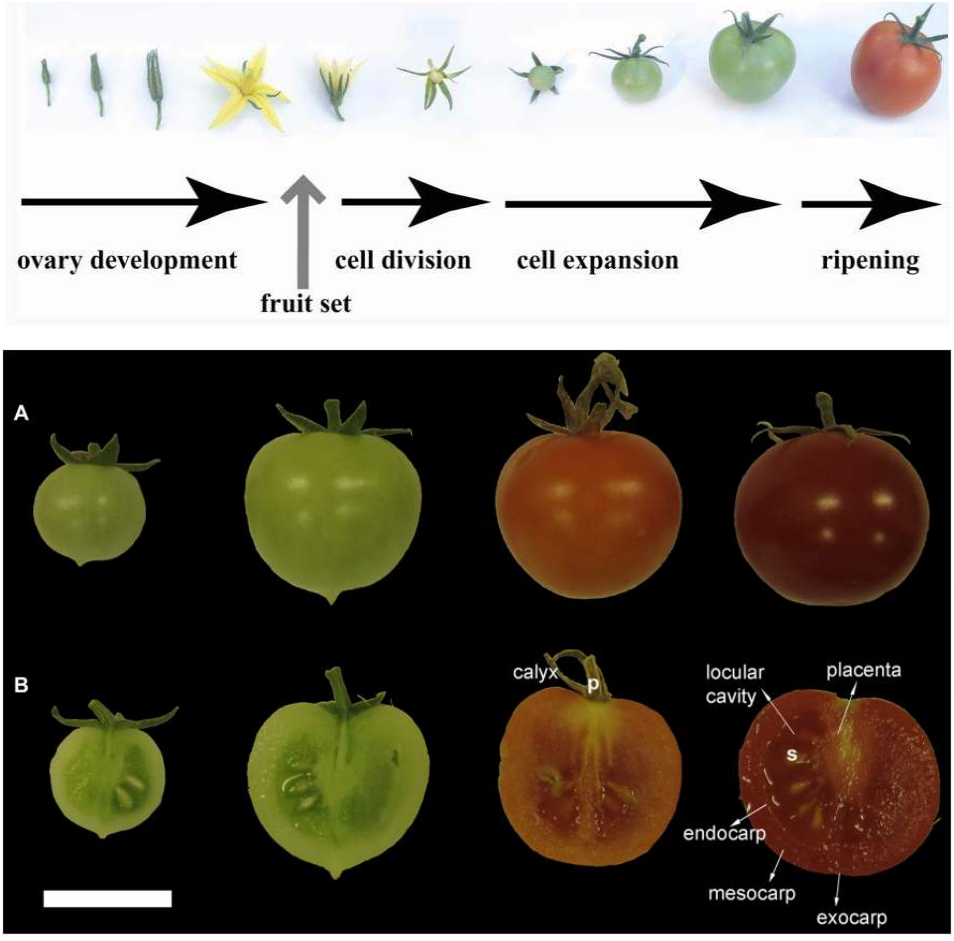

Figure 1. Different stages of tomato fruit development and anatomical details. (A) Tomato fruit development can be divided into different stages: IG, immature green; MG, mature green; BR, orange-breaker; and RR, red ripening stages are shown.(B) Transverse sections of fruits corresponding to the developmental stages shown in (A). p, pedicel; s, seed. Scale bar: 2 cm (Pesaresi et al., 2014)

Ethylene and tomato fruit ripening

Fruit development and maturation is tightly controlled by hormone homeostasis (Pandolfini, 2009). Indeed, several findings indicate that manipulation of hormone

homeostasis is able to induce fruit development and ripening (Pesaresi et al., 2014). According to the presence or absence of autocatalytic ethylene production, fruit can be divided into two types: climacteric and non-climacteric fruit (Bouzayen et al., 2010). Gaseous plant hormone ethylene plays a major role in the ripening of climacteric fruits. Tomato is a climacteric fruit and its ripening is dependent on ethylene burst. There are two systems of ethylene production in plants. System 1 is characterized by a negative feedback regulation by ethylene itself (auto-inhibition). System 1 acts during vegetative growth and during stress responses but also in young fruit at immature green stages. In system 1, exogenous ethylene inhibits synthesis and inhibitors of ethylene perception can stimulate ethylene production. In contrast, System2 is characterized by a positive feedback regulation by ethylene. System 2 functions during floral senescence and fruit ripening where it can stimulate the ethylene synthesis and where inhibitors of ethylene perception inhibit ethylene production (McMurchie et al., 1972).

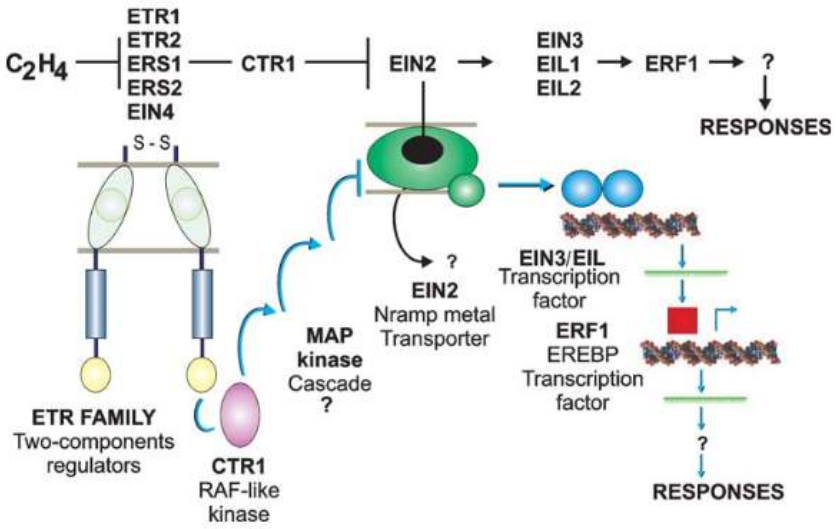

Figure 2. Genetic interactions and biochemical identities of the ethylene signal transduction pathway components. (from Bleecker and Kende, 2000).

Ethylene regulation of fruit ripening has been described for more than fifty years. So far, direct evidences demonstrating that ethylene mediates fruit ripening at the physiological, biochemical and molecular levels have been accumulated. These include ethylene biosynthesis, ethylene perception by the receptors (ETRs), signal

transduction cascade involving both positive and negative regulators (CTR, EIN2,EIN3 etc.) and finally regulation of target gene expression by transcription factors such as ethylene response factors (ERFs) (Fig 2) (Bapat et al., 2010).

Ethylene synthesis

During ethylene biosynthesis, S-adenosylm ethionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, and then, ACC is converted to ethylene by ACC oxidase (ACO) (Fig 3). There are at least 12 ACS and 7 ACO genes in the tomato genome, with temporal and tissue-specific patterns of expression (Klee and Giovannoni, 2011).

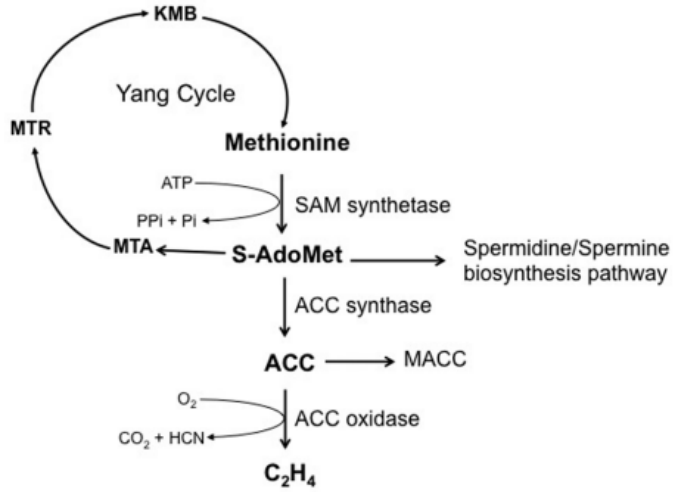

Figure 3. The ethylene biosynthetic pathway. (*from Arc et al., 2013*)

Some studies show that system 1 relies on the expression of LeACS6 and LeACS1A. Expression of LeACS6 decreases rapidly at the onset of ripening during the transition from system1 to system 2. (Barry et al., 2000; Nakatsuka et al., 1998). LeACS1A is induced during the transition from system1 to system 2 (Barry et al., 2000). LeACS2 and LeACS4 are responsible for the activation of system 2 (Barry et al., 2000; Nakatsuka et al., 1998), since both ACS genes are not expressed in green fruit but are induced at the onset of ripening (Barry et al., 2000; Nakatsuka et al., 1998). In addition, LeACO1, LeACO3, and LeACO4 are expressed at low levels in

green fruit (System 1), but the transcripts of both genes increase at the onset of ripening as the fruit transition to system 2. During ripening, LeACO1 and LeACO4 dispaly sustained expression, whereas the increase in LEACO3 expression is transient (Barry et al., 2000; Nakatsuka et al., 1998). So during ethylene biosynthesis, ACS and ACO are key genes for the control of ethylene production in fruits. Previous studies showed that down-regulation of ACS2 or ACO1 results in inhibited or delayed ripening in tomato (Hamilton et al., 1990; Oeller et al., 1991; Gray et al., 1992).

Ethylene perception

Ethylene is perceived by a specific receptor (ETR1) identified for the first time in Arabidopsis, and it was by that time the first receptor of a plant hormone to be isolated (Bleecker et al., 1988; Guzmán and Ecker, 1990; Chang et al., 1993). Subsequently, all members of the ethylene receptor gene family were isolated in Arabidpsis and then in other plant species like the tomato. Based on structural similarity, the ethylene receptors have been classified into two subfamilies (Guo and Ecker, 2004). In tomato, subfamily 1 comprises LeETR1, LeETR2 and NR (LeETR3) that share three N-terminal membrane-spanning domains and a conserved carboxy terminus histidine (His) kinase domain. Subfamily 2 lacks a complete His kinase domain and possesses an additional transmembrane-spanning domain at the N terminus (Klee, 2004; Cara and Giovannoni, 2008). In addition, all the tomato receptors possess a receiver domain at the carboxy terminus except NR (O'Malley et al., 2005; Barry and Giovannoni, 2007). The tomato ethylene receptors are differentially expressed in organs and tissues at various stages of development, but none of them seem to have strict organ-specificity (Pech et al., 2012; Barry and Giovannoni, 2007). Exogenous application of ethylene to fruit didn't induce the transcript levels of LeETR1, LeETR2 and LeETR5, but the mRNA levels of LeETR3/Nr, LeETR4 and LeETR6 increased during ripening (Kevany et al., 2007; Tieman and Klee, 1999; Wilkinson et al., 1995). Characterization of the individual functions of members of the ethylene receptor genes family was attempted via down-regulation of specific receptor isoforms using antisense suppression (Hackett et

al., 2000; Tieman et al., 2000; Whitelaw et al., 2002). Suppression of LeETR1 gene resulted in plants with shorter internodes and reduced rates of floral abscission (Whitelaw et al., 2002). Down regulation of NR resulted in slightly delayed fruit ripening with reduced rates of ethylene synthesis and slower carotenoid accumulation (Tieman et al., 2000). Meanwhile, the expression of the LeETR4 was induced in the NR antisense lines, suggesting that LeETR4 compensates for loss of NR. In contrary, down-regulation of LeETR4 lead to enhanced ethylene sensitivity, exaggerated triple response, increased floral abscission, and accelerated fruit ripening (Tieman et al., 2000). Interestingly, these phenotypes of LeETR4 antisense lines could be recovered by overexpression of a NR transgene, indicating that these two receptors are functionally redundant. In addition, suppression of LeETR4 and LeETR6 expression also leads to accelerated fruit ripening but severely affected plant growth (Kevany et al., 2007), while fruit-specific suppression of LeETR4 resulted in early-ripening fruit without affecting plant growth (Kevany et al., 2008). Interestingly, it is well admitted that the ethylene receptors act as negative regulators of ethylene action, since the antisense inhibition of NR gene was able to restore normal ripening to the tomato Nr mutant (Hackett et al., 2000).

Besides post-translational regulation, the ethylene perception is also controlled by the Arabidopsis RTE1 who acts as a negative regulator of the ethylene response. RTE1 promotes ETR1 receptor signaling, facilitating the ability of ETR1 to suppress ethylene responses in the absence of ethylene. Green-Ripe (GR) protein, a tomato homologue of RTE1, is identified in tomato. The *Gr* mutant fails to ripen as a consequence of inhibition of ethylene responsiveness due to overexpression of GR in this mutant (Fig 4) (Barry et al., 2005b). The GR protein is proposed to interact with and regulate the ethylene receptor(s) possibly via receptor-copper interaction (Zhou et al., 2007; Kendrick and Chang, 2008).

Interaction of Ethylene Receptors with CTR proteins

The Arabidopsis CTR1 protein is similar to the mammalian RAF serine/threonine MAP kinase kinas kinase (MAP3K) and acts as a negative regulator of ethylene

response (Kieber et al., 1993). In Arabidopsis, the CTR1 interacts with ethylene receptors ETR1 and ERS2 through the C-terminal domains of ethylene receptors and the N-terminus of CTR1. CTR1 is co-localized with the receptors to the ER membrane (Clark et al., 1998; Gao et al., 2003). This interaction between the receptors and CTR1 is essential for CTR1 function in repressing the downstream ethylene response (Gao et al., 2003; Zhong et al., 2008). In Arabidopsis, kinase activity is important for negative regulation in the absence of ethylene, as loss of CTR1 kinase activity leads to constitutive ethylene responses. So far, four CTR1 homologs are identified from tomato: tCTR1 (also known as ER50), tCTR2, tCTR3, and tCTR4 (Zegzouti et al., 1999; Leclercq et al., 2002; Adams-Phillips et al., 2004b, 2004a). The evidences for functional conservation between Arabidopsis and tomato *CTR* genes are: (i) Phylogenetic analysis indicates that tCTR1, tCTR3, and tCTR4 are closely related to Arabidopsis CTR1, (ii) three different tomato *CTR* genes can partially or completely complement the Arabidopsis *ctr1* mutant (Leclercq et al., 2002; Adams-Phillips et al., 2004b), (iii) CTR1, 3, and 4 show differential expression in various plant tissues (Adams-Phillips et al., 2004a, 2004b; Leclercq et al., 2002) and these tomato CTRs display ability to bind one or more of the tomato ethylene receptors in model experiments (Zhong et al., 2008). Among the tomato CTR proteins, the more divergent is tCTR2 shown to be implicated in disease resistance, stress responses that are known to be mediated by ethylene (Lin et al., 2009).

Ethylene signaling downstream of CTR

EIN2 (ETHYLENE INSENSITIVE2) is a membrane protein that functions downstream of CTR1 (Solano et al., 1998). When ethylene binds to the ethylene receptors, the inhibitory signal from CTR1 is switched off allowing EIN2 to activate the ethylene response through downstream transcription factors such as EIN3 and other EIN3-like proteins (EILs). EIN3 proteins subsequently regulate other ethylene-responsive genes in the transcription cascade. There are four EIL genes in tomato and only LeEIL4 is up-regulated during tomato fruit ripening (Tieman et al., 2001a; Yokotani et al., 2003). Furthermore, it has been suggested that these four

LeEILs function redundantly, as down-regulation of a single LeEIL did not result in changes in ethylene responses (Tieman et al., 2001b; Chen et al., 2004).

EILs and EIN3 proteins are post-transcriptionally regulated in response to the ethylene signaling pathway (Chao et al., 1997; Kendrick and Chang, 2008). In Arabidopsis, EBF (EIN3-binding F-box) proteins are proved to negatively regulate ethylene signaling via mediating the degradation of EIN3/EIL proteins through 26S proteasome (Potuschak et al., 2003; Guo and Ecker, 2003). In tomato, two F-box proteins SlEBF1 and SlEBF2 are identified and found to be regulated by both ethylene and auxin (Yang et al., 2010). Silencing of SlEBF1and SlEBF2 expression causes a constitutive ethylene response phenotype and accelerates fruit ripening (Yang et al., 2010). In addition, EIN3 protein stability can be further regulated by MAPK phosphorylation (Yoo et al., 2008). Previous studies suggested that the simultaneous activation of the MAPKK9 cascade and the inhibition of the CTR1 pathway control EIN3 levels (Yoo et al., 2009).

Ethylene Response Factors

Ethylene Response Factors (ERFs) are the last components of the ethylene transduction pathway and are responsible of the installation of the secondary response (Pirrello et al., 2012). ERFs are part of AP2 (APETALA2)/ERF super-family which also contains AP2 and RAV family genes (Riechmann et al., 2000; Riechmann and Meyerowitz, 1998; Sakuma et al., 2002). In Arabidopsis the ERF subfamily contains 65 members and is divided into 5 subclasses based on the conservation of the AP2 domain (Nakano et al., 2006). In tomato the ERF subfamily comprises 9 subclasses (Pirrello et al., 2012). Based on functional analysis of 28 tomato ERFs and through testing their ability to activate or repress transcriptional activity of target genes, it was suggested that functional activity is conserved among ERF proteins sharing the same structural features (Pirrello et al., 2012). The ERFs show tissue-specific expression patterns and bind the GCC box, a conserved motif of the *Cis*-acting element found in the promoters of ethylene-responsive genes (Ohme-Takagi and Shinshi, 1995; Solano et al., 1998), though some ERFs were shown to also bind other types of *cis*-elements

(Tournier et al., 2003). Using a dominant repressor strategy, it was recently shown that Sl-ERF.B3, a member of the ERF gene family in tomato, is involved in mediating fruit ripening and ethylene response (Liu et al., 2014a). Besides ripening, ERFs proteins are involved in a wide range of plant processes, including response to wounding, biotic stress, salt stress. ERFs have been also associated with the brassinosteroids, jasmonic acid, and salicylic acid signaling pathways (Pan et al., 2010, 2012; Park et al., 2001; Sasaki et al., 2007; Taketa et al., 2008; Oñate-Sánchez et al., 2007; Lorenzo et al., 2003; Chen et al., 2002; Brown et al., 2003; Zhang et al., 2005; Pirrello et al., 2012, 2006).

Ripening is driven by key transcriptional regulators in the tomato

A major breakthrough in dissecting the transcriptional control of tomato ripening was the identification of three pleiotropic non-ripening mutants, ripening-inhibitor (*rin*), non-ripening (*nor*), and Colorless non-ripening (*Cnr*) (Barry and Giovannoni, 2007). These mutant loci all harbor transcription factors (Thompson and others 1999). These three ripening transcriptional factors mutants severely block the ripening process and the fruit fail to produce elevated ethylene (Fig 4). The fruits remain firm and green for an extended period and do not ripen by application of exogenous ethylene (Vrebalov et al., 2002; Eriksson et al., 2004).

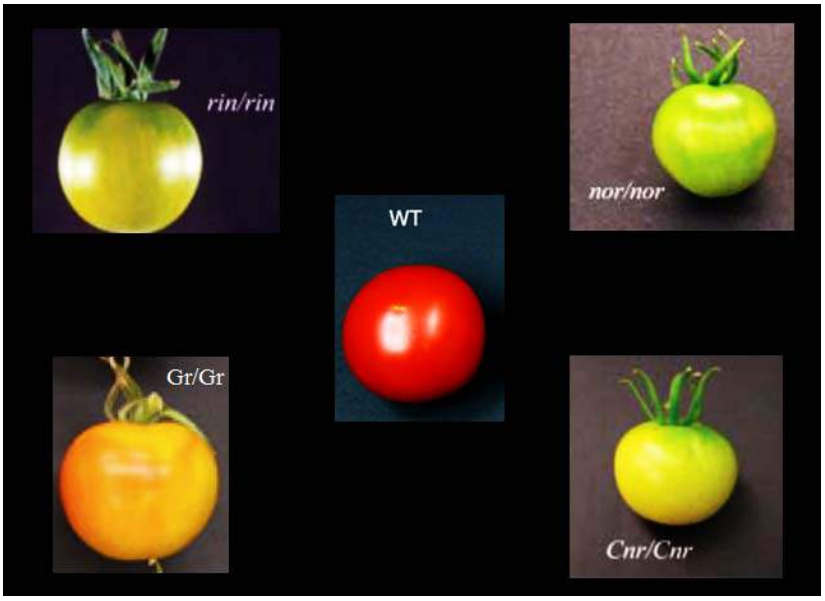

Figure 4. Normal and mutant tomato fruit. (from Giovannoni, 2004, 2007)

RIN is encoded by a member of the SEPALLATA4 (SEP4) clade of MADS-box genes. The *rin* mutation disrupts the function of RIN-MADS (Vrebalov et al., 2002). In addition, *RIN-MADS* lies very close to another *MADS-box* gene, Macrocalyx (MC), which is also silenced in *rin* plants. Antisense repression of RIN-MADS and MC confirmed that only RIN-MADS is necessary for tomato ripening. Several independent groups have described a plethora of direct targets for RIN-MADS (Ito et al., 2008; Fujisawa et al., 2011; Martel et al., 2011). Chromatin immune-precipitation experiments also show that MADS-RIN directly controls the expression of a wide range of other ripening-related genes, targeting the promoters of genes involved in the biosynthesis and perception of ethylene, such as (i) *LeACS2*, *LeACS4*, *NR* and *E8*; (ii) cell wall metabolism, such as polygalacturonase (PG), galactanase (TBG4), Endo-(1,4)-β-mannanase 4, LeMAN4; and expansins (LeEXP1); (iii) carotenoid formation, such as phytoene syn-thase (PSY1); (iv) aroma biosynthesis, such as lipoxygenase (Tomlox C), alcohol dehydroge-nase (ADH2), and hydroperoxidelyase (HPL); and (v) the generation of ATP, such as phos-phoglycerate kinase (PGK) and the promoter of *MADS-RIN* gene itself (Fujisawa et al., 2011; Martel et al., 2011; Qin et al., 2012). MADS-RIN is also involved in suppressing the expression of most ARF genes (Kumar et al., 2011) and therefore auxin-related gene expression. NOR is a member of the NAC-domain transcription factor family (Giovannoni, 2007), and nor mutant causes retardation of tomato fruit ripening with a phenotype similar to the *rin* mutant (Giovannoni, 2004). The promoter of *NOR* is also a target for MADS-RIN. CNR is encoded by an SBP-box gene, targets of which are likely to include the promoters of the SQUAMOSA clade of MADS-box genes (Thompson et al., 1999; Cardon et al., 1999; Manning et al., 2006; Vogel et al., 2010). The transcription of *CNR* can be positively stimulated by RIN-MADS. The demethylation of the CNR promoter is necessary for RIN-MADS binding. In *cnr* mutants the promoter remains hypermethylated preventing RIN-MADS from binding to it (Zhong et al., 2013).

Transcriptomic studies suggested that many more transcription factors are

potentially involved in the regulation of ripening (Vriezen et al., 2008; Pascual et al., 2009). In tomato, *ACO1*, encoding the enzyme performing the conversion of ACC into ethylene, is regulated by LeHB1, a tomato homeobox protein. LeHB1 can stimulate ethylene synthesis by activating *ACO1* expression. LeHB1 is highly expressed in developing fruits and decreased at the onset of ripening (Lin et al., 2008). Suppression of LeHB1 inhibits fruit ripening and greatly reduces *ACO1* expression levels. The promoter of *LeHB1* gene is also targeted by MADS-RIN. FUL1 (TDR4) and FUL2 (MBP7) are MADS-box transcription factors of SQUAMOSA clade (Hileman et al., 2006; Bemer et al., 2012). The FUL1 is up-regulated during fruit ripening, while FUL2 only shows a minor increase during fruit ripening. The FUL1 and FUL2 function redundantly. The down-regulation of both FUL1 and FUL2 results in ripening phenotype, and this is independent of ethylene (Bemer et al., 2012). The promoter of FUL1 is targeted by MADS-RIN and FUL1 protein can form heterodimers with MADS-RIN. TAGL1 is a member of AGAMOUS (AG) clade of MADS-box transcription factors. TAG1 is up-regulated during tomato fruit ripening. TAGL1 can activate the promoter of ACS2. Down-regulation of TAGL1 results in yellow-orange fruits and lower ethylene levels which due to the depression of ACS2. TAGL1 can form heterodimers with MADS-RIN. TAGL1 regulate lycopene accumulation in a RIN-dependent manner, while it regulates cell wall modification in a RIN-independent manner (Itkin et al., 2009; Vrebalov et al., 2009). AP2A belongs to the AP2/ETHYLENE RESPONSE FAC-TOR (ERF) family of transcription factors (Karlova et al., 2011; Chung et al., 2010). AP2A functions as a negative regulator of fruit ripening in tomato. Tomato *APETALA2a* gene (Karlova et al., 2011) controls fruit ripening by regulating genes involved in ethylene and auxin signaling pathway and down-regulation of AP2A results in rapid softening with incerased ethylene production and early ripening (Chung et al., 2010). Moreover, AP2A RNAi fruits show elevated levels of *GH3* transcripts indicating a link between AP2A and auxin-related gene expression (Karlova et al., 2011). In addition, AP2A can form a negative-feedback loop with CNR based on the following observations: (1) expression of CNR is induced in the AP2A RNAi fruit, and (2) CNR can bind to the promoter of

AP2 *in vitro*.

The role of other hormones in fruit ripening

While the prominent role of ethylene in regulating climacteric fruit ripening is now largely accepted, it has long been considered that other plant hormones, mainly Auxin, Abscissic Acid (ABA), Jasmonic Acid and Cytokines, are likely required for both the attainment of competence to ripen and the coordination of subsequent steps of fruit ripening (Abdel-Kader et al., 1966; Sun et al., 2012; Jiang et al., 2000; Jia et al., 2011; Zhang et al., 2009; Vendrell, 1985; Manning, 1994; Cohen Jerry, 1996; Davies et al., 1997; Aharoni, 2002; Davey and Van Staden, 1978). Depending on the fruit type, these phytohormones can have either agonistic or antagonistic effects on ripening. Auxin is among the first to be assigned a role in the ripening of fleshy fruits based on the observation that exogenous auxin treatment delays fruit ripening (Vendrell, 1985; Manning, 1994; Cohen Jerry, 1996; Davies et al., 1997; Aharoni et al., 2002). In tomato, crosstalk between indole-3-acetic acid (IAA) and ethylene during ripening has been reported. Ethylene production can be concomitant with an increase of IAA and auxin-signaling components can be up-regulated by ethylene and vice versa (Jones et al., 2002; Trainotti et al., 2007). In the tomato, 22 ARFs have been identified (Zouine et al., 2014) and the accumulation of some ARF transcripts has been reported to be under ethylene regulation during tomato fruit development suggesting that auxin signaling may influence the control of climacteric fruit ripening (Jones et al., 2002). More direct evidence for the involvement of auxin came later with approaches based on reverse genetics strategies (Jones et al., 2002; Liu et al., 2005; Sagar et al., 2013). For instance, SlARF4 plays a role in fruit development and ripening mainly by controlling sugar metabolism and the down-regulation of this ARF member results in ripening phenotypes such as enhanced firmness and chlorophyll content leading to dark green fruit and blotchy ripening (Jones et al., 2002; Guillon et al., 2008; Sagar et al., 2013). Nevertheless, the role of auxin in fruit ripening remains poorly understood and the underlying mechanisms and contribuiting factors are unknown.

Considering that literature reports reviewing the role of auxin in fruit development and ripening are scarce, it was decided to dedicate a specific section of the introduction to this topic. This review covers auxin signaling and response mechanisms that are the main issues dealt with in Chapter II and Chapter III of the thesis manuscript. It also addresses the interactions between auxin and components of other hormone signling. The last part deals with the role of auxin in fruit development. Moreover, given the originality of its content and considering that critical reviews on this topic is still missing, we decided to transform this part of the introduction section into a manuscript that will be submitted for publication in a refereed journal.

Review article

Review article on auxin and fruit development

(Draft manuscript)

Auxin signaling and fruit development

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Keywords: auxin, miRNA, ARF, AuxIAA, TPL, flower development, fruit set, fruit ripening

The plant hormone auxin regulates many aspects of fruit development including fruit set, growth and ripening. Auxin coordinates plant development through the regulation of a specific set of auxin-regulated genes that are appropriate for the desired developmental process. Auxin is first perceived by the TIR1/AFB receptors and then converted into a signal leading to the transcriptional control of auxin-responsive genes. The auxin response is mediated by three main players: (i) Aux/IAAs which act both as part of the hormone perception complex and as transcriptional repressors, (ii) Auxin Response Factors (ARFs) that modulate auxin-dependent gene transcription through the binding to target promoters, and (iii) the Topless which works as co-repressors. The expression of ARFs and TIR1/AFBs genes is also regulated at the post-transcriptional level by small RNAs (miRNAs or tasi-RNAs). The precise spatial and temporal expression of all these factors is critical to the coordination of fruit development and ripening. The present paper aims at reviewing the most recent knowledge on auxin signaling components and their involvement in the process of fruit development and ripening. It also highlights how these components interact with other plant hormones signaling in the context of fruit development.

Introduction

Fruit is a typical organ of the angiosperms and derives from specific tissues of the flower, often ovaries and in some cases accessory tissues. Based on their mature morphology, fruits can be fleshy or dry (Pabón-Mora and Litt, 2011). Dry fruits are characterized by harden pericarp constituting a coat that becomes dry at maturity and in many cases splits to release the mature seeds. By contrast, in fleshy fruit the wall becomes soft and fleshy as it matures. Ripe fleshy fruits become attractive for animals which play an essential role for seed dispersal. Evolutionary studies show that plant species bearing fleshy fruit evolved from ancestral dry fruit bearing species, suggesting common development and ripening mechanisms between the two fruit types (Knapp, 2002) such as fruit set, fruit growth, maturation, and ripening/senescence (Gillaspy et al., 1993; Picken, 1984; Harborne, 1971; Nitsch et al., 1960; Bergervoet et al., 1996; Czerednik et al., 2012; Gray et al., 1992; Mao et al., 2000; Szymkowiak and Irish, 1999; Adams-Phillips et al., 2004). Fruit set initiates the fruit development and is dependent on the successful pollination and fertilization of the ovary. Subsequently, the fruit enters the growth phase which includes cell division and cell expansion. When the fruit reaches its final size and becomes mature, it undergoes the ripening/senescence process (Seymour et al., 2013). The plant hormone auxin regulates these last steps of fruit development (de Jong et al., 2009; De Jong et al., 2009; Ruan et al., 2012). Exogenous auxin is able to induce fruit set, stimulate fruit growth and inhibit fruit ripening (Aharoni et al., 2002a; Davies et al., 1997; Manning et al., 2006; Vendrell, 1985). Auxin coordinates these processes through the regulation of a specific set of auxin-regulated genes. In order to be converted into a signal resulting in the transcriptional control of auxin-responsive genes, auxin is first perceived by the TIR1/AFB receptors. As depicted in Figure 1, the auxin response is known to be mediated in its downstream part by three types of transcriptional regulators: (i) the repressors Aux/IAAs, (ii) the transcriptional factors ARFs, and (iii) the co-repressors Topless (Pierre-Jerome et al., 2013; Quint and Gray, 2006; Weijers and Friml, 2009). Moreover, the expression of ARFs and TIR1/AFBs genes is also

regulated at the post-transcriptional level by small ARNs (miRNAs or tasi-RNAs) (Zouine et al., 2014; Xing et al., 2011; Si-Ammour et al., 2011; Chen et al., 2011, Vidal et al., 2010;). The present review aims at providing an overview of auxin signaling components and their involvement in the process of fruit development and ripening. It also highlights how these components interact with other plant hormones signaling in the context of fruit development.

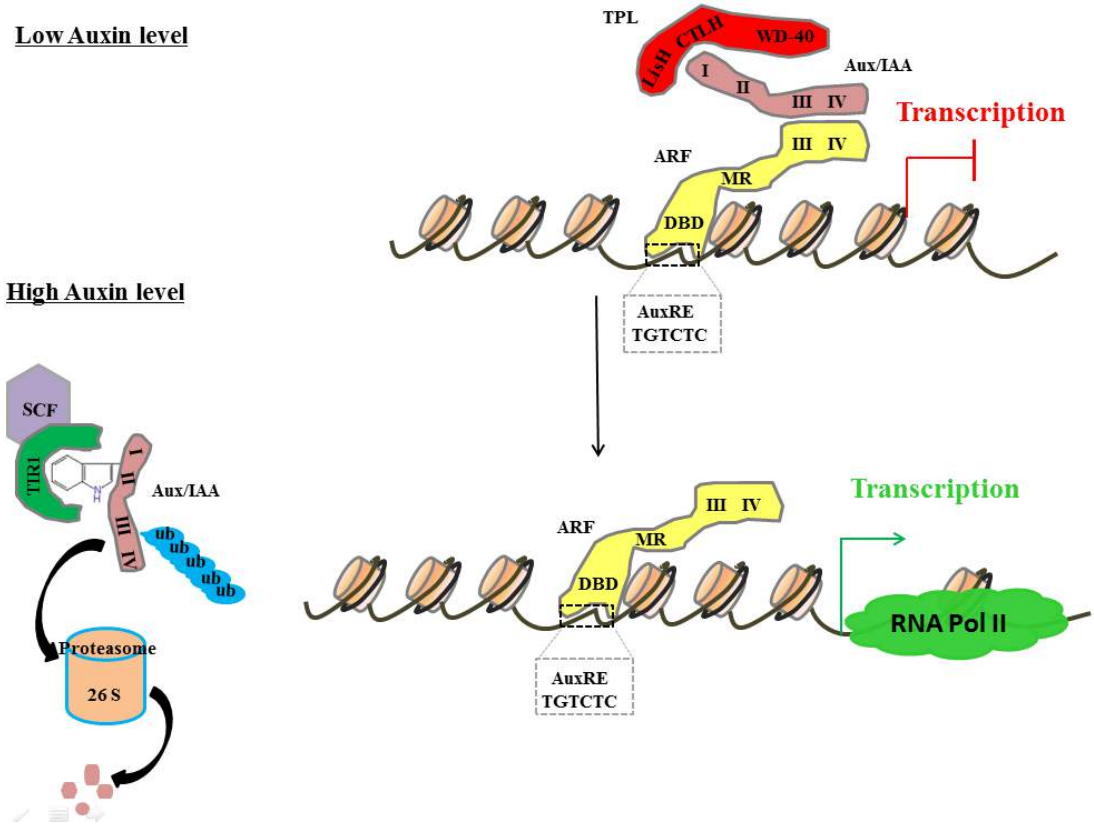

Figure 1. The TIR1 auxin signaling pathway. In the absence of auxin, Aux/IAA proteins form dimers with ARFs to inhibit their activity by recruiting the TPL co-repressors. In the presence of auxin, Aux/IAAs bind to the SCF-TIR1 complex and get subsequently ubiquitinated and degraded by the 26 S proteasome. The ARF is then released and can regulate the transcription of its target auxin responsive genes.

The TIR1/AFB receptor family in fruit development

Auxin perception results in the degradation of Aux/IAA transcriptional repressors (Mockaitis and Estelle, 2008). Auxin binds a hydrophobic pocket within the F-box protein of the SCF and acts as a molecular glue to promote high-affinity binding of an Aux/IAA protein, thus inducing its ubiquitination and degradation (Yu et al., 2013; Kepinski and Leyser, 2005; Dharmasiri et al., 2005b, 2005a; Tan et al., 2007). The

auxin receptor TIR1 contains a leucine-rich-repeat (LRR) motif that is responsible for the properties of SCF^{TIR1}. The D170E and M473L mutations in the LRR motif increase the affinity between TIR1 and Aux/IAAs and enhance the activity of the SCF^{TIR1} complex (Yu et al., 2013). TIR1 also contains a highly conserved F-box domain that interacts with CUL1, ASK1 or ASK2, and RBX1 to form SCF^{TIR1} (Ruegger et al., 1998; Gray et al., 2001). In Japanese plum (*Prunus salicina* L.), substitution of the conserved amino acid residue Pro61 to a Ser in the *F*-box domain of the TIR1-like auxin-receptor results in a reduced percption of the hormone (El-Sharkawy et al., 2014).

In Arabidopsis, besides TIR1, there are five other auxin signaling F-box proteins (AFB1-5). TIR1 and AFB1-3 function redundantly, as single mutations do not cause dramatic development defects while combing *tir1* and *afb1-3* mutations results in a severely reduced auxin response (Parry et al., 2009a; Kepinski and Leyser, 2005; Dharmasiri et al., 2005b, 2005a). AFB4 appears to be a negative regulator of auxin signaling, since AFB4 loss-of-function leads to growth defects consistent with auxin hypersensitivity (Hu et al., 2012; Greenham et al., 2011). AFB5 binds picloram, an auxin mimicking compound, with much higher affinity than TIR1, probably as a result of amino acid substitutions within the auxin-binding pocket (Irina et al., 2012; Walsh et al., 2006). These six auxin receoptors have overlapping functions and are essential for Arabidopsis growth and development (Dharmasiri et al., 2005b). Reducing the number of TIR1/AFB proteins in the plant results in increasing resistance to exogenous auxin. In the *tir1/afb* triple and quadruple mutants, anther dehiscence and pollen maturation occur earlier than in wild type, causing the release of mature pollen grains before the completion of filament elongation (Cecchetti et al., 2008). TIR1 functions during fruit development and ripening have been reported in flesh fruit producing plants. In tomato, there are at least three TIR1/AFB genes (Ben-Gera et al., 2012) and mining RNAseq expression data indicates that SlTIR1 displays constant high expression levels from flower to ripe fruit while SlAFB1 and SlAFB2 show very low expression level during flower throughout fruit ripening (Figure 2). In tomato, *SlTIR1* plays an important role in flower-to-fruit transition and its overexpression

results in parthenocarpic fruit formation and altered transcript levels of a number of auxin-responsive genes (Ren et al., 2011). Three TIR1/AFB genes have been reported in plum where the TIR1-like auxin-receptors (AFB) are thought to be involved in the regulation of plum fruit development since the contrasted fruit development and ripening of two plum cultivars depends on their differential sensitivity to auxin termined by the allelic forms of the TIR1-like auxin receptor gene (El-Sharkawy et al., 2014).

Auxin signaling is also regulated by miR393 which targets TIR1 transcripts (Si-Ammour et al., 2011; Vidal et al., 2010; Chen et al., 2011). The miR393 is encoded by MIR393a and MIR393b in Arabidopsis and Rice (Chen et al., 2011; Bian et al., 2012) and post-transcriptionally regulates TIR1/AFB (Parry et al., 2009b; Bian et al., 2012). The expression of miR393 can be induced by exogenous IAA treatment and over-expression of miR393 leads to auxin resistant phenotypes (Bian et al., 2012; Xia et al., 2012; Chen et al., 2011; Parry et al., 2009b). Loss of miR393 expression results in abnormalities in leaves and cotyledons and also in elevated expression of the primary Aux/IAA genes in Arabidopsis (Windels et al., 2014). Overexpression of a miR393-resistant form of TIR1 (mTIR1) in Arabidopsis, enhanced auxin sensitivity and led to pleiotropic effects on plant development including inhibition of primary root growth, overproduction of lateral roots, altered leaf phenotype and delayed flowering (Chen et al., 2011). In rice, over-expression of OsmiR393 results in increased tillers and early flowering (Xia et al., 2012).

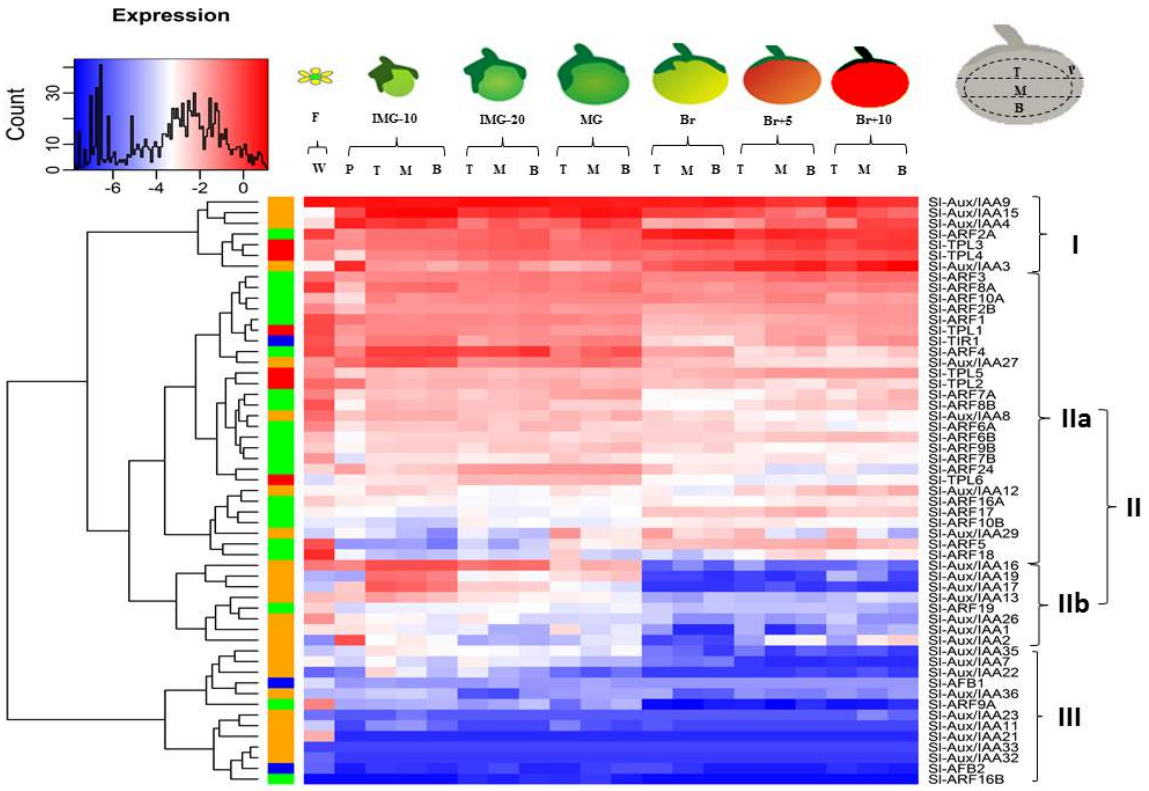

Figure 2. The expression profile of auxin signaling components in tomato from fruit initiation to ripening. The heatmap data shows that the auxin signaling components can be divided into three groups. Group I: genes exhibiting very high expression levels from flower to ripe fruit. Group II contains two subgroups, (i) IIa group genes showing constantly moderate expression levels from flower to ripe fruit, and (ii) IIb group genes displaying moderate expression levels from flower to mature green fruit that largely decreases during fruit ripening. Finally, group III contain genes with very low expression levels from flower to ripe fruit. W: whole flower; P: fruit pericarp; T: top section of the fruit; M: middle section of the fruit; B: bottom section of the fruit. F: flower; IMG-10: immature green fruit at 10 days post-pollination; IMG-20: immature green fruit at 20 days post-pollination; MG: mature green fruit; Br: breaker fruit; Br+5: breaker plus 5 days fruit; Br+10: breaker plus 10 days fruit.

The Aux/IAA co-repressor family in fruit development

Aux/IAAs function as transcriptional repressors of auxin-regulated genes and regulate the early response of the auxin signaling (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002; Tiwari et al., 2001, 2004). Typical Aux/IAA proteins are short-lived, nuclear-localized and have four conserved motifs named Domains I, II, III and IV (Audran-Delalande et al., 2012; Reed, 2001; Liscum and Reed, 2002). Domain I is the repressor domain responsible for recruiting the co-repressor Topless to inhibit the activity of ARFs. Mutation in domain I results in auxin-related phenotypes

(Szemenyei et al., 2008; Causier et al., 2012a; Tiwari et al., 2004; Li et al., 2011b; Lokerse and Weijers, 2009). Domain II contributes to the protein instability through interacting with F-box protein TIR1 (Dharmasiri et al., 2005b; Kepinski and Leyser, 2005; Tan et al., 2007). Mutations in domain II lead to elevated Aux/IAA accumulation and auxin-related phenotypes (Liscum and Reed, 2002; Reed, 2001; Uehara et al., 2008). Domains III and IV are required for protein-protein interaction with ARFs (Ulmasov et al., 1997b; Kim et al., 1997; Muto et al., 2006; Okushima et al., 2005b; Remington et al., 2004; Ulmasov et al., 1999c). Some predicted proteins lack one or more of these domains and the localization of some ARFs is not restricted to the nucleus (Wu et al., 2014; Gan et al., 2013; Nigam and Sawant, 2013; Ludwig et al., 2013; Audran-Delalande et al., 2012; Song et al., 2009; Jain et al., 2006; Reed, 2001; Ainleysq et al., 1988). For example, in tomato, Sl-IAA32 lacks domain II and Sl-IAA33 only contains a weakly conserved domain III. The repression activity of Sl-IAA32 is not affected by the lack of domain II and Sl-IAA32 protein is localized in nucleus and also in other compartments of the cell (Audran-Delalande et al., 2012; Wu et al., 2012).

Aux/IAAs belong to a large multigenic family and are found in all plants. In Arabidopsis, this gene family comprises 29 members (Liscum and Reed, 2002) while it contains 31 in rice (Jain et al., 2006) and maize (Wang et al., 2010) , 29 in cucumber (Wu et al., 2014), 9 in Gossypium hirsutum (Han et al., 2012), 25 in Tomato (Audran-Delalande et al., 2012). Aux/IAAs regulate many aspects of plant development as well as fruit development and ripening (Wang, 2005, 2009; Liu et al., 2011; Bassa et al., 2012; Deng et al., 2012a, 2012b; Tatsuki et al., 2013). Aux/IAA genes show a specific expression pattern during fruit development and ripening. In *Gossypium hirsutum*, GhAux4, GhAux5, GhAux6, GhAux 7 and GhAux8 show higher expression in ovules while GhAux 9 and GhAux 16 display highest expression during fibers development (Nigam and Sawant, 2013). In cucumber, CsIAA3 and CsIAA6 mRNAs accumulate during ovary and young fruit development in contrast to CsIAA17 and CsIAA23 that show a relative high expression during whole fruit development (Wu et al., 2014). In tomato, SlAux/IAA3, SlAux/IAA 4, SlAux/IAA 9,

SlAux/IAA15 show the highest expression levels from flower to ripe fruit process. SlAux/IAA 27, SlAux/IAA8 exhibit a constantly moderate expression level through out this proecss, while other SlAux/IAAs genes show very low levels of expression during fruit development and ripening (figure 2) (Wu et al., 2012; Audran-Delalande et al., 2012). In addition, *SlAux/IAAs* can be responsive to both auxin and ethylene two hormones important for fruit development and ripening (Audran-Delalande et al., 2012). The FaAux/IAA1 and FaAux/IAA2 from strawberry show high levels of transcripts accumulation at the green and early stages of fruit development and then decline at the turning and ripe stages. Auxin treatment on the late white fruits induces the expression of FaAux/IAA1 and FaAux/IAA2 (Liu et al., 2011). LcAux/IAA1 from litchi is induced in the abscission zone (AZ) after the treatment of girdling plus defoliation which promotes litchi fruitlet abscission implying its role in abscission (Kuang et al., 2012). Aux/IAA mutants exhibit multiple reduced auxin response phenotypes on seed, flower and fruit. Tomato Sl-IAA9 antisense lines exhibit early fruit initiation resulting in parthenocarpy fruit (Wang et al., 2005 and 2009). SlIAA15 down-regulated lines show decreased flower number and reduced fruit set efficiency (Deng et al., 2012a). Under-expression of Sl-IAA27 results in altered fruit shape and smaller fruit with reduced seed number and fruit set efficiency (Bassa et al., 2012). Aux/IAAs function redundantly in Arabidopsis so only gain-of-function mutants display altered auxin response phenotypes (Fukaki et al., 2002, 2005, 2006, 2007; Uehara et al., 2008; Overvoorde et al., 2005). Some At-Aux/IAA gain of function mutants display phenotypes related to fruit development. The stamen of AtIAA16-1 mutant is unable to reach the stigma before dehiscence resulting in the absence of seeds in mutant fruits (Enders et al., 2013). The pIAA8::GFP-mIAA8 mutant shows abnormal flower phenotypes with short petal, sepal, stamen and bent stigma as a result of mutated domain II of IAA8 (Wang et al., 2013).

The Auxin Response Factor (ARFs) family in fruit development

The Auxin Response Factors (ARFs) are transcription factors that regulate auxin signaling through binding to the promoter of auxin-responsive genes and interacting

with repressor Aux/IAAs (Guilfoyle et al., 1998; Tiwari et al., 2003). The first Arabidopsis ARF is originally identified by a yeast one-hybrid screen using the auxin-responsive element TGTCTC as a bait sequence (Ulmasov et al., 1997a, 1995). Typically ARFs possess three domains, an N-terminal DNA binding domain (DBD), a variable middle region (MR) and a C-terminal dimerization domain (CTD) (Guilfoyle et al., 1998; Tiwari et al., 2003; Zouine et al., 2014). The DBD domain is a plant specific B3 type domain found in many types of plant transcription factors (Guilfoyle et al., 1998). The ARF DBD domain has been shown to bind the TGTCTC Auxin Response Elements (AuxREs) on the promoter of auxin-regulated genes to allow activation or repression of the transcription of these target genes (Ulmasov et al., 1999a). The activity of ARF as activator or repressor is determined by the composition of the ARF middle region. ARFs with AD type middle region are rich in glutamine(Q), serine (S), and leucine (L) residues and function as activators whereas ARFs with RD type middle region that are rich in proline (P), serine (S), threonine (T), and glycine (G) residues function as repressors (Guilfoyle et al., 1998; Tiwari et al., 2003; Ulmasov et al., 1999a). The ARF C-terminal dimerization domain (CTD) is also found in Aux/IAA proteins referred to as domain III and IV. The ARF CTD domain is responsible for forming ARF homodimers or Aux/IAA-ARF heterodimers (Ulmasov et al., 1999c; Guilfoyle and Hagen, 2007).

There are 23 ARFs in Arabidopsis, 25 in rice (Oryza sativa), 39 in Populus trichocarpa, 24 in sorghum (Sorghum vulgare), 31 in *Brassica rapa* and Maize, 51 in Soybean and 22 in tomato (Kalluri et al., 2008; Wang et al., 2007; Xing et al., 2011; Shen et al., 2010; Wu et al., 2011; Sato et al., 2001; Mun et al., 2012; Wang et al., 2012; Ha et al., 2013; Zouine et al., 2014). So far, all the ARFs studied in different species are shown to be targeted to the nucleus (Kalluri et al., 2008; Wang et al., 2007; Xing et al., 2011; Shen et al., 2010; Wu et al., 2011; Sato et al., 2001; Mun et al., 2012; Wang et al., 2012; Ha et al., 2013; Zouine et al., 2014). Atypical ARF contains the canonical domains (B3, MR, and CTD) though some ARFs lack the CTD domain whereas some others contain only the DBD domain but whether or not these ARFs are functionally active remains to be elucidated (Kalluri et al., 2008; Wang et al., 2007; Xing et al.,

2011; Shen et al., 2010; Wu et al., 2011; Sato et al., 2001; Mun et al., 2012; Wang et al., 2012; Ha et al., 2013; Zouine et al., 2014). The repression and activation activities of tomato ARFs were asessed using a single cell system co-transfected with a reporter construct harboring the synthetic DR5 auxin-responsive promoter fused to the GFP coding sequence and an effector construct allowing the expression of an ARF protein (Zouine et al., 2014). Interactions between ARFs and Aux/IAAs was performed by yeast two hybrid system indicating that activator ARFs show strong ability to interact with most Aux/IAA proteins in contrast to repressor ARFs which display weak or no affinity to Aux/IAAs (Shen et al., 2010). The ARFs lacking the CTD domain do not interact with Aux/IAAs but they are still capable to repress or activate transcription on the DR5 promoter (Zouine et al., 2014). *ARF* genes encoding proteins with only the DBD domain are predicted to be pseudogenes (Wang et al., 2007; Zouine et al., 2014). The expression of *ARFs* can be induced or repressed by exogenous auxin and ethylene consistent with the presence in many ARF promoters of auxin and ethylene cis-regulatory elements, which suggests that ARFs possess the ability to mediate both auxin and ethylene responses (Zouine et al., 2014). Several ARFs are found to be post-transcriptionally regulated by microRNAs or transacting small interfering RNAs, siRNAs (Zouine et al., 2014; Xing et al., 2011). A single small RNA can potentially regulate different ARFs. That is, in Arabidopsis and tomato, ARF10, ARF16 and ARF17 are negatively regulated by mir160 (Liu et al., 2007; Mallory et al., 2005; Wang et al., 2005b) and overexpression of miR160 in Arabidopsis leads to root tip defects similar to that displayed by *arf10 / arf16* double mutant (Wang et al., 2005b). Overexpression of an *ARF10* gene resistant to mir160 in tomato results in narrow leaflet blades, sepals and petals, and abnormally shaped fruit (Hendelman et al., 2012). ARF6 and ARF8 are targeted by mir167 (Nagpal et al., 2005; Wu et al., 2006). And inhibition of mir167 results in impaired organogenesis throughout the plant (Gutierrez et al., 2009). Mutations in the miR167 target sites of ARF6 and ARF8 leads to overaccumulation of transcripts corresponding to these two genes and results in arrested ovule growth and defective anthers unable to release pollen (Wu et al., 2006). Overexpression of miR167 leads to floral development defects and female sterility in

tomato which may give rise to parthenocarpic fruit (Liu et al., 2014b). ARF2, ARF3 and ARF4 are post-transcriptionally regulated by TAS3 ta-siRNA, whose formation involves miR390 (Williams et al., 2005). The repression of TAS3 drastically impairs the normal development of flowers and leaves (Fahlgren et al., 2006; Garcia et al., 2006; Hunter et al., 2006).

In the last period, an increasing number of studies pointed to the role of ARFs in regulating fruit development and ripening. ARFs are shown to regulate dry fruit development in Arabidopsis (Ellis et al., 2005; Rensing et al., 2008; Lim et al., 2010b; Okushima et al., 2005; Schruff et al., 2006; Sessions and Zambryski, 1995; Tantikanjana and Nasrallah, 2012; Liu et al., 2014), and fleshy fruit in tomato (Jones et al., 2002; Guillon et al., 2008; De Jong et al., 2009; de Jong et al., 2011; Hendelman et al., 2012; Sagar et al., 2013). *ARF* genes show specific expression patterns during flower and fruit development (Kalluri et al., 2008; Wang et al., 2007; Xing et al., 2011; Shen et al., 2010; Wu et al., 2011; Sato et al., 2001; Mun et al., 2012; Wang et al., 2012; Ha et al., 2013; Zouine et al., 2014). In Arabidopsis, some ARF loss-of-function mutants shows phenotypes on both flowers and fruit. *At-ARF2* mutants display abnormal flower morphology, delayed development related to aging including initiation of flowering, rosette leaf senescence, floral organ abscission and silique ripening, and also seeds with increased size and weight (Ellis et al., 2005a; Hughes et al., 2008; Lim et al., 2010; Okushima et al., 2005a; Schruff et al., 2006). The At-ARF2 homologue in maize, ZmARF25, affects cell proliferation and its down-regulation results in reduced organ size in Arabidopsis (Li et al., 2014). *At-ARF3* mutant shows impaired gynoecium and floral development (Sessions and Zambryski, 1995; Tantikanjana and Nasrallah, 2012) whereas At-ARF6 and At-ARF8 were shown to regulate both stamen and gynoecium development (Liu et al., 2014; Glazińska et al., 2014). Arabidopsis *arf6 / arf8* double mutant display infertile closed buds with short petals, short stamen filament and undehisced anthers that do not release pollen (Goetz et al., 2007; Jay et al., 2011; Nagpal et al., 2005). Mutations in At-ARF8 result in the formation of seedless perthenocarpic fruit and Sl-ARF8 may

control tomato fruit initiation in a similar manner than At-ARF8 (Liu et al., 2014; Goetz et al., 2007). In addition, At-ARF8 can interact with BREp to affect petal growth. At-ARF8 mutant (*arf8-3*) produces larger petals than wild type due to increased cell number and expansion (Varaud et al., 2011). In tomato, SlARF2A shows a very high expression level from flower to ripe fruit process. With the exception of SlARF9A and SlARF16B, all other tomato ARFs display a constantly moderate expression level during fruit development and ripening (figure 2). In tomato, SlARF7 shows high levels expression in mature flowers and unpollinated mature ovaries. It is down-regulated within 48 hours after pollination. Down-regulation of SlARF7 causes parthenocarpic fruit with heart-shaped and a rather thick pericarp that can be interpreted as an auxin response phenotype (de Jong et al., 2011; De Jong et al., 2009). SlARF10 is important for early fruit development and outgrowth of auxin-mediated blade, because increasing mSlARF10 levels in tomato results in narrow leaflet blades, sepals and petals, and abnormally shaped fruit (Hendelman et al., 2012). SlARF4 plays a role in fruit development and ripening, fruit tissue architecture and also sugar metabolism. Down-regulation of SlARF4 enhances fruit firmness as a result of the pectin fine structure, increases chlorophy II content associated with a higher number of chloroplasts leading to dark green fruit and blotchy ripening, and also increases sugar content in the fruit (Guillon et al., 2008; Jones et al., 2002; Sagar et al., 2013). Down-regulation of ARF6 and ARF8 by miRNA 167 in tomato leads to floral development defects and female sterility (Liu et al., 2014).

The transcriptional co-repressors topless (TPLs) in fruit development

Topless (TPLs) is a transcriptional regulator co-repressors of the GROUCHO family (Liu and Karmarkar, 2008). Canonical TPL proteins possess three conserved domains: Lissencephaly (LisH) domain, C-terminal to LisH (CTLH) domain and two WD40-repeat domains (Kieffer et al., 2006; Liu and Karmarkar, 2008). LisH domain and CTLH domain are responsible for the interaction between the TPL/TPR co-repressors and partner transcription factors (Gallavotti et al., 2010; Szemenyei et

al., 2008). In Arabidopsis, the first TPL gene is identified as responsible for the semi-dominant *tpl-1* embryo development mutation resulting in altered polarity, ranging from fused cotyledons to complete replacement of the shoot with a second root (Long et al., 2006, 2002). Subsequently, 5 TPL/TPR family members were isolated (TPL, TPR1, TPR2, TPR3, TPR4). As a quintuple loss of function, in which all five TPL/TPR genes are inactivated by mutation or RNA interference, is required to phenocopy the tpl-1 phenotype, it was concluded that the five *TPL/TPR* genes function redundantly (Long et al., 2006). So far, the characterization of topless family members in plant species carrying fleshy fruit only occured in tomato. Six *SlTPLs* genes were isolated all encoding proteins that localize to nucleus with the exception of Sl-TPL6. Tomato *TPL* genes show different expression patterns (figure 2) with *SlTPL3* and *SlTPL4* displaying constant and high expression levels during fruit development and ripening suggesting their putative role in these proceses (Hao et al., 2014).

Topless is also reported to be related to meristem maintenance, floral induction, biotic stress, and circadian oscillator mechanism (Causier et al., 2012a; Liu and Karmarkar, 2008; Pauwels et al., 2010a; Szemenyei et al., 2008; Zhu et al., 2010). In maize, the transcription factor RAMOSA1 (RA1) controls the development of inflorescences, branches, tassel and ear by regulating the axillary meristems. The *ra1* and *ra2* mutants display an increasing long branches formation in ears leading to lower yield. The *REL2* gene, which encodes a topless protein, is strongly expressed in inflorescence, branch and spikelet-pair meristems and floral organs. REL2 interacts with RA1 protein. *Rel2* mutants enhance the phenotypes of the *ra1* and *ra2* mutant (Gallavotti et al., 2010). OsREL2, the REL2 homologue in rice, exhibits a relatively low expression through the rice inflorescence development. The *rel2* rice mutant shows shorter and decreased number of branches resulting in reduced grains yield (Kwon et al., 2012). In rice, the genes giving high grains yield elevate the numbers of branches and spikelet (Ikeda-Kawakatsu et al., 2009; Miura et al., 2010; Jiao et al., 2010; Ookawa et al., 2010). ASP1, a TPL-related protein, shows a strong expression in the branches and spikelet meristems and the lateral organ primordia of the spikelet. Moreover, the

asp1 mutant produces lower number of normal spikelet and shorter, bleached abnormal branches leading to a lower grains yield (Yoshida et al., 2012). The WUSCHEL gene is responsible for floral meristem integrity in Arabidopsis (Laux et al., 1996). The interaction between WUS and TPLs is essential for the WUS function. TOE1 and TOE2, repressors of the flowering-time gene interact with all five TPL/TPRs. The flowering delay in the 35S::TOE1 lines is abolished in the *tpl-1* mutant background. TPLs also interact with other repressors of the flowering-time gene such as: TEM1, AP2, AGL15 (Causier et al., 2012).

TPL/TPR proteins can use multiple chromatin-remodeling mechanisms to induce transcriptional repression (Causier et al., 2012). In particular, they induce local chromatin compaction at target sites through association with chromatin remodelers such as histone deacetylases (HDACs). Histone acetylation is largely correlated with gene expression (Figure 3); therefore, removal of these modifications by HDACs generally leads to repression of transcription (Shahbazian and Grunstein, 2007). In Arabidopsis, TPL acts through HDA19 and interactions between TPR1 and HDA19 can be observed in pull-down experiments from plant extracts. Mutations in HDA19 increase the penetrance of *tpl-1* and display similar apical defects (Gonzalez et al., 2007; Long et al., 2006; Sridhar et al., 2004). Besides histone deacetylases, large interactome studies in Arabidopsis show that TPL/TPR proteins interact with some histone methyltransferases such as SDG19 (SUVH3); PKR1; EMF1, VRN5(Causier et al., 2012). SDG19 also called SUVH3 is a SET domain protein catalyzing the methylation of histone H3 Lys residue 9 resulting in nucleosome compaction and gene silencing (Pontvianne et al., 2010; Zhao and Shen, 2004). PKR1 is a protein related to the PICKLE (PKL) CHD3/Mi-2-like chromatin remodeler (Ogas et al., 1999; Zhang et al., 2008), which represses the expression of seed-associated genes during germination by promoting the methylation of histone H3 Lys residue 27 (Ogas et al., 1999; Zhang et al., 2008). EMF1 is a component of Polycomb Repressive Complex 1 PRC1 (Calonje et al., 2008), while VRN5 is a component of Polycomb Repressive Complex 2 PRC2 (Greb et al., 2007). PRC2 catalyzes the trimethylation of histone H3 on lysine 27 (H3K27 trimeth) (Cao et al., 2002). PRC1 binds to this mark through its

subunit POLYCOMB (PC) and catalyzes the mono-ubiquitylation of lysine 118 of histone H2A (H2AK118ub) (Wang et al., 2004) (Figure 3). The sequence of these events finally leads to gene silencing through the mechanisms involving chromatin compaction (Figure 3).

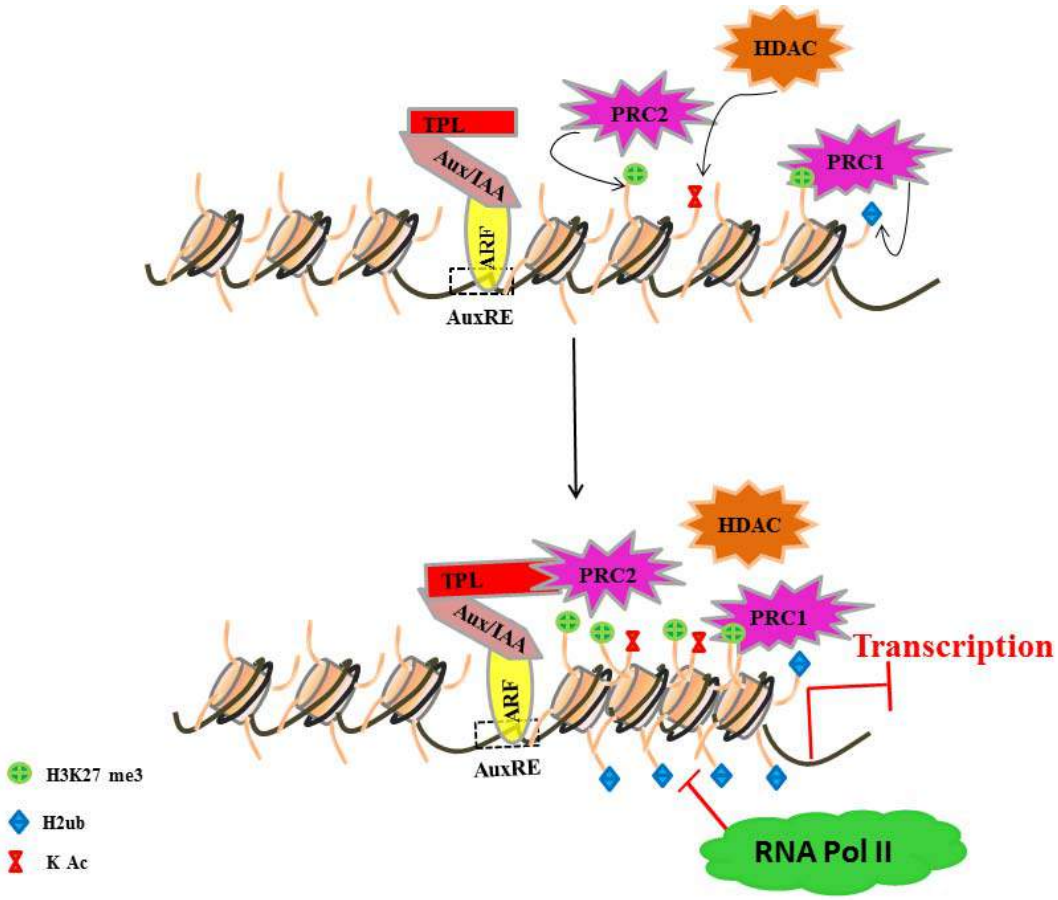

Figure 3. Gene silencing through the mechanisms involving chromatin compaction. PRC2 induces H3K27me3. H3K27me3 recruits PRC1 that ubiquitylates H2AK119 promoting chromatin compaction and gene silencing. Deacetylation of the target gene by HDACs generally leads to chromatin compaction. PRC2 associates with histone deacetylases, reinforcing transcriptional repression and providing functional synergy to stable silencing of target genes.

So far, these interaction data are only described in Arabidopsis which produces dry fruit. However, studies on the components of PRC2 complex homologues in tomato showed that mutation in some of these components lead to fruit phenotypes that are related to auxin. SlEZ1 is one component of PRC2 and SlEZ1 RNAi plants exhibit abnormal flower morphology and fruits with small size and fewer seeds and increased number of locules (How Kit et al., 2010). SlFIE, another component of PRC2,

interacts with EZ2 and its down-regulation results in flowers with increased sepal and petal numbers, fused ovule and pistil and parthenocarpic fruit (Liu et al., 2012).

Auxin signaling components affect other plant hormone responses

It well known that fruit development and ripening rely not only on auxin (Figure 4) but also on the combined action of other plant hormones such as gibberellin, abscisic acid, ethylene and brassinosteroid (Ziosi et al., 2009; Kondo and Fukuda, 2001; Ziosi et al., 2008; Carbonell-Bejerano et al., 2011; Serrani et al., 2007; Jia et al., 2011a; Chai et al., 2012; Symons et al., 2006; Motyka et al., 2003; Li et al., 2011a; Jia et al., 2011b; Zaharah et al., 2013; Jiang et al., 2000). Most likely, from flower initiation to fruit ripening, auxin functions through cooperating with these plant hormones (Ziosi et al., 2009; Kondo and Fukuda, 2001; Ziosi et al., 2008; Carbonell-Bejerano et al., 2011; Serrani et al., 2007; Jia et al., 2011a; Chai et al., 2012; Symons et al., 2006; Motyka et al., 2003; Li et al., 2011a; Jia et al., 2011b; Zaharah et al., 2013; Jiang et al., 2000). As described above, auxin signaling components have been reported to be involved in these hormones signaling pathway. The Aux/IAAs and ARFs can be induced or reduced by auxin and by other phytohormones such as gibberellin (GA) (de Jong et al., 2011; De Jong et al., 2009), ethylene (Zouine et al., 2014; Audran-Delalande et al., 2012), jasmonate acid (JA) (Nagpal et al., 2005), abscise acid (ABA) (Wang et al., 2011) and brassinosteroid (BR) (Walcher et al., 2008). Fruit set can be triggered by application of auxin and gibberellin (Ruiz-rivero et al., 2007; McAtee et al., 2013; Jong et al., 2009) and auxin appears to act at least partly through gibberellin, as it can induce gibberellin biosynthesis early during fruit development (Ruiz-rivero et al., 2007). SlARF7, acts as a modifier of both auxin and gibberellin responses, and regulates part of the auxin and GA signaling pathways. Down-regulation of SlARF7 results in parthenocarpic fruit as a result of both increased auxin and gibberellin response during fruit growth (de Jong et al., 2011; De Jong et al., 2009) (Figure 4). Abscisic acid (ABA) is thought to be related to the expansion phase in tomato (Gillaspy et al., 1993; McAtee et al., 2013) and the ABA-deficient mutants produce smaller fruit (Nitsch et al., 2012). Application of

exogenous ABA promotes starch hydrolysis (Sun et al., 2012), enhances the onset of breaker stage and accelerates tomato ripening (Zhang et al., 2009a). In the dry dehiscent fruit Arabidopsis, increased ABA levels promotes silique maturation and dehiscence (Kanno et al., 2010; Kou et al., 2012). The mutation of domain II in At-IAA16 results in reduced response to auxin and ABA and also in impaired plant growth and fertility (Enders et al., 2013). The expression of At-ARF2 can be induced by ABA and *At-ARF2* mutant shows enhanced ABA sensitivity indicating that At-ARF2 links ABA and auxin signaling (Wang et al., 2011). At-ARF10 and At-ARF16 are required to control the expression of Abscisic Acid Insensitive3 (ABI3) which is a major downstream component of ABA signaling regulating seed dormancy and ABA inhibition of seed germination. The over-expression of miR160 leads to plants with enhanced seed dormancy (Liu et al., 2013) (Figure 4).

The gasous plant hormone ethylene is a crucial component of normal ripening in climacteric fruit (Gapper et al., 2013) and exogenous ethylene can accelerate fruit ripening and silique abscission (Lelievre et al. 1997) . In Arabidopsis, ARF19 is induced by ethylene and contributes to ethylene sensitivity through a cross-talk between auxin and ethylene signaling (Li et al., 2006). At-ARF2 and SlARF2 are reported to regulate hook curvature, a typical ethylene response of etiolated seedling (Chaabouni et al., 2009a and 2009b; Li et al., 2004). In the developing siliques of *arf2-6* mutant, the expression of the ethylene synthesis genes *ACS2*, *ACS6* and *ACS8* is impaired suggesting that At-ARF2 might play a role in connecting auxin and ethylene signaling (Okushima et al., 2005) (Figure 4).

Brassinosteroids have a role in fruit ripening of strawberry and grape (Chervin et al., 2004; Carbonell-Bejerano et al., 2011; Zaharah et al., 2013; Chai et al., 2012; Symons et al., 2006). Brassinosteroids have also been reported to affect cell expansion during fruit growth and may have a role in fruit set (Fu et al., 2008). The homone cis-regulatory elements Up at Dawn (HUD)-type E-box and AuxRE-related TGTCT are both necessary for auxin and brassinosteroids response and treatment with both hormones enhances the binding of At-ARF5 and brassinosteroid insentitivel-EMS suppressors target promoters (Walcher et al., 2012). At-ARF2 interacts with the

brassinosteroid regulated BIN2 Kinase and At-ARF2 are supposed to integrate the auxin and brassinosteroid pathway (Walcher et al., 2008). The key transcription factors in the BR signaling pathway BZR1 can bind to the promoter of both IAA19 and ARF7. The BR regulates the growth of Arabidopsis hypocotyles through auxin signaling components IAA19 and ARF7 (Zhou et al., 2013). In rice, BR and auxin are implicated in grain yield. OsARF19 is induced by auxin and BR and can direct the expression of OsCH3-5 and OsBRI1 by binding to their promoters. OsARF19 overexpressing lines are sensitive to BR treatment and alter the expression of genes related to BR signaling (Zhang et al., 2014) (Figure 4).

The plant hormone Jasmonate (JA) modulates anther dehiscent, fruit ripening and plant resistance to insect (McAtee et al., 2013; Ziosi et al., 2008, 2009). The Arabidiosis double mutant of *arf6 arf8* shows delay in the elongation of floral organs and inhibition of the opening of flower buds with a decreased levels of JA indicating that At-ARF6 and At-ARF8 modulate flower development through mediating JA levels (Nagpal et al., 2005). The At-ARF6 and At-ARF8 interact with At-IAA8 and the pIAA8::GFP-mIAA8 mutant also shows similar abnormal flower phenotypes with decreased JA levels indicating that the At-IAA8 regulate floral organ development by changing JA levels via its interaction with ARF6/8 proteins (Wang et al., 2013) (Figure 4).

Though there is not enough data supporting that the TOPLESS (TPL) transcriptional co-repressor is involved in fruit development and ripening, the screening for Topless interacting partners indicated that TPL proteins might be involved in multiple plant hormones signaling pathways (Figure 4). Aux/IAAs interact with TPL to form a complex that represses the transcriptional function of ARFs (Peer, 2013). In Arabidopsis, there are 5 TPL/TPR family members interacting with 20 out of the 29 AtIAA proteins (Causier et al., 2012). Some repressor ARFs, like ARF2 and ARF9, can also interact directly with TPL/TPRs (Causier et al., 2012b). In the moss *Physcomitrella patens*, there are 2 PpTPL members. The moss Aux/IAAs interact with all the TPL/TPRs but only with repressor ARFs (Causier et al., 2012). In tomato, 6 TPLs members are isolated and shown to interact with most of the Sl-Aux/IAAs.

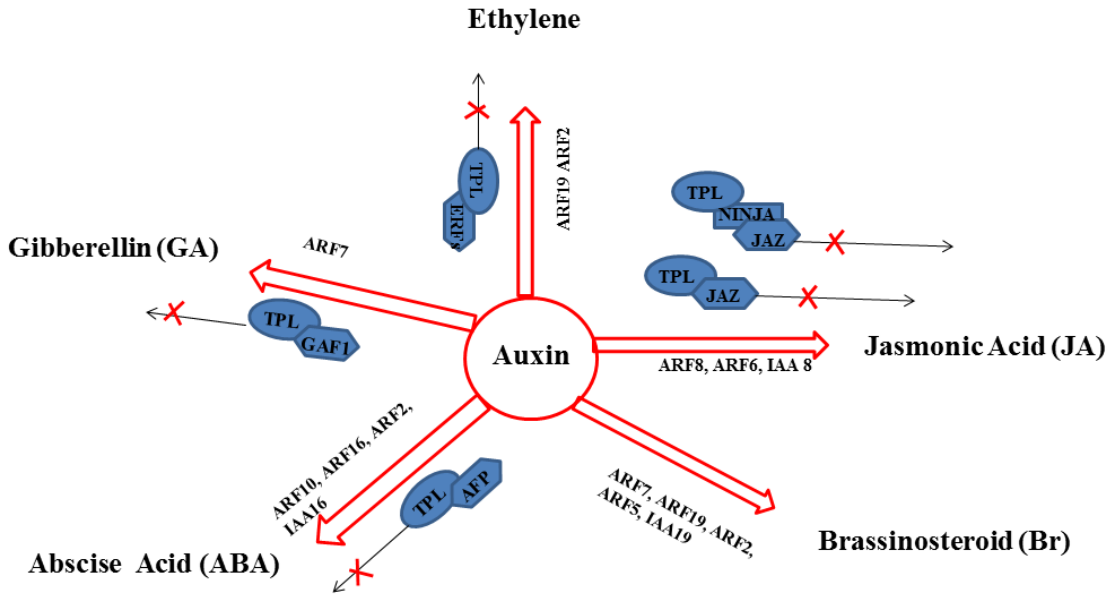

Figure 4. Auxin signaling components affect other plant hormone responses. Auxin can affect Jasmonic Acid responses via ARF6, ARF8, IAA8 and TPL which can interact with JAZ to modulate transcription of JA regulated genes. Auxin can potentially affect Etylene responses via ARF2, ARF19, and TPL which is able to interact with ERFs. Auxin may also affect Gibberellic Acid responses via ARF7 and TPL which interacts with GAF1. Auxin affects Abscisic Acid responses via ARF16, ARF10, ARF2, IAA16 and TPL which interacts with AFP. Finally, Auxin can affect responses to Brassinosteroids by ARF2, ARF5, ARF7, ARF19 and IAA9.

Topless seem to be a central component of hormone-dependent inhibition of gene transcription. Indeed, in Arabidopsis, JAZ, the transcriptional regulators of JA signaling pathway interact with TPLs through an adapter protein NINJA indicating that JAZ represses gene expression by recruiting TPLs (Santner and Estelle, 2007; Pauwels et al., 2010) On the other hand, AFPs, the negative regulators of ABA signaling, interact with TPLs indicating that TPLs are involved in ABA signaling pathway (Lopez-molina et al., 2003; Causier et al., 2012). Ethylene response factors ERFs which are induced by ethylene, high-salt conditions, drought stress, and pathogen attack interact with TPLs. Finally, DELLAs are negative regulators of GA signaling and GAF1, the DELLA binding transcription factor, interacts with TPLs to modulate gene expression (Fukazawa et al., 2014; Causier et al., 2012). All of these plant hormones are important for the fruit from initiation to ripening. Taking together

these data support the idea that Topless is a common player mediating multiple hormone signaling and resposes. Moreover, given the role of the above described hormones in fruit development and ripening, it is likely Topless is also a major player in fruit development.

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Introduction to Chapter II

Introduction to Chapter II

Apart from ethylene, auxin also plays a role during fruit ripening by interplay with ethylene or other hormones. Auxin response facotrs (ARFs), as one of the important components for auxin signaling, are well known for their involvement in the regulation of plant development processes, including the auxin signaling and crosstalk between auxin and ethylene. It has been reported that mRNA accumulation of ARF (auxin response factor) family genes were regulated by ethylene during tomato fruit development in a tissue-specific manner suggesting that auxin signaling may influence ripening control of climacteric fruits (Jones et al., 2002). The *SlARF4* is one member of *ARFs* family. It plays a role in fruit development and ripening and also sugar metabolism, down-regulation of *SlARF4* enhances fruit firmness, increases chlorophyll content associated with a higher number of chloroplasts leading to dark green fruit and blotchy ripening, and also increases sugar content in the fruit (Sagar et al., 2013). In tomato, 22 *ARFs* have been identified. Several *ARFs* can be regulated by both ethylene and auxin, which imply their potential contribution to the convergence mechanism between the signaling pathways of these two hormones (Zouine et al., 2014). Among all *ARFs*, *SlARF2* displays the most prominent transcript accumulation during fruit development and ripening (Zouine et al., 2014). It has also shown that SlARF2 can be modulated by auxin and ethylene via IAA3 and HLS protein to regulate hypocotyl bending (Chaabouni et al., 2009a,b) but the role of ARF2 during fruit ripening remains to be clearly established. To address the function of SlARF2 homologs during fruit ripening, we generated transgenic lines that were either specifically silenced for *SlARF2A* or *SlARF2B* or silence for both. In my thesis, chapter II will describe the role of SlARF2 during fruit development and ripening through the following part: The identification, expression pattern, auxin and ethylene response, the physiological molecular analyses of the SlARF2 mutant.

Chapter II

Auxin Response Factor SlARF2, a new component of the regulatory mechanism controlling fruit ripening in tomato

(Manuscript in preparation)

Title page

Auxin Response Factor SlARF2, a new component of the regulatory mechanism controlling fruit ripening in tomato

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Running Title: SlARF2A and SlARF2B regulate fruit ripening

Key words: Auxin response factor (ARF), ethylene, auxin, tomato, SlARF2A, SlARF2B, fruit ripening

ABSTRACT

Ethylene is a major regulator of climacteric fruit ripening whereas the putative role of other phytohormones in this process remains poorly understood. The present study brings auxin into the mechanism regulating tomato fruit ripening via addressing the physiological significance of *SlARF2* (Auxin Response Factor), encoding a downstream component of auxin signaling and responses. In the tomato, SlARF2 is encoded by two genes, *SlARF2A* and *SlARF2B*, both shown here to act as transcriptional repressors and to exhibit distinct responsiveness to ethylene and auxin and a marked ripening-associated pattern of expression. Specific down-regulation of either SlARF2A or SlARF2B resulted in ripening defects while simultaneous silencing of both genes led to more severe ripening inhibition phenotypes suggesting a functional redundancy among the two orthologs. *SlARF2* under-expressing fruits produced less climacteric ethylene and the expression of key regulators of ripening, such as RIN, CNR, NOR and TAGL1 was dramatically down-regulated in SlARF2 under-expressing lines. While exogenous ethylene treatment failed to reverse the non-ripening phenotype, molecular analysis revealed a disturbed pattern of expression of ethylene signaling and biosynthesis genes. Altogether, the data further extend our knowledge on the role of auxin in fleshy fruit development and set SlARF2 as a new component of the regulatory network controlling the ripening process in tomato.

INTRODUCTION

Fruit ripening is a complex, genetically programmed process that is associated with dramatic metabolic and textural transformation including color changes, fruit softening, accumulation of sugar and production of flavor and aroma compounds (Alexander and Grierson, 2002; Adams-Phillips et al., 2004a; Giovannoni, 2004). Ultimately, the ripening process leads to fruit withering allowing dispersal of the seeds. Based on their type of ripening mechanism, fleshy fruits can be divided into climacteric and non-climacteric (Oeller et al., 1991; Theologis et al., 1993; Gray et al., 1992; Ayub et al., 1996). Climacteric fruit ripening is characterized by autocatalytic increase in ethylene biosynthesis (Lelievre et al., 1997), and it is widely accepted that this hormone is the main trigger and coordinator of the ripening process. Accordingly, several genes involved in ethylene metabolism and signaling have been shown to be essential for fruit ripening in tomato and reducing ethylene production via suppression of ethylene biosynthesis genes, ACC synthase (ACS) and ACC oxidase (ACO), leads to the inhibition of fruit ripening (Hamilton et al., 1990; Oeller et al., 1991; Nakatsuka et al., 1998). Likewise, the tomato Never-ripe (*Nr*) mutant, bearing an altered allele of the ethylene receptor gene *ETR3*, also shows non-ripening phenotype due to reduced ethylene sensitivity (Rick and Butler, 1956; Lanahan et al., 1994). On the other hand, silencing of either LeETR4 or LeETR6 with a fruit-specific promoter causes enhanced ethylene sensitivity and early ripening phenotype (Kevany et al., 2008, 2007). EIN3-Binding Factors, EBF1and EBF2, are F-BOX proteins responsible for the degradation of EIN3 protein, a downstream component of ethylene signaling. Repression of tomato SlEBF1/SlEBF2 causes constitutive ethylene responses and early fruit ripening (Yang et al., 2010). In concert with ethylene, the control of fruit ripening also involves other key regulators, some of which have been functionally characterized. For example, silencing of the homeobox protein LeHB1 results in delayed ripening (Lin et al., 2008) and *MADS-box* genes like *RIPENING-INHIBITOR* (*RIN*) and *TOMATO AGAMOUS-LIKE 1* (*TAGL1*) are proved to dramatically affect

fruit ripening (Vrebalov et al., 2002; Ito et al., 2008; Itkin et al., 2009; Vrebalov et al., 2009). *COLORLESS NON-RIPENING* (*CNR*), a SQUA-MOSA promoter binding protein (SBP), is shown to directly influence the expression of *RIN* or other *MADS-box* genes during fruit ripening (Manning et al., 2006; Pech et al., 2012). The *rin* and *cnr* mutants produce fruits that remain firm and green for an extended period, deficient in ethylene production and unable to ripen upon exogenous ethylene (Tigchelaar and McGlasson, 1978; Manning et al., 2006).

Without minimizing the role of ethylene, it has long been considered that other plant hormones are likely required for both the attainment of competence to ripen and the coordination of subsequent steps of fruit ripening. In this regard, old physiologists used to mention that the control of such a highly coordinated and complex process is driven by a subtle hormonal balance. Auxin is among the first to be assigned a role in the ripening of fleshy fruits as adding auxin to mature fruit has been shown to delay ripening (Vendrell, 1985; Manning, 1994; Davies et al., 1997; Cohen Jerry, 1996; Aharoni et al., 2002b). More direct evidence for the involvement of auxin came later with approaches based on reverse genetics strategies (Davey and Van Staden, 1978; Rolle and Chism, 1989; Jones et al., 2002; Liu et al., 2005; Wang et al., 2005, 2009; Ireland et al., 2013; Sagar et al., 2013). Auxin signaling is known to regulate the expression of target genes mainly through two types of transcriptional regulators, namely, Aux/IAA and Auxin Response Factors (ARF). ARFs can be either transcriptional activators or repressors through direct binding to the promoter of auxin-responsive genes (Ulmasov et al., 1997b; Guilfoyle et al., 1998; Ulmasov et al., 1999b; Guilfoyle and Hagen, 2007; Ulmasov et al., 1999d; Audran-Delalande et al., 2012; Li et al., 2012; Zouine et al., 2014). In the tomato, 22 ARFs have been identified (Zouine et al., 2014) and the accumulation of some ARF transcripts has been reported to be under ethylene regulation during tomato fruit development suggesting that auxin signaling may influence the control of climacteric fruit ripening (Jones et al., 2002). Recently, it was shown that SlARF4 plays a role in fruit development and ripening mainly by controlling sugar metabolism and the down-regulation of this ARF member resulted in ripening phenotypes such as

enhanced firmness and chlorophyll content leading to dark green fruit and blotchy ripening (Jones et al., 2002; Guillon et al., 2008; Sagar et al., 2013).

To further extend our knowledge on the role of ARFs in fleshy fruit development and ripening the present work addresses the physiological significance of SlARF2 which displays a marked ripening associated pattern of expression. Because SlARF2 is encoded by two genes in the tomato, *SlARF2A* and *SlARF2B*, transgenic lines either specifically silenced in one of the two orthlogs or simultaneously for both genes were generated. In both SlARF2A and SlARF2B down-regulated lines, fruits produced less ethylene than wild type and failed to ripen normally. The expression of key regulators of fruit ripening, such as *RIN*, *CNR*, *NOR* and *TAGL1* was down-regulated in *SlARF2* under-expressing lines suggesting that this *ARF* gene is a new component of the regulatory network controlling the ripening process in tomato.

METHODS

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* L. *cv* MicroTom) seeds were sterilized, washed by sterile water 5 times, and sown in Magenta vessels containing 50ml of 50% Murashige and Skoog (MS) medium added 0.8% (w/v) agar, pH 5.9. The transgenetic plant were transferred to soil and grown under standard greenhouse conditions (Sagar et al., 2013). Conditions in the culture chamber room were set as follows: 14-h-day/10-h-night cycle, 25/20 °C day/night temperature, 80% relative humidity, 250 $mol.m^{-2}.s^{-1}$ intense light (Liu et al., 2014a).

Plant transformation

Three cDNA fragments specific to *SlARF2A* or *SlARF2B* or both were cloned into pHellsgate 12 vector independently, with primers in the Supplemental Table 1.Transgenic plants were generated by Agrobacterium-mediated transformation according to Bird (Bird et al., 1988) with minor changes: 6 days old cotyledons were used for the transformation; the duration of subcultures for shoot formation was

reduced to 15 days; and the kanamycin concentration was 70 mgL^{-1}. The constructs were under the transcriptional control of the CamV 35S and the Nos terminator (Sagar et al., 2013) .

Sequence structure and promoter Analysis

The structure of the *SlARF2A* and *SlARF2B* were determinate using *in silico* approaches (software: Fancy Gene V1.4). Protein domains were first predicted on the prosite database protein (http://prosite.expasy.org/) (Hao *et al.*, 2014). Promoter sequences of *SlARF2A* and *SlARF2B* genes were analyzed using PLACE signal scan search software (http://www.dna.affrc.go.jp/PLACE/signal scan.html).

Flower emasculation and cross assay

Flower buds of *DR5:GUS* transgenic plants were emasculated before dehiscence of anthers (closed flowers) to avoid accidental self-pollination. Cross-pollination was performed on *DR5:GUS* emasculated flowers with pollen from wild type, *SlARF2A RNAi*, *SlARF2B RNAi*, and *SlARF2AB RNAi* plants independently.

Subcellular localization of SlARF2A and SlARF2B

For localization of SlARF2A and SlARF2B proteins, two CDS sequences were cloned by Gateway technology as a C-terminal fusion in frame with green fluorescent protein (GFP) into the pGreen-GFP vector, and expressed under the control of the 35S CaMV promoter. The pGreen-GFP empty vector was used as the control. Protoplasts were obtained from tobacco suspension-cultured (*Nicotiana tabacum*) BY-2-cells and transfected according to the method described previously (Leclercq et al., 2005). GFP localization by confocal microscopy was performed as described previously (Audran-Delalande et al., 2012).

Transient expression using a single cell system

For co-transfection assays, the coding sequence of SlARF2A and SlARF2B were seperately cloned into the pGreen vector and expressed under the control of the 35S

CaMV promoter. The synthetic DR5 promoter containing AuxRE and the promoter of *SlARF2B* were cloned in frame with GFP reporter gene in pGreen vector independently. Protoplasts were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2-cells and transfected according to the method described previously (Leclercq et al., 2005). After 16 h of incubation in the presence or absence of 2.4-D (50 µM), GFP expression was analyzed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA, USA) as indicated in Hagenbeek and Rock (2001). All transient expression assays were repeated at least three times.

Gus staining and analysis

To visualize GUS activity, transgenic lines bearing the promoter of *DR5* fused with GUS constructs were incubated with GUS staining solution (0.1% Triton X-Gluc, pH7.2, 10 mM EDTA) at 37°C overnight. After GUS staining, samples were decolorized using several washes of graded ethanol series (Sagar et al., 2013).

Auxin, ethylene, and 1-MCP treatment

For auxin treatment on light grown seedlings, 21-day-old *DR5::GUS* seedlings were soaked in liquid MS medium with or without (mock treatment) 20 µM IAA for 2 hours. For auxin treatment on fruit, mature green fruits were injected with 20 µM IAA and kept for 6 hours at room temperature. For ethylene treatment on fruit, mature green fruits were treated with air or ethylene gas (50 $\mu L.L^{-1}$) for 5 hours. For 1-MCP treatment, 1.0 $mg.L^{-1}$ 1-MCP was applied into the breaker stage fruits for 16 hours. For qPCR expression analysis, the tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Ethylene production and ethylene response

Fruits from different developmental stages were harvested and incubated in opened 125-ml jars for 3 hours to remove the wound ethylene production caused by picking. Jars were then sealed and incubated at room temperature for 2 hours, and1 ml of

headspace gas was injected into an Agilent 7820A gas chromatograph equipped with a flame ionization detector (Agilent, Santa Clara, CA, USA). Samples were compared with 1 ml L^{-1} ethylene standard and normalized for fruit weight. For ethylene response assay, mature green fruits from wild-type and *SlARF2AB RNAi* lines were treated by 10 ml L^{-1} ethylene for 3 days, 2 hours and 3 times per day.

Firmness measurement

Fifteen fruits from each line of the *SlARF2AB RNAi* and wild type were harvested at the Breaker (Br) stage. The firmness was then assessed using Harpenden calipers (British Indicators Ltd, Burgess Hill, UK) as described by Ecarnot et al., (2013).After the first measurement, these fruits were kept at the room temperature for measuring the firmness day by day.

Color measurement

Twenty fruits for each line of the *SlARF2AB RNAi* and wild type were harvested at the Br stage. The hue angle values were calculated according to the methods previously described (Sagar et al., 2013). After measurement, these fruit were kept at the room temperature and were measured day by day until fruits got fully red.

RNA Extraction and Quantitative RT-PCR

Different stage fruits were harvested, the pericarp were frozen in the liquid nitrogen, stored in the -80 °C. Total RNA extraction, DNA contamination removing, cDNA generation of tomato tissues (root, stem, leaves, bud, flower, mature green fruit, breaker fruit, and red fruit) and qRT-PCR were performed according to methods previously described (Audran-Delalande et al., 2012; Pirrello et al., 2006). The primer sequences are listed in the Supplemental Table 3. Actin was used as the internal reference. Three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to real-time PCR analysis in triplicate.

Accession number

The sequences of genes used for the qPCR can be found in the website (http://solgenomics.net/) under the following solyc numbers: *Sl-ERF.A1* (Solyc08g078180), *Sl-ERF.A2* (Solyc03g093610), *Sl-ERF.A3* (Solyc06g063070), *Sl-ERF.B1* (Solyc05g052040), *Sl-ERF.B2* (Solyc02g077360), *Sl-ERF.B3* (Solyc05g052030), *Sl-ERF.C1* (Solyc05g051200), *Sl-ERF.C2* (Solyc04g014530), *Sl-ERF.C3* (Solyc09g066360), *Sl-ERF.C6* (Solyc03g093560), *Sl-ERF.D1* (Solyc04g051360), *Sl-ERF.D2* (Solyc12g056590), *Sl-ERF.D3* (Solyc01g108240), *Sl-ERF.D4* (Solyc10g050970), *Sl-ERF.E1* (Solyc09g075420), *Sl-ERF.E2* (Solyc09g089930), *Sl-ERF.E3* (Solyc06g082590), *Sl-ERF.E4* (Solyc01g065980), *Sl-ERF.F1* (Solyc10g006130), *Sl-ERF.F2* (Solyc07g064890), *Sl-ERF.F3* (Solyc07g049490), *Sl-ERF.F4* (Solyc07g053740), *Sl-ERF.F5* (Solyc10g009110), *Sl-ERF.G1* (Solyc01g095500), *Sl-ERF.G2* (Solyc06g082590), *Sl-ERF.H1* (Solyc06g065820), *PSY1* (Solyc03g031860), *PDS* (Solyc03g123760), *ZDS* (Solyc01g097810), *β-LCY1* (Solyc04g040190), *β-LCY2* (Solyc10g079480), *CYC-β* (Solyc06g074240), *ACS2* (Solyc01g095080), *ACS4* (Solyc05g050010), *ACO1* (Solyc07g049530), *E4* (Solyc03g111720), *E8* (Solyc09g089580), *PG2a* (Solyc10g080210), *RIN* (Solyc05g012020), *CNR* (Solyc02g077850), *NOR* (Solyc10g006880), *HB1* (Solyc02g086930), *TAGL1* (Solyc07g055920), *AP2a* (Solyc03g044300), *EIN2* (Solyc09g007870), *EIL2* (Solyc01g009170), *EIL3* (Solyc01g096810), *ETR1* (Solyc12g011330), *ETR2* (Solyc07g056580), *ETR3* (NR) (Solyc09g075440), *ETR4* (Solyc06g053710), *ETR5* (Solyc11g006180), *ETR6* (Solyc09g089610), *CTR1* (Solyc10g083610), *ACS1* (Solyc08g081550), *ACS3* (Solyc02g091990), *ACS6* (Solyc08g008100), *FUL1* (Solyc06g069430), *FUL2* (Solyc03g114830), *SGR1* (Solyc08g080090), *ACO2* (Solyc12g005940), *ACO3* (Solyc07g049550), *ACO4* (Solyc02g081190). The solyc number of Sl-ARFs can be found in the publication of Zouine (Zouine et al., 2014).

RESULTS

Sl-ARF2 is encoded by two genes in the tomato with distinct expression patterns

It was recently shown that some members of the *ARF* gene family, encoding auxin transcriptional mediators, can play a critical role in regulating the ripening of tomato fruit (Jones et al., 2002; Sagar et al., 2013). To gain better insight on the putative involvement of members of the ARF family in the ripening process of fleshy fruits, *in silico* mining of the available tomato expression databases was performed revealing that among all *ARFs*, *SlARF2* displays the most prominent transcript accumulation during fruit development and ripening (Zouine et al., 2014). This prompted a more thorough molecular and functional characterization of this ARF member. In contrast to Arabidopsis, ARF2 is encoded by two genes in the tomato named *SlARF2A* (Solyc03g118290.2.1) and *SlARF2B* (Solyc12g042070.1.1) and located in chromosome 3 and 12, respectively (Zouine et al., 2014). The two genomic clones share similar structural organization with, however, 14 exons in *SlARF2A* but only 13 in *SlARF2B*. The full-length cDNAs of the two *SlARF2* genes were isolated by RT-PCR amplification indicating that the corresponding coding sequences (CDS) are 2541 bp and 2490 bp long with deduced protein sizes of 847 amino acids and 830 amino acids, respectively (Table 1). Pairwise comparison of the two SlARF2 protein sequences revealed 83.3% amino acid identity. Search for protein domains in Expasy database (http://prosite.expasy.org/) indicated the presence of highly conserved domains typical of ARFs in the two proteins: DBD domain (DNA Binding Domain) and dimerization domain (protein/protein domain III and IV) (Figure 1A). Analysis of a 2 kb promoter sequence using PLACE/signal search tool (http://www.dna.affrc.go.jp/PLACE/signalscan.html) revealed the presence of putative Ethylene Response (ERE) and Auxin Response (AuxRE) elements in both *SlARF2A* and *SlARF2B* promoters (Figure 1A).

Assessing transcript accumulation by quantitative-RT-PCR confirmed that the two *SlARF2* genes show distinctive ripening-associated patterns of expression (Figure 1B). *SlARF2A* and *SlARF2B* are expressed in all plant tissues tested including root, leaf, stem, flower and fruit with a higher transcript accumulation for *SlARF2A* in both vegetative and reproductive tissues. Noteworthy, the transcript levels corresponding to the two *ARF2* genes undergo a net up-regulation at the onset of fruit ripening (Figure

1B). The expression studies suggested that the tomato SlARF2A and SlARF2B are likely to play an active role in flower and fruit development especially as related to ripening.

Table 1. Main structural features of the tomato SlARF2A and SlARF2B.

Nomenclature	***Gene***			***Predicted Protein***		***Domains***	
SlARF2	***iTAG Gene ID***	***Exons***	***Introns***	***Length***	***MW (kDa)***	***DBD***	***Dimerization domain***
SlARF2A	Solyc03g118290.2.1	15	14	847 aa	94.01358	146-248	721-803
SlARF2B	Solyc12g042070.1.1	14	13	830 aa	92.46828	128-230	704-785

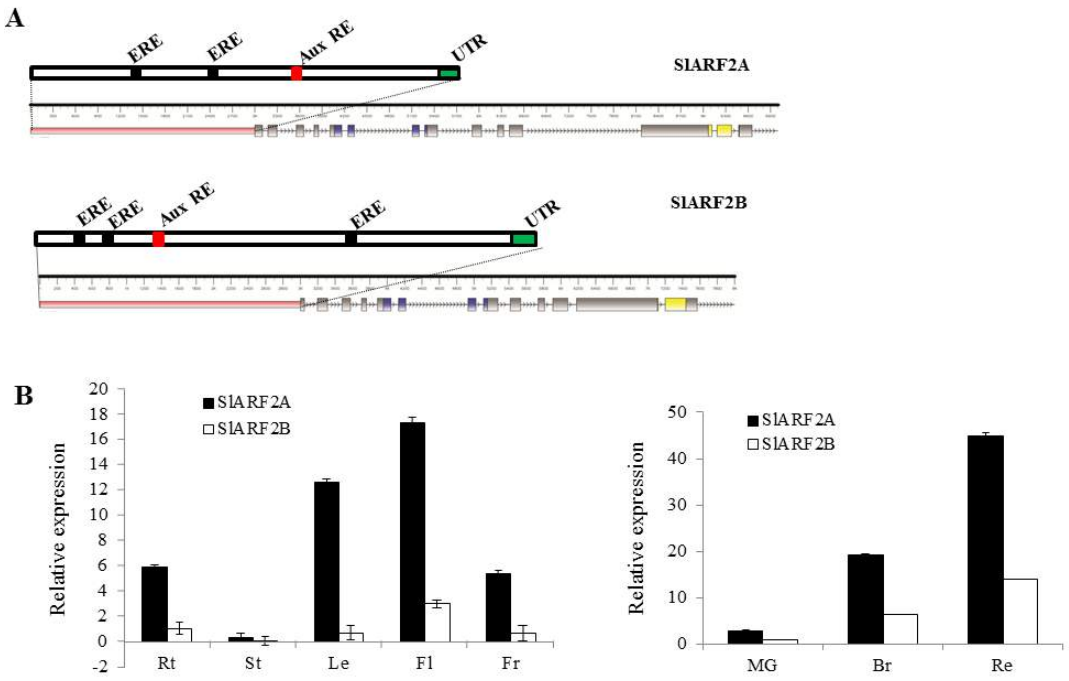

Figure 1 Structural features and expression patterns of tomato SlARF2A and SlARF2B genes. (A) Genomic structure analysis of *SlARF2A* and *SlARF2B* Gene were performed by using Fancy gene V1.4. The pink portion represents the promoter region; the strandlines represent the introns; the gray boxes indicate the exons; the yellow boxes are the dimerization regions; blue box means the DBD domain; ERE, ethylene responsive element; Aux RE, auxin regulation element. (B) Expression pattern of *SlARF2A/2B* was monitored by (Quantitative Real-Time RT-PCR) qPCR. Total RNA was extracted from leaf (Le), stem (St), root (Rt), flower (Fl), fruit (Fr), mature green fruit (MG), breaker fruit (Br) and red fruit (Re). The relative mRNA levels of *SlARF2A* in root and at the mature green (MG) stage were standardized to 1.0, referring to the *Sl-Actin* gene as an internal control. The error bars represent ±SE of three independent trials.

SlARF2A and _SlARF2B_ are differentially regulated by auxin and ethylene.

The presence of conserved AuxRE and ERE *cis*-regulatory elements in the promoter region of both genes and the induced expression of *SlARF2A* and *SlARF2B* in developmental processes known to be regulated by both hormones prompted the investigation of their responsiveness to auxin and ethylene. Genes known to be ethylene (E4, E8) and auxin (GH3, SAUR) responsive were used as control to validate the efficiency of hormone treatment. Transcript accumulation assessed by RT-qPCR in mature green fruit indicated that *SlARF2A* but not *SlARF2B* is responsive to exogenous ethylene treatment (Figure 2A), and that this ethylene-induced expression is repressed by 1-MCP, the inhibitor of ethylene perception (Figure 2B). By contrast, *SlARF2B* expression was up-regulated by auxin in mature green fruit, while *SlARF2A* showed no responsiveness to auxin treatment (Figure 2C).

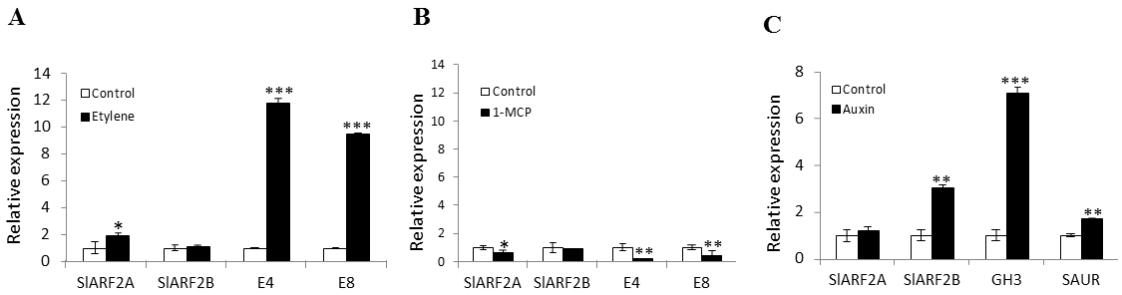

Figure 2. Auxin and ethylene responsiveness of SlARF2A and SlARF2B genes. (A) Quantitative RT-PCR analysis of SlARF2A and SlARF2B after ethylene treatment. The wide-type mature green fruits were treated or untreated with 50 ml L^{-1} ethylene for 5 hours. (B) Quantitative RT-PCR analysis of *SlARF2A* and *SlARF2B* after 1-MCP treatment. The wide-type breaker fruits were treated or untreated with 1-MCP (1.0 mg L^{-1})for 16 hours. (C) Quantitative RT-PCR analysis of *SlARF2A* and *SlARF2B* after auxin treatment. The wide-type mature green fruits were treated with 20 μM IAA or buffer (control) for 6 hours. The relative mRNA levels of SlARF2A/SlARF2B in control were standardized to 1.0, referring to the Sl-Actin gene as an internal control. The error bars represent ±SE of three independent trials. *0.01 < P < 0.05, ** 0.001<P<0.01, ***P < 0.001 (Student's t-test). E4, E8: ethylene response genes; GH3, SAUR: auxin response genes.

SlARF2A and SlARF2B are targeted to the nucleus where they act as transcriptional repressor of auxin-responsive genes

The subcellular localization of the SlARF2A and SlARF2B proteins was assessed

using translational fusion to the Green Fluorescent Protein (GFP) in a transient expression assay in tobacco protoplasts. Microscopy analysis clearly showed that SlARF2A/2B:GFP fusion proteins exclusively localized into the nucleus (Figure 3A), consistent with a putative role in transcriptional regulation activity. The ability of SlARF2A/2B proteins to regulate the activity of auxin-responsive promoters was then evaluated in a single cell system. A reporter construct, consisting of the synthetic auxin-responsive promoter DR5 fused to GFP (Ottenschläger et al., 2003), was co-transfected into tobacco protoplasts with an effector construct allowing the constitutive expression of SlARF2A or SlARF2B protein. As expected the DR5-driven GFP expression was strongly enhanced by auxin (2,4-D) treatment. However, the presence SlARF2A or SlARF2B proteins strongly inhibited this auxin-induced activity of DR5 promoter, clearly demonstrating that SlARF2A and SlARF2B act *in vivo* as a transcriptional repressor of auxin-dependent gene transcription (Figure 3B).

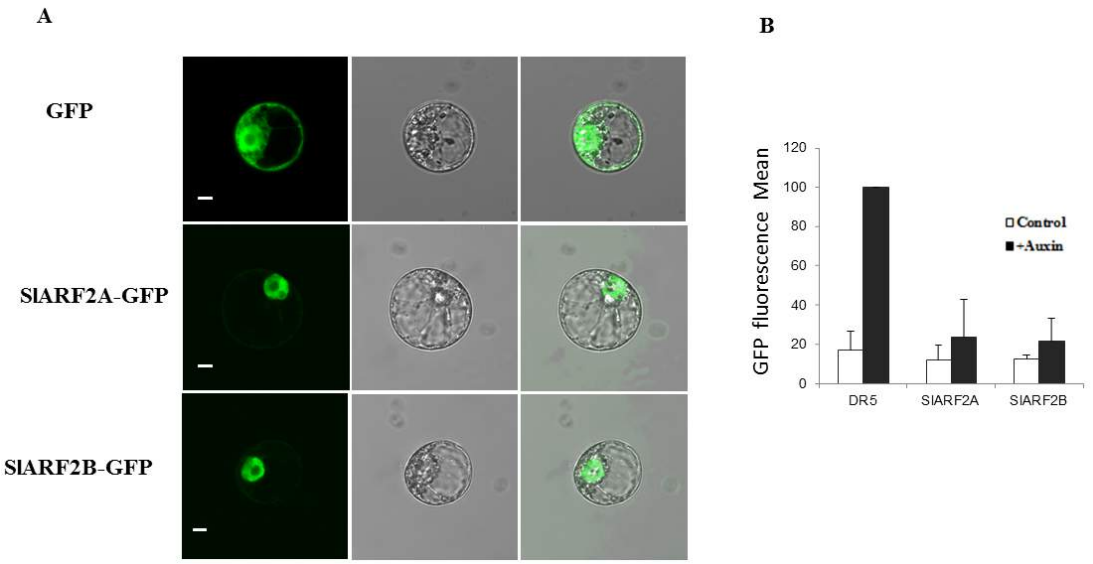

Figure 3. Subcellular localization and functional analysis of SlARF2A and SlARF2B by signal cell system. (A) SlARF2A/2B-GFP fusion proteins were transiently expressed in BY-2 tobacco protoplasts and subcellular localization was analyzed by confocal laser scanning microscopy. The merged pictures of the green fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown in the right panels. The scale bar indicates 10 µm. The top is control cells expressing GFP alone. The middle is cells expressing the SlARF2A-GFP fusion protein. The bottom is cells expressing the SlARF2B-GFP fusion

protein. (B) SlARF2A/2B protein represses the activity of DR5 *in vivo*. Tobacco protoplasts were transformed either with the reporter construct (DR5::GFP) alone or with both the reporter and effector constructs (35S-SlARF2A/2B) and incubated in the presence or absence of 50 µM 2,4-D. GFP fluorescence was measured 16 h after transfection. A mock effector construct lacking SlARF2A/2B was used as a control for the co-transfection experiments. Transformations were performed in triplicate. Mean fluorescence is indicated in arbitrary unit (a.u.) ± standard error.

Generation of *SlARF2A RNAi*, *SlARF2B RNAi*, and *SlARF2AB RNAi* lines in tomato

To gain insight on the physiological significance of SlARF2, transgenic lines under-expressing the two paralogs were generated in the MicroTom tomato genetic background. To this purpose, dedicated RNAi constructs were designed to selectively target either *SlARF2A* or *SlARF2B* allowing the generation of transgenic lines specifically silenced in only one the two *SlARF2* genes (Figure 4A). Transgenic RNAi lines were also obtained where both paralogs are simultaneously silenced. Repression of *SlARF2A* and *SlARF2B* in the RNAi lines was confirmed by qPCR analyses in seedling tissues showing that the accumulation of SlARF2A or SlARF2B transcripts was selectively reduced in the appropriate silenced lines whereas in the SlARF2A/2B double knockdown lines both *SlARF2* genes were significantly down-regulated (Figure 4B). Noteworthy, the down-regulation of *SlARF2A* in the RNAi lines is compensated by an increased expression of *SlARF2B*, while such a compensation mechanism does not occur in the *SlARF2B* RNAi lines. To check whether SlARF2A may be directly involved in the transcriptional regulation of *SlARF2B*, a GFP reporter construct driven by the *SlARF2B* promoter was co-transfected into tobacco protoplasts with an effector construct allowing constitutive expression of SlARF2A. The data (Figure 4C) show that the presence of SlARF2A inhibits the expression of the *GFP* reporter gene driven by the *SlARF2B* promoter, revealing the ability of SlARF2A to repress the transcriptional activity of *SlARF2B in vivo*.

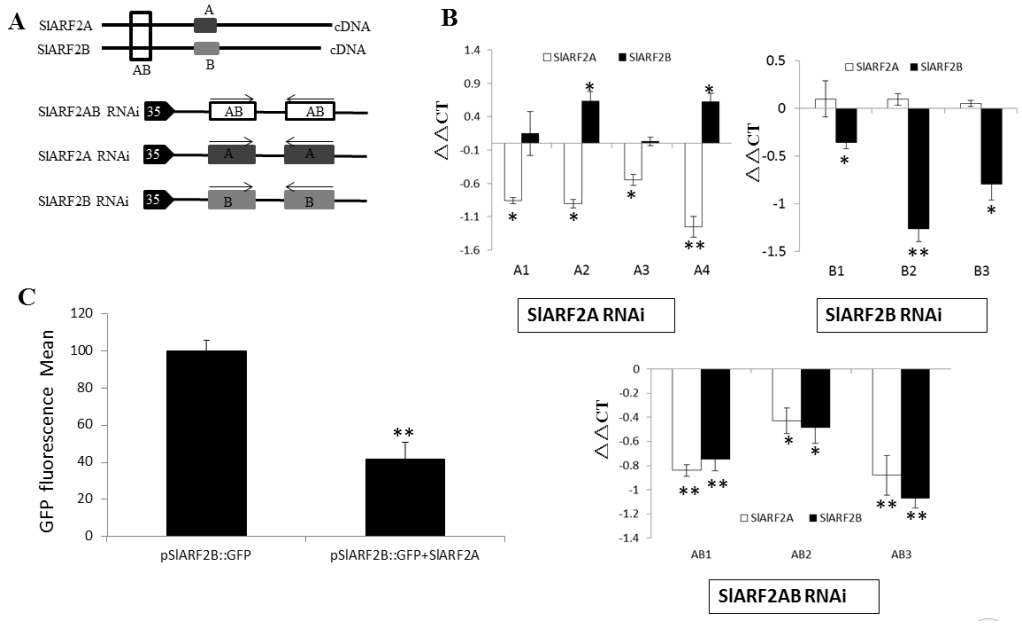

Figure 4. Expression pattern of *SlARF2A* and *SlARF2B* in *SlARF2 RNAi* transgenetic lines. (A) SlARF2A RNAi, SlARF2B RNAi and SlARF2AB RNAi constructs. AB= specific fragment in the DBD binding domain for both SlARF2A and SlARF2B used for SlARF2AB RNAi construct. A= specific fragment in the middle region (RD) of SlARF2A used for SlARF2A RNAi construct, B= specific fragment in the middle region (RD) of SlARF2B used for SlARF2B RNAi construct. (B) Expression of SlARF2A and SlARF2B in *RNAi* transgenic lines analyzed by quantitative RT-PCR. Expression of SlARF2A/SlARF2B in wild type was taken as reference, the *SlActin* gene as an internal control. (C) SlARF2A represses the activity of SlARF2B promoter. Tobacco protoplasts were transformed either with the reporter construct (pSlARF2B::GFP) alone or with both the reporter and effector constructs (35S-SlARF2A) and GFP fluorescence was measured 16 h after transfection. A mock effector construct lacking SlARF2A was used as a control for the co-transfection experiments. Transformations were performed in triplicate. Mean fluorescence is indicated in arbitrary unit (a.u.) ± standard error. * p-value<0.05, ** p-value<0.01(Student's t-test).

Down-regulation of *SlARF2* results in enhanced expression of auxin-responsive genes

To address whether SlARF2A and SlARF2B are involved in auxin responses *in planta*, genetic crosses were performed between the three types of *SlARF2* RNAi lines and a tomato line expressing the GUS reporter gene under the control of the auxin-responsive DR5 promoter. In the wild-type background, the basal expression of the DR5-driven GUS was low but displayed a net increase upon exogenous auxin treatment (Figure 5A). By contrast, the basal expression of the GUS reporter gene was dramatically high in the SlARF2AB RNAi background in the absence of auxin

treatment indicating that under-expression of SlARF2 results in enhanced expression of auxin-responsive genes. Interestingly, such an increase in GUS expression was not observed neither in the SlARF2A RNAi nor in SlARF2B RNAi background, suggesting that the two genes are functionally redundant and can compensate for each other (Figure 5A). Assessing GUS transcript accumulation by qPCR confirmed the higher expression of the DR5-driven GUS only in the SlARF2A/B RNAi background but not in the SlARF2A and SlARF2B RNAi lines (Figure 5B).

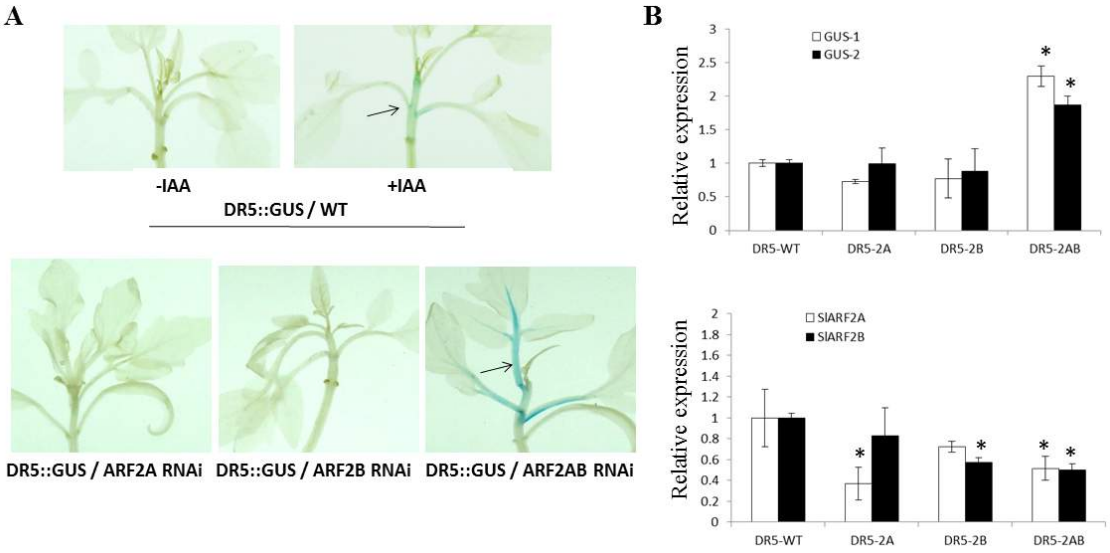

Figure 5. Down-regulation of ARF2A and ARF2B affects auxin response in *planta*. (A) Expression of the GUS reporter gene under the control of the auxin-inducible DR5 promoter. (Upper panel) In planta expression of the GUS reporter gene driven by DR5 in WT genetic background in the absence (left) or presence (right) of auxin treatment. Seedlings were treated with auxin (IAA 20µM for 3 hours) or with a mock solution without auxin. Expression of the GUS reporter gene driven by DR5 in ARF2A (left), ARF2B (middle) and ARF2AB (right) genetic background. (B) Expression of GUS (Upper panel) and SlARF2A/2B (bellows) genes in crossed lines analyzed by quantitative RT-PCR in seedlings. The relative mRNA levels of GUS-1/GUS-2 (Upper panel) and SlARF2A/2B (bellows) in wild type were standardized to 1.0, referring to the *SlActin* gene as an internal control. The error bars represent ±SE of three independent trials. *0.01 < P < 0.05. DR5-WT= DR5::GUS/WT; DR5-2A= DR5::GUS/ARF2A RNAi; DR5-2B= DR5::GUS/ARF2B RNAi; DR5-2AB = DR5::GUS/ARF2AB RNAi. GUS-1 and GUS-2 refer to the use of two pair of primers for the GUS gene.

SlARF2 RNAi fruits display spiky and blotchy ripening phenotype

Considering the high expression and ripening-associated pattern of both *SlARF2A* and *SlARF2B*, we sought to analyze the fruit phenotypes of SlARF2A and SlARF2B

single and double knockdown tomato lines. In both, *SlARF2A* and *SlARF2B* RNAi single knockdown lines the fruit exhibited dark green spots at immature and mature green stages, and then displayed a spiky pattern of ripening with yellow/orange spots on the skin which remain till the full mature stage (Figure 6A). The double silenced lines exhibited more severe ripening defects with yellow and orange patches never reaching the typical red color of the wild type or out-segregating lines, again suggesting that SlARF2A and SlARF2B may have a redundant function in fruit ripening (Figure 6A). We noted that fruit color of the *SlARF2AB RNAi* lines never get fully red. Assessing the time period from anthesis to breaker stage revealed no significant or little delay (2 to 3 days delay) in the onset of ripening between wild type and double knockdown lines (Figure 6B). So the delay most happened during the ripening stage (Figure 6C). Interestingly, full ripening cannot be recovered upon exogenous ethylene treatment of the *SlARF2A/B* RNAi double knockdown fruits which suggests a possible alteration in ethylene perception or response (Figure 6D).

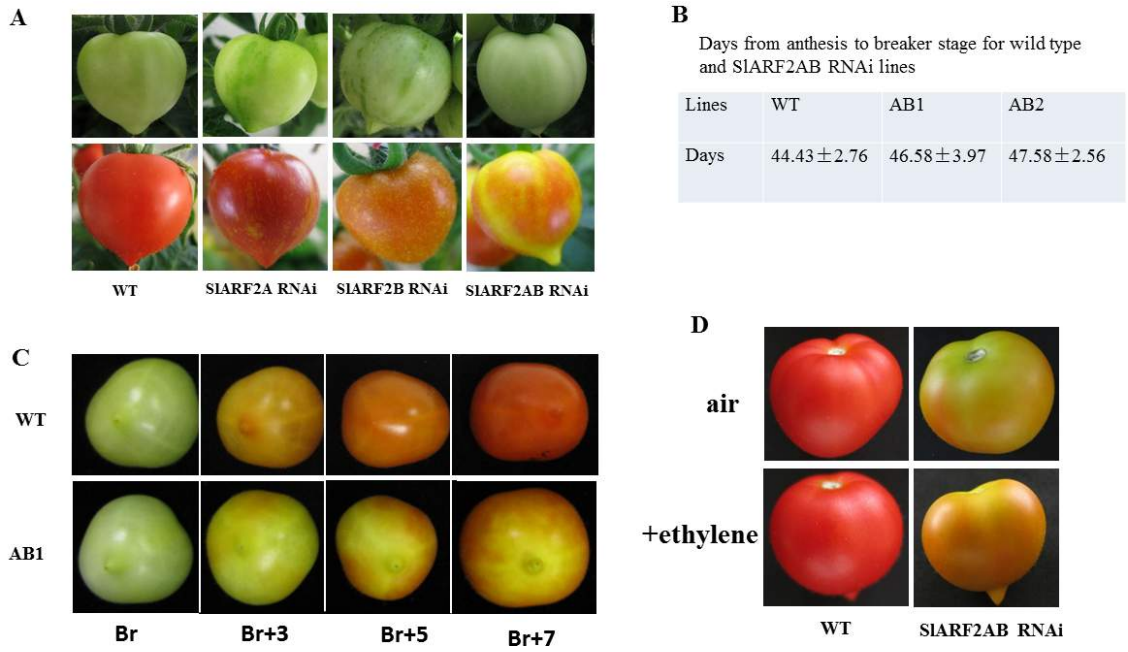

Figure 6. Altered ripening phenotypes of SlARF2 down-regulated mutant. (A) Ripening phenotype of *SlARF2A RNAi; SlARF2B RNAi; SlARF2AB RNAi* fruit at mature green stage and ripe stage. The *SlARF2A/SlARF2B RNAi* mutants show spiky phenotype at mature green stage and ripe stage fruits, *SlARF2AB RNAi* mutant displays blotchy phenotype only at ripe stage fruit. (B) The days of fruit from anthesis to breaker stage in wild type and SlARF2AB

RNAi lines. The *SlARF2AB RNAi* mutant displays similar days with wild type. (C) Different stages of fruit ripening of wild-type (WT) and *SlARF2AB RNAi* lines. Fruits from transgenic lines show delayed color development, never reaching a full red color. Br, breaker stage; Br+3, 3 days post-breaker stage; Br+5, 5 days post-breaker stage; Br+7, 7 days post-breaker stage. (D) Effect of ethylene treatment on wild type (WT) and SlARF2AB RNAi fruit. Mature green fruits from wild type and SlARF2AB RNAi mutant were treated or untreated (air) with 10 ppm ethylene for 3 days, 2 hours and 3 times per day. 7 days after treatment, wild type fruits treated or untreated both reached full red, SlARF2AB RNAi fruits treated or untreated still keep orange sectors on the fruit surface.

SlARF2A and SlARF2B affect ethylene production and perception on fruit.

The ripening defect phenotype prompted us to assess the climacteric ethylene production in the *SlARF2AB RNAi* lines. Compared to wild type, ethylene production is significantly low throughout ripening and occurs with 2 to 3 days delay with regard to anthesis stage (Figure 7). Important to mention, the increase in ethylene production occurs at the breaker stage in both wild type and *SlARF2* down-regulated lines. Assessing the expression of ethylene biosynthesis genes by qPCR revealed that reduced transcript levels corresponding to *ACO1*, *ACS2*, *ACS3* and *ACS4* in the *SlARF2A/B* RNAi lines at Breaker (Br), Breaker+2 (Br+2), and Breaker+8 (Br+8) stages (Figure 8A). Since exogenous ethylene treatment cannot reverse the phenotype (Figure 6D), the reduced ethylene production cannot account for the ripening defects, we therefore examined the expression of ethylene receptor genes. The data show that *ETR3* (*NR*) and *ETR4* transcript levels are dramatically lower in the transgenic lines compared to wild type at all stages of fruit ripening (Br, Br+2, and Br+8) and that the expression of other receptor genes (*ETR1*, *ETR2*, and *ETR5*) is down-regulated at the breaker+8 stage which may result in a defect in ethylene perception (Figure 8B). Also, the expression of *EIN-like* genes (*EIN2*, *EIL2* and *EIL3*), which encode major components of ethylene transduction pathways, was also down-regulated during ripening of *SlARF2A/B RNAi* fruit. Noteworthy, the expression of a high number of *ERF* genes (Figure 9), which are known to mediate ethylene responses, was also altered showing either down-regulation (*SlERF.A1*, *SlERF.A2*, *SlERF.A3*, *SlERF.C1*, *SlERF.C3*, *SlERF.C6*, *SlERF.D1*, *SlERF.D2, SlERF.D4, SlERF.E1, SlERF.E2, SlERF.E3, SlERF.E4*) or up-regulated (*SlERF.B1, SlERF.B2, SlERF.B3, SlERF.D3,*

SlERF.F2). These data strongly suggest that ethylene responses are likely to be disturbed in the transgenic lines.

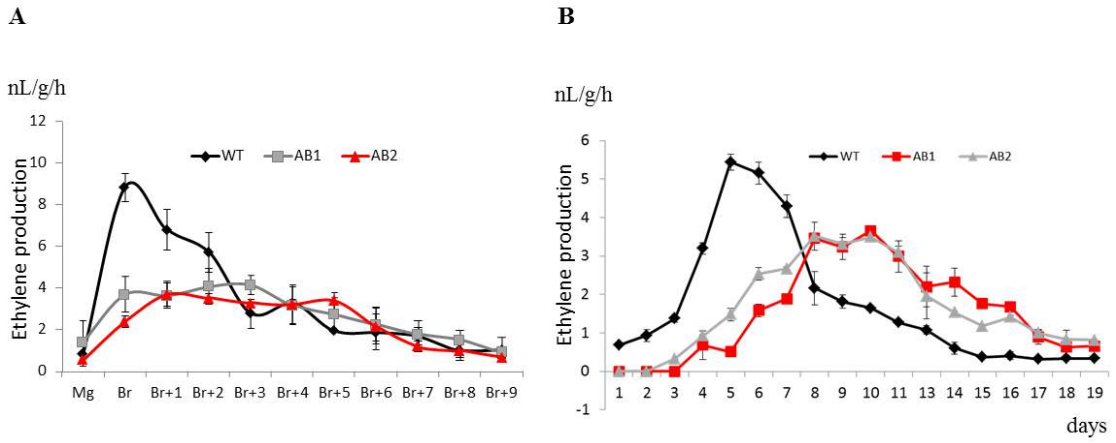

Figure 7. Ethylene production of SlARF2AB RANi fruit. (A) Ethylene production of wild-type and SlARF2AB RNAi fruits was assessed at different ripening stages. Mg, mature green stage; Br, breaker stage; Br+1, 1 day post breaker stage; Br+2, 2 days post breaker stage; Br+3, 3 days post breaker stage. (B) Ethylene production of wild-type and *SlARF2AB RNAi* fruits were measured at different ripening stages indicated as days post mature green stage. Values represent means of at least 10 individual fruits. Vertical bars represent SD. AB1= SlARF2AB RNAi line 311; AB2= SlARF2AB RNAi line 223.

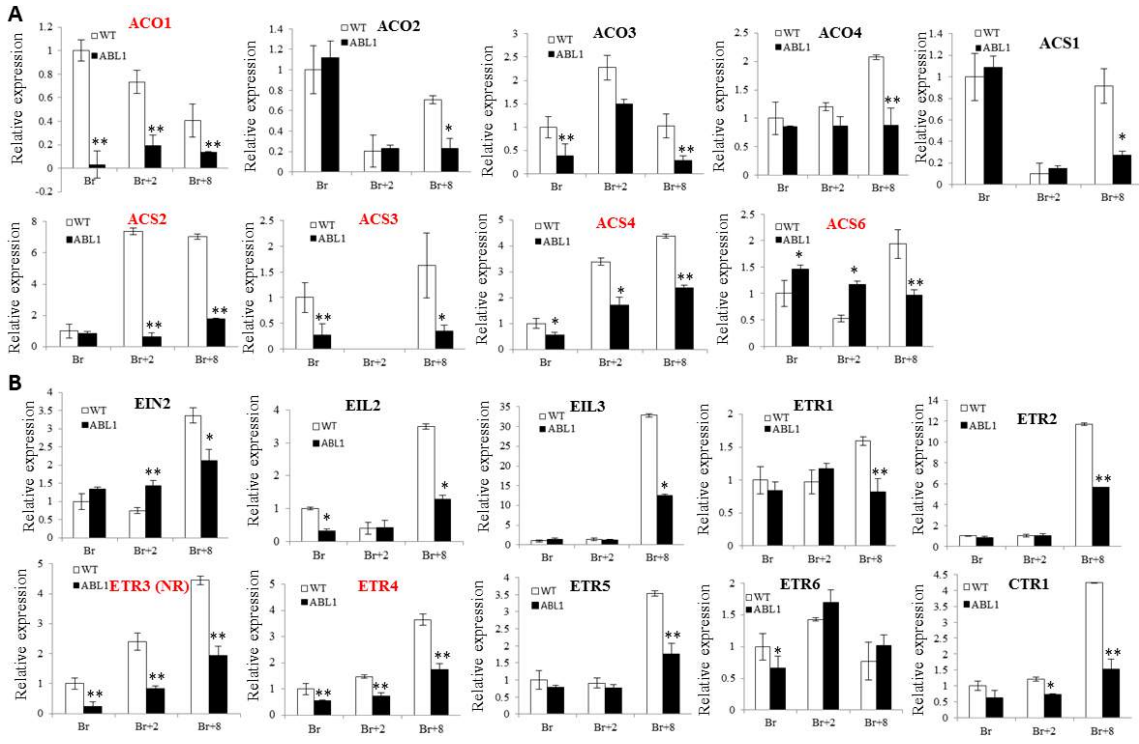

Figure 8. The expression of some ethylene synthesis and ethylene perception genes is altered in SlARF2AB RNAi plants. (A) Relative expression of ethylene synthesis pathway gens in different stages of SlARF2AB RNAi fruit compared with wild type. Total RNA was

extracted from different developmental stages of fruit. The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the SlActin gene as an internal control. Error bar means ±SD of three biological replicates. Br= breaker stage; Br+2= 2 days post breaker stage; Br+8= 8 days post breaker stage. * p-value<0.05, ** p-value<0.01. ABL1 is SlARF2AB RNAi line 311. *ACO1, ACO2, ACO3, ACO4* aminocyclopropane-1-carboxylic acid oxidase; *ACS1, ACS2, ACS3, ACS4, ACS6* aminocyclopropane-1-carboxylic acid synthases. (B) Relative expression of ethylene perception genes in different stages of SlARF2AB RNAi fruit compared with wild type. Total RNA was extracted from different developmental stages of fruit. The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the SlActin gene as an internal control. Error bar means ±SD of three biological replicates. Br= breaker stage; Br+2= 2 days post breaker stage; Br+8= 8 days post breaker stage. * p-value<0.05, ** p-value<0.01. ABL1 is SlARF2AB RNAi line 311. EIN2 ethylene signaling protein; EIL2, EIL3, EIL4 EIN3-like proteins; ETR1, ETR2, ETR3 (NR, never-ripe), ETR4, ETR5, ETR6 ethylene receptors; CTR1 ethylene-responsive protein kinase.

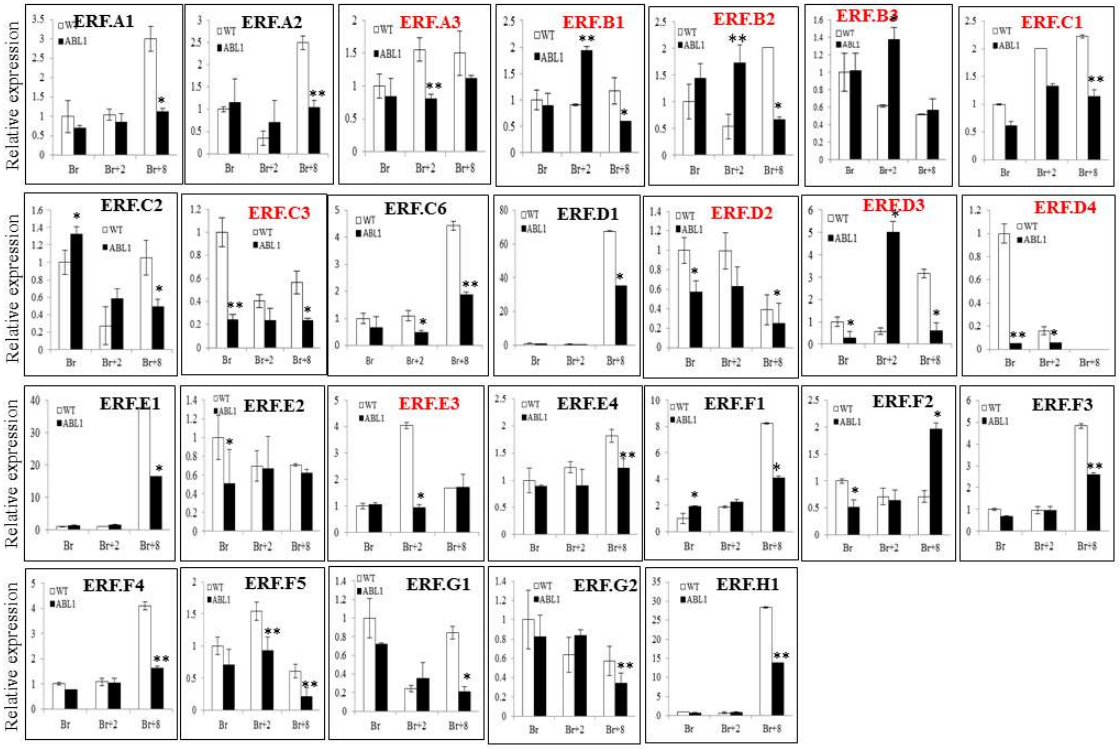

Figure 9. The expression of ERFs family genes in wild type and *SlARF2AB RNAi* plants. Relative expression of ERFs family genes in different stages of SlARF2AB RNAi fruit compared with wild type. Total RNA was extracted from different developmental stages of fruit. The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the SlActin gene as an internal control. Error bar means ±SD of three biological replicates. Br= breaker stage; Br+2= 2 days post breaker stage; Br+8= 8 days post breaker stage. * p-value<0.05, ** p-value<0.01. ABL1 is SlARF2AB RNAi line 311.

SlARF2AB RNAi fruit shows decreased color change and softening.

The fruit color saturation assessed by Hue angle indicative of color intensity indicated that red pigment accumulation was reduced in *SlARF2AB RNAi* down-regulated lines compared to wild type (Figure 10A). Accordingly, the expression of genes involved the carotenoid pathway was altered. *PSY1*, a key regulator of flux through the carotenoid pathway was significantly down-regulated in the *SlARF2AB RNAi* knockdown fruits at all ripening stages from Breaker to Breaker+8 (Figure 10B). Lower levels of phytoene desaturase (PDS) and phytoene synthase (ZDS) transcripts was also observed at Br+2 stage in the SlARF2AB RNAi fruit. By contrast, transcripts corresponding to lycopene beta cyclase genes (*β-LCY1*, *β-LCY2*) displayed higher accumulation than in wild-type at all ripening stages tested and lycopene β-cyclases (CYCB) was also up-regulated at Br and Br+2 stages in *SlARF2AB RANi* fruit (Figure 10B). On the other hand, *SlARF2AB RNAi* fruits maintained higher firmness than wild type throughout ripening (Figure 11A). In line with the delayed softening phenotype, transcript accumulation of a major fruit polygalacturonase gene, PG2A, involved in ripening-related cell wall metabolism, was significantly reduced at Br, Br+2, and Br+8 stages in *SlARF2AB RNAi* fruits (Figure 11B).

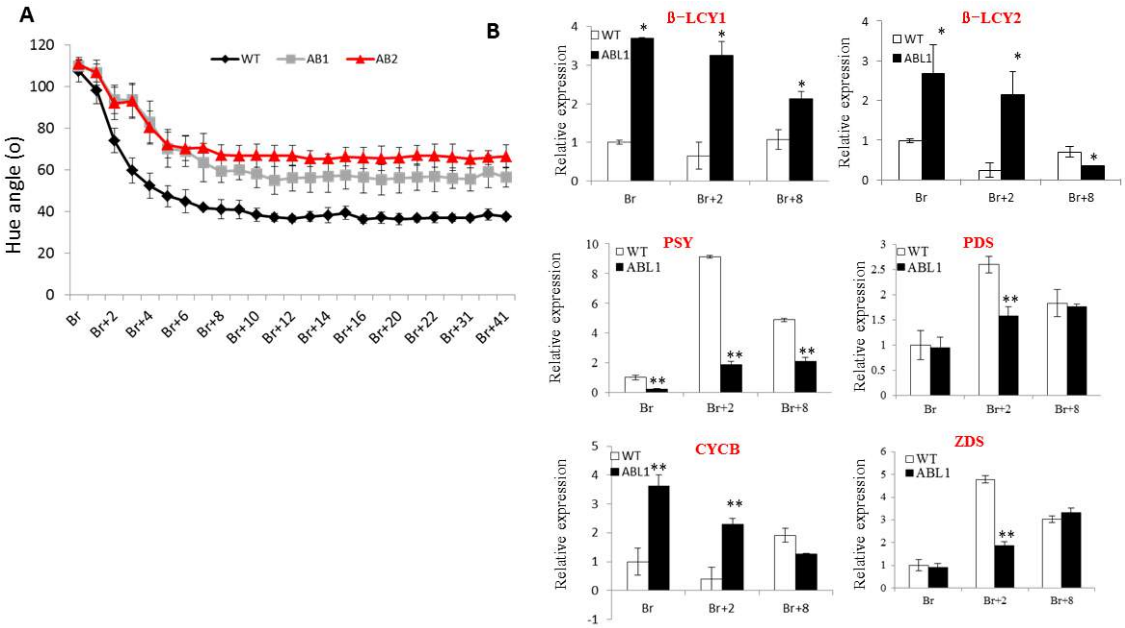

Figure 10. Alter pigment accumulation in SlARF2AB RANi fruit. (A) Changes in hue angle in WT and SlARF2AB RNAi lines during different ripening stages. AB1= SlARF2AB RNAi line 311; AB2= SlARF2AB RNAi line 223. (B) Expression of carotenoid biosynthesis genes in wild-type (WT) and SlARF2AB RNAi tomato lines. Total RNA was extracted from different developmental stages of fruit. The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the SlActin gene as an internal control. Error bar means ±SD of three biological replicates. Br= breaker stage; Br+2= 2 days post breaker stage; Br+8 = 8 days post breaker stage. * p-value<0.05, ** p-value<0.01. ABL1 is SlARF2AB RNAi line 311. PSY1 phytoene synthase; PDS phytoene desaturase; ZDS, carotenoid desaturases; ß-LCY1, ß-LCY2, CYC-ß lycopene b-cyclases.

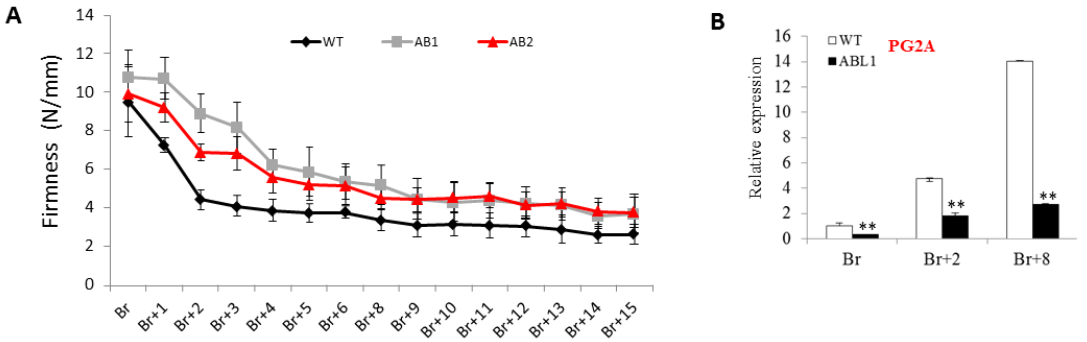

Figure 11. Altered Firmness in SlARF2AB RANi Fruit. (A) Fruit firmness of wild-type and SlARF2AB RNAi fruits. Fruits were harvested at the breaker stage, kept at room temperate and the firmness was measured day by day. A total of 15 fruits was used for each measurement and the error bars represent ±SD. AB1= SlARF2AB RNAi line 311; AB2= SlARF2AB RNAi line 223. (B) Relative expression of polygalacturonase gene PG2A in different stages of SlARF2AB RNAi fruit compared with wild type. Relative mRNA level in WT at the breaker (Br) stage was standardized to 1.0, referring to Sl-Actin gene as an internal control. Error bars represent ±SD of three biological replicates. Br = breaker stage fruit; Br+2 = 2 days post breaker stage; Br+8 = 8 days post breaker stage. * p-value<0.05, ** p-value<0.01. ABL1 is SlARF2AB RNAi line 311.

Expression of ripening-related and ripening regulator genes is altered in SlARF2 down-regulated lines.

The expression of key regulatory genes assessed at the transcript accumulation level was strongly reduced throughout ripening in the *SlARF2 RNAi* lines. Compared to wild type fruit, transcript levels of *RIN* and *CNR* genes were significantly lower at Br, Br+2 and Br+8 stages (Figure 12). Likewise, the *NOR* gene displayed reduced expression at Br and Br+8 stages, *TAGL1* at Br and Br+2 stages and *FUL1*, *FUL2* at Br+2 and Br+8 stages. The altered expression of these genes in the *SlARF2AB RNAi*

fruits is consistent with the dramatically altered ripening of transgenic fruits. Also, the low expression of *E8* and *E4*, two ethylene-responsive and ripening associated genes, was consistent with the altered expression of ethylene biosynthesis and signaling genes. By contrast, mRNA levels of *LeHB-1*, another ripening regulator gene, did not display significant change in SlARF2AB RNAi fruits compared to wild type (Figure 12).

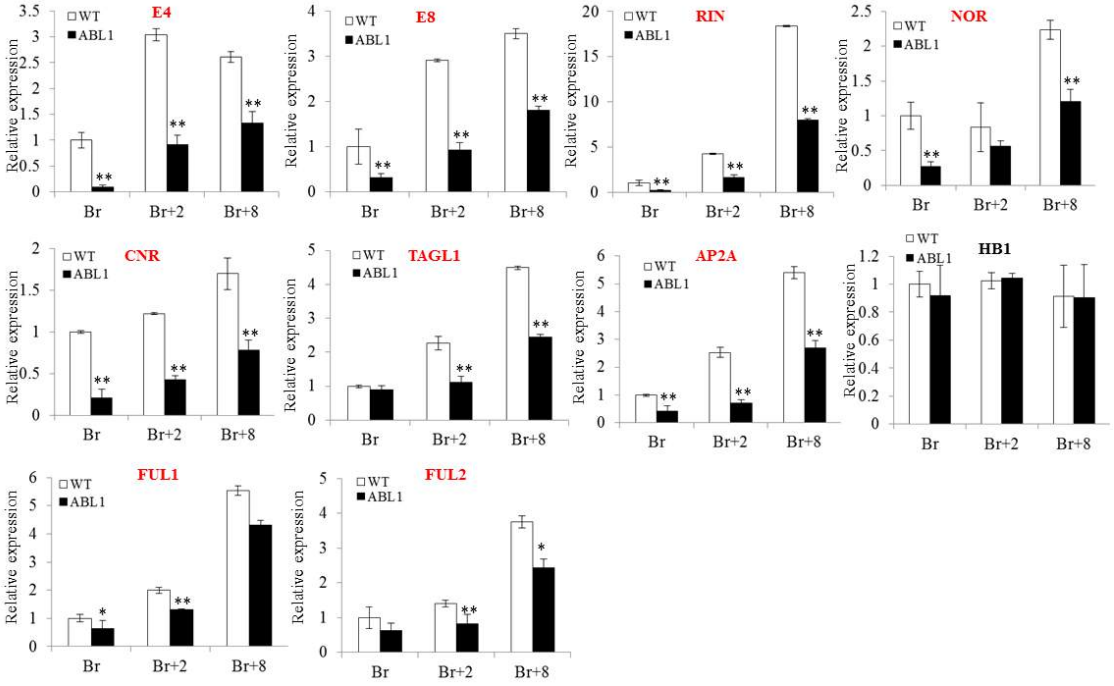

Figure 12. The expression of a number of ripening related genes is altered in SlARF2AB RNAi plants. Expression of ripening regulator genes in wild-type (WT) and *SlARF2AB RNAi* lines during tomato fruit ripening. Total RNA was extracted from the indicated developmental stages of fruit. The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the SlActin gene as an internal control. Error bar means ±SD of three biological replicates. Br = breaker stage; Br+2 = 2 days post breaker stage; Br+8 = 8 days post breaker stage. * p-value<0.05, ** p-value<0.01. AP2a, APETALA2/ERF gene; CNR, colorless non-ripening; HB-1, HD-Zip homeobox; NOR, non-ripening; RIN, ripening inhibitor; TAGL1, tomato AGAMOUS-LIKE 1. FUL1, FUL2 MADS domain transcription factor; E4, E8 ethylene response genes.

DISCUSSION

While ethylene is known as the key hormone regulating climacteric fruit ripening, the impact of down-regulating *SlARF2* described in the present study brings new evidence supporting the role of auxin in the control of this developmental process. In contrast to

Arabidopsis, in the tomato SlARF2 is encoded by two genes, *SlARF2A* and *SlARF2B*, both displaying a ripening-induced pattern of expression (Zouine et al., 2014). Single knockdown of either *SlARF2A* or *SlARF2B* resulted in spiky fruit phenotype, while simultaneous down-regulation of the two genes leads to a severe delay or almost complete inhibition of ripening, indicating that both genes contribute to tomato fruit ripening. Genetic crosses between *SlARF2 RNAi* tomato lines and line expressing the GUS reporter driven by the auxin-responsive DR5 promoter indicated that single repression of *SlARF2A* or *SlARF2B* did not induce significant increase in GUS expression while simultaneous down-regulation of both *SlARF2* genes resulted in strongly enhanced expression of DR5:GUS similar to that observed upon exogenous auxin treatment (Figure 5A, B). These data indicate that *in planta*, SlARF2 acts as a repressor of auxin-dependent gene transcription and suggest that SlARF2A and SlARF2B are functionally redundant. Moreover, down-regulation of SlARF2A is compensated by an up-regulation of SlARF2B suggesting a coordinated expression of the two *ARF* genes. The transient expression assay in a single cell system revealed the ability of SlARF2A to repress the activity of *SlARF2B* promoter indicating therefore that this latter gene is under direct regulation by SlARF2A.

Down-regulation of *SlARF2* genes impairs normal fruit ripening likely via altering components of ethylene metabolism, signaling and response. In support of this idea, *SlARF2A/B* RNAi fruits produce less climacteric ethylene than wild type (Figure 7A, B), consistent with the lower expression of *ACC oxidase* (*ACO*) and *ACC synthase* (*ACS*) genes whose expression is instrumental for the triggering of the climacteric ripening (Nakatsuka et al., 1998; Barry et al., 2000). Indeed, transition from auto-inhibitory system1 to auto-catalytic system 2 is associated with an increased expression of *LeACS1A*, *LeACS2*, *LeACS4*, *LeACO1*, *LeACO3*, and *LeACO4* genes (Lincoln et al., 1993; Nakatsuka et al., 1998; Barry et al., 2000). Moreover, repression of genes belonging to these two families blocked fruit ripening in tomato (Hamilton et al., 1990; Oeller et al., 1991; Gray et al., 1992; Nakatsuka et al., 1998). In line with the reduced ethylene production, the expression of ethylene responsive genes *E4* and *E8* is also reduced in the SlARF2AB RNAi fruit (Figure 12). Importantly, treatment

with exogenous ethylene was unable to restore normal fruit ripening suggesting that ethylene signaling and response is also impaired in SlARF2 knockdown lines. Consistent with this hypothesis, ethylene receptor genes such as Nr (SlETR3), SlETR4, and SlETR6 displayed altered expression pattern in the transgenic lines compared to wild type which may lead to the fruit insensitivity to exogenous ethylene. Importantly, down regulation of NR receptor results in slightly delayed fruit ripening with reduced rates of ethylene synthesis and slower carotenoid accumulation (Tieman et al., 2000). However, it has been reported that reducing *NR* expression via RNA antisense strategy results in up-regulation of LeETR4 as a compensation mechanism for the loss of NR (Tieman et al., 2000). In the SlARF2 under-expressing fruit, both SlETR3/NR and SlETR4 were down-regulated simultaneously (Figure 8B), which may explain the more sever delayed fruit ripening in *SlARF2AB RNAi* lines compared to *NR* antisense lines. It is now widely accepted that modulation of the expression of ethylene-regulated genes is mediated by ERFs (Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000; Zhang et al., 2009b; Lee et al., 2012; Pirrello et al., 2012). In particular, it was shown that SlAP2a, a tomato *APETALA2/ERF* gene, is a negative regulator of fruit ripening (Chung et al., 2010; Karlova et al., 2011). More recently the expression of a dominant repression version of another tomato ERF gene, SlERF.B3, leads to a dramatic delay in fruit ripening (Liu et al., 2014a). Interestingly, the expression of a number of ERFs is disturbed in *SlARF2AB RNAi* fruits suggesting an altered ethylene response that may contribute to the ripening defect phenotype.

Tomato genes encoding ripening-inhibitor (RIN), non-ripening (NOR) and colorless non-ripening (CNR) are considered to be master regulators of the ripening process and mutation in the corresponding loci dramatically impairs fruit ripening (Vrebalov et al., 2002; Tigchelaar and McGlasson, 1978; Manning et al., 2006). Some of the main features of these non-ripening mutants are also observed in *SlARF2* knockdown lines such as enhanced fruit firmness, low ethylene production and incapacity to ripen in response to exogenous ethylene. Interestingly, the expression *RIN*, *NOR* and *CNR* genes were significantly down-regulated during fruit ripening of *SlARF2AB RNAi* lines (Figure 12). Considering that *RIN*, *NOR*, and *CNR,* were reported to play a

crucial role for the attainment of competence to ripen by acting up-stream of ethylene in the ripening cascade, (Lincoln and Fischer, 1988; Yokotani et al., 2004; Barry et al., 2000; Griffiths et al., 1999; Thompson et al., 1999; Yen et al., 1995; Yokotani et al., 2009; Barry and Giovannoni, 2007), our data strongly suggest that SlARF2 affects fruit ripening through down-regulation of these master transcriptional regulators. *SlARF2AB RNAi* fruit showed yellow-orange fruits and low expression level of ACS2 concomitant to a reduced expression of *AGAMOUS-like 1* (*TAGL1*), *FRUITFUL* (*FUL*) orthologs *FUL1* and *FUL2* encoding ripening-related MADS domain transcription factors. Suppression of *TAGL1* resulted in yellow-orange fruits and lower ethylene levels due to the down-regulation of *ACS2* (Itkin et al., 2009; Vrebalov et al., 2009) and simultaneous suppression of *FUL1* and *FUL2* resulted in ripening defects (Bemer et al., 2012). These phenotypes strikingly recall those displayed by SlARF2 down-regulated lines. It has been reported that TAGL1, FUL1, and FUL2 interact with RIN (Leseberg et al., 2008; Martel et al., 2011) forming higher order complexes that regulate tomato fruit ripening (Wang et al., 2014). The phenotypes and the associated gene expression patterns support the hypothesis that down-regulation of SlARF2 impairs ripening through interfering with the MADS-box regulatory network. So far, the function characterization of SlARF2 in tomato was limited to its putative role in apical hook formation thus suggesting its involvement in the interplay between auxin and ethylene (Salma et al., 2009; Chaabouni et al., 2009). This is in agreement with our data showing that the promoters of SlARF2A and SlARF2B harbor conserved motifs corresponding to auxin and ethylene responsive elements. The ethylene responsiveness of *SlARF2B* and *SlARF2A* is further supported by their induced expression by exogenous auxin and ethylene treatment in mature green fruit, and conversely by their repression by 1-MCP treatment in breaker fruit. Down-regulation of SlARF2 leads to altered expression of transcription factors known to mediate both ethylene (ERFs) and auxin (ARFs) responses and results in the changes in auxin responsive and ethylene responsive genes expression suggesting that SlARF2A and SlARF2B might be involved in the crosstalk between auxin and ethylene.

A typical feature of tomato fruit undergoing ripening is the accumulation of lycopene which accounts for the red color whereas b-carotene, conferring an orange color, does not accumulate at this stage (Fraser et al., 1994; Rosati et al., 2000). The *SlARF2AB RNAi* fruit displayed a yellow-orange sectors reflecting increased accumulation of b-carotene and degraded lycopene. The relative abundance of lycopene is caused by the up-regulation of the phytoene synthase gene (*PSY1*) and down-regulation of *LCYB* and *CYCB* (Fraser et al., 1994; Ronen et al., 1999, 2000; Alba et al., 2005). PSY1 is the first rate-limiting enzyme in the plant carotenoid biosynthetic pathway whose transcript accumulations is induced by ethylene (Vrebalov et al., 2002; Martel et al., 2011; Barry et al., 2005; Adams-Phillips et al., 2004a; Bramley et al., 1992). Repression of *PSY1* inhibit total carotenoid accumulation resulting in mature yellow fruit with little lycopene or b-carotene (Bramley et al., 1992). LCYB and CYCB are responsible for the conversion of lycopene into b-carotene, which turns the fruit orange (Ronen et al., 2000; Rosati et al., 2000). During fruit ripening, transcript accumulations of both genes is repressed by the elevated ethylene thus leading to the accumulation of lycopene and resulting in the red color of the ripe fruit (Vrebalov et al., 2009). The *SlARF2AB RNAi* fruit produced less ethylene than wild type and exhibited low levels of *SlPSY1* transcripts and high levels of *SlLCYB* and *SlCYCB*, which promotes the accumulation of b-carotene rather than lycopene thus causing the orange-yellow sectors on *SlARF2AB RNAi* fruit.

The altered ripening phenotypes associated with the under-expression of *SlARF2* genes are consistent with previous work showing that the coordinated expression of some ARF and Aux/IAA genes in the tomato is instrumental to normal fruit ripening (Jones et al., 2002; Guillon et al., 2008; Sagar et al., 2013). As depicted in the model proposed (Figure 13), in addition to the crucial role devoted to ethylene, the data support a higher order of complexity of the mechanism underlying the control of fleshy fruit ripening which, henceforth, should be seen as a multi-hormonal process. In particular, auxin seems to take an active part in the control of tomato fruit ripening and this action is mediated at least partly by ARF transcription factors. Given the severe ripening defects displayed by the under-expressing lines, SlARF2 likely acts at

the level of the master regulators of ripening like RIN, NOR and CNR or alternatively via the control of ethylene biosynthesis and response, even though it cannot be ruled out that it may impacts ripening at both levels of regulation. Overall, the work reinforces the concept where, beside ethylene, ripening relies most likely on the interplay between different hormones signaling. While an increasing number of evidence supports now the role of auxin in fleshy fruit ripening, there is little doubt that the involvement of other hormones signaling is required for a proper tuning of this complex developmental process. Moreover, ethylene and auxin regulation of *SlARF2* points out to the interconnection between hormone signaling pathways and may give a hint on the complexity of the signaling networks underlying the big diversity of fruit ripening feature among different plant species.

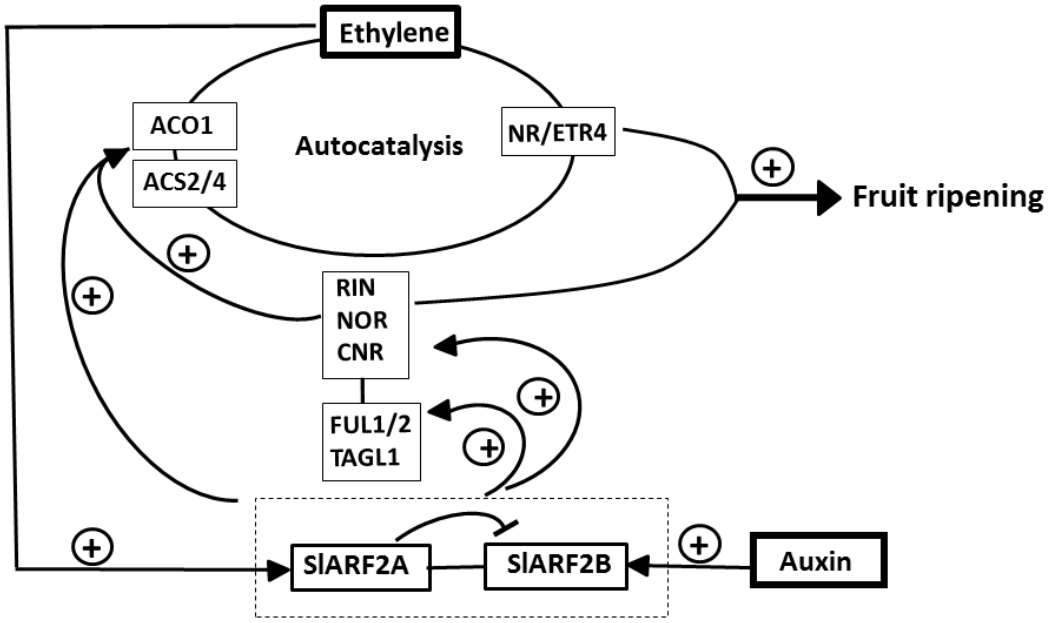

Figure 13. A synthetic model depicting the position of SlARF2 in the network regulatory mechanism controlling fruit ripening. SlARF2A and SlARF2B mediate tomato fruit ripening by positively regulating key ethylene biosynthesis genes (*ACO1*, *ACS2/4*) and through modulating the major regulators of fruit ripening such as RIN, NOR, and CNR transcription factors known to affect ripening by positively regulating ACO1 and ACS2/4. SlARF2A is up-regulated by ethylene while SlARF2B is up-regulated by auxin. SlARF2A negatively regulates the expression of SlARF2B, thus down-regulation of SlARF2A is compensated by an up-regulation of SlARF2B. SLARF2 also modulates the expression of FUL1/2 and TAGL1. It has been postulated that RIN forms a complex with FUL1/2 to regulate fruit ripening in an ethylene-independent manner. RIN binds to TAGL1 to regulate the fruit ripening in an ethylene-dependent way.

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Introduction to Chapter III

Introduction to Chapter III

In plants, the transcriptional co-repressors play a very important role during plant development (Krogan and Long, 2009; Liu and Karmarkar, 2008). Co-repressors are transcriptional regulators that are incapable of independent DNA binding, being recruited directly or indirectly by DNA-binding transcription factors to repress target gene expression (Liu and Karmarkar, 2008). TOPLESS/TOPLESS-RELATED (TPL/TPR) is one of the co-repressors families (Liu and Karmarkar, 2008) which emerges as key players in gene repression in several mechanisms especially in auxin perception. More studies in *Arabidopsis* imply that TOPLESS family (TPLs) is recruited by some specific IAAs to repress the function of ARFs (Causier et al., 2012b; Szemenyei et al., 2008).

Our lab has been studying the tomato fruit development for many years. Auxin is one of these important hormones involved in the fruit development. Topless as a co-repressor are predicted to regulate the auxin signaling pathway which suggests its role on fruit development. In order to check whether tomato SlTPLs is also involved in auxin signaling pathway and to further study its role on fruit development, we characterize the topless family genes in tomato, a model plant for fleshy fruit.

The characterization results of the topless genes family in tomato have been published by the Journal of Experimental Botany. In the following chapter III I will present you the topless publication: Genome-wide identification, phylogenetic analysis, expression profiling, and protein-protein interaction properties of TOPLESS gene family members in tomato. In the complementary results to the chapter III we describe the potential interaction partners of ARF and TPL and propose a model for the transcription repression mechanism.

Chapter III

Genome-wide Identification, phylogenetic analysis, expression profiling and protein-protein interaction properties of the *TOPLESS* gene family members in tomato

(*Journal of Experiment Botany, 65(4),1013–1023*)

Journal of Experimental Botany, Vol. 65, No. 4, pp. 1013–1023, 2014
doi:10.1093/jxb/ert440 Advance Access publication 7 January, 2014
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Genome-wide identification, phylogenetic analysis, expression profiling, and protein–protein interaction properties of *TOPLESS* gene family members in tomato

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Received 10 September 2013; Revised 19 November 2013; Accepted 26 November 2013

Abstract

Members of the *TOPLESS* gene family emerged recently as key players in gene repression in several mechanisms, especially in auxin perception. The *TOPLESS* genes constitute, in 'higher-plant' genomes, a small multigenic family comprising four to 11 members. In this study, this family was investigated in tomato, a model plant for Solanaceae species and fleshy fruits. Six open reading frames predicted to encode topless-like proteins (SlTPLs) containing the canonical domains (LisH, CTLH, and two WD40 repeats) were identified in the tomato genome. Nuclear localization was confirmed for all members of the SlTPL family with the exception SlTPL6, which localized at the cytoplasm and was excluded from the nucleus. *SlTPL* genes displayed distinctive expression patterns in different tomato organs, with *SlTPL1* showing the highest levels of transcript accumulation in all tissues tested except in ripening fruit where *SlTPL3* and *SlTPL4* were the most prominently expressed. To gain insight into the specificity of the different TOPLESS paralogues, a protein–protein interaction map between TOPLESS and auxin/indole-3-acetic acid (Aux/IAA) proteins was built using a yeast two-hybrid approach. The PPI map enabled the distinction of two patterns: TOPLESS isoforms interacting with the majority of Aux/IAA, and isoforms with limited capacity for interaction with these protein partners. Interestingly, evolutionary analyses of the *TOPLESS* gene family revealed that the highly expressed isoforms (*SlTPL1*, *SlTPL3*, and *SlTPL4*) corresponded to the three *TPL*-related genes undergoing the strongest purifying selection, while the selection was much weaker for *SlTPL6*, which was expressed at a low level and encoded a protein lacking the capacity to interact with Aux/IAAs.

Key words: Aux/IAA, auxin signalling, co-repressor, multigenic family, protein–protein interactions, *Solanum lycopersicum*, tomato, TOPLESS.

Abbreviations: AD, activating domain; ARF, auxin response factor; Aux/IAA, auxin/indole-3-acetic acid; BD, binding domain; EST, expressed sequence tag; NLS, nuclear localization signal; PPI, protein-protein interaction; qRT-PCR, quantitative reverse transcription-PCR; Y2H, yeast two-hybrid; YFP, yellow fluorescent protein.

Subcellular localization of SlTPL proteins

For localization of the SlTPL proteins, the *SlTPL* coding sequences were cloned using Gateway technology as a C-terminal fusion in frame with yellow fluorescent protein (YFP) into the pEarlyGate104 vector and expressed under the control of the 35S cauliflower mosaic virus promoter. The empty vector pEarleyGate104 was used as a control. Protoplasts were obtained from tobacco (*Nicotiana tabacum*) suspension-cultured BY-2-cells and transfected according to a method described previously (Leclercq *et al.*, 2005). YFP localization by confocal microscopy was performed as described previously (Audran-Delalande *et al.*, 2012).

Expression analysis of SlTPL genes

Total RNA extraction, removal of DNA contamination, cDNA generation of eight tomato tissues (root, stem, leaves, bud, flower, mature green fruit, breaker fruit, and red fruit), and quantitative reverse transcription-PCR (qRT-PCR) were performed according to methods previously described (Audran-Delalande *et al.*, 2012; Pirrello *et al.*, 2006). The primer sequences were as follows: TPL1F: 5′-TGTTCGT TCTAGGAGACTAACCAG-3′ and 5′-TPL1R: AAGACAAACCTTCCCTTC CGA-3′; TPL2F: 5′-CC TGTAAATACGCCT CTTGCT-3′ and TPL2R: 5′-ACTGGTTGG AATGGACTGTG-3′; TPL3F: 5′-CACTTTCTGCTCCAATAA CCT-3′ and TPL3R: 5′-TCCA TCTGTCAACCCAACTG-3′; TPL4 F: 5′-CCTTCTAACC CAAGCTCCAG-3′ and TPL4R: 5′-AT AAACTCCGCCATCAGTA AGTC-3′; TPL5F: 5′-CGTCTATT GTAACCCATCCA CTC-3′ and TPL5R: 5′-AGAAGTTACACCAT GAGGACCC-3′; and TPL6F: 5′-ACTG GACTAGCATTCTCT AACAC-3′ and TPL6R: 5′-TTGAATT CCACA CCACTATCTG AG-3′. Actin was used as an internal reference. The relative fold differences (with *SlTPL6* as a reference gene) for each sample were calculated using the formula $2^{-\Delta\Delta Ct}$. Three independent RNA isolations were used for cDNA synthesis and, each cDNA sample was subjected to real-time PCR analysis in triplicate.

Bioinformatic analyses

SlTPL genes were searched using BLAST queries on the Genomic (Chromosome v.2.40) and transcript database (cDNA itag 2.4) available on the SGN website (http://solgenomics.net/tools/blast/index.pl). Exons and introns were deduced from the ITAG 2.3 annotation. For *SlTPL5* (Solyc07g008040), the 'predicted annotation' missing the N-terminal extremity was completed with an additional exon (from position 2754093 to 2754173 on SL2.40ch07 chromosome 7 annotation). Protein domains were first predicted on the prosite database protein (http://prosite.expasy.org/). Prediction of the WD40 segments was refined using the PF00400.27 Pfam Hidden Markov Model with an i-value threshold at 0.1. For i-values > 0.1, the prediction of WD40 position was deduced from the sequence alignment of the different *TPL* isoforms.

Nuclear localization signal (NLS) analysis prediction was performed with 'cNLS Mapper' (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Kosugi *et al.*, 2009). NLS prediction scores >5.0 were considered positive.

Evolutionary analyses

Phylogenetic analyses and distance matrices were built using the MEGA5 package (Tamura *et al.*, 2011). Full-length amino acid sequences were aligned using the ClustalW algorithm. For the overall phylogeny, an initial tree encompassing sequences from *Physcomitrella patens*, *Selaginella moellendorffii*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Arabidopsis thaliana*, *Solanum lycopersicon*, *Nicotiana benthamiana*, *Populus trichocarpa*, *Glycine max* and *Mimulus guttatus* was performed using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together was calculated in the bootstrap test (500 replicates). The topology was further confirmed using the maximum-likelihood method. Ultimately, a simplified tree was performed by limiting the number of genomic sets as the topology remained unchanged. Trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The following genome annotations were used for phylogenetic analyses: *Physcomitrella patens* (Phypa1_1.FilteredModels; Rensing *et al.*, 2008); *Selaginella moellendorffii* (Lycophyte Selmo1_GeneModels_FilteredModels3; Banks *et al.*, 2011); *A. thaliana* (TAIR10; Swarbreck *et al.*, 2008); *Populus trichocarpa* (Eudicot Populus.trichocarpa.v2.0; Tuskan *et al.*, 2006); *V. vinifera* (12X March 2010 release, Glycine max Glyma1_pacId; Schmutz *et al.*, 2010); *O. sativa* [MSU Rice Genome Annotation (Osa1) Release 6.1; Ouyang *et al.*, 2007]; *Z. mays* (ZmB73_4a.53_working_translations; Schnable *et al.*, 2009); *Sorghum bicolor* (Sorbi1_GeneModels_Sbi1_4_aa; Paterson *et al.*, 2009); *Solanum lycopersicon* (ITAG2.3_release; Sato *et al.*, 2012); *Brassica rapa* (Chiifu-401–42; Wang *et al.*, 2011); *Eucalyptus grandis* (Egrandis_201; http://www.jgi.doe.gov/); *M. guttatus* (Mguttatus_140; http://www.jgi.doe.gov/); *N. benthamiana* (Niben.genome.v0.4.4; Pallas *et al.*, 2012); *Solanum tuberosum* (PGSC_DM_v3.4; Xu *et al.*, 2011).

Protein–protein interaction (PPI) assay of SlTPLs and SlIAAs by Y2H assay

Tomato *TPL* genes were amplified and cloned into the pDBD (BD-TPLs) vector (Clontech). Similarly, *SlIAA* target genes [*IAA1* (JN379431), *IAA3* (JN379433), *IAA4* (JN379434), *IAA7* (JN379435), *IAA8* (JN379436), *IAA9* (JN379437), *IAA11* (JN379438), *IAA12* (JN379439), *IAA14* (JN379441), *IAA15* (JN379442), *IAA16* (JN379443), *IAA17* (JN379444), *IAA19* (JN379445), *IAA22* (JN379447), *IAA26* (JN379449), *IAA27* (JN379450) and *IAA29* (JN379451)] were inserted in pGAD (AD-IAAs) vectors (Clontech). Diploids were selected on medium lacking Trp and Leu, and interactions were validated by the use of *HIS3* and *ADE2* reporter genes on medium lacking Trp, Leu, His, and Ade. Manipulation and analysis of the Y2H assay followed the manufacturer's instructions (Clontech Yeast Protocols Handbook), and all experiments were repeated three times independently. For *SlTPL1* genes lacking LisH, the coding sequence was truncated at nucleotide position +112.

Results

Identification and cloning of TPL-related genes in the tomato genome

An *in silico* search was performed on the tomato genome and transcript databases (http://www.solgenomics.net/) using Arabidopsis *TPL* and *TPR* sequences as queries for BLAST searches. While the initial screen identified nine ORFs predicted to encode putative TPL-like proteins (SlTPLs), only six corresponded to full-length proteins containing all canonical motifs that define the TPL proteins (Table 1). The full-length cDNA of the six *SlTPL*s was further confirmed by RT-PCR amplification, indicating that the corresponding coding sequences range from 3396 to 3669 bp with deduced protein sizes ranging from 1131 to 1222 aa (Table 1).

Structural analysis of the six *SlTPL* genes showed that they displayed similar numbers of introns (23–25) and exons (24–26), except for *SlTPL6*, which was longer than the other *TPL* members (Table 1). Pairwise comparison of the six SlTPL protein sequences showed that the percentage identity among family members ranged from 44 to 75%. Protein domain searches in the Pfam database (http://pfam.sanger.ac.uk/) indicated

that all SlTPLs displayed the conserved LisH and CTLH domains and had two domains containing several WD40 repeats: WD40-repeat-1 and WD40-repeat-2 with seven and five WD40 segments, respectively (Fig. 1 and Supplementary Fig. S1 available at *JXB* online). The CTLH domain and the WD40-repeat-1 were separated by a proline-rich region.

The tomato *TPLs* were distributed on four chromosomes: two *SlTPLs* (Solyc03g116750 and Solyc3g117360) on chromosome 3, two (Solyc08g076030.2.1 and Solyc08g029050.2.1) on chromosome 8, one (Solyc01g100050.2.1) on chromosome 1 and one (Solyc07g008040.2.1) on chromosome 7. There were three additional truncated TPL sequences lacking the LisH and CTLH domains, with two located on chromosome 3 (Solyc03g117370 and Solyc03g117410) and one on chromosome 1 (Solyc05g016070).

The number of 'full-length' *TPL* genes in tomato fell in the range found in other plant genomes, which varies in angiosperms from four members in monocots to 11 members in soybean (Fig. 2). It is noteworthy that a high number of isoforms is often observed in organisms having undergone recent whole-genome duplication or polyploidization events (e.g. *G. max*, *N. benthamiana* and *B. rapa*).

SlTPL nomenclature and phylogenetic analyses

To adopt a nomenclature consensual with that of *Arabidopsis* TPL and TPR proteins, we carried out phylogenetic analyses on different TPL-like proteins or cDNAs from different plant sequenced genomes comprising moss, fern, and various angiosperm sequences (see Materials and methods). The phylogenetic trees (Fig. 3A) allowed the individualization of four branches. Three branches looked well defined in all dicot plants: the first branch contained AtTPL, AtTPL1, AtTPR4, Solyc3g117360.2.1 (named SlTPL1), Solyc03g117360.2.1 (named SlTPL4), and Solyc07g008040.2.1 (named SlTPL5); the second branch, absent in *Arabidopsis* yet present in *Eucalyptus* (Eucgr.K00093.1|PACid:23601479) and grapes (GSVIVT01024440001), contained Solyc08g076030 (named SlTPL2), rice ASP1 protein, and moss or lycophyte TPL-like proteins; and the third branch contained AtTPR2, AtTPR3, and Solyc01g100050.2.1 (named SlTPL3). Lastly, Solyc08g029050.2.1 (named SlTPL6) appeared as an outgroup branch in the phylogenetic tree (Fig. 3A). The robustness of the tree topology was assessed either with a bootstrap test (Fig. 3A) or by changing the number of genomes used

Table 1. *Main structural features of the tomato SlTPL family members*

Nomenclature	Gene			Predicted protein		Domains		
SlTPLs	iTAG Gene ID	Exons	Introns	Length (aa)	MW (kDa)	LisH	CTLH	WD-40 repeats
SlTPL1	Solyc03g117360.2.1	25	24	1131	124.676	4–36	34–92	411–632/832/957
SlTPL2	Solyc08g076030.2.1	25	24	1136	124.60	4–36	34–92	341–668/834–959
SlTPL3	Solyc01g100050.2.1	25	24	1132	124.676	4–36	34–92	343–669/871–955
SlTPL4	Solyc03g116750.2.1	26	25	1133	124.318	4–36	34–92	413–634/839–964
SlTPL5	Solyc07g008040.2.1	24	23	1134	124.82	4–36	34–92	398–639/881/965
SlTPL6	Solyc08g029050.2.1	33	32	1222	134.181	3–35	33–91	531–664/934–1060

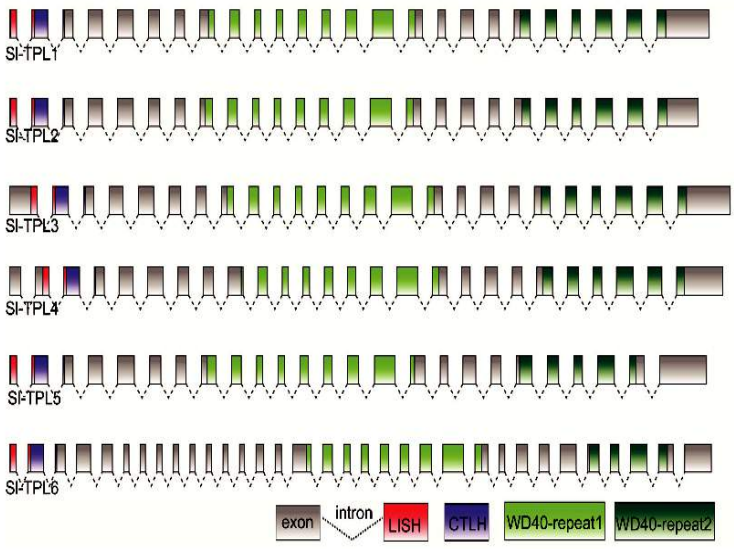

Fig. 1. Gene structure of the six tomato *TPL* genes. Grey boxes represent exons, dotted lines represent introns, the red box is the LisH domain, the blue box is the CTLH domain, the light green boxes are the WD40-repeat 1 and the dark green boxes are the WD40-repeat 2. The figure was produced using FancyGene software (http://bio.ieo.eu/fancygene/). (This figure is available in colour at *JXB* online.)

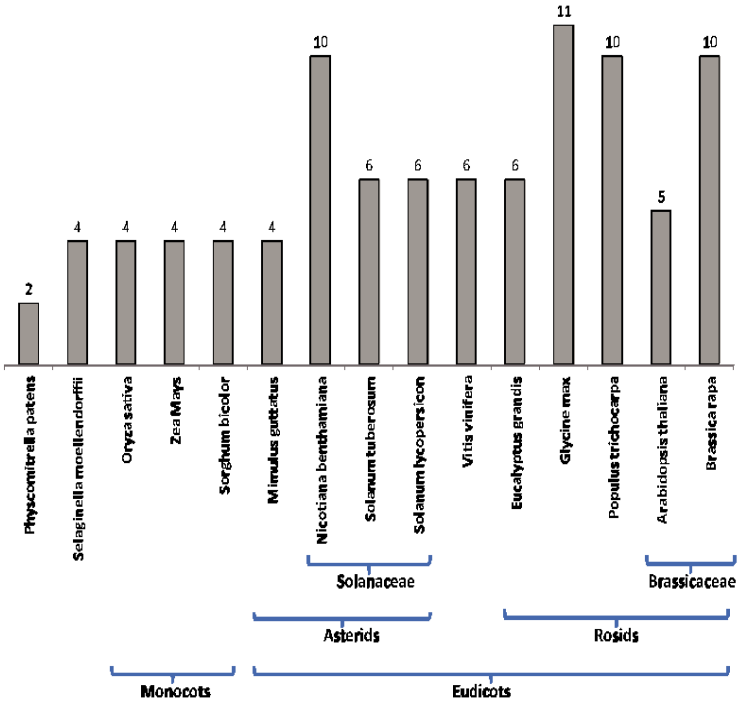

Fig. 2. Inventory of *TPL* genes in different plant genomes. Only *TPL* genes containing the four canonical domains (LisH, CTLH and two WD40 repreats) were considered. The major taxons are shown below.

in the phylogeny and the portion of the aligned sequence (N-terminal, C-terminal, or conserved domains) or the clustering method (neighbour-joining or maximum-likelihood method). The vast majority of the nodes presented in Fig. 3A remained unchanged.

To understand further the TPL phylogeny, and notably to characterize the SlTPL6 outgroup, the presence of TPL 'orthologues' was investigated in Asterid genomes belonging either to the Solanaceae family (*Solanum tuberosum* and *N. benthamiana*) or to the Lamiales order (*M. guttatus*). An SlTPL6 homologue was found in all Asterids, supporting the view that SlTPL6 homologues form a distinct clade (Fig. 3B). Within this SlTPL6 clade, the length of the branches suggested that these isoforms had evolved faster than other TPLs. This observation was supported by sequence divergences: the amino acid substitution rates calculated within the Solanaceae orthology groups varied from 2.6 to 6.3% for SlTPL1–5 and reaching 22.7% for the SlTPL6 (Table 2). Moreover, a neutrality test (*dS*/*dN* values) calculated on Solanaceae orthologues suggested that the purifying selection exerted by evolution on the *SlTPL6* family is much weaker than the selection pressure exerted on other *TPL* genes.

Subcellular localization of SlTPLs

The subcellular localization of the SlTPL proteins was assessed by a transient expression assay in tobacco protoplasts using a translational fusion between each of the SlTPL proteins and YFP. Microscopy analysis showed that SlTPL1–5–YFP fusion proteins localized exclusively to the nucleus (Fig. 4) whereas SlTPL6 was localized at the cytoplasm and excluded from the nucleus. This result is in agreement with the *in silico* prediction of a conserved NLS for the five nuclear SlTPL1–5 proteins, while SlTPL6 NLS scores were below the 5.0 threshold value (Supplementary Table S1 available at *JXB* online). Altogether, the nuclear localization of the majority of SlTPLs was consistent with their putative role in transcriptional regulation activity.

Expression analyses

In order to study the spatio-temporal expression pattern of the six *SlTPL* genes, qRT–PCR was performed on eight different plant tissues and organs. Three *SlTPL* members (*SlTPL1*, *SlTPL3*, and *SlTPL4*) displayed significantly higher levels of expression than the three remaining paralogues. *SlTPL1* and *SlTPL4* were found to be highly expressed in flowers and vegetative tissues (roots, stems, and leaves) and in developing flowers (buds and during anthesis) but with reduced expression in ripening fruit, while *SlTPL3* expression remained constant and high during fruit ripening (Fig. 5). This preferential expression of *SlTPL1*, *SlTPL3*, and *SlTPL4* is coherent with their estimated expression in two public databases (RNAseq database: http://ted.bti.cornell.edu and ESTs database: http://solgenomics.net/). Although less expressed, *SlTPL2* was found preferentially in leaves and developing flowers; the levels of *SlTPL5* transcripts were low in all tissues; *SlTPL6* expression was restricted to roots and stems (Fig. 5b).

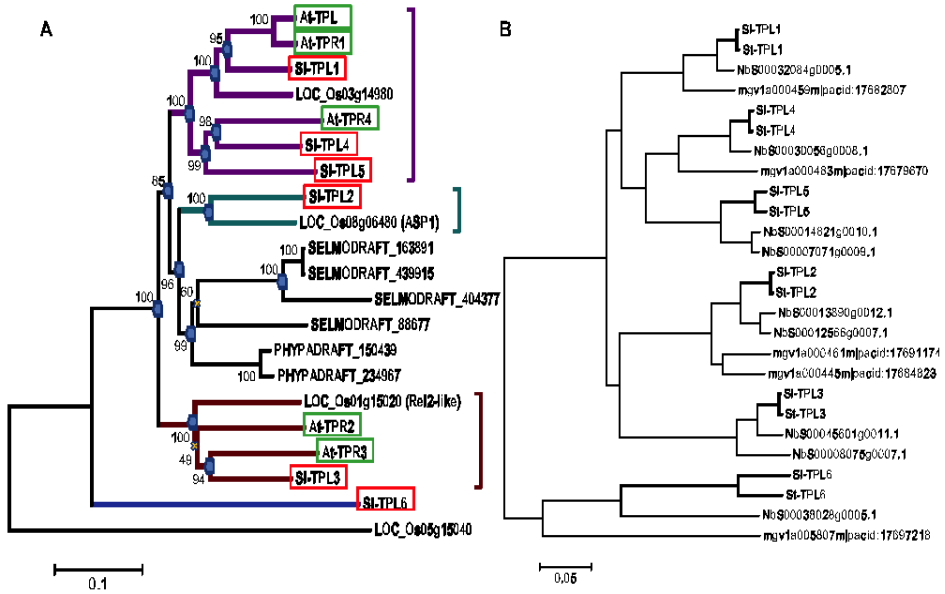

Fig. 3. Phylogenetic trees of some plant and tomato TPL proteins. (A) Representative phylogenetic tree of TPL proteins from land plants: moss (*P. patens*, PHYPADRAFT_xxx), lycophyte (*Selaginella moellendorffii*, SELMODRAFT_xxx), rice (LOC_Os-xxx), tomato (red boxes) and *Arabidopsis* (green boxes). The coloured brackets emphasize the main branches conserved among angiosperms. The present tree was obtained after alignment of full-length TPL sequences using ClustalW and clustering with the neighbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses including additional genome sets (*Z. mays*, *Sorghum bicolor*, *Populus trichocarpa*, *G. max*, *V. vinifera* and *M. guttatus*) or using the maximum-likelihood clustering method displayed similar topologies, the majority of the nodes being conserved (blue circles) while only few nodes (yellow crosses) were unstable. (B) Phylogenetic tree of TPL proteins among Asterid and Solanaceaous species. The tree was built using sequences from four genomes: *Solanum lycopersicon*, *Solanum tuberosum*, *N. benthamiana* and *M. guttatus*. (This figure is available in colour at *JXB* online.)

Table 2. *Evolutionary features of TOPLESS-related genes in Solanaceous species*

Mean distance was expressed as the proportion of amino acid or nucleic acids positions different after pairwise alignment. dS/dN values were calculated using the codon-based test of purifying selection performed on each pair of orthologous sequences from Solanum lycopersicon and Solanum tuberosum. The variance of the difference was computed using the bootstrap method (500 replicates). Analyses were conducted using the Nei and Gojobori (1986) method.

		SlTPL1	SlTPL2	SlTPL3	SlTPL4	SlTPL5	SlTPL6
Mean distance (*Solanum*/*Nicotiana*)	Amino acids	0.026	0.041	0.032	0.029	0.063	0.227
	Nucleic acids	0.055	0.050	0.054	0.054	0.067	0.154
Neutrality test (*Solanum*)	*dS/dN*	7.08	6.66	6.98	7.62	6.19	3.645

Examination of PPIs in the framework of auxin mediation

The differential expression of *SlTPL* genes evokes the critical question of functional redundancy within the TPL family. In a recent paper, Causier *et al.* (2012*a*) compared the PPI patterns of different *Arabidopsis* TPL proteins using a high-throughput Y2H screen both on a whole-plant and on a transcription factor library. In the present work, we focused on the interactions with the Aux/IAA family by performing an exhaustive targeted analysis of Aux/IAA–TPL interactions. The six SlTPL proteins were fused to a binding domain (BD) and used as bait in a Y2H test with 17 different SlIAA proteins fused to an activating domain (AD). After monitoring the yeast growth on two auxotroph selective media, two patterns of TPL could clearly be defined (Fig. 6A, B): SlTPL1, SlTPL2, SlTPL4, and SlTPL5 interacted with the majority of SlIAAs and grew in all the selective media, and SlTPL3 and SlTPL6 exhibited only limited growth when co-expressed with Aux/IAA–AD fusion proteins. Contrary to other SlIAAs, SlIAA29 failed to show interaction with any of the SlTPLs. With the exception of SlIAA12 and SlIAA15, the Aux/IAAs did not harbour any obvious specificity towards the 'TPL' clade (SlTPL1, SlTPL4, and SlTPL5), sharing high

similarity with AtTPL. In addition, SlTPL2, which belongs to a distinct clade of SlTPLs (1, 4, and 5), also exhibited a broad capacity to interact with the majority of SlIAAs. As a control, we performed a Y2H test with truncated SlTPL1 or SlTPL5 (ΔLisH-TPL) (Fig. 6C) lacking the LisH domain shown previously to be essential for TPL–WUS or TPL–Aux/IAA interactions (Kieffer *et al.*, 2006; Szemenyei *et al.*, 2008). Contrary to all SlTPLs BD fusions assayed, a complete lack of growth was observed when co-expressing BD–ΔLisH-TPL proteins with BD–Aux/IAAs (Fig. 6B).

Discussion

The present study addressed the structural, evolutionary, and functional features of the tomato *TPL* family. TPL proteins have been primarily defined as a major component of the auxin transduction and response pathway, but the present data sustain the hypothesis of a functional diversification of these regulatory proteins. While mainly focusing on the *TPL* family in tomato, a plant model for Solanaceae and fleshy fruit research, the data also addressed the comparative features of this gene family within Plant kingdom at the evolutionary level, shedding new light on their functional diversification.

The structure of the *SlTPL* family is representative of that found in angiosperms where these proteins belong to a small multigenic family comprising five to 11 members. In the tomato, six full-length *SlTPL* genes were identified, as well as additional three pseudogenes with incomplete coding sequences. Among the six *SlTPL* genes, five were highly conserved (*SlTPL1–5*), while the last gene (*SlTPL6*) was more distant. With the exception of poplar genomes and genomes having undergone recent polyploidization (i.e. soybean, *B. rapa*, and *N. benthamiana*), the number of *TPL* isoforms ranges from four to six members, suggesting that the number of genes remains stable in this family and that usually, after a whole-genome duplication event, duplicated copies of *TPL* genes are not retained. The phylogenetic analysis of *TPL* genes enabled the distinction of three major clades gathering homologues in the majority of angiosperm genomes. The last clade, containing the distant *SlTPL6*, displays only clear homologues in closely related taxa (Asterids). Interestingly, highly diverging sequences of TPL-related proteins have also been found in other genomes such as the *AtTPR-like* gene (At2g25420; Causier *et al.*, 2012b) and in poplar, but no clear relationship could be established with *SlTPL6*. Contrary to angiosperm TPL proteins, TPL from *Physcomitrella patens* and *Selaginella moellendorffii* clustered in a same branch, indicating the existence of ancestral divergences occurring before angiosperm radiation.

The functionality of *SlTPL* genes was addressed through three approaches: expression analysis, subcellular localization, and establishment of an interaction map between SlTPL and SlIAA proteins. The expression patterns of different *SlTPLs* revealed the tissue specificity of various isoforms and suggested a functional specialization of *SlTPL* isoforms. For example, *SlTPL1* is highly expressed in vegetative organs (stems, roots) and flowers, while the expression of *SlTPL3* and *SlTPL4* prevails in fruit. Moreover, the overall intensity of gene expression evaluated by qPCR demonstrated a distinction between a group of three isoforms (*SlTPL1*, *SlTPL3*, and *SlTPL4*) that are highly expressed, *SlTPL2*, which is moderately expressed in the leaves and flowers, and third group made of two isoforms (*SlTPL5* and *SlTPL6*) that displayed very low levels of expression. In agreement with our data, the prevalence of *SlTPL1*, *SlTPL3*, and *SlTPL4* transcripts was also observed in expressed sequence tag (EST) and RNAseq expression databases (http://ted.bti.cornell.edu), whereas the expression of *SlTPL6* was again found to be very low (no EST and few RNAseq reads). Interestingly, the overall expression level negatively correlated with the amino acid substitution rate. Indeed, after defining orthology groups among *Solanaceous TPLs*, we found that the highly expressed isoforms (*SlTPL1*, *SlTPL3*, and *SlTPL4*) showed the highest

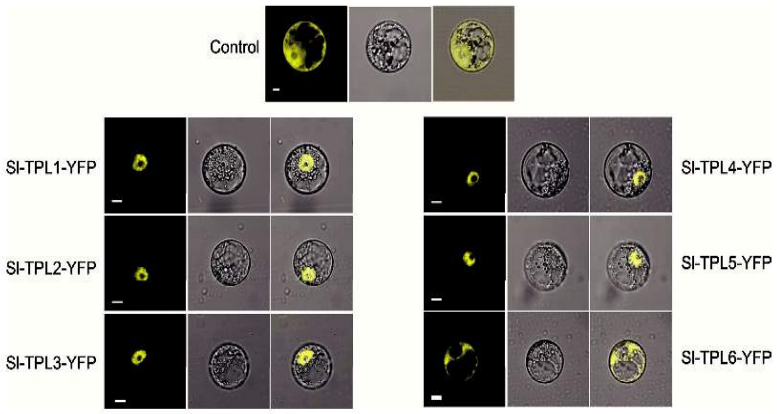

Fig. 4. Subcellular localization of tomato TPL proteins. SlTPL–YFP fusion proteins were transiently expressed in BY-2 tobacco protoplasts and subcellular localization was analysed by confocal laser-scanning microscopy. The merged pictures of the yellow fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown (right panels). The empty vector pEarleyGate104 was used as a control. Bar, 10 μm. (This figure is available in colour at *JXB* online.)

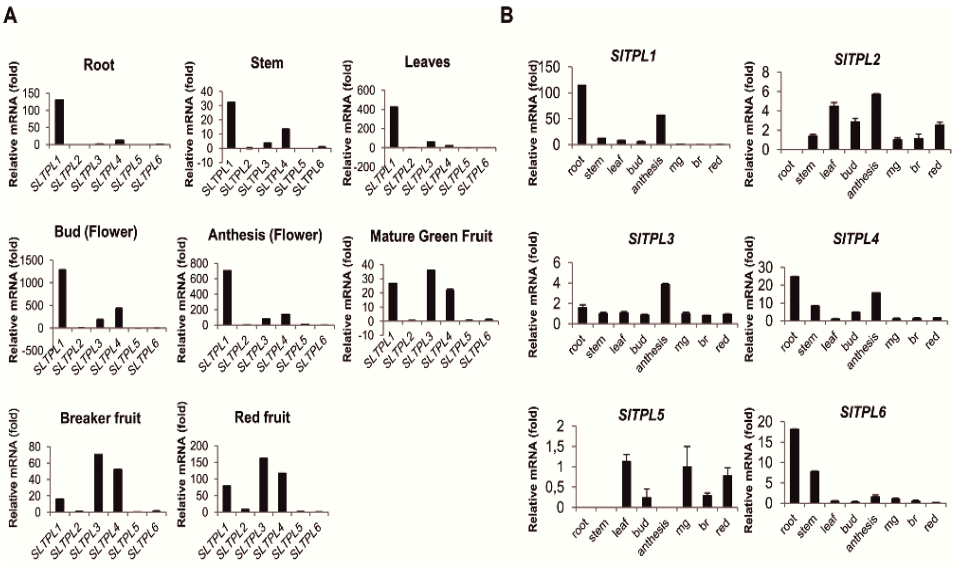

Fig. 5. Real-time PCR expression profiles of six tomato *TPL* genes. (A) Expression patterns of *SlTPL* genes in various tomato tissues. Relative mRNA levels of each *SlTPL* gene in different tissues were normalized against actin. The results were expressed using *SlTPL6* as a reference (relative mRNA level 1). Values represent the best experiment among three independent biological repetitions. Bars indicate the standard deviation of three experimental repetitions. (B) Expression patterns in different tomato tissues of each *SlTPL* gene. The relative mRNA level of each *SlTPL* gene was normalized against actin. mg, Mature green fruit; br, breaker fruit; red, red fruit. The results were expressed using the mature green fruit as a reference (relative mRNA level 1). Values represent the best experiment among three independent biological repetitions. Bars indicate the standard deviation of three experimental repetitions.

amino acid sequence conservation (<3.2% difference within *Solanaceous* sequences), while sequences were less conserved within the SlTPL6 orthology group (22.7% difference within *Solanaceous* sequences). The moderately expressed *SlTPL2* and *SlTPL5* displayed intermediate substitution rates (4 and 6% differences, respectively). This correlation was also supported by a neutrality test (*dS*/*dN* values) performed between potato and tomato pairs of othologues. The high substitution rate within the *SlTPL6* orthology group was interpreted as an indication that the *SlTPL6* subfamily undergoes a reduced purifying selection. By contrast, broadly expressed *SlTPL* isoforms are under a stronger purifying selection. Such a correlation between gene expression level and amino acid substitution rate has already been observed in genome-wide comparisons of expression patterns and protein evolution in *Arabidopsis*-related plants and in the Poaceae family (Wright *et al.*, 2004; Slotte *et al.*, 2011; Davidson *et al.*, 2012). Indeed, this correlation is consistent with *A. thaliana* expression data (AtGenExpress), *At-TPL* being expressed more than other *AtTPR*s and *AtTPL* orthologues remaining highly conserved either in *Arabidopsis lyrata* or in *B. rapa*.

The subcellular localization established a second discrimination criterion among SlTPLs. YFP fusion proteins of SlTPL1–5 isoforms all migrated exclusively to the nucleus, as observed with other TPL proteins from *Arabidopsis* (Long *et al.*, 2006), maize (Gallavotti *et al.*, 2010), and rice (Yoshida *et al.*, 2012). By contrast, the SlTPL6–YFP fusion protein displayed a divergent subcellular targeting, this isoform being targeted to the cytosol. This divergent localization is in line with the lower scores calculated by the NLS prediction tool for SlTPL6. This observation, in addition to the low expression level and the high substitution rate, supports the view of either a partial loss of functionality or divergent functionality regarding SlTPL6.

The first established function of TPL proteins is related to their role in auxin signalling via interaction with Aux/IAA partners (Szemenyei *et al.*, 2008). To check whether this role is conserved among all SlTPLs isoforms and gain insight on either functional redundancy or potential functional diversification among family members in tomato, a comprehensive PPI study was carried out between all SlTPLs and SlIAA members using a Y2H screen. This targeted interactome study revealed two distinct patterns of interaction for tomato TPLs: four isoforms (SlTPL1, SlTPL2, SlTPL4, and SlTPL5) displayed a broad capacity for interaction with the majority of SlIAAs, and the remaining two isoforms (SlTPL3 and SlTPL6) showing a more restricted interaction capacity. It is noteworthy that a large number of SlIAAs showed positive interaction with SlTPLs, consistent with the outcome of Y2H screens performed in *Arabidopsis* where 20 out of the 29 AtAux/IAAs were able to interact with AtTPLs (Szemenyei *et al.*, 2008; Arabidopsis Interactome Mapping Consortium, 2011; Causier *et al.*, 2012*a*). Interestingly, neither SlIAA29 nor its *Arabidopsis* homologue At IAA29 (AT4G32280.1) interacted with TPL proteins, although SlIAA29 exhibits a repressor activity (Audran-Delalande *et al.*, 2012). On the other hand, the limited interaction capacity displayed by SlTPL6 adds another distinctive feature to this isoform,

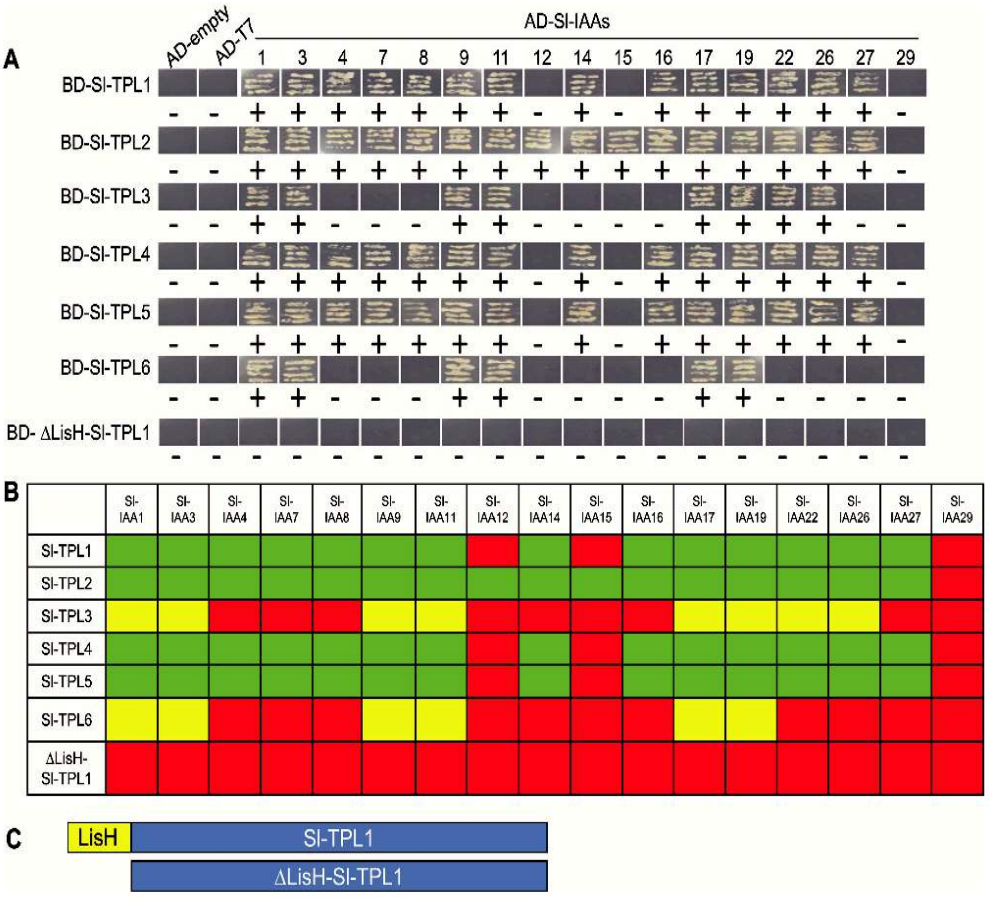

Fig. 6. PPI maps between SlTPLs and SlIAAs established by a Y2H screen. (A) Yeast growth of co-transformed BD–TPLs and AD–IAAs. The yeast clones grown on selected medium lacking Trp, Leu, His, and Ade (TLHA) were scratched again on a TLHA plate. After 3–4 d, the growth of the yeast strains confirmed a positive interaction, as shown. AD–empty vector and AD–T7 vector were used as negative controls. (B) Schematic representation of the interaction map between SlTPLs and SlIAAs. Green indicates that the yeast grew quickly, less than 4 or 5 d after co-transformation, indicating a strong interaction between the SlTPL and SlIAA partners. Yellow indicates that the yeast grew slowly 7–8 d after co-transformation, indicating a weak interaction between the tested SlTPL and SlIAA. Red indicates that there was no interaction detected between the tested SlTPLs and SlIAAs. (C) Truncated form of SlTPL1 protein lacking the N-terminal LisH domain N-terminal used as a negative control. (This figure is available in colour at *JXB* online.)

which has already diverged from other family members by its low expression level, high amino acid substitution rate and different subcellular localization. Altogether, the cumulative distinctive features support the idea that SlTPL6 has partially lost its ancestral function and may have gained new functionality.

In previous Y2H screens performed in *Arabidopsis* by Causier *et al.* (2012*a*), AtTPR3 and AtTPR2, closely related to SlTPL3, both displayed the capacity to interact with various Aux/IAA proteins. However, a closer look at the interaction map published by Causier *et al.* (2012*a*) could also suggest differences in specificity between AtTPL and AtTPR2 or AtTPR3, with the two latter notably interacting with partners displaying partial repression domains. Such hypothesis opens the possibility that At-TPR2, At-TPR3 and the closely related SlTPL3 display a specialization alternative to auxin signalling. The development of quantitative PPI methods such as Förster resonance energy transfer or surface plasmon resonance may provide deeper insight on discriminating interaction features among various TPL isoforms.

Functional redundancy among *Arabidopsis* TPL family members is supported by the absence of obvious phenotypes in single loss-of-function mutants of *AtTPL/TPR* genes and by the requirement for downregulation of all five *AtTPL-TPRs* in order to phenocopy the dominant mutation *tpl-1* (Long *et al.*, 2006). However, this assumption is in contrast to the situation prevailing in rice and maize, where genetic evidence seems to support a more specialized functionality for *TPL* genes. Thus, in rice (Yoshida *et al.*, 2012), a single recessive mutation in *Asp1*, a *TPL-like* gene close to *SlTPL2*, exhibited several pleiotropic phenotypes, such as altered phyllotaxy and spikelet morphology. While these phenotypes suggest a close association of *Asp1* with auxin action, they clearly reveal that the specialization of TPL-related proteins in some organisms can differ from that in *Arabidopsis*. Further evidence sustaining a diversified function for TPL

proteins is provided by maize *rel2* mutants affected in a *TPL-like* gene closely related to *SlTPL3* and *AtTPR3* (Gallavotti *et al.*, 2010). A better clarification of the putative specialized functionality among tomato TPLs might be addressed by a reverse genetics approach. Simultaneous downregulation of *SlTPL1* and *SlTPL4* would uncover the importance of the TPL family in vegetative development and auxin action. Likewise, specific downregulation of *SlTPL3* would be of particular interest to unravel the role of TPL co-repressors in flower and fruit biology.

Altogether, these data shed new light on structural, evolutionary, and some functional features of the tomato *TPL* gene family that suggest functional diversification of these regulatory proteins. Of particular interest, the setup of a comprehensive TPL–Aux/IAA interaction map and the differential subcellular targeting of some SlTPLs proteins would provide important clues towards designing appropriate strategies for the elucidation of both redundant and specific roles of *TPL* genes.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Multiple sequence alignment of full-length SlTPL proteins.

Supplementary Table S1. NLS prediction scores computed with cNLS Mapper (Kosugi *et al.*, 2009).

Acknowledgements

This work was carried out in the GBF laboratory, part of the TULIP 'Laboratoire d'Excellence' (LABEX) (ANR -10-LABX-41) and supported by the European Integrated Project EU-SOL (FOOD-CT-2006-016214). The work benefited from the networking activities within the European COST Action FA1106. YH and XW were supported by the China Scholarship Council.

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Complementary results

It is proposed that Aux/IAA recruits TPL to repress ARF activity. In *Arabidopsis*, it is proved that 20 of 29 Aux/IAAs interact with TPL/TPRs, ARFs activators interact with most of the Aux/IAAs while ARFs repressors show a very limit ability to interact with Aux/IAAs. But some ARF repressors can interact with topless directly such as ARF2 and ARF9. In order to check this interaction results are also conserved in tomato, we performed Y2H to check the interaction among topless, ARFs and Aux/IAAs. In addition, in *Arabidopsis*, it is reported that there are other topless interaction partners involved in histone methylation mediated by PRC1 and PRC2 polycomb-complex. In this complementary results we also investigate the interactions between topless and PRC1 and PRC2 complex components.

1. PPIs between whole Sl-ARFs and Sl-Aux/IAAs

We used Y2H to check the interaction between whole Sl-ARFs and Sl-Aux/IAAs family members. The Sl-Aux/IAAs and Sl-ARFs members were cloned into pGAD vector and pGBD vector respectively.

BD-IAA1, IAA 3, IAA 4, IAA 7, IAA 8, IAA 9, IAA 11, IAA 12, IAA 14, IAA 15, IAA 16, IAA 17, IAA 19, IAA 22, IAA 26, IAA 27, IAA 29

AD-ARF1, ARF 2a, ARF 2b, ARF 3, ARF 4, ARF 5, ARF 6, ARF 7, ARF 8a, ARF 9a, ARF 9b, ARF 10a, ARF 10b, ARF 16a, ARF 16b and ARF 17.

Figure 7 is the interaction results between whole Sl-ARFs and Sl-Aux/IAAs family members. ARF activators (ARF5, 6, 7, 8) interact with most of the Aux/IAAs except Aux/IAA 11. ARF repressors (ARF1, 2a, 2b, 4, 16a) interact with few members Aux/IAAs. The other ARF repressors ARF (3, 9a, 9b, 10a, 10b, 16b, 17) do not interact with any other Aux/IAA.

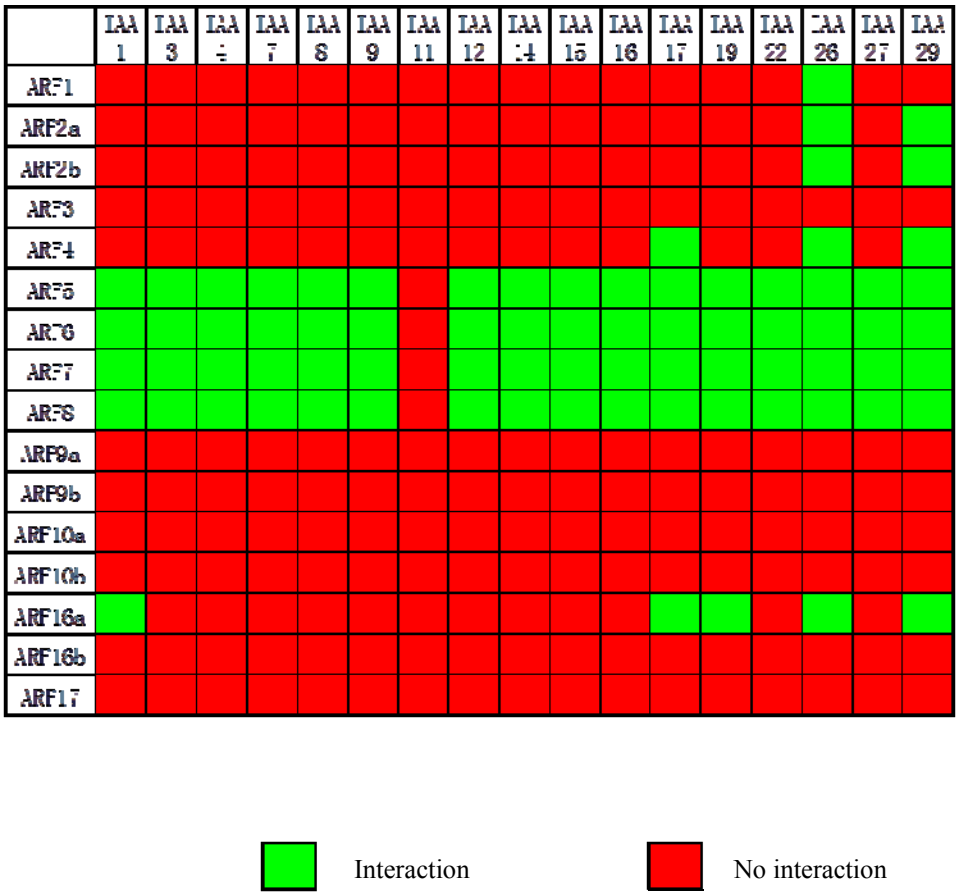

Figure 7 The interaction map between whole Sl-ARFs and Sl-Aux/IAAs. The green grid meas there is interaction between the two proteins. The red grid presents on interaction between the two proteins.

2. PPIs between whole Sl-ARFs and Sl-TPLs

We used Y2H to check the interaction between whole Sl-ARFs and Sl-TPLs family members. The Sl-ARFs and Sl-TPLs members were cloned into pGAD vector and pGBD vector respectively.

BD-TPL1, TPL2, TPL3, TPL4, TPL5, TPL6

AD-ARF1, ARF 2a, ARF 2b, ARF 3, ARF 4, ARF 5, ARF 6, ARF 7, ARF 8a, ARF 9a, ARF 9b, ARF 10a, ARF 10b, ARF 16a, ARF 16b and ARF 17.

Figure 8 is the interaction results between whole Sl-ARFs and Sl-TPLs family members. ARF activators (ARF5, 6, 7, 8) do not interact with any of the Sl-TPLs. ARF repressors (ARF1, 3, 4, 9a, 9b, 10a, 10b, 16a, 16b, 17) at least interact with one of the Sl-TPL1, 2, 4. There is on interaction between any of the Sl-ARFs members

and Sl-TPL 3, 5, 6.

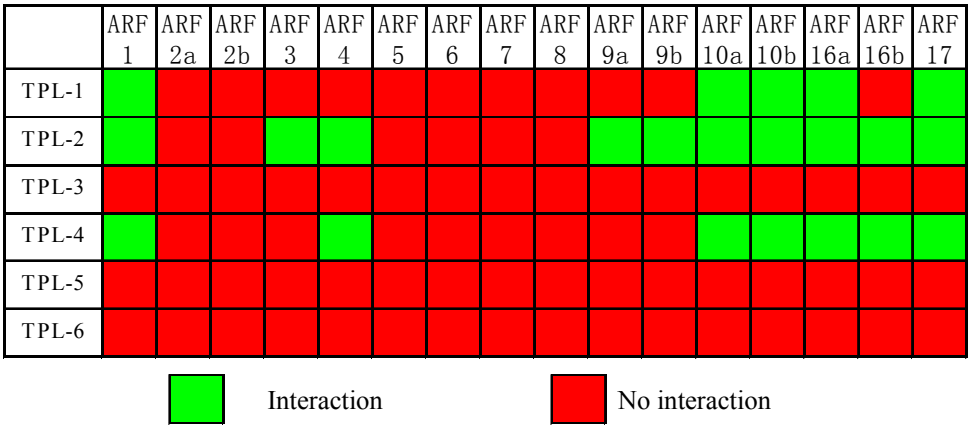

Figure 8. The interaction map between whole Sl-ARFs and Sl-TPLs. The green grid meas there is interaction between the two proteins. The red grid presents on interaction between the two proteins.

3. PPs between Sl-TPL and different truncated ARF4 proteins.

SlARF4 and truncated SlARF4 (ARF4-I; ARF4-I-II; ARF4-II; ARF4-III-IV; ARF4-II-III-IV; figure 9) were cloned into pGAD vector. Sl-TPLs members were cloned into pGBD vector. We performed Y2H to test the interaction between the Sl-ARF4, truncated SlARF4 and Sl-TPLs.

BD-TPL1, 2, 3, 4, 5, 6

AD-ARF4, ARF4-I, ARF4-I-II, ARF4-II, ARF4-III-IV, ARF4-II-III-IV.

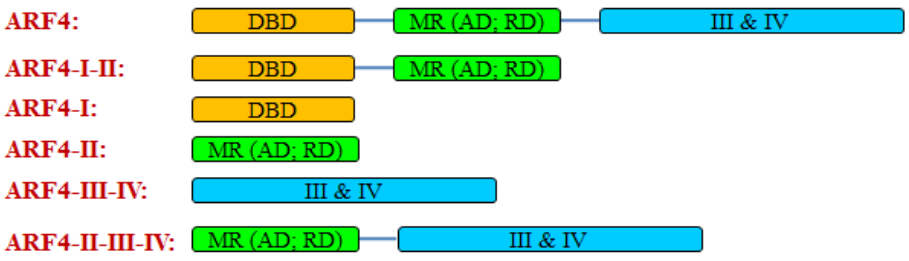

Figure 9. The construction of truncated ARF4.

Figure 10 is the interaction results between SlARF4 and truncated SlARF4 and Sl-TPLs members. The truncated ARF4 only containing domain I and domain II show the similar interaction results with full length ARF4. They both interact with SlTPL2

and SlTPL4. The other truncated ARF4 did not interact with any of the Sl-TPLs. The ARF4 only containing DBD domain or RD domain did not interact with any of the Sl-TPLs. This result indicates that the DBD and RD domain are both necessary for the interaction between SlARFs and SlTPLs.

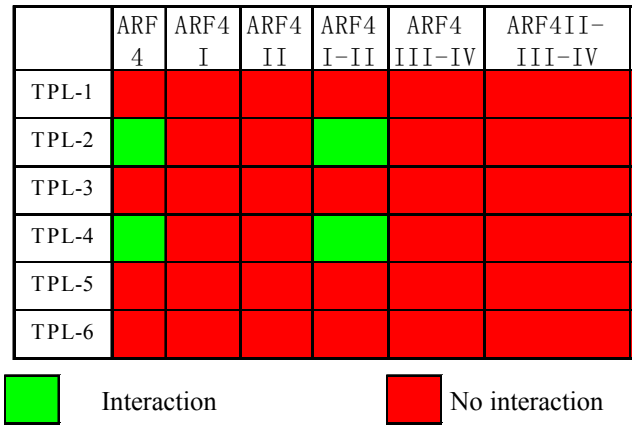

Figure 10. The interaction map between SlARF4, truncated SlARF4 Sl-TPLs. The green grid meas there is interaction between the two proteins. The red grid presents on interaction between the two proteins.

4. PPs between Sl-TPL and PRC1 PRC2 polycomb complex components.

In *Arabidopsis*, the PRC1 component EMF1 interact with TPL and TPR3, PRC2 component VRN5 interact with TPL. In order to find whether there is interaction between PRC1 PRC2 components and topless in tomato, we isolated the homologues of the PRC1 PRC2 components (figure 11) in tomato and put them into the pGAD vector. Sl-TPLs family members were cloned into pGBD vector. Y2H was performed to test the interactions between the components of the polycomb complex and Sl-TPLs.

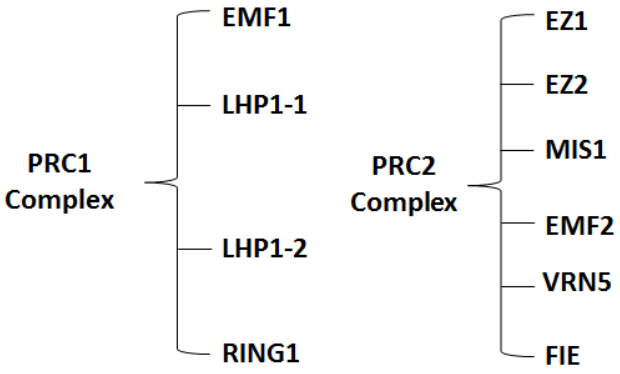

Figure 11. The components of PRC1 and PRC2 polycomb complex.

BD-TPL1, TPL 2, TPL 3, TPL 4, TPL 5, TPL 6

AD-EMF1, LHP1-1, LHP1-2, RING1, EZ1, EZ2, MIS1, EMF2, VRN5, FIE

Figure 12 is the interaction result between the components of PRC1 and PRC2 and Sl-TPLs. The PRC1 component EMF1 interacts with SlTPL2 while LHP1-2 interacts with the TPL1, 2, 4. The PRC2 component VRN5 interacts with SlTPL2 and SlTPL4. There is on interaction between the other components of the polycomb complex and SlTPLs.

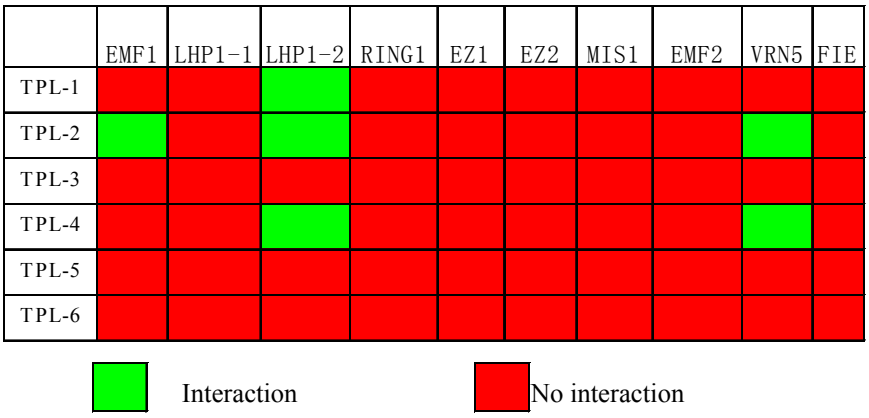

Figure 12. The interaction map between components of PRC1 and PRC2 and Sl-TPLs. The green grid meas there is interaction between the two proteins. The red grid presents on interaction between the two proteins.

Complementary discussion

Topless as co-repressors in TIR1-auxin-dependent and independent regulation of

ARFs.

It is proposed that Aux/IAA recruits TPL to repress ARF activity in *Arabidopsis* (Szemenyei et al., 2008; Causier et al., 2012b). In order to test the hypothesis is also appeared in tomato; the interactions among these three components were performed by Y2H. The interaction results show that the ARF activators interact with most of the Aux/IAA which show similary results the *Arabidopsis* (Causier et al., 2012b). In tomato, TPLs interact with most of the Aux/IAAs, while TPLs do not interact with any of the ARF activators. These results indicate that for ARF activators, Aux/IAA recruits TPL to repress the ARF activity. For the ARF repressors, Some ARF repressors interact with few numbers of Aux/IAAs. Most ARF repressors can interact with TPLs directly. In *Arabidopsis*, some ARF repressors can also interact with TPL/TPR, such as At-ARF2 and At-ARF9 (Causier et al., 2012b). These results suggest that for ARF repressors, TPL can be recruited by Aux/IAA or ARF to repress ARF activity. All of these results may get a point to the repression mechanism of topless acts as co-repressors in TIR1-auxin-dependent and independent ARF-mediated repression.

The ARF DBD domain and RD domain are both necessary for the interaction between ARFs and TPLs.

In order to find out the crucial domain for the interaction between ARF and TPL, the ARF repressor SlARF4 was fist investigated (Zouine et al., 2014). A typical ARF possess four conserved domains: DBD domain, MR domain, CTD domain containing domain III and IV (Guilfoyle et al., 1998; Tiwari et al., 2003; Zouine et al., 2014). SlARF4 was divided into 5 different truncated proteins. The interaction results show that only the truncated ARF4 with the DBD and MR domain can interact with Sl-TPL2 and Sl-TPL4 the same interaction results with the full length ARF4. The DBD domain is responsible for recognizing and interacting with the auxin response element in the genomic DNA (Guilfoyle et al., 1998). The MR domain is deciding the ARF activation or repression ability. ARF with AD type middle region that is rich QSL is activator, while ARF with RD type middle regions that is rich in SPL is

repressors (Guilfoyle et al., 1998; Ouellet et al., 2001; Tiwari et al., 2003; Ulmasov et al., 1999). The ARF activators did not interact with TPLs while most of ARF repressors interact with SlTPL1, SlTPL2, SlTPL4 which suggest that this middle region may account for the no interaction between ARF activators and TPLs. Moreover, the truncated ARF4 only contains middle region did not interact with TPLs indicating that both the DBD and RD are necessary for the interaction.

Topless represses the gene expression by multiple chromatin-remodeling mechanisms

Topless acts as a co-repressor inhibits the gene expression by changing the chromatin state from active to inactive (Long et al., 2006; Liu and Karmarkar, 2008; Krogan and Long, 2009; Krogan et al., 2012). Histone acetylation is largely correlated with gene expression; therefore, removal of these modifications by HDACs generally leads to repression of transcription (Shahbazian and Grunstein, 2007). In *Arabidopsis*, TPL acts through HDA19, the interactions between TPR1 and HDA19 can be observed in pull-down experiment from plant extracts. Mutations in HDA19 increase the penetrance of *tpl-1* and display similar apical defects (Gonzalez et al., 2007; Krogan et al., 2012; Long et al., 2006; Sridhar et al., 2004; Zhu et al., 2010).

Besides histone deacetylases, large interactome studies in *Arabidopsis* show that TPL/TPR proteins interact with some histone methyltransferases such as EMF1, VRN5. EMF1 is a component of Polycomb Repressive Complex 1 (PRC1) (Calonje et al., 2008), while VRN5 is a component of Polycomb Repressive Complex 2 (Greb et al., 2007). PRC2 catalyze the trimethylation of histone H3 on lysine 27 (H3K27 trimeth) (Cao et al., 2002). PRC1 binds to this mark through its subunit POLYCOMB (PC) and catalyzes mono-ubiquitylation of lysine 118 of histone H2A (H2AK118ub) (Wang et al., 2004). The sequence of these events finally leads to gene silencing through the mechanisms involving chromatin compaction. In the complementary results, the EMF1 and VRN5 homologues in tomato also interact with SlTPLs. Moreover, LHP1, another component of PRC1 complex, also interact with SlTPLs. In addition, in *Arabidopsis*, TPL/TPR proteins interact with some histone

methyltransferases such as SDG19 (SUVH3); PKR1. SDG19 also called SUVH3 is a SET domain protein catalyzing the methylation of histone H3 Lys residue 9 resulting in nucleosome compaction and gene silencing (Pontvianne et al., 2010; Zhao and Shen, 2004). PKR1 is a protein related to the PICKLE (PKL) CHD3/Mi-2-like chromatin remodeler (Ogas et al., 1999), which repressed the expression of seed-associated genes during germination by promoting the methylation of histone H3 Lys residue 27 (Zhang et al., 2008).

These results indicate that topless represses gene expression by recruiting chromatin-remodeling factors to induce local chromatin compaction at target sites so that the RNA polymerase II cannot bind to the target sites to start the gene transcription.

General conclusion and perspectives

Auxin signaling and epigenetic control of gene expression: future prospect

The data supporting the idea that, besides ethylene, auxin plays also a role in fleshy fruit ripening are the main outcome of the thesis research project. The study aims to better decipher the mechanisms underlying the auxin control of fruit ripening. To do so, the first task was dedicated to the characterization of components of auxin signaling such as ARFs, Aux/IAAs, and TPLs known to be essential in mediating the hormone action via the regulation of transcriptional activity of auxin-responsive genes. While a specific focus was made on the functional characterization of SlARF2 to uncover its role in tomato fruit ripening, an important part of the thesis work was also devoted to the isolation of the tomato topless genes to subsequently allow establishing their interaction map with members of the Aux/IAA family. Overall, the data bring new insight on the molecular players involved in auxin signaling and in the interplay between auxin and ethylene. In this regard, the outcome of the thesis opens new avenues towards a better understanding of the multi-hormonal control of fruit development.

The work also provides original clues on the link between hormone signaling and epigenetic regulation of gene expression. This issue represents a challenging but promising perspective that is being now addressed in the GBF laboratory. The last section of my thesis report, entitled Conclusion and Perspectives, attempts to outline future developments of the topic related to the link between auxin signaling and chromatin remodeling components, building on the initial data generated within my thesis research project.

The link between auxin signaling and epigenetic control of gene expression: TOPLESS, the missing part?

Topless gene family emerged as key players in gene repression in several mechanisms especially in auxin perception. In Arabidopsis, TPL is recruited by Aux/IAA to suppress the expression of auxin-responsives genes in the absence of auxin (Szemenyei et al., 2008). In order to better define the relationship between Sl-TPLs and Aux/IAA in tomato as a reference species for fruit research, we first sought to isolate all members of the topless family genes. Six Sl-TPL (Sl-TPL1, 2, 3, 4, 5, 6) genes have identified in the tomato, all of them encode proteins bearing the TOPLESS canonical domains (LiSH, CTLH and two WD40 repeats). They display similar numbers of introns and exons except Sl-TPL6 who is longer than the remaining gene family members. Functional characterization revealed that, with the exception of Sl-TPL6, all Sl-TPLs proteins are nuclear localized, consistent with their transcriptional repression activity via interaction with Aux/IAAs. Expression profiling assessed at the transcript levels showed that Sl-TPL1, Sl-TPL3 and Sl-TPL4 display the highest expression, Sl-TPL2 is moderately expressed while Sl-TPL5 and Sl-TPL6 are weakly expressed. This suggests that Sl-TPL1, 3, 4 are potentially the most active during plant development. Sl-TPL1 is highly expressed in vegetative organs (stems, roots) and flowers, while the expression of Sl-TPL3 and Sl-TPL4 is prevailing in fruit. This differential pattern of expression may suggest a functional specialization among Sl-TPL isoforms. Interactions studies between Sl-TPLs and Aux/IAA support the involvement of most Sl-TPLs in auxin signaling and a functional redundancy among family members. This is in line with the functional redundancy previously reported for Arabidopsis TPLs where single loss-of-function mutants of all five At-TPL/TPRs didn't display obvious phenotypes (Long et al., 2006). However, this assumption is contrasting with the situation prevailing in rice and maize where genetic evidences seem to support a more specialized functionality for TPL genes. That is, in rice (Yoshida et al., 2012), a single recessive mutation in Asp1, a TPL-like gene close to Sl-TPL2, exhibited several pleiotropic phenotypes, such as altered phyllotaxy and spikelet morphology. Further evidence sustaining a diversified function for TPL proteins is provided by maize rel2 mutants affected in a TPL-like gene (Gallavotti et al., 2010). A better clarification of the putative specialized functionality among tomato

TPLs is now being addressed by the GBF group using a reverse genetics approach. Given the distinctive expression pattern of SlTPL3 in reproductive tissues, specific down-regulation of this gene would be of particular interest to unravel the role of TPL co-repressors in flower and fruit biology.

So far, the most accepted paradigm states that Aux/IAAs recruit co-repressors TPLs to block ARF activity which leads to the transcriptional inhibition of auxin-responsive genes. Our study of the interactions between Aux/IAAs, ARFs and TPLs in tomato shows that TPLs interact with most of the Aux/IAAs, while they only interact with repressor ARFs. By contrast, activator ARFs directly interacts with Aux/IAAs but not with TPLs. In Arabidopsis, similar interaction results are reported (Causier et al., 2012b). These data suggest that TPLs may repress the ARF-dependent transcriptional activity either by binding directly to a repressor ARF or by binding to an Aux/IAA that is bound to an activator ARF (Figure 13).

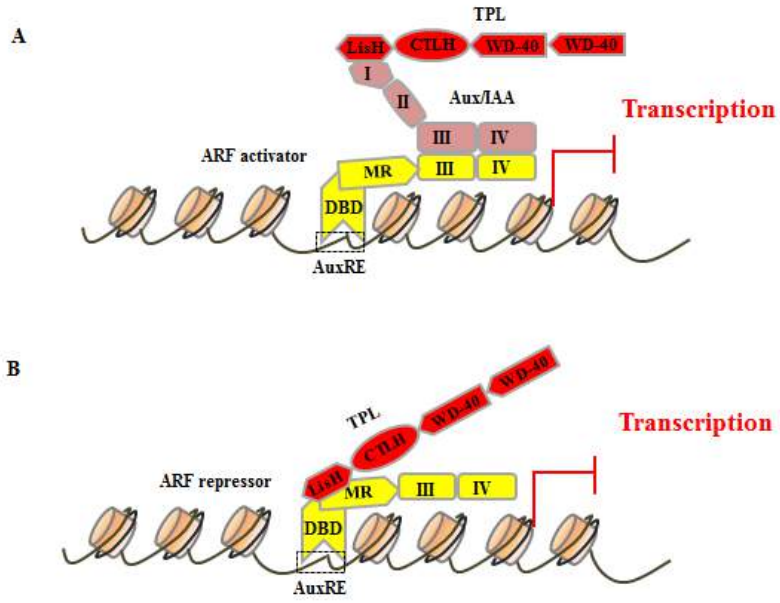

Figure 13. ARF activator and ARF repressor repression model. (A) ARF activators interact with Aux/IAA through domain III and domain IV, and Aux/IAA recruit TPLs via domain I. They form a complex to inhibit gene transcription. This modle is dependent of auxin. **(B)** ARF repressors interact with TPLs directly to from a complex to inhibit gene transcription. This modle is independent of auxin.

Previous studies suggested that TPL/TPR proteins can use multiple chromatin-remodeling mechanisms to induce transcriptional repression (Causier et al., 2012b). TPL acts through the recruitment of histone deacetylases (HDACs) into transcription complexes (Figure 14), leading to a change in the chromatin state that shifts from active to inactive (Long et al., 2006; Liu and Karmarkar, 2008; Krogan and Long, 2009; Krogan et al., 2012). In particular, it has been postulated that TPLs induce local chromatin compaction at target sites through an association with histone deacetylases (HDACs). Histone acetylation is largely correlated with gene expression and removal of these modifications by HDACs generally leads to repression of transcription (Shahbazian and Grunstein, 2007) as depicted in Figure 15. It has been suggested that TPL acts in Arabidopsis through HDA19 and interactions between TPR1 because HDA19 can be observed in pull-down experiment from whole plant extracts. Furthermore, mutations in HDA19 increase the penetrance of tpl-1 and display similar apical defects (Gonzalez et al., 2007; Long et al., 2006; Sridhar et al., 2004).

In tomato, interaction between TPLs and HDACs could not be detected by yeast-2-hybrid (unpublished data from my colleague GUOJIAN HU). Accordingly, while the interaction between TPR1 and HDAC19 was found in pull-down experiment from Arabidopsis plant extracts, this interaction was not detected by Yeast-2-hybrid (Gonzalez et al., 2007; Long et al., 2006; Sridhar et al., 2004). It seems that the yeast-2-hybrid system is not suited for assessing the interaction between TPLs and HDACs which should be investigated by another approach such as pull-down assay or Bimolecular Fluorescence Complementation (BIFC).

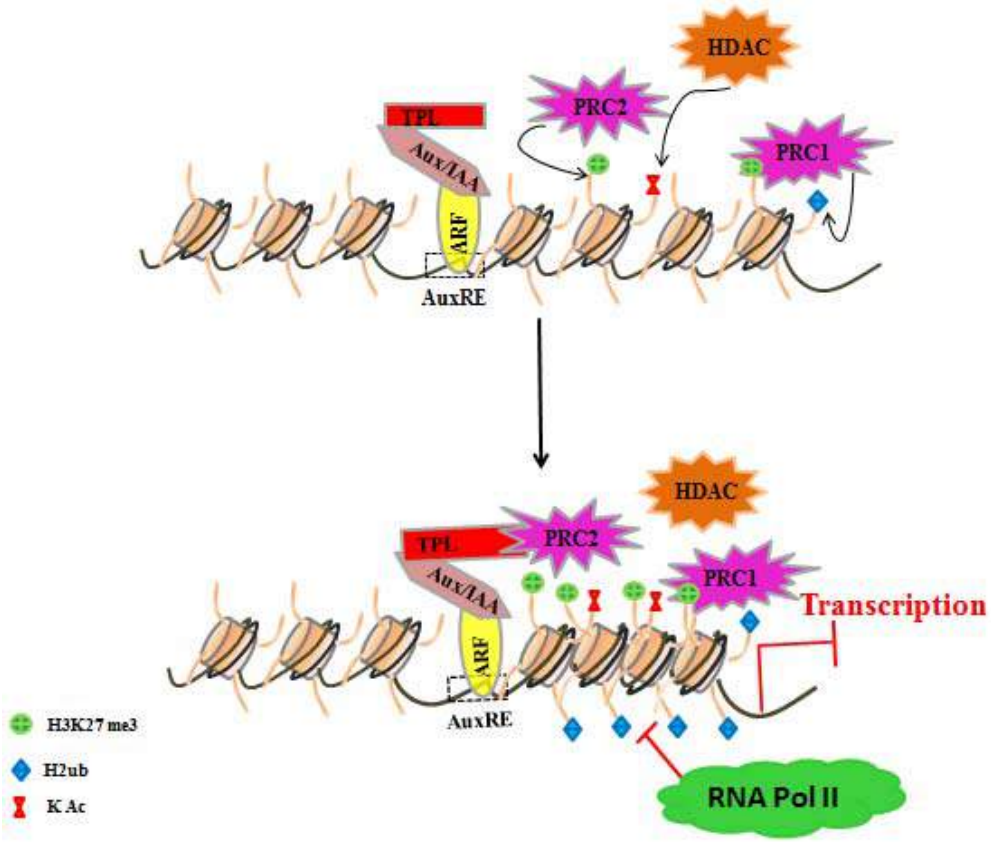

Figure 14. Gene silencing through the mechanisms involving chromatin compaction. PRC2 induces H3K27me3 type methylation. H3K27me3 mark recruits PRC1 which ubiquitylates H2AK119 thus promoting chromatin compaction and gene silencing. Deacetylation of the target gene by HDACs generally leads to chromatin compaction and PRC2 associates with histone deacetylases, reinforcing transcriptional repression and providing functional synergy to stable silencing of target genes.

Beside histone deacetylases, large interactome studies in Arabidopsis showed that TPL/TPR proteins can interact with some histone methyltransferases such as SDG19 (SUVH3); PKR1; EMF1, VRN5 (Causier et al., 2012b). SDG19, also called SUVH3, is a SET domain protein catalyzing the methylation of histone H3 Lys at residue 9 resulting in nucleosome compaction and gene silencing (Pontvianne et al., 2010; Zhao and Shen, 2004). PKR1 is a protein related to the PICKLE (PKL) CHD3/Mi-2-like chromatin remodeler (Ogas et al., 1999; Zhang et al., 2008), shown to repress seed-associated gene expression during germination through promoting the methylation of histone H3 Lys residue 27 (Ogas et al., 1999; Zhang et al., 2008). EMF1 is a component of Polycomb Repressive Complex 1 (PRC1) (Calonje et al.,

2008), while VRN5 is a component of Polycomb Repressive Complex 2 (Greb et al., 2007). As featured in Figure 14, PRC2 catalyzes the trimethylation of histone H3 on lysine 27 (H3K27 trimeth) (Cao et al., 2002) allowing PRC1 to bind to this mark through its subunit POLYCOMB (PC) and to catalyze mono-ubiquitylation of lysine 118 of histone H2A (H2AK118ub) (Wang et al., 2004). The sequence of these events finally leads to gene silencing through the mechanisms involving chromatin compaction (Figure 14). In tomato, we isolated the components of PRC1 and PRC2 and checked their interactions with TPLs. The results show that TPLs can interact with one component of PRC2 and two components of PRC1 (see complementary results). It seems that TPLs may recruit PRC1 and PRC2 to repress gene transcription. These preliminary data brings the first block for a study investigating the physiological significance of these interactions (Figure 14).

SlARF2, a major regulator of fruit ripening: is it also linked to epigenetic control of gene expression?

Among all ARF members in the tomato, Sl-ARF2 is the most highly expressed during fruit ripening and this feature provided the starting point towards addressing its putative role in fruit ripening. The data generated in my thesis work indicate that SlARF2 is encoded by two genes in the tomato, *SlARF2A* and *SlARF2B*, both encoding active transcriptional repressors. Furthermore, SlARF2A is shown to be ethylene-responsive while SlARF2B is up-regulated by auxin. To address, the role of Sl-ARF2 in fruit ripening, we generated transgenic lines that were either specifically silenced for SlARF2A or SlARF2B or simultaneously silenced for both genes. Suppression of either SlARF2A or SlARF2B alters ripening but the double repression led to dramatic inhibition of the ripening process. Ethylene synthesis and perception and pigment accumulation were altered in the down-regulated lines. Key genes encoding regulators of ripening (RIN, NOR, CNR), of ethylene signaling and carotenoid pathway are misexpressed in the Sl-ARF2 deficient lines. The expression patterns of a number of *ERFs* genes was also altered suggesting disturbed ethylene responses in the transgenic lines. Altogether, the data indicate that SlARF2 is a major

regulator of fruit ripening.

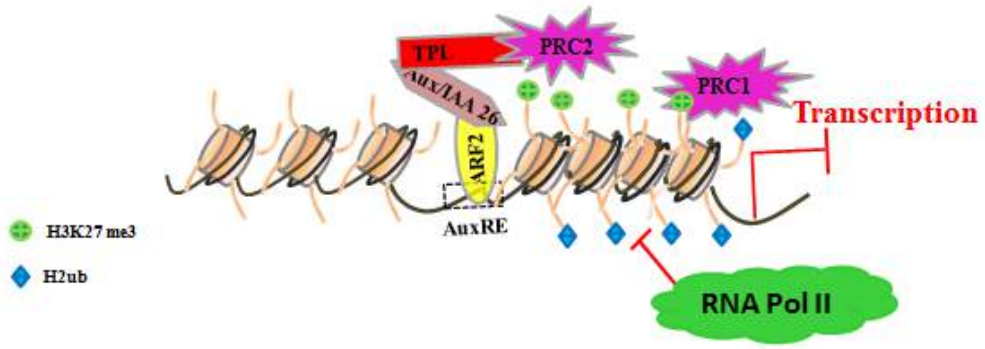

Figure 15. Model of SlARF2 repress the auxin response gene transcription. SlARF2 mediates epigenetic gene silencing by interacting at the protein level with the polycomb complex.

To date, the mechanisms by which repressor ARFs inhibit the transcription of their target genes is unknown. It was recently shown (How Kit et al., 2010) that repression of *SlEZ2* gene, a tomato enhancer of zeste, leads to a decrease in the trimethylation of lysine 27 on histone H3 and to pleiotropic effect on sporophyte development. In our SlARF2 down-regulated lines, we observed similar phenotypes, suggesting that SL-ARF2 and SlEZ2 might be involved in the same control mechanism of gene expression. EZ is one components of PRC2 complex, which is responsible for catalyzing the trimethylation of histone H3on lysine 27 (H3K27 trimeth). In Arabidopsis, large interactome studies showed that TPL/TPR proteins interact with EMF1 and VRN5 proteins which belong to PRC1 and PRC2 complexes, respectively.

Considering that ARFs bind to Aux/IAAs which recruits TPL to suppress the expression of auxin-responsives genes in the absence of auxin (see above and Szemenyei et al., 2008), our working hypothesis is that SlARF2 could recruit PRC1 and PRC2 complex through TPL proteins to repress gene expression (Figure 15). On the other hand, the interaction map established within my thesis work indicated that SlARF2 interacts with Sl-IAA26 and Sl-IAA29. Meanwhile, we also showed that

Sl-IAA 26 interacts with SlTPL1, 2, 3, 4, 5 in contrast to Sl-IAA 29 that displayed no interaction with any of the TPLs (Hao et al., 2014). The emerging question here is to know whether and how these components link to the main players of epigenetic modulation of gene expression. We cloned homologs of PRC1 and PRC2 components in tomato and preliminary data confirmed that some Topless protein can interact with PRC1 and PRC2 complex (complementary data Figure 12). The next step will be to assess whether SlARF2 can be part of a complex formed by PRC1, PRC2 and TPLs.

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Supplementary Data for Chapter II

Table S1 List of primers used in the expression studies

Gene Name	Primer Sequence
Sl-Actin	F 5'-TGTCCCTATTTACGAGGGTTATGC-3'
	R 5'-CAGTTAAATCACGACCAGCAAGAT-3'
SlARF2A	F 5'-GCAAGGTCAAGAGTTATCGA-3'
	R 5'-CATTGGTTTCTGAGACAAGTC-3'
SlARF2B	F 5'-TTTAACGAGTATCCAACCTTCC-3'
	R 5'-GGGTTTAGGCATAATTTCTCCA-3'
GUS-1	F 5'-TACCGTACCTCGCATTACCC-3'
	R 5'-GCAGCAGTTTCATCAATCACC-3'
GUS-2	F 5'-ACCGATACCATCAGCGATCTC-3'
	R 5'-GTACCTTCTCTGCCGTTTCC-3'
Sl-ERF.B3	F 5'-CGGAGATAAGAGATCCAAGTCGAA-3'
	R 5'-CTTAAACGCTGCACAATCATAAGC-3'
Sl-ERF.A1	F 5'-ACCGGATCCTGTTAGAGTTGGA-3'
	R 5'-CGACGCCGATGAACAATG-3'
Sl-ERF.A2	F 5'-CGGTATCATCAGCTTCGGAAA-3'
	R 5'-TCTCAACTTCTAATTCGGCTTGCT-3'
Sl-ERF.A3	F 5'-GCGAAATGGATCAACAGTTACCA-3'
	R 5'-ATTAGACGACTGAAGCTTGAATTCC-3'
Sl-ERF.B1	F 5'-GAATGATGACGGAATTGTAATGAAGA-3'
	R 5'-TTCCACAATCCCAAATTGAAGA-3'
Sl-ERF.B2	F 5'-AGTTTGCAGCGGAGATTCGT-3'
	R 5'-TGCCCTGTCATATGCCTTTG-3'
Sl-ERF.C1	F 5'-TTCTTCGTGTCGAAAATACTAAGTTCAGT-3'
	R 5'-ACTCTAAATTCTTCAAGAAATCCAGAACA-3'

Sl-ERF.C2	F 5'-ATCATTACCATGGAATGATCAACATT-3'
	R 5'-CCGTCTATAACTTTCTTTCGAGGTTAA-3'
Sl-ERF.C3	F 5'-CAAGAAGTTTCCTCAATCTCTCATGTAT-3'
	R 5'-CCGAGATGAATAATCCATTTGATTT-3'
Sl-ERF.C6	F 5'-GGGAAATACGCTGCGGAAA-3'
	R 5'-TTTCGAACGTACCTAGCCATACTCT-3'
Sl-ERF.D1	F 5'-GGCAGCTGAAATAAGAGATCCATATAA-3'
	R 5'-CTAGCAGCCCCTTCAGCAGTAT-3'
Sl-ERF.D2	F 5'-ACACAAGTAGCACCAGCACCACTA-3'
	R 5'-ACCCCAAAAAAAGCAAGAAAATT-3'
Sl-ERF.D3	F 5'-ATTCATTTTCGGGTTGTGCAGTA-3'
	R 5'-CGACTATAATGATTTCTGCCGAACT-3'
Sl-ERF.D4	F 5'-GTTGCTGCTTTAACCAATGTGATTAT-3'
	R 5'-CTTCCGGTACGCGAAACAAG-3'
Sl-ERF.E1	F 5'-GTTCCTCTCAACCCCAAACG-3'
	R 5'-TTCATCTGCTCACCACCTGTAGA-3'
Sl-ERF.E2	F 5'-ACTTCGTGAGGAAACCCTGAAC-3'
	R 5'-GTTACTAATATAAGTCATGTTGGGCTGAA-3'
Sl-ERF.E3	F 5'-GCATTTGCGATCTGAAGTTGTT-3'
	R 5'-CAAATGGCTTGACATCGACTTG-3'
Sl-ERF.E4	F 5'-AGGCCAAGGAAGAACAAGTACAGA-3'
	R 5'-CCAAGCCAAACGCGTACAC-3'
Sl-ERF.F1	F 5'-ACGAGCTTTCTTCTTTTCTCTCTCTAAA-3'
	R 5'-GAAACTCGATATCCTTCTGTAAAATCTTC-3'
Sl-ERF.F2	F 5'-TTGATACCACTGCTTACCTAGTTTTTCT-3'
	R 5'-TATCTTCTATGGCTCCTTCCTCTTCT-3'
Sl-ERF.F3	F 5'-AGTAGTAAGGTGACCCGGATGAAG-3'
	R 5'-CACCGATCATCCACCACAGA-3'
Sl-ERF.F4	F 5'-GAGCTAATGGCTGATTTTTGTATATAAGTTC-3'

	R 5'-AAATGGTAGAAACAGCACGAGAAAG-3'
Sl-ERF.F5	F 5'-TGGAGCGAAAGCGAAAACTAA-3'
	R 5'-GTCTGACTCGGACTCCGATTG-3'
Sl-ERF.G1	F 5'-GAAGAAAGCGATCGATTTGAAGA-3'
	R 5'-TTTTCCCCATGGCCTCTGT-3'
Sl-ERF.G2	F 5'-CGGTGGAGATAAAAGCGAAAAC-3'
	R 5'-CCACTTCGCAGAACCCTAGATT-3'
Sl-ERF.H1	F 5'-AGATGCAGCAAGAGCATATGATG-3'
	R 5'-TTGGGTTGTATGGGAAATTAGTTCT-3'
PSY1	F 5'-GGAAAGCAAACTAATAATGGACGG-3'
	R 5'-CCACATCATAGACCATCTGTTCC-3'
PDS	F 5'-GGTCACAAACCGATACTGCT-3'
	R 5'-AAACCAGTCTCGTACCAATCTC-3'
ZDS	F 5'-AGTGGTTTCTGTCTAAAGGTGG-3'
	R 5'-ACCGAGCACTCATGTTATCAC-3'
β-LCY1	F 5'-GTCCACTTCCAGTATTACCTCAG-3'
	R 5'-TGTCCTTGCCACCATATAACC-3'
β-LCY2	F 5'-CGGGTTATATGGTAGCAAGGA-3'
	R 5'-CAGATGCCGATAACTCATTACC-3'
CYC-β	F 5'-TGTTATTGAGGAAGAGAAATGTGTGAT-3'
	R 5'-TCCCACCAATAGCCATAACATTTT-3'
ACS1	F 5'-TCGTTTCGAAGATTGGATGA-3'
	R 5'-CAACAACAACAAATCTAAGCCATT-3'
ACS3	F 5'-CCCTTGTCCACAAATCCAGA-3'
	R 5'-ACAGAGTGCACCCTCTAACATTT-3'
ACS6	F 5'-CTCCTATGGTCCAAGCAAGG-3'
	R 5'-CGACATGTCCATAATTGAACG-3'
ACS2	F 5'-TGTTAGCGTATGTATTGACAACTGG-3'
	R 5'-TCATAACATAACTTCACTTTTGCATTC-3'

ACS4	F 5'-CTCCTCAAATGGGGAGTACG-3'
	R 5'-TTTTGTTTGCTCGCACTACG-3'
ACO1	F 5'-GCCAAAGAGCCAAGATTTGA-3'
	R 5'-TTTTTAATTGAATTGGGATCTAAGC-3'
ACO2	F 5'-TTTATTACAAAGTGTGCGTCCCTA-3'
	R 5'-CTCATTTTTGGGTATTAAAATATGTGT-3'
ACO3	F 5'- GGAGCCTAGGTTTGAAGCAA-3'
	R 5'- AAACAAATTCCCCCTTGAAAA-3'
ACO4	F 5'- TGATCAAATTGCAAGTGCTTAAA-3'
	R 5'- ACCACACAACAATCACACACA-3'
E4	F 5'-GACCACTCTAAATCGCCAGG-3'
	R 5'-TTCCTGAGCGGTATTGCTTT-3'
FUL1	R 5'-GTTTTGCCACAACAACTGGACTC-3'
	R 5'-CTTGCTGCTGTGAAGAACTACC-3'
FUL2	R 5'-AATGGAGAAGTAGAAGGATCATCG-3'
	R 5'-GATAACATAATATTGTCCGCTTGC-3'
SGR1	R 5'-TGCCAAGAACATATACACTGAC-3'
	R 5'-GTTATACCAACCTTGCAACTGAG-3'
E8	F 5'-TGGCTCCGAATCCTCCCAGTCT-3'
	R 5'-GTCCGCCTCTGCCACTGAGC-3'
PG2a	F 5'-TCAAGGGCACAAGTGCAACAAAGG-3'
	R 5'-TGCACGTAGCCTCTGATGGTTT-3'
RIN	F 5'-ATGCAGCACCATCAACACAT-3'
	R 5'-CTCCAAATTCAAAGCATCCA-3'
CNR	F 5'-GCCAAATCAAGCAATGATGA-3'
	R 5'-TCGCAACCATACAGACCATT-3'
NOR	F 5'-AGAGAACGATGCATGGAGGTTTGT-3'
	R 5'-ACTGGCTCAGGAAATTGGCAATGG-3'
HB-1	F 5'-CAATCGGAGGAAGATGATGG-3'

	R 5'-TGTTCATGGTGCTGCTCTTC-3'
TAGL1	F 5'-ACTTTCTGTTCTTTGTGATGCT-3'
	R 5'-TTGGATGCTTCTTGCTGGTAG-3'
AP2a	F 5'-AACGGACCACAATCTTGAC-3'
	R 5'-CTGCTCGGAGTCTGAACC-3'
EIN2	F 5'-GTGTGCTGAATAAGTTTAGTGGAG-3'
	F 5'-TGCTGTACAATAGAAGAATGGAGG-3'
EIL2	F 5'-TGAAGATGATGGAAGTCTGTAAGG-3'
	F 5'-CCACTCCCTGAGATTATCCGA-3'
EIL3	F 5'-ACAGGACTTCAAGAAACAACCA-3'
	F 5'-GTGTTGTGCTCATAGTTGATCTG-3'
ETR1	F 5'-GGAAGAACATTGGCATTGGAAG-3'
	F 5'-CCAACTGGATTTTGGTGTCGT-3'
ETR2	F 5'-TTGGAGGAATCAATGAGGGC-3'
	F 5'-TCATTACGCGCACGAACAG-3'
NR	F 5'-TGCTGTTCGTGTACCGCTTT-3'
	F 5'-TCATCGGGAGAACCAGAACC-3'
ETR4	F 5'-ATGGCTGTCGTTCTTGGGC-3'
	F 5'-TGGAGGAGTGAGTGTGGATGC-3'
ETR5	F 5'-GTGCTCTGGGCCCTTCACTA-3'
	F 5'-GAACTTACGCACCCTCAATGC-3'
ETR6	F 5'-TCAAAAAGCCGGTGATCTCG-3'
	F 5'-GCACCCATTTGAACGGAAAA-3'
CTR1	F 5'-CGATTTGAACATGACAGGGAG-3'
	F 5'-AAGGGATTGAGATGGAAGATGG-3'

Annex

SlARF4, an Auxin Response Factor Involved in the Control of Sugar Metabolism during Tomato Fruit Development[1][C][W]

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Successful completion of fruit developmental programs depends on the interplay between multiple phytohormones. However, besides ethylene, the impact of other hormones on fruit quality traits remains elusive. A previous study has shown that down-regulation of *SlARF4*, a member of the tomato (*Solanum lycopersicum*) auxin response factor (*ARF*) gene family, results in a dark-green fruit phenotype with increased chloroplasts (Jones et al., 2002). This study further examines the role of this auxin transcriptional regulator during tomato fruit development at the level of transcripts, enzyme activities, and metabolites. It is noteworthy that the dark-green phenotype of antisense *SlARF4*-suppressed lines is restricted to fruit, suggesting that *SlARF4* controls chlorophyll accumulation specifically in this organ. The *SlARF4* underexpressing lines accumulate more starch at early stages of fruit development and display enhanced chlorophyll content and photochemical efficiency, which is consistent with the idea that fruit photosynthetic activity accounts for the elevated starch levels. *SlARF4* expression is high in pericarp tissues of immature fruit and then undergoes a dramatic decline at the onset of ripening concomitant with the increase in sugar content. The higher starch content in developing fruits of *SlARF4* down-regulated lines correlates with the up-regulation of genes and enzyme activities involved in starch biosynthesis, suggesting their negative regulation by SlARF4. Altogether, the data uncover the involvement of ARFs in the control of sugar content, an essential feature of fruit quality, and provide insight into the link between auxin signaling, chloroplastic activity, and sugar metabolism in developing fruit.

The fruit developmental process is controlled by an intricate interplay between multiple phytohormones that influences the overall fruit quality. However, with the exception of ethylene, which has been shown to control many ripening-associated metabolic pathways such as those leading to pigment and aroma volatile production, the impact of other hormones on fruit quality traits remains poorly known (Pech et al., 2012). Auxin is, however, an important phytohormone for initiation of fleshy fruit development, since it was shown to play a key role in triggering fruit set upon flower fertilization (Pandolfini et al., 2002; Wang et al., 2005; de Jong et al., 2009a, 2009b). Auxin is also essential in determining final fruit size through the control of cell division and cell expansion (Devoghalaere et al., 2012). In support of the potential role of auxin in fruit development is the finding that the highest auxin concentrations in different parts of the plant were found in developing fruit (Müller et al., 2002). Auxin was shown to repress amyloplast development in tobacco (*Nicotiana tabacum*) cells, and the accumulation of the major enzymes for starch biosynthesis is affected by auxin, including ADP-Glc pyrophosphorylase (AGPase) small subunit genes, granule-bound starch synthase (STS),

[1] This work was supported by the Laboratoire d'Excellence (grant no. ANR–10–LABX–41; carried out in the Génomique et Biotechnologie des Fruits laboratory) and by the Eu-Sol European integrated project.

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www.plantphysiol.org/cgi/doi/10.1104/pp.113.213843

and starch-branching enzyme (SBE) transcripts (Miyazawa et al., 1999).

It is well established that auxin modulates plant development through transcriptional regulation of target genes (Ulmasov et al., 1999) and that the regulation of auxin-responsive genes is mediated by two gene families, *Auxin Response Factor* (*ARF*) and *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*; Ulmasov et al., 1999; Guilfoyle and Hagen, 2007, 2012; Audran-Delalande et al., 2012). ARFs can either activate or repress transcription of auxin-responsive genes. Auxin is known to regulate various aspects of plant development, including apical dominance, tropisms, and vascular patterning, and plays a crucial role in cell division and cell expansion during the developmental stages spanning and subsequent to the fruit set (Abel and Theologis, 1996; Inzé and De Veylder, 2006). Even though the direct role of ARFs during fruit ripening remains to be clearly established, experimental evidence supporting such a hypothesis was provided by the down-regulation of *DEVELOPMENTALLY REGULATED12* (*DR12*), a tomato (*Solanum lycopersicum*) *ARF* gene now named *SlARF4*, which results in enhanced fruit firmness and increased chlorophyll content associated with a larger number of chloroplasts, leading to dark-green fruits at preripening stages (Jones et al., 2002). Taken together, these findings suggest the ability of auxin to regulate sugar accumulation during fruit development via *SlARF4*. A number of studies have demonstrated the role of specific ARFs in early stages of fruit development such as fruit set (Wang et al., 2005; Goetz et al., 2006; de Jong et al., 2009b), but the putative role of these transcriptional regulators in controlling some ripening-related events and the overall quality of the fruit remains largely unknown.

Tomato organoleptic quality is strongly influenced by the increase in total sugar and acidity in mature fruit (Bucheli et al., 1999), while the sugar/organic acid ratio is considered an important indicator of the flavor and nutritional quality of fruits (Davies and Hobson, 1981; Bassi and Selli, 1990; Salles et al., 2003). It is well accepted that fruit growth comprises three main stages (Ho and Hewitt, 1986), with the first stage being characterized by an intense mitotic activity leading to an increase in cell number. During this stage, starch, which represents the major carbon reserve in the fruit, reaches a maximal accumulation (Ho, 1996). The second stage corresponds to cell enlargement associated with the degradation of starch into soluble sugars (Davies and Cocking, 1965; Schaffer and Petreikov, 1997). The last stage corresponds to a slow growth phase comprising the fruit-ripening phase, characterized by intensive metabolic changes that lead to Glc and Fru accumulation (Carrari et al., 2006). All three growth stages are essential for final sugar accumulation in the fruit, and early studies have shown that the level of soluble solids in ripe tomato fruit is related to the starch level in immature and mature green fruit (Davies and Cocking). At the physiological and molecular levels, sugar accumulation in tomato fruit is the consequence of various linked physiological processes that are genetically programmed under multihormonal control (Bouzayen et al., 2010).

To further address the link between auxin signaling and sugar metabolism, this study carries out metabolic and transcriptomic analyses of antisense and cosuppressed transgenic lines for the *SlARF4* gene, showing that *SlARF4* controls chlorophyll accumulation specifically in the fruit. The data support the hypothesis that fruit photosynthetic activity accounts for the photoassimilate production and therefore for the elevated starch levels in the transgenic fruit.

RESULTS

SlARF4 Genomic Structure and Expression Pattern

SlARF4, formerly named *DR12*, is the first *ARF* gene isolated and characterized in the tomato (Jones et al., 2002). The *SlARF4* coding sequence is 2,436 bp long, and the genomic clone is composed of 12 exons and 11 introns (Fig. 1A). The derived protein contains 811 amino acids, sharing the three highly conserved domains (DNA-binding domain and protein/protein domains III and IV) that are typical of the ARF family (Guilfoyle and Hagen, 2007). In silico analysis of the 1.8-kb promoter sequence performed using the PLACE signal scan search tool (http://www.dna.affrc.go.jp/PLACE/signalscan.html) identified several cis-elements, including the canonical auxin response element (AuxRE), TGTCTC, at position –220, a sugar starvation element, TATCCA, at position –960, and an auxin induction element, ACTTTA, at position –977. This latter sequence has been shown to be involved in mediating tissue-specific and auxin-inducible expression of the *rolB* oncogene (Baumann et al., 1999).

The expression pattern of the *SlARF4* gene in tomato 'Micro-Tom' was analyzed by real-time PCR to assess its transcript accumulation in roots, leaves, stems, flowers, and fruit 8 DPA and at mature green, breaker, and red ripe stages. The data reveal ubiquitous expression in all tissues tested, with the highest levels of *SlARF4* transcript accumulation found in flowers and young fruit 8 DPA. During fruit development, the transcript levels decrease dramatically, showing the lowest levels at the ripening stages (Fig. 1B). The expression pattern of *SlARF4* was also assessed in planta using a promoter-GUS fusion construct (pro*ARF4*::GUS) stably introduced into tomato lines. GUS staining performed on pro*ARF4*::GUS homozygous lines revealed a strong expression in the pericarp and vascular tissues of young fruit 15 and 25 DPA. Thereafter, the *SlARF4* expression dramatically decreases throughout ripening, with the GUS staining being no longer detectable at 55 DPA (Fig. 1C). The presence of the canonical AuxRE TGTCTC in the promoter region of *SlARF4* (Fig. 1A) prompted the investigation of the auxin responsiveness of the *SlARF4* promoter. Quantitative PCR (qPCR) analyses indicated that exogenous auxin treatment induces *SlARF4* transcript accumulation up to nearly 8-fold in light-grown seedlings

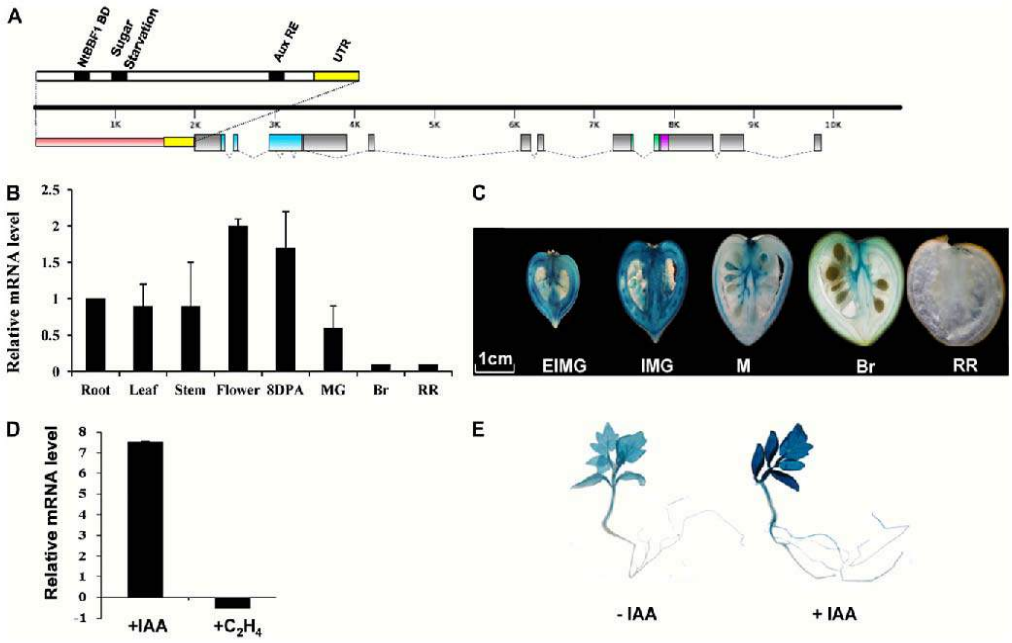

Figure 1. Structural features and expression patterns of the *SlARF4* gene. A, Genomic structure of the *SlARF4* gene. The pink portion represents the promoter region, the gray dots represent the introns, the gray boxes represent the exons, the yellow boxes represent the untranslated regions, the blue box represents the DNA-binding domain, the green box represents domain III, and the purple box represents domain IV. The putative cis-acting elements found in the promoter region are indicated by black bars. B, Expression pattern of *SlARF4* monitored by qPCR. Expression in the root was taken as reference. C, Expression pattern of *SlARF4* revealed by the expression of the GUS reporter gene driven by the *SlARF4* promoter during fruit development and maturation. D, Auxin and ethylene regulation of *SlARF4* expression. qPCR analysis of *SlARF4* transcript levels in RNA samples extracted from 3-week-old light-grown seedlings treated with buffer (control), auxin (20 μM IAA for 2 h), or ethylene (50 μL L^{-1} for 5 h). E, Auxin-responsiveness of *SlARF4* promoter revealed by the expression of the GUS reporter gene driven by the *SlARF4* promoter in seedlings treated with auxin (20 μM IAA for 2 h). UTR, Untranslated region; DBD, DNA-binding domain; 8DPA, fruit at 8 DPA; MG, fruit at mature green stage; Br, fruit at breaker; RR, red ripe fruit.

when compared with untreated plants (Fig. 1D). The auxin responsiveness is then confirmed using transgenic lines expressing the GUS reporter gene driven by the *SlARF4* promoter (Pro*ARF4*::GUS), where 2-h auxin treatment resulted in a strong induction of *SlARF4* promoter activity in tomato seedlings (Fig. 1E). In contrast, qPCR analysis revealed no significant change in *SlARF4* expression upon ethylene treatment of light-grown seedlings (Fig. 1D).

SlARF4 Acts as a Repressor of Auxin Response

To better characterize the function of the *SlARF4*-encoded protein, the ability of this protein to regulate the activity of auxin-responsive promoters in a single-cell system was evaluated. A reporter construct, consisting of the synthetic auxin-responsive promoter DR5 fused to a GFP coding sequence (Ottenschläger et al., 2003), was cotransfected into tobacco protoplasts with an effector construct, allowing a constitutive expression of the SlARF4 protein. Transient expression experiments using this dedicated single-cell system revealed that the DR5-driven GFP expression was enhanced by auxin (2,4-D) treatment in the absence of the effector construct providing the SlARF4 protein. However, the presence of the SlARF4 protein strongly inhibited the auxin-induced activity of the DR5 promoter, clearly demonstrating the ability of SlARF4 to act in vivo as a transcriptional repressor of auxin-dependent gene transcription (Fig. 2A).

Down-Regulation of the *SlARF4* Gene in Tomato

A previous study has shown that down-regulation of *DR12*/*SlARF4* in tomato results in a dark-green fruit phenotype that is associated with a dramatic increase in chloroplast number (Jones et al., 2002). To gain insight into the physiological significance of the *SlARF4*-encoded protein, transgenic lines expressing either sense

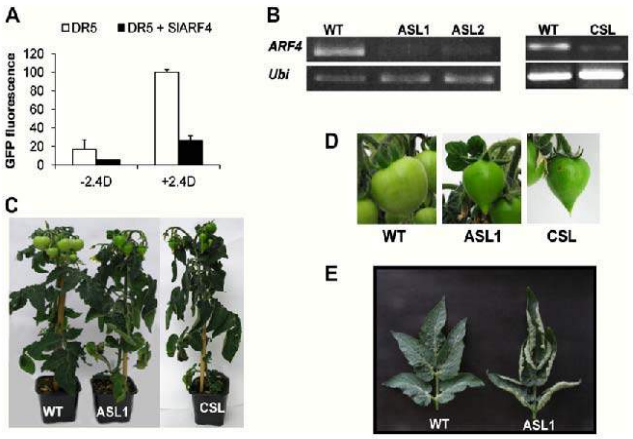

Figure 2. Altered phenotypes of *SlARF4* down-regulated plants. A, SlARF4 protein represses in vivo the activity of the synthetic promoter DR5. Tobacco protoplasts were transformed either with the reporter construct (DR5::GFP) alone or with both the reporter and effector constructs (35S-*SlARF4*) and incubated in the presence or absence of 50 μM 2,4-D. GFP fluorescence was measured 16 h after transfection. A mock effector construct lacking *SlARF4* was used as a control for the cotransfection experiments. Transformations were performed in triplicate. Mean fluorescence is indicated in arbitrary units ± SE. B, Expression of *SlARF4* in transgenic lines analyzed by semiquantitative real-time PCR analysis in leaves. In each PCR reaction, the internal reference ubiquitin (*Ubi*) gene was coamplified with the *SlARF4* gene. C, Wild-type and *SlARF4* antisense plants at the same stage of development (6-week-old plants). D, Dark-green and heart-shaped phenotype of *SlARF4* down-regulated fruit at 35 DPA compared with wild-type fruit at the same stage. E, Upward-curled leaf phenotype of *SlARF4* down-regulated fruit. The leaves of *SlARF4* down-regulated lines exhibit severe in-rolling along the longitudinal axis of the leaf compared with wild-type plants grown in the same conditions at the same stage. WT, Wild type. [See online article for color version of this figure.]

or antisense constructs of the *SlARF4* gene were generated using the tomato 'Micro-Tom' genotype, and several homozygous lines corresponding to independent transformation events were obtained. Several independent antisense lines (ASLs) and cosuppressed sense lines displayed substantial down-regulation of *SlARF4* (Fig. 2B) and reproduced the same phenotypes as those previously described for *DR12* ASLs within the genetic background of tomato 'Kemer' (Jones et al., 2002). Importantly, the phenotypes of the transgenic lines are very consistent and independent of the genetic background, since they are reproducible between the *DR12* ASL lines generated in the Kemer cultivar and those obtained in the Micro-Tom cultivar used in this study. That is, *SlARF4* down-regulated lines display severe upward leaf curling along the longitudinal axis of the leaf (Fig. 2E) and dark-green fruits at the preripening stages, with a slightly heart-shaped phenotype (Fig. 2, C and D). Although, overall, more than 10 independent lines showing the above-described phenotypes were generated, three down-regulated lines, ASL1, ASL2, and cosuppressed line1 (CSL1), showing the strongest phenotypes were selected for deep molecular and physiological characterization.

Physiological and Biochemical Characterization of Transgenic Lines

The impact of *SlARF4* silencing on fruit and leaf development was investigated at the biochemical and physiological levels in the two selected transgenic lines. The assessment of color parameters in *SlARF4* down-regulated fruits at 35 DPA, corresponding to the mature green stage, indicated that the hue angle values, indicative of color saturation, are higher than in the wild type, thus confirming the observed dark-green phenotype (Fig. 3A). Furthermore, measurement of color saturation (chroma, which is indicative of color intensity) provided significantly higher values in *SlARF4* down-regulated fruit than in wild-type fruit (Fig. 3B). Chlorophyll quantification in 35-DPA fruits indicated that *SlARF4* down-regulated fruits accumulate higher amount than the wild type (Fig. 3C), although no increase in chlorophyll accumulation was found in leaves (data not shown). The dark-green phenotype and the associated elevated chlorophyll content in the fruit tissues may potentially confer higher photosynthetic performance to the transgenic fruit. This hypothesis was assessed by measuring the photochemical potential in wild-type and *SlARF4* antisense or cosuppressed leaves and fruits. In fruits, the photochemical potential was more important in *SlARF4* down-regulated lines than in the wild type (Fig. 3D), whereas no significant differences were observed for leaves (Fig. 3E). Because sugar is the main product of chloroplast activity, it became relevant to assess whether the enhanced chlorophyll content and higher photochemical potential in *SlARF4* down-regulated fruits results in higher sugar accumulation. Indeed, Brix determination in fruits at 55 DPA indicates that total soluble

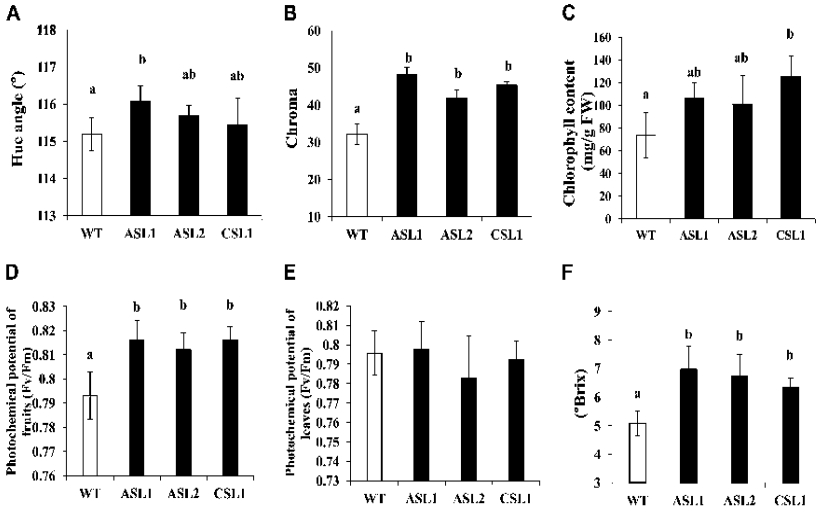

Figure 3. Physiological and biochemical analysis of *SlARF4* down-regulated lines. Color parameters measured in wild-type and *SlARF4* down-regulated fruits. A, Hue angle. B, Chroma. C, Chlorophyll content in wild-type and *SlARF4* down-regulated fruit. D and E, Potential photochemical efficiency of fruits (D) and leaves (E) of wild-type and *SlARF4* down-regulated plants. Fruits were analyzed at the same stage of development. F, Total soluble solids content measured in fruit of wild-type and *SlARF4* down-regulated plants at 55 DPA. Small letters show significant difference using ANOVA at $P < 0.05$. WT, Wild type, F_v/F_m, maximum photochemical efficiency of PSII in the dark-adapted state, FW, fresh weight.

solids showed significantly higher values in *SlARF4* down-regulated fruit than in the wild type (Fig. 3F).

SlARF4 Down-Regulation Leads to Enhanced Sugar Accumulation in Fruit

In further characterizing the effects of *SlARF4* down-regulation on fruit biology and quality, increased starch levels in green fruit were observed. Performing an iodine-staining experiment to uncover whether starch accumulation localizes to a particular tissue in the fruit revealed that the blue-purple color, indicative of the presence of starch, was mainly found in the pericarp tissue, with more intense staining found in *SlARF4* down-regulated fruit than in the wild type (Fig. 4A).

The changes in sugar metabolism occurring in the *SlARF4* down-regulated lines were assessed by following sugar and starch content at different stages of fruit development and ripening (15, 25, 35, 45, and 55 DPA). Starch content declined steadily throughout fruit development in both wild-type and *SlARF4* down-regulated lines when expressed on fresh weight (Fig. 4B). Starch accumulated over the early stages of fruit development and then underwent rapid degradation starting at the preripening stages. However, comparatively, in *SlARF4* down-regulated fruit, starch content stayed above the levels found in the wild type, particularly at early stages (15 and 25 DPA) of fruit development (Fig. 4B). Because starch degradation is known to be the main source of soluble sugars, we assessed the impact of underexpressing *SlARF4* on Glc and Fru contents. The levels of Glc and Fru became significantly higher in the *SlARF4* down-regulated fruit than in the wild type (Fig. 4, C and D) as fruit development advanced toward ripening (stages 35, 45, and 55 DPA). This difference, which represents 30 and 50 μmol g^{-1} fresh weight in CSL1 and ASL1, respectively, can be explained at least in part by the higher levels of transient starch accumulation in the transgenic lines.

Expression Profiling of Starch Biosynthesis Genes in the Tomato

To gain more insight into the mechanism by which sugar metabolism is impacted in *SlARF4* down-regulated lines, we investigated the expression pattern of starch biosynthesis genes. Starch biosynthesis is known to involve a series of enzyme-catalyzed processes (Smith, 1999; Liang et al., 2001; James et al., 2003) belonging to three separate enzyme families (Fig. 5), AGPase, STS, and SBE (Yelle et al., 1988; Schaffer and Petreikov, 1997). Building on the annotated tomato genome sequence, genome-wide in silico screening allowed for the identification of all members of the three enzyme families involved in starch synthesis in tomato. The tomato genome contains three genes encoding the large AGPase subunit, *SlAGPaseL1* (*L1*), *SlAGPaseL2* (*L2*), and *SlAGPaseL3* (*L3*), and one gene encoding the small subunit,

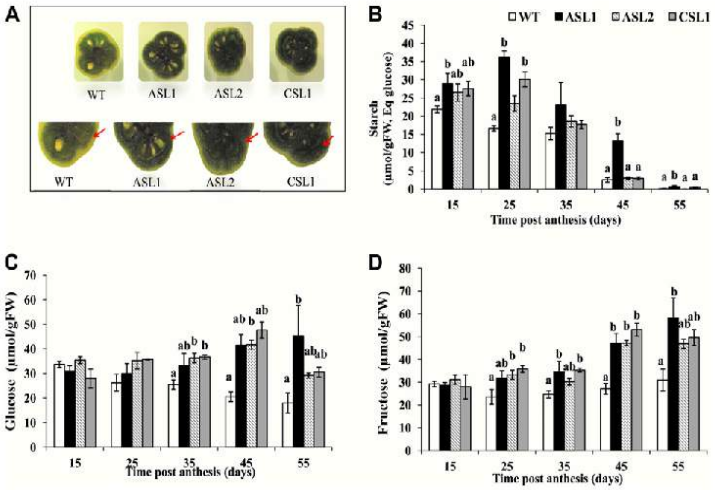

Figure 4. A, Starch content evaluated by Lugol staining in wild-type and *SlARF4* down-regulated 35-d-old fruits. Red arrows show the starch accumulation in the pericarp of the fruit revealed by blue-purple color indicative of starch reaction with iodine. B to D, Starch and soluble sugar contents in wild-type and *SlARF4* down-regulated fruit during development and maturation (15, 25, 35, 45, and 55 DPA). For each developmental stage, the samples consist of a mixture of six different fruits, and the data represent the mean ± SE of three independent biological repeats. Small letters show significant difference using ANOVA at $P < 0.05$. FW, Fresh weight. [See online article for color version of this figure.]

SlAGPaseS (*S1*). STS enzymes are encoded by seven genes, *SlSTS1* to *SlSTS7*, and SBE enzymes are encoded by a small gene family made up of three members, *SlSBE1*, *SlSBE2*, and *SlSBE3* (Fig. 5). However, the lack of a reference expression pattern for starch synthesis genes in the tomato prompted us to establish their expression profile in wild-type tomato fruit. Transcript accumulation was assessed for all members of *AGPase* (Fig. 6A), *STS* (Fig. 6B), and *SBE* (Fig. 6C) gene families by qPCR throughout fruit development (15, 25, 35, and 45 DPA). With respect to the *AGPase* family, *L1* and *S1* show the highest level of expression concomitant with the starch accumulation phase (15–35 DPA). However, the expression of *S1* dramatically decreases as fruit ripening proceeds (45 DPA), suggesting that the regulation of the AGPase activity may take place primarily at the level of the small subunit. Among the seven *SlSTS* genes, transcripts were detected only for *SlSTS1*, *SlSTS2*, *SlSTS3*, and *SlSTS6* (Fig. 6B), suggesting that *SlSTS4*, *SlSTS5*, and *SlSTS7* genes may contribute to starch synthesis in nonfruit tissues. *SlSBE1* and *SlSBE2* display fruit-associated expression at early stages of fruit development (15 and 25 DPA), with no contribution of the *SlSBE3* gene at any of the fruit developmental stages tested (Fig. 6C). Moreover, the expression of *SlSTS* and *SlSBE* genes was undetectable at late stages of fruit development (35–55 DPA).

SlARF4 Down-Regulation Alters the Expression of Starch Biosynthetic Genes and the Corresponding Enzyme Activities

Comparative expression analysis of starch biosynthesis genes assessed in the wild type and ASL1 indicated a significant up-regulation of all *AGPase* genes (Fig. 6A) at the preripening stages lasting 25 DPA and thereafter; during the ripening phase, transcript accumulation of *L1* remained higher in ASL1 than in the wild type. In contrast, the expression of *L2* and *S1* was similar in wild-type and transgenic lines at late stages of fruit development (35 and 45 DPA). It was noteworthy that the expression of *L3* undergoes strong down-regulation at the ripening stage in ASLs (Fig. 6A).

Assessing the transcript levels of *SlSTS* genes (Fig. 6B) revealed that the expression of *STS2* and *STS6* was induced at 15 DPA, while that of *STS3* was clearly repressed (Fig. 6B). At 25 DPA, the expression of *STS1* and *STS6* was induced in the *SlARF4* down-regulated lines, while that of *STS3* remained repressed. At 15 DPA, the expression of *SlSBE1* was repressed and that of *SlSBE2* induced; while at 25 DPA, *SlSBE2* was strongly repressed (Fig. 6C).

Overall, the data indicate that down-regulation of *SlARF4* leads to an increase in transcript levels for *SlAGPase* genes at the preripening stages of fruit development concomitant with the observed starch accumulation at the same stages. To further unravel the impact of *SlARF4* down-regulation on the expression of *SlAGPase* genes, the corresponding enzyme activity was assessed at different stages of fruit development and ripening (Fig. 7). In line with the increase in transcript accumulation, SlAGPase activity was greater in the *SlARF4* down-regulated fruits than in wild-type fruit, especially at the preripening stages, providing a good correlation between transcript levels and enzyme activity. The SlAGPase activity dramatically decreased at the onset of fruit ripening in both wild-type and transgenic lines, though it remained significantly higher in *SlARF4* down-regulated fruits (Fig. 7).

Expression Analysis of *SlGLK* Genes

Considering that the chlorophyll and starch phenotypes of *SlARF4* ASLs are reminiscent of those described

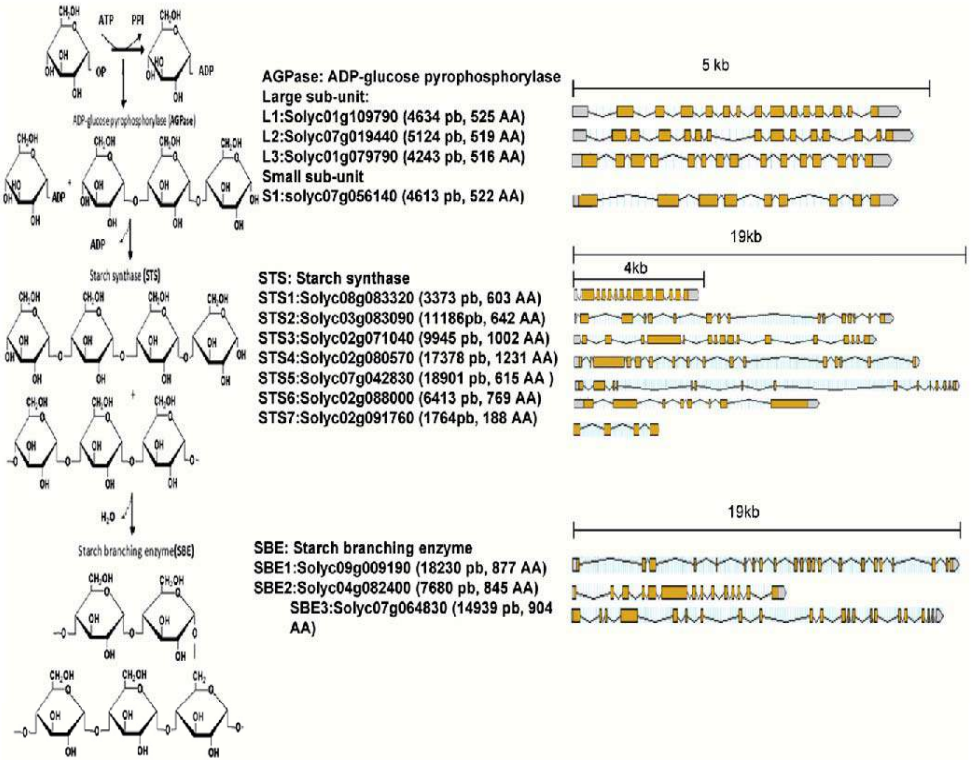

Figure 5. Gene structure of the different enzymes involved in the biosynthesis of starch in tomato fruit. Left, Steps of starch biosynthesis. Center, Different members of the three enzyme families involved in AGPase, STS, and SBE. Genomic and protein lengths are also indicated. Right, Representation of the genomic structure of each gene showing the introns and exons. [See online article for color version of this figure.]

in tomato *GOLDEN2-LIKE* (*SlGLK*) overexpressing lines (Powell et al., 2012), we addressed the putative link between the expression of this MYB-type transcription factor and the phenotypes displayed by the *SlARF4* transgenic lines. Two *GLK* genes (*SlGLK1* and *SlGLK2*) are present in the tomato genome, and it was reported that in most domesticated genotypes the *SlGLK2* gene bears the *uniform ripening* (*u*) mutation that results in light-green fruit phenotype. Transcript accumulation analysis revealed that *SlGLK2* expression is up-regulated in transgenic lines underexpressing *SlARF4* (data not shown). However, verifying the sequence of the *SlGLK2* gene in the Micro-Tom cultivar revealed that this genotype carries the inactive *u* allele of *SlGLK2*, which rules out the possibility that the dark-green phenotype of *SlARF4* antisense fruit may result from the up-regulation of *SlGLK2*. We then checked whether ARF4 might regulate the expression of *SlGLK1*, whose expression has been reported to be low in the fruit tissues. The data presented in Figure 8B show an enhanced accumulation of *SlGLK1* transcripts in *SlARF4*-ASL fruit tissues, suggesting that the down-regulation of *ARF4* expression results in the up-regulation of *SlGLK1*, which in turn may increase chlorophyll accumulation. In support of this hypothesis, we found that the promoter region of the *SlGLK1* gene contains two perfectly conserved canonical ARF binding sites, the so-called TGTCTC box (Fig. 8A).

DISCUSSION

Auxin has long been reported to be involved in fruit development, and exogenous application of auxin was shown to disturb normal fruit ripening in many crop species (Vendrell, 1985; Cohen, 1996). Moreover, the link between auxin biosynthesis or signaling and sugar accumulation in the fruit tissues has been highlighted by a number of studies (Pandolfini et al., 2002; Wang et al., 2009), though the mechanisms by which this hormone impacts sugar metabolism and therefore fruit quality remain poorly understood. Previous work demonstrated that *DR12/ARF4*, a member of the tomato *ARF* gene family of transcription factors, is involved in the

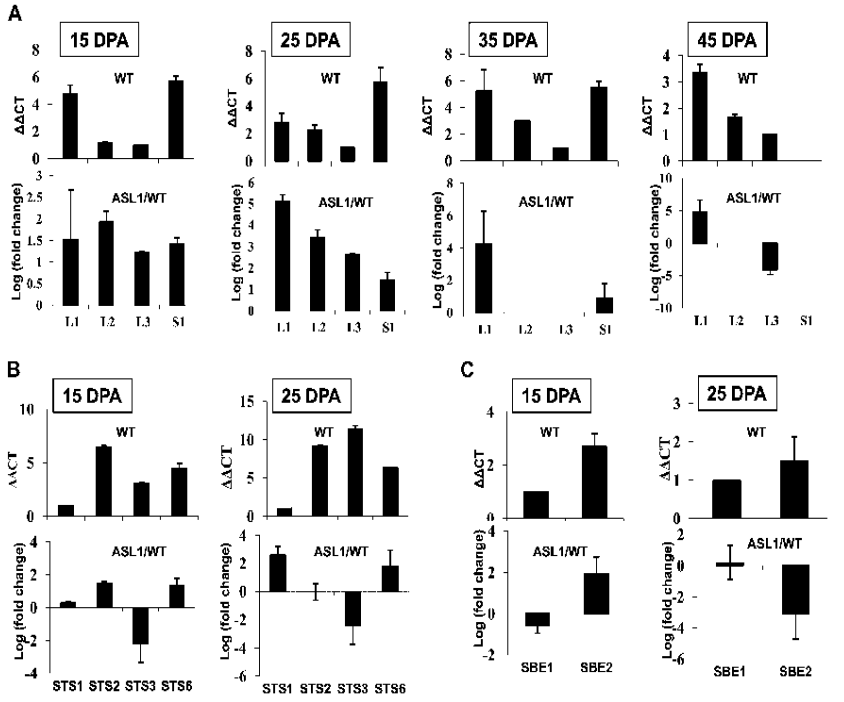

Figure 6. Expression profile of *SlAGPase* genes in wild-type and ASL1 tomato fruits. The levels of transcripts were assessed in tomato fruit by qPCR at 15, 25, 35, and 45 DPA for (A) *SlAGPaseL1* (*L1*), *SlAGPaseL2* (*L2*), *SlAGPaseL3* (*L3*), and *SlAGPaseS* (*S1*); at 15 and 25 DPA for (B) *SlSTS1* (*STS1*), *SlSTS2* (*STS2*), *SlSTS3* (*STS3*) and *SlSTS6* (*STS6*); and at 15 and 25 DPA for (C) *SlSBE1* (*SBE1*) and *SlSBE2* (*SBE2*). ΔΔCT refers to the fold difference in the expression of *SlAGPase*, *SlSTS*, and *SlSBE* relative to the isoforms *L3*, *STS1*, and *SBE1*, respectively. Levels of *STS4*, *STS5*, *STS7*, and *SBE3* were not detectable. Log (fold change) refers to the expression of *SlAGPase*, *SlSTS*, and *SlSBE* isoforms in ASL1 relative to the expression of the same isoform in the wild type. The data represent mean values obtained with three replicates. WT, Wild type.

regulation of fruit development; that is, transgenic tomato plants with decreased *SlARF4* mRNA levels produced dark-green fruit at immature stages, with increased chlorophyll content, a larger number of chloroplasts, and unusual cell division at late stages of fruit development, as well as blotchy ripening and enhanced fruit firmness (Jones et al., 2002; Guillon et al., 2008). In further characterizing the role of this auxin transcriptional regulator, the current study addresses more specifically the impact of down-regulation of *SlARF4* on sugar metabolism throughout fruit development. Both metabolic and transcriptomic data lead to the conclusion that *SlARF4* underexpressing lines accumulate more starch at early stages of fruit development and more sugar at the ripening stages. Overall, the data provide insight into the link between auxin signaling, chloroplastic activity in the fruit tissues, and sugar metabolism.

Several tomato mutants such as *dark green*, *high pigment1*, and *high pigment2* (Sanders et al., 1975; Jarret et al., 1984) displayed fruit phenotypes similar to those showed by *SlARF4* down-regulated lines with regard to high chlorophyll content. However, in contrast to these mutants where the dark-green phenotype can be observed in both leaf and fruit tissues, the enhanced chlorophyll content in *SlARF4* underexpressing plants is restricted to immature fruits. This feature suggests that *SlARF4* controls chlorophyll accumulation specifically in the fruit. Furthermore, the enhanced chlorophyll content in *SlARF4* down-regulated fruits correlates with a higher photochemical efficiency compared with wild-type fruits, supporting the idea that fruit photosynthetic activity may account, at least partially, for photoassimilate production and therefore for the elevated starch levels in the transgenic fruit. Consistent with this idea, cells in developing fruit were shown to contain photosynthetically active chloroplasts (Piechulla et al., 1987), suggesting that photosynthesis may provide a significant contribution to both metabolism and growth of the fruit organ. This hypothesis is further supported by global transcriptomic profiling of transgenic lines altered in auxin response owing to down-regulation of *SlIAA9*, an *Aux/IAA* gene, which revealed that the activation of photosynthesis-related genes is a major phenomenon in developing tomato fruit (Schauer et al., 2006; Wang et al.,

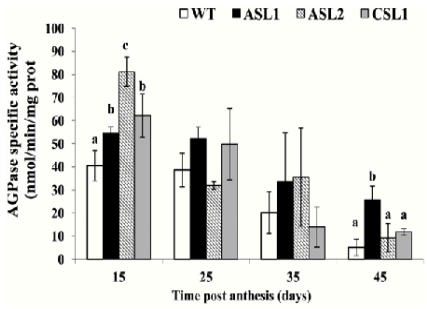

Figure 7. Specific activity of *SlAGPase*. The AGPase specific activity was quantified in wild-type (WT) and SlARF4 down-regulated fruits (ASL1, ASL2, and CSL1). The data represent the mean ± SE of six replicates. Small letters show significant difference using ANOVA at $P = 0.05$.

2009; Matas et al., 2011). In tomato, photosynthesis in developing fruit can contribute up to 20% of the fruit photosynthate, and light-harvesting electron transfer and CO_2 fixation proteins are conserved in the active state in fruit tissue (Blanke and Lenz, 1989; Hetherington et al., 1998; Carrara et al., 2001; Matas et al., 2011). Yet, the prevailing idea is that fruit growth and metabolism are predominantly supported by photoassimilate supply from the source (Ruan et al., 2012), and in this regard, our data cannot rule out that the higher sugar content observed in the transgenic lines could also arise from a more efficient import of photoassimilate into fruit. Indeed, altering auxin sensitivity via down-regulation of tomato *IAA9* has been reported to promote the development of vascular bundles (Wang et al., 2005), which may enhance sink strength and sugar supply to the fruit.

Starch is the end product of photosynthesis and the predominant carbohydrate reserve in many plants, and in addition to being important for plant development, starch biosynthesis is also a critical factor for fruit quality. The regulation of starch synthesis has received much attention in tomato fruit (Beckles et al., 2001a, 2001b), and it has been reported that the reaction catalyzed by AGPase is the limiting step for starch biosynthesis in potato (*Solanum tuberosum*) tubers (Tiessen et al., 2002), a Solanaceae species close to tomato. Indeed, modifying AGPase activity and properties has a direct impact on starch levels in plants (Tsai and Nelson, 1966; Smidansky et al., 2002; Berger et al., 2004; Hädrich et al., 2012). Of particular note, the enhanced starch content in *SlARF4* down-regulated fruit correlates well with the up-regulated expression of key genes involved in starch biosynthesis, especially genes coding for AGPase. The data revealed a net increase, compared with wild-type fruit, in both transcript accumulation and enzyme activity for SlAGPase at the preripening stages in the *SlARF4* down-regulated fruit. The expression of *SlAGPase* is highly correlated with the accumulation of starch in both wild-type fruits and *SlARF4* down-regulated fruits. The presence of three conserved motifs in the promoter region of *SlAGPase* (Supplemental Table S1) corresponding to putative AuxREs is supportive of a direct regulation of *AGPase* gene expression by SlARF4. Together, the data strongly suggest that *SlARF4* controls starch accumulation in fruit mainly by repressing the expression of the *SlAGPase* gene. In the same way, previous studies showed a negative effect of auxin on the expression of the *SlAGPase* gene (Miyazawa et al., 1999). *SlARF4* down-regulated fruit displayed higher soluble solids (Brix) at the ripening stages, likely owing to the overaccumulation of starch

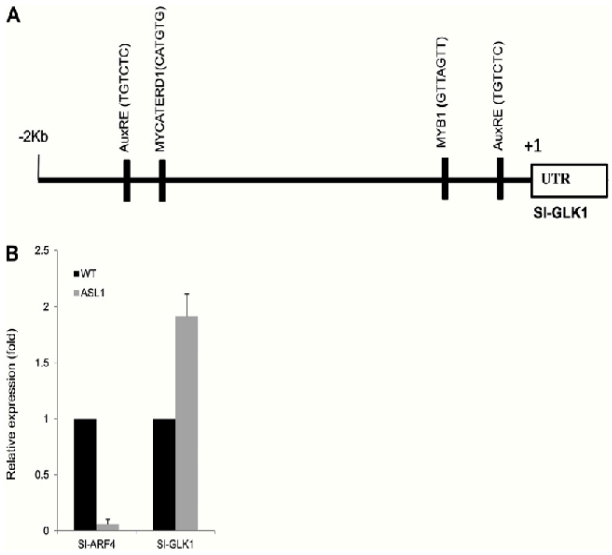

Figure 8. SlARF4 is a possible repressor of the *SlGLK1* gene. A, *SlGLK1* promoter sequence analysis. The promoter region of SlGLK1 was analyzed for putative cis-acting elements. The identified sites are represented by black bars: MYB-binding site (GTTAGTT), AuxRE (TGTCTC), and MYC box (CATGTG). B, Expression pattern of *SlGLK1* and *SlARF4* monitored by qPCR in down-regulated fruit compared with the wild type at 25 DPA. The relative mRNA level for each gene was normalized with respect to the actin housekeeping gene. The results were expressed using the wild type as a reference for each gene (relative mRNA level 1). WT, Wild type.

in green fruit that could be degraded into soluble sugars. This is in agreement with previous work stressing the decisiveness of starch content at green fruit stage in the determination of soluble solid content at the ripening stage (Schaffer et al., 2000; Baxter et al., 2005). In addition to auxin, it was recently reported that malate levels impact starch metabolism (Centeno et al., 2011); however, the putative link between auxin regulation of carbohydrate accumulation and malate metabolism is still to be elucidated.

Expression pattern revealed by the ProARF4::GUS fusion reporter construct uncovered a significant expression of *SlARF4* in all tissues analyzed, with the highest level of expression observed in flower and pericarp and in vascular tissues of young fruit. *SlARF4* expression reaches a maximum at 25 DPA and then decreases at the end of ripening. In addition, the up-regulation of *SlARF4* expression by auxin suggests an auxin control of this gene. These findings are in accordance with previous studies showing that auxin concentration increases at the beginning (10 to 25 DPA) of fruit development (Müller et al., 2002). Using a single-cell approach, we showed that similar to its Arabidopsis (*Arabidopsis thaliana*) ortholog, *SlARF4* is also able to strongly repress in vivo the activity of the synthetic DR5 auxin-responsive promoter. Taken together, these data suggest that *SlARF4* is involved in the auxin regulation of young fruit development by repressing the expression of auxin-responsive genes.

It was recently reported that the *u* mutation of the *SlGLK2* gene is responsible for the light-green phenotype in cultivated tomato varieties (Powell et al., 2012), and that overexpression of *SlGLK2* and its paralog *SlGLK1* leads to dark-green fruit similar to those described in down-regulated *SlARF4*. However, even though the expression of *SlGLK2* was found to be significantly enhanced in *SlARF4* down-regulated lines, it cannot account for the dark-green phenotype of the transgenic fruit, since the cv Micro-Tom variety bears the inactive *u* allele of *SlGLK2*. Interestingly, transcript accumulation of the *SlGLK1* gene was also significantly enhanced in *SlARF4* transgenic lines, suggesting that down-regulation of *SlARF4* leads to derepression of the *SlGLK1* gene in the fruit tissue, which may be responsible for the increase in chlorophyll accumulation. This hypothesis is further supported by the presence of two perfectly conserved canonical ARF binding sites, the so-called TGTCTC box, in the promoter region of the *SlGLK1* gene. The data could also suggest that in wild-type tomato, SlARF4 may act through the transcriptional repression of *SlGLK1* gene expression in fruits. It is noteworthy that many of the phenotypes displayed by *SlGLK* overexpressing lines are shared by the antisense *SlARF4* plants, including the increased number of green fruit chloroplasts (Jones et al., 2002) and enhanced sugar accumulation. The possible ability of the SlARF4 protein to repress the transcriptional activity of the *SlGLK* promoter supports the idea that these transcription factors may control the photosynthetic activity in the fruit through a common route. Overall, the current study brings insight into the ability of auxin to control starch accumulation during fruit development and therefore to impact fruit quality. The data also shed some light on the molecular actors involved in auxin action and define *SlARF4* as a major player in mediating the auxin control of sugar metabolism in tomato fruit.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tomato (*Solanum lycopersicum* 'Micro-Tom') plants were grown under standard greenhouse conditions. Conditions in the culture chamber room were set as follows: 14-h-day/10-h-night cycle, 25°C /20°C day/night temperature, 80% relative humidity, and 250 mol m^{-2} s^{-1} intense light. Seeds were sterilized, rinsed in sterile water, and sown in Magenta vessels containing 50 mL of one-half-strength Murashige and Skoog 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) Suc, and 0.8% (w/v) agar, pH 5.9.

Plant Transformation

To generate *SlARF4* overexpressing plants, the forward 5′-ATGGAAATTGATCTGAATCATGC-3′ and reverse 5′-TCAAATCCTGATTACAGTTGGAGATG-3′ primers were used to amplify the 2,436 bp of full-length *SlARF4* coding sequence. Two *SlARF4* antisense constructs were made, one corresponding to the 5′ region (5′ untranslated region and DNA-binding domain) of ARF4 and the other to the 3′ region. The forward 5′-ATGGAAATTGATCTGAATCATGC-3′ and reverse 5′-TGGCTGTCCAGTACTGATGGTG-3′ primers were used to amplify the 1,300-bp 5′ sequence. The forward 5′-CATGTCGATTTCGTTGTACCTTAC-3′ and reverse 5′-CCACATAGTTTTCATCATACAAGC-3′ primers were used to amplify the 1.6-kb nucleotide 3′ sequence. These two fragments were then cloned into the pGA643 binary vector in the antisense orientation under the transcriptional control of the *Cauliflower mosaic virus* 35S (35SCaMV) promoter and the nopaline synthase terminator. All transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation according to Wang et al. (2005). All experiments were carried out using homozygous lines from F3 or later generations.

Isolation and Cloning of the *SlARF4* Promoter

PCR was performed on the genomic DNA of tomato 'Micro-Tom' (10 ng μL^{-1}). PCR primers are detailed in Supplemental Table S2. The corresponding amplified fragment was cloned into the pMDC162 vector containing the GUS reporter gene using Gateway technology (Invitrogen). The cloned *SlARF* promoter was sequenced from both sides using vector primers in order to see whether the end of the promoter is matching with the beginning of the reporter gene. Sequence results were carried out using the Vector NTI (Invitrogen) and ContigExpress software by referring to ARF promoter sequences. Transgenic plants were generated by *A. tumefaciens*-mediated transformation according to Wang et al. (2005).

Transient Expression Using a Single-Cell System

For cotransfection assays, the coding sequence of *SlARF4* was cloned into the pGreen vector and expressed under the control of the 35SCaMV promoter. Protoplasts were transformed either with 10 μg of the reporter vector alone containing the DR5 synthetic AuxRE fused to the GFP reporter gene (Ottenschläger et al., 2003) or in combination with 10 μg of the SlARF4 construct as the effector plasmid, allowing for the constitutive expression of the SlARF4 protein. Protoplasts were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) Bright Yellow-2 cells and transfected according to the method described previously (Leclercq et al., 2005). After 16 h of incubation in the presence or absence of 2,4-D (50 μM), GFP expression was analyzed and quantified by flow cytometry (FACSCalibur II, BD Biosciences) as indicated in Audran-Delalande et al. (2012). All transient expression assays were repeated at least three times with similar results.

GUS Staining and Analysis

Tissues from transgenic lines transformed with the *SlARF4* promoter-GUS fusion construct (Pro*ARF4*::GUS) were taken and put in GUS staining solution

(100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA). Vacuum was made twice for 15 min. Tissues were then incubated in GUS staining solution at 37°C overnight. Samples were then decolorated using several washes of graded ethanol series.

Auxin and Ethylene Treatment

For qPCR expression studies, 21-d-old tomato seedlings were harvested and treated with auxin (20 μM IAA for 2 h). The tissues were then immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction. For GUS analysis, 21-d-old tomato Pro*ARF4*::*GUS*-transformed seedlings were incubated for 2 h in one-half-strength Murashige and Skoog buffer with or without 20 μM IAA. Tissues were then immediately incubated in GUS staining buffer. Ethylene treatments were performed for 5 h in sealed glass boxes. Five-day-old etiolated seedlings were treated with 50 μL L^{-1} ethylene, and control seedlings were exposed to air alone. The tissues were immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from tissues was extracted using a plant RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was then DNase-treated with Ambion (Invitrogen) to remove any contaminating genomic DNA. Complementary DNA synthesis was done by reverse transcription of first strand complementary DNA from 2 μg of total RNA using Omniscript (Qiagen) according to the manufacturer's instructions. Gene-specific primers were designed by Primer3 software (version 0.4.0). Primers sequences are listed in Supplemental Table S2). The relative transcript abundance was monitored on an ABI PRISM 7900HT sequencer using SYBR Green PCR Master Mix (Applied Biosystems). The relative expression for each gene of interest was calculated using the comparative threshold cycle values and the SlActin (forward 5′-TGTCCCTATCTACGAGGGTTATGC-3′, reverse 5′-AGTTAAATCACGACCAGCAAGAT-3′) as an internal standard, as described previously (Pirrello et al., 2006).

Chlorophyll Fluorescence Parameter Measurements

Chlorophyll fluorescence parameters were measured with a PAM-2000 pulse-amplitude modulation fluorometer (Walz). The measurements were made on fruits at 35 DPA. The chlorophyll fluorescence parameter measurements were done according to the method described in detail by Maury et al. (1996).

Color Measurement

L, a, and b values (International Commission on Illumination) were measured on fruit with a Konica Minolta CR-200 Chroma Meter at 35 DPA. The chromameter was calibrated against a standard white tile. The different color indexes were calculated according to the following equations: Hue = $\tan^{-1}$ (b/a), if a > 0 and 180 + $\tan^{-1}$ (b/a), if a < 0; Chroma = $(a^2 + b^2)^{0.5}$.

Starch was colored in situ with Lugol solution (Sigma-Aldrich) by dipping tomato halves for 10 s, then removing the excess stain by gently tipping onto a paper tissue. The starch was then revealed by turning the pale-brown color of the iodine solution to a dark-blue color.

Fruit Brix Measurement

Breaker and breaker-plus-10-d fruit tissue was homogenized in a razor blade and centrifuged for 1 min at 12,000 rpm. The soluble solids (Brix) content of the resulting juice was measured on the MASTER-20T portable refractometer (Atago).

Chemicals and Enzymes

ADP-Glc, AMP, ATP, 6-aminocaproic acid, benzamidine, Bradford reagent, Fru-6-P, Glc-1-P, Glc-1,6-bisP, phenazine ethosulfate, Suc, thiazolyl blue tetrazolium bromide, Tricine, Triton X-100, amyloglucosidase, catalase, and NAD glyceraldehyde-3-P dehydrogenase were purchased from Sigma-Aldrich. Dithiothreitol, leupeptin, NAD^+, NADH, $NADP^+$, NADPH, and α-amylase were purchased from Roche.

Extraction and Assay of Enzymes

Samples were powdered under liquid nitrogen and stored at –80°C until use. Aliquots of approximately 20 mg fresh weight were extracted as in Gibon et al. (2009). Assays were prepared in 96-well polystyrene microplates (Sarstedt) using a robotized platform (Hamilton). Absorbances were read at 340 nm in MP96 readers (SAFAS). ADP-Glc pyrophosphorylase and NADP-glyceraldehyde-3-P dehydrogenase were assayed as described in Gibon et al. (2004).

Extraction and Assay of Metabolites

Metabolites were extracted twice with 80% (v/v) ethanol and once with 50% (v/v) ethanol as described in Geigenberger et al. (1996). Chlorophylls were then determined as in Arnon (1949), Suc, Glc, and Fru as in Geigenberger et al. (1996), and starch as in Hendriks et al. (2003). Extractions and assays were performed using a robotized platform and absorbances were read at 340 nm (carbohydrates) and at 645 and 665 nm (chlorophylls) in a Xenius reader (SAFAS). Extractions were performed using 1.1-mL Micronic tubes (VALDEA Biosciences) with screw caps and assays using 96-well polystyrene microplates (Sarstedt).

Sequence Structure and Promoter Analysis

The structure of *SlARF4* was determined using in silico approaches (fancyGENE software version 1.4). Promoter sequences of *SlARF4*, *SlAGPase*, *SlSTS*, *SlSBE*, and *SlGLK2* genes were analyzed using PLACE signal scan search software (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. In silico analysis of SlAGPase and SlGLK1 gene promoters.

Supplemental Table S2. PCR primers of all genes analyzed in the article.

ACKNOWLEDGMENTS

The work benefited from the networking activities within the European Cooperation in Science and Technology Action FA1106. We thank L. Lemonier (Université de Toulouse, Institut National Polytechnique-Ecole Nationale Supérieure Agronomique de Toulouse, Laboratoire de Génomique et Biotechnologie des Fruits) and D. Saint-Martin (Université de Toulouse, Institut National Polytechnique-Ecole Nationale Supérieure Agronomique de Toulouse, Laboratoire de Génomique et Biotechnologie des Fruits) for tomato cultures and genetic transformation, the "plateforme de microscopie TRI" (Fédération de Recherches) for microscopy analyses, and D. Combes (Laboratoire d'Ingénierie des Systémes Biologiques et des Procédés-Institut National Polytechnique de Toulouse) for his help in carbohydrate analysis.

Received January 6, 2013; accepted January 18, 2013; published January 22, 2013.

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