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CHARACTERIZATION OF AUXIN-ETHYLENE INTERACTIONS DURING THE TOMATO FRUIT DEVELOPMENT - ROLE OF SI-IAA17 GENE

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Résumé

Les interactions entre l'auxine et l'éthylène sont complexes et contrôlent divers processus de développement des plantes tels que l'élongation racinaire ou la différentiation des racines secondaires. Mais, il existe peu d'études montrant le rôle des interactions entre ces deux hormones au cours du développement et de la maturation des fruits. Le changement de couleur des fruits chez la tomate est une caractéristique de la maturation qui est associée à la fois à la dégradation des chlorophylles et à l'accumulation des caroténoïdes. Dans ce travail, l'application exogène d'auxine et d'éthylène a montré l'impact de ces deux hormones sur la maturation de la tomate et en particulier sur le changement de couleur des fruits. Nous avons montré que l'acide-indol acétique (IAA) retarde la transition du vert à l'orange/rouge, alors que l'éthylène, apporté sous la forme d'acide 1-aminocyclopropane-1-carboxylique (ACC), son précurseur, accèlère la coloration des fruits. Par contre, l'inhibition de l'auxine par le PCIB, un antagoniste de l'auxine, provoque les mêmes effets que l'éthylène. L'analyse des caroténoïdes montre que l'ACC comme le PCIB augmente la teneur en lycopène et diminue la teneur en carotène alors que l'IAA provoque l'effet inverse.

L'étude de l'accumulation des ARNs messagers de plusieurs gènes clés de la voie de biosynthèse des caroténoïdes a montré que le gène *β-lcy* codant pour la lycopène cyclase joue un rôle clé dans le contrôle de la biosynthèse et de l'accumulation des pigments et que son expression est fortement dépendante de l'équilibre auxine-éthylène. D'autre part, nos résultats ont montré que le gène *rin* joue un rôle important dans le contrôle de l'expression des gènes clés de la voie de biosynthèse des caroténoïdes.

Pour avoir une meilleure vision des gènes différentiellement exprimés par l'auxine et l'éthylène au cours de la maturation, l'analyse du transcriptome des fruits traités par de l'ACC et de l'IAA a été réalisée par RNA-Seq au laboratoire. Parmi les facteurs de transcriptions étudiés, le gène *Sl-IAA17*, un membre de la famille des AUX/IAA, est fortement affecté par l'auxine et l'éthylène. La caractérisation fonctionnelle du gène

Sl-IAA17 pendant le développement du fruit a été réalisée en créant des lignées transgéniques sous exprimant ce gène en mettant en œuvre la stratégie des ARNs interférents. Ces lignées présentent un phénotype caractéristique produisant des fruits de plus gros qualibre que celui des fruits sauvages. Les analyses histologiques des tissus des fruits ont montré que ce phénotype est associé à un péricarpe plus épais. En microscopie, nous avons constaté que l'augmentation de l'épaisseur du péricarpe dans les lignées transgéniques n'était pas due à un plus grand nombre de cellules mais à l'augmentation de la taille des cellules. Enfin, nous avons observé que l'expansion des cellules dans les fruits transgéniques est étroitement couplée avec des niveaux de ploïdie plus élevés que dans les fruits sauvages, ce qui suggère une stimulation du processus endoreduplication. Ces résultats démontrent très clairement l'existance d'une étroite relation entre la signlisation de l'auxine, le contrôle de la taille du volume cellulaire et le processus d'endoreduplication.

En conclusion, les résultats présentés fournissent des connaissances nouvelles sur les interactions entre l'auxine et l'éthylène au cours du développement du fruit et en particulier au cours de la transition fruit immature - fruit mature. De plus, ils apportent des éléments nouveaux sur la connaissance du rôle de la voie de signalisation de l'auxine dans le contrôle du développement des fruits charnus et en particulier sur la fonction de certains membres des AUX/IAA sur la détermintion du volume et du poids des fruits.

Abstract

The interaction between auxin and ethylene are complex and control various processes of plant development, such as root elongation or differentiation of secondary roots. But there are few studies showing the role of interactions between these two hormones during development and maturation of the fruit. The color change in the tomato fruit is a feature of the maturation that is associated with the degradation of the chlorophyll and carotenoid accumulation. In this work, the application of exogenous auxin and ethylene showed the impact of these two hormones in the tomato ripening and in particular the change of fruit color. We have shown that indole-acetic acid (IAA) delays the transition from green to orange / red, while ethylene, supplied as 1-aminocyclopropane-1-carboxylic acid form (ACC), its precursor, accelerated this transition. However the auxin inhibition by p-chlorophenoxy isobutyic acid (PCIB), an auxin antagonist, caused the same effects similar to ethylene. The carotenoid analysis showed that the ACC and PCIB increase the lycopene content and reduced the carotene content while IAA causes the opposite effect.

The study of the accumulation of mRNAs for several key genes of the carotenoid biosynthetic pathway has shown that the gene *β-lcy* encoding lycopene cyclase plays a key role in the control of biosynthesis and accumulation of pigments and that its expression is highly dependent on the auxin-ethylene balance. In addition, our results showed that the *rin* gene plays an important role in controlling the expression of the key carotenoid biosynthetic pathway genes.

To get a better view of differentially expressed genes by auxin and ethylene during ripening, transcriptome analysis of fruits treated with ACC and IAA was performed by a preliminary RNA-Seq approach. Among the transcription factors studied in the laboratory, the gene *Sl-IAA17*, a member of the family of Aux/IAA was affected by auxin and ethylene. Functional characterization of *Sl-IAA17* gene during fruit development was performed by creating transgenic lines under-expressing this gene by RNAi. These lines display a phenotype producing bigger fruit than wild type. Histological analysis of the tissues showed that fruit phenotype is associated with a thicker pericarp. By microscopy, we observed that

increasing the thickness of the pericarp in the transgenic lines was not due to a greater number of cells but to the increase in cell size. Finally, we observed that cell expansion in transgenic fruit is tightly coupled with higher ploidy levels than wild fruits, suggesting a stimulation of the endoreduplication process. These results clearly demonstrate the existence of a close relationship between the auxin signal, the control cell size, fruit volume and the endoreduplication process.

In conclusion, the results provide new insights into the interactions between auxin and ethylene during fruit development and in particular during the transition immature fruit, mature fruit. In addition, they provide new information on the understanding of the role of the signalling pathway of auxin in controlling the development of fleshy fruits and in particular on the basis of certain members of the AUX/IAA on regulating volume and fruit weight.

摘要

生长素和乙烯的互作在植物的生长和发育过程中扮演着非常重要的角色，包括根的生长发育，根毛的形成等，但生长素和乙稀互作机制在果实成熟发育过程中的研究较少。番茄果实颜色由绿色变成红色是成熟的重要表型之一，这一过程通常是由于叶绿素的降解和类胡萝卜素的累积共同决定的。在本研究中，外源生长素 (IAA),乙烯 (ACC) 和生长素抑制剂 (PCIB) 处理显著影响了果实的成熟过程和颜色变化。IAA 延缓了果实由绿色到红色的颜色变化过程而 ACC 和 PCIB 则加快了果实颜色的变化。另外，与对照组果实相比，ACC 和 PCIB 提高了番茄红素的含量并降低了胡萝卜素的含量,而 IAA 处理组则与之相反，降低了番茄红素含量而升高了胡萝卜素含量。

对类胡萝卜素合成途径中的所有合成基因进行定量 PCR 分析，结果显示，番茄红素环化酶可能在外源激素处理过程中对色素合成和累积有重要调控作用，该蛋白的表达受到生长素和乙烯的共同调控。另外，定量结果显示 RIN 对胡萝卜素合成途径中的关键酶有重要调控作用。

为了全面分析和研究生长素-乙烯的互作对果实成熟发育过程的影响，本实验室利用 RNAseq 技术对不同激素处理 48h 后基因组范围内的基因表达差异进行了通量分析，结果表明，*Sl-IAA17* 基因的表达受到生长素和乙烯的共同调控。故本研究同时对 Sl-IAA17 在番茄果实生长发育成熟过程中的功能进行了分析研究。利用 RNAi 技术沉默 Sl-IAA17 蛋白的表达后，和野生型植株的果实相比，转基因抑制表达株的果实明显变小。通过组织学和显微镜切片分析证明，抑制表达株果实的外果皮厚度增加，细胞发育过程中，抑制表达株果实细胞大小明显大于野生型果实，但细胞数量没有明显区别。最后，通过对染色体水平的检测分析表明，抑制表达 *Sl-IAA17* 影响调控了果实发育过程中核内复制过程，从而提高了转基因表达株果实的染色体水平。以上结果充分证明了生长素信号通路与细胞膨胀，果实体积大小及和染色质复制过程有着密切关系。

总之，本研究结果进一步分析明确了生长素和乙烯在果实发育过程中的互作关系，特别是在果实转色期的互作调控关系。另外，首次提出了生长素信号途径在果实发育过程通过核染色体复制调控果实大小，为进一步更全面的分析生长素在果实发育过程中的调控机制奠定了坚实的基础。

Publications

Articles:

Liyan Su, Carole Bassa, Corinne Audran, Catherine Cheniclet, Christian Chevalier, Mondher Bouzayen, Jean-Paul Roustan, Christian Chervin*. The auxin response *Sl-IAA17* transcriptional repressor controls fruit size via the regulation of the endoreduplication-related cell expansion. In press in Plant and Cell Physiology 2014. *doi: 10.1093/pcp/pcu124*

Liyan Su, Eduardo Purgato, Gianfranco Diretto, Saïda Danoun, Mohamed Zouine, Zhengguo Li, Giovanni Giuliano, Mondher Bouzayen, Jean-Paul Roustan and Christian Chervin*. Carotenoid accumulation in ripening tomatoes is modulated by the ethylene-auxin balance. In preparation

Liyan Su, Zhengguo Li*, Jing Fan, XueGao, Yingwu Yang, Wei Deng, Yanwei Hao. Cloning of *CsCAB* Gene from Navel Orange and Construction of Its Plant Expression Vectors. Acta Agriculturae Boreali-Sinica 2010 2: 40-43

Poster presentation:

Liyan Su, Carole Bassa, Corinne Audran, Isabelle Mila, Catherine Cheniclet, Christian Chevalier, Mondher Bouzayen, Jean-Paul Roustan, Christian Chervin. An auxin transcriptional repressor controls fruit size via endoreduplication and cell expansion (2014). The 3rd Annual Meeting of the FA1106-COST Action "Quality Fruit", 2014, Chania, Maich (Crete), Greece

Liyan Su, Wanping BIAN, Jean-Paul ROUSTAN, Christian CHERVIN. Ethylene/auxin modulates the lycopene/carotene pathway in tomato fruit **(**2012). La journée de l'école doctorale SEVAB, 2012, Toulouse, France

Abbreviations

A. thaliana: *Arabidopsis thaliana*

ABA: Abscisic acid

ABP1: Auxin binding protein 1

ACC: 1-aminocyclopropane-1-carboxylic acid

AD: Activation domain

AFB: AUXIN RECEPTOR F-BOX

ARF: Auxin response factors

AuxREs: Auxin response elements

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

BD : Binding domain

bp : Base pair

cDNA: Complementary deoxyribonucleic acid

CTR: Constitutive Triple Response

EAR: Ethylene-responsive element binding factor-associated Amphiphilic Repression

EIN: Ethylene insensitive

ERF: Ethylene response factor

ER: Endoplasmic reticulum

EST: Expressed sequence tags

ETR: Ethylene Triple Response

GA: Gibberellic acid or gibberellin

GFP: Green Fluorescent Protein

IAA: Indole-3-acetic acid

IFs: Transcriptional factors

NAA: α-Naphthalene acetic acid

NLS: Nuclear localization signal

ORF: Open reading Frame

PCIB: p-chlorophenoxy isobutyic acid

PCR: Polymerase chain reaction

qRT-PCR: Quantitative reverse transcription Polymerase chain reaction

RIN: Ripening Inhibitor

RNAi: RNA interference

RNAseq: RNA Sequencing

SCF: SKP1-Cullin-F-box

Sl-IAA: Solanum lycopersicum Auxin/Indole-3-Acetic Acid

S. lycopersicum: *Solanum lycopersicum*

Taq: Thermus aquaticus DNA polymerase

TIR1: Transport Inhibitor Resistant 1

WT: Wild-type

YFP: Yellow Fluorescent Protein

Content

Objective of the Thesis

Ethylene and auxin are two important hormones and play a crucial crosstalk during many plant development aspects. Although it starts to be deciphered in root tissues in other plant species (Muday et al., 2012), there is still less knowledge about the interplay of auxin and ethylene during fruit developmental process. Tomatoes, as a model of climacteric fruit has a strong requirement for ethylene to ripen (Paul et al., 2012), and auxin has also important roles (Vendrell, 1985).

My Ph.D study aimed at studying the ethylene-auxin interactions during fruit ripening processes, especially with regard to colour changes which are caused by the carotenoid accumulation. Then, I also aimed at identifying the key genes involved in the carotenoid pathway, which could be induced or repressed by ethylene or auxin. After this, we focused on the functional characterization of an Aux/IAA transcription factor, *Sl-IAA17*, which was shown to be expressed during the fruit development and ripening, in preliminary studies.

In summary, our thesis will start with a review of auxin/ethylene and metabolic and molecular events that have been described so far during the plant development. Then by using tomato as model plant shown that how ethylene-auxin crosstalk worked on fruit colour changing. In the end, a new role of *Sl-IAA17* gene in regulating tomato fruit size development has been unraveled.

Chapter I: General introduction

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1. Tomato as a model plant

1.1 History and Significance

The *Solanaceae* family contains a lot of crops, including potato, tobacco, eggplant, tomato. Most of them have a great value in modern agriculture. Among them, tomato (*Solanum lycopersicum* L.) has a long history as an important fresh vegetable source (Fig.1A) (Matsukura et al., 2008). The tomato appeared in France in the early sixteenth century, but it is originated from South America. From now, there are more than 7500 different varieties tomato be cultivated in the whole world (Fig.1B) and the production is considerable (Fig.1C). Different varieties display different character in fruit size and fruit color. For example, the diameter of fruit from 5 mm to 10 cm, and the color of the fruit contains red, green, yellow, black, orange (Allen, 2008 and http://en.wikipedia.org/wiki/Tomato). The reason for the tomato dominant position is because it's one of the most nutritious foods for human, and it's also an important source for vitamin C and the vitamin A precursor, β-carotene. Several studies shown that lycopene (one of the most important pigments in tomato) has great significance to balance and improve the body's immune system and has a great function in anti-cancer (Hernández et al., 2007; Ray et al., 2011).

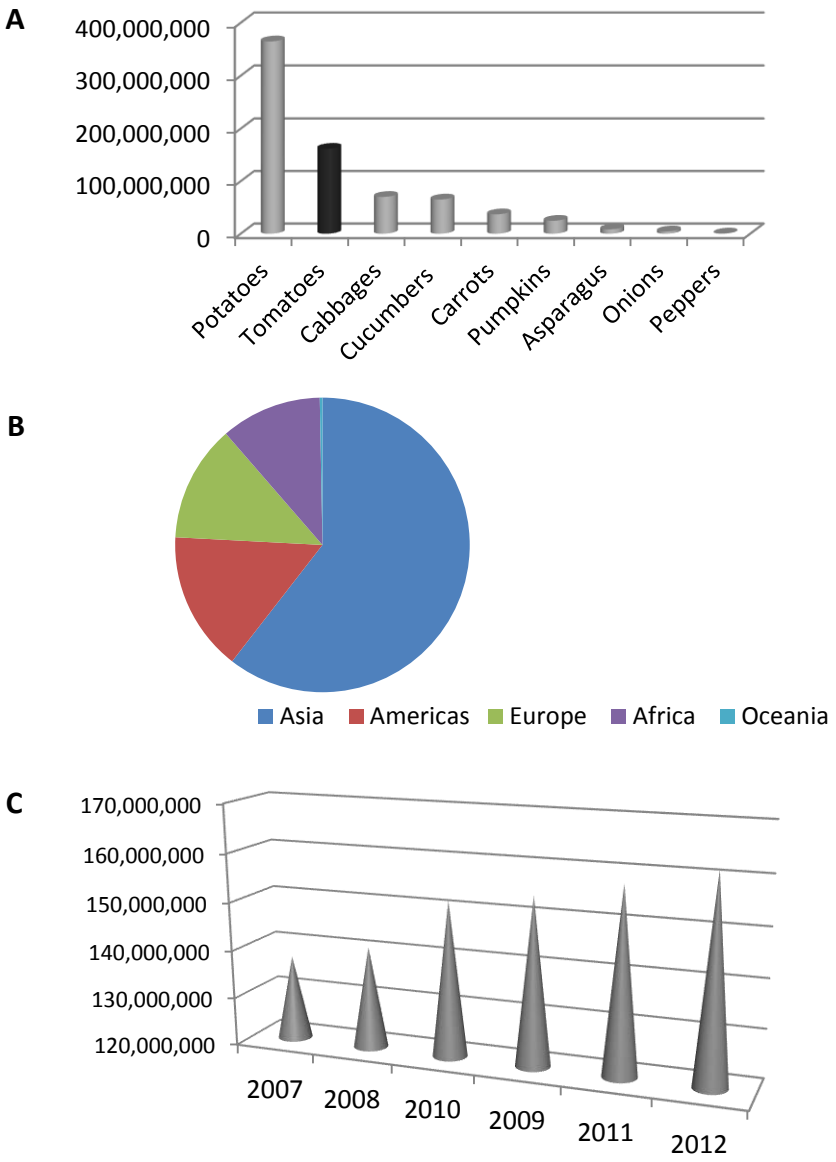

Fig. 1: The world production according to FAOSTATA): Production (tons) of primary vegetables in 2012; **B)**: Total production (tons) of tomato in the different continents (2012); **C)**: Tomatoes production (tons) from 2007 to 2012.

1.2 Plant characteristics

As an important agronomical and economic crop, tomato is also famous as a classic model plant that is widely used for genomic research in biology (Giovannoni, 2001). Compared to other model plants, tomato has particular characters. Firstly, compared to *Arabidopsis*, tomato can provide interesting features for fleshy fruit research, like the color changing, sugar synthesis, the formation of acidity, texture, and aroma volatiles during the

fruit ripening. Secondly, tomato is a simple diploid plant, the number of the chromosomes is 12 pairs. From now, more and more information about tomato has been published, a relatively compact genome (900 Mb) has been sequenced (Sato et al., 2012), an abundant RNAseq data, and a range of biological resources and tools (e.g. EST database, TILLING resources, genetic and physical maps), which provide a convenient set of tools for the researchers using tomato as a model plant. Lastly, highly efficient transformation protocols and a relatively short reproductive cycle (3-4 generations per year) make the tomato one of the best choice for genetic and genomic studies.

1.3 Micro Tom

During my PhD, Micro Tom was the variety I used for my research, which is a dwarf cultivar of tomato created for ornamental purposes in the beginning (Scott and Harbaugh, 1989; Meissner et al., 1997; Emmanuel and Levy, 2002) (picture of the plant Fig. 2A). The leaves of Micro Tom are small with deformed leaflets and present a deep green color (picture of micro leaf Fig. 2B). The fruit size of Micro Tom is from 10mm to 30mm, and it's has short reproductive cycle (60 to 90 days) (picture of micro fruit Fig. 2C).

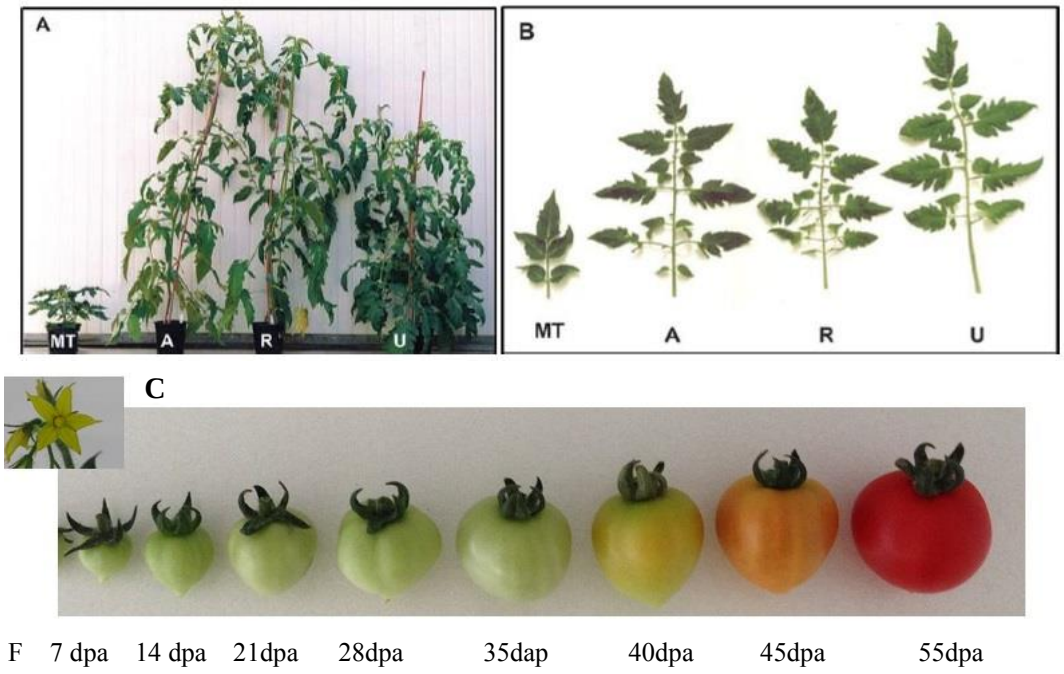

Fig. 2: Plants of MicroTom, Ailsa Craig, Rutgers, and UC-82A): Entire plants at the time of flowering (~ 2 months old). **B)**: Fifth leaf from the base. MT, Micro-Tom; A, Ailsa-Craig; R, Rutgers, U, UC-82 (Liu, 2013) **C)**: Different stages of fruit ripening development. F: Flower; dpa: days post anthesis.

The phonotype of Micro Tom is ascribed to two major recessive mutations, one is DWARF (*d*) and MINIATURE (*mnt*), and the other one is SELF PRUNING (*sp*) gene (Meissner et al., 1997; Marti et al., 2006). The *d* gene encodes cytochrome P450 protein, which worked as brassinosteroid biosynthetic enzyme (Bishop et al., 1996). The deep-green leaves and shortened internode phenotype of Micro Tom are induced by reduced brassinosteroid content (Marti et al., 2006). It is suggested that *mnt* mutation associated with gibberellin (GA) signalling without affecting GA metabolism, which has not been well characterized (Marti et al., 2006). *Sp* is an orthologous of the *Arabidopsistfl1* (Terminal flower 1) gene, which is involved in continuous growth of the shoot apical meristem (Pnueli, 1998).

1.4 Fruit color and size

Fruit color

The color of the fruit could be an important factor to attract customer, which change occurs during fruit ripening (Fig. 2C). In recent years, thanks to the use of advanced biotechnology, the molecular mechanisms of fruit color change become more and more understood.

The tomato fruit color is depends on the content of different pigments. The color change from green to red in tomato is associated with both chlorophyll degradation and carotenoid accumulation, which have been studied in numerous tomato mutants affected in pigment biosynthetic pathways (Bramley 2002; Giuliano et al., 2008). Additionally, Egea et al. have proved that the transition started in the nascent chromoplast by a sharp accumulation of carotenoids while the chlorophyll level was still high and confirmed that all chromoplasts derived from pre-existing chloroplasts (2011). Carotenoid is the most important pigment family for the fruit color change during the tomato ripening. The carotenoid biosynthesis pathway in tomato is well described in Fig. 3 (Ronen G, 1999; Giuliano et al., 2008; Ampomah-Dwamena et al., 2009). The first committed step is the condensation of two molecules GGPPs form the colourless phytoene, a reaction catalyzed by phytoene synthase (PSY). Then, phytoene is desaturated to the colourless zeta-carotene by phytoene desaturase (PDS). The production of red lycopene in tomato is catalyzed by the zeta-carotene desaturase (ZDS) and carotenoid isomerases (CRTISO) (Ampomah-Dwamena et al., 2009; Giuliano et al., 2008). The formation of δ-carotene and γ-carotene are catalyzed by ε-lycopene cyclases (ε-LCY) and β-lycopene cyclases (β-LCY), and the orange α-carotene and β-carotene are synthetized by β-lycopene cyclases. Finally, these carotenes are transformed to lutein and zeaxanthin by the β-carotene hydroxylase (β-CRTR). Up to now, the mechanism of carotenoid biosynthetic pathway and accumulation are not well understood during the ripening process (Rosati et al., 2000; Fantini et al., 2013). Therefore, the regulatory and metabolic network between the genes involved in carotenoid pathways and the tomato ripening is a crucial point in the future.

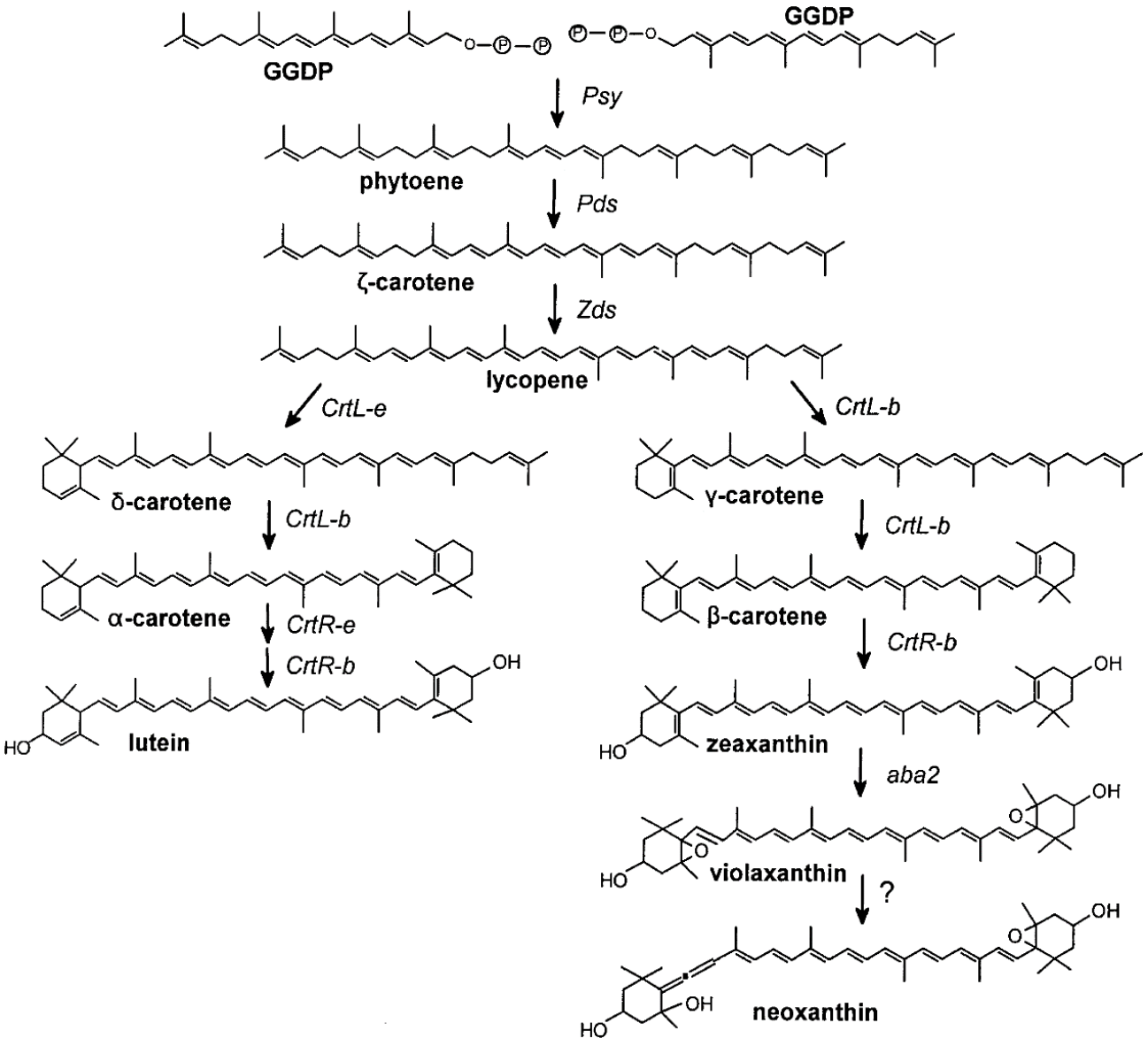

Fig.3: Pathway of carotenoid biosynthesis in plants. Cyclization of lycopene marks a branching point of the pathway to either a- or b-carotene. Enzymes are indicated by their gene assignment symbols: aba2, zeaxanthin epoxidase; CrtL-b, lycopene b-cyclase; CrtL-e, lycopenee-cyclase; CrtR-b, b-ring hydroxylase; CrtR-e, e-ring hydroxylase; Pds, phytoene desaturase; Psy, phytoene synthase; Zds, z-carotenedesaturase; GGDP, geranylgeranyl diphosphate. (Reprinted from Ronen et al., 1999)

Fruit size

Fruit size is one of the most important fruit quality traits (Tanksley, 2004). Tomato fruit development could divide into 3 phases (Gillaspy et al., 1993). Phases I is the stage for ovary development, which is the key step to decide fruit set; Phases II is for ovary developed via cell division, cell division provides the building blocks, setting the number of cells that will make up an organism; Phases III, the cells begin to expansion, which activity then determines its final size, in the end, the cell could reach an hundreds times size compare to their initial (Cheniclet et al., 2005).

In the early stage, there is no answer about the molecular determinants of cell size control, but cyclin-dependent kinase inhibitory kinase WEE1 and the anaphase promoting complex activator CCS52A have been proved involved in control cell size and the endo-reduplication process during the fruit development in tomato (Chevalier et al., 2011). Later, it's has been proved that the large size of the fruit in the pericarp or loculars related to the arrest of mitotic activity and DNA ploidy level in the nuclear (Chevalier, 2007), which induced by endo-reduplication (Mathieu-Rivet et al., 2010 and herein). Endo-polyploidy is a widespread process in eukaryotes that leads to an increase in the cell ploidy level. Endo-reduplication means that the normal mitotic cell division was replaced by an altered cell cycle where mitosis is ignored, and it's exists in many cell types (Chevalier et al., 2011 and herein). Although the adaptive value and physiological role of endo-reduplication remains unclear, numbers of functional hypotheses have presented by researchers: endo-reduplication is thought to provide a means to sustain growth under adverse environmental conditions, such as genotoxic stress (Hase et al., 2006; Adachi et al., 2011), saline stress (Ceccarelli et al., 2006), water deficit (Cookson et al., 2006), and low temperature (Barow, 2006).

Fruit size/weight in tomato has been proved genetically under the control of nearly 30 quantitative trait loci (QTLs) (Grandillo et al., 1999). The gene *fw2.2* (for fresh weight locus no.2 on chromosome 2) is the only fruit size-controlling locus to be cloned and characterized at molecular level (Frary et al., 2000), which regulated negatively the mitotic activity of developing fruit and induced a modulation of the final fruit size. FW2.2 appears to be a part of cell-cycle control signal transduction pathway play a role after fruit set and upstream of the

endoreduplication (Cong and Tanksley, 2006).

2. Ethylene

2.1 Ethylene biosynthetic pathway

Fruit could be divided into two groups depending on the existence of ethylene-associated respiratory peak: 1) Climacteric fruit, such as tomato, apple, pear, presenting a ripening-associated increase in respiration and in ethylene production, and 2) non-climacteric fruit, such as orange, grape, strawberry lacking the ethylene and respiratory peaks.

Ethylene (C_2H_4) exists in all advanced plants and many data proved the complex function of ethylene during the tomato fruit ripening (Bouzayen et al., 2010). The production of ethylene in tomato is based on an "autocatalytic synthesis" system (McMurchin et al., 1972), in which the ethylene produced is promoting its own synthesis (Bouzayen et al., 2010).

The biosynthetic pathway of ethylene has been well established (Yang and Hoffman, 1984). Ethylene is synthesized from the amino acid methionine via S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane -1-carboxylic acid (ACC) (Fig. 4). Two major enzymes, ACC synthase (ACS) which converts SAM into ACC, and ACC oxidase (ACO), which converts ACC into ethylene, are involved in the ethylene biosynthetic pathway.

The role of ethylene during the fruit ripening process has been demonstrated clearly by own-regulating *ACO* and *ACS* genes in transgenic plants, as the ethylene-suppressed lines showed strongly delayed ripening in tomato fruit (Oeller et al., 1991; Picton et al., 1993). There are numerous changes happening during the fruit ripening, and sets of ethylene- related genes were identified during the tomato ripening process, including pigment biosynthesis enzymes, genes encoding cell wall-degradation, and regulation of acidity and sugar (Zegzouti et al., 1999; Bennett and Labavitch, 2008; Pirrello et al., 2009 and cited herein). Moreover, ethylene has numerous effects on fruit growth, developmental process and storage life, still including seed germination and cell elongation, flowering and root nodulation, programmed cell death, and response to abiotic stresses and pathogen attacks (Johnson and Ecker, 1998;

Bleecker and Kende, 2000; Lin et al., 2009).

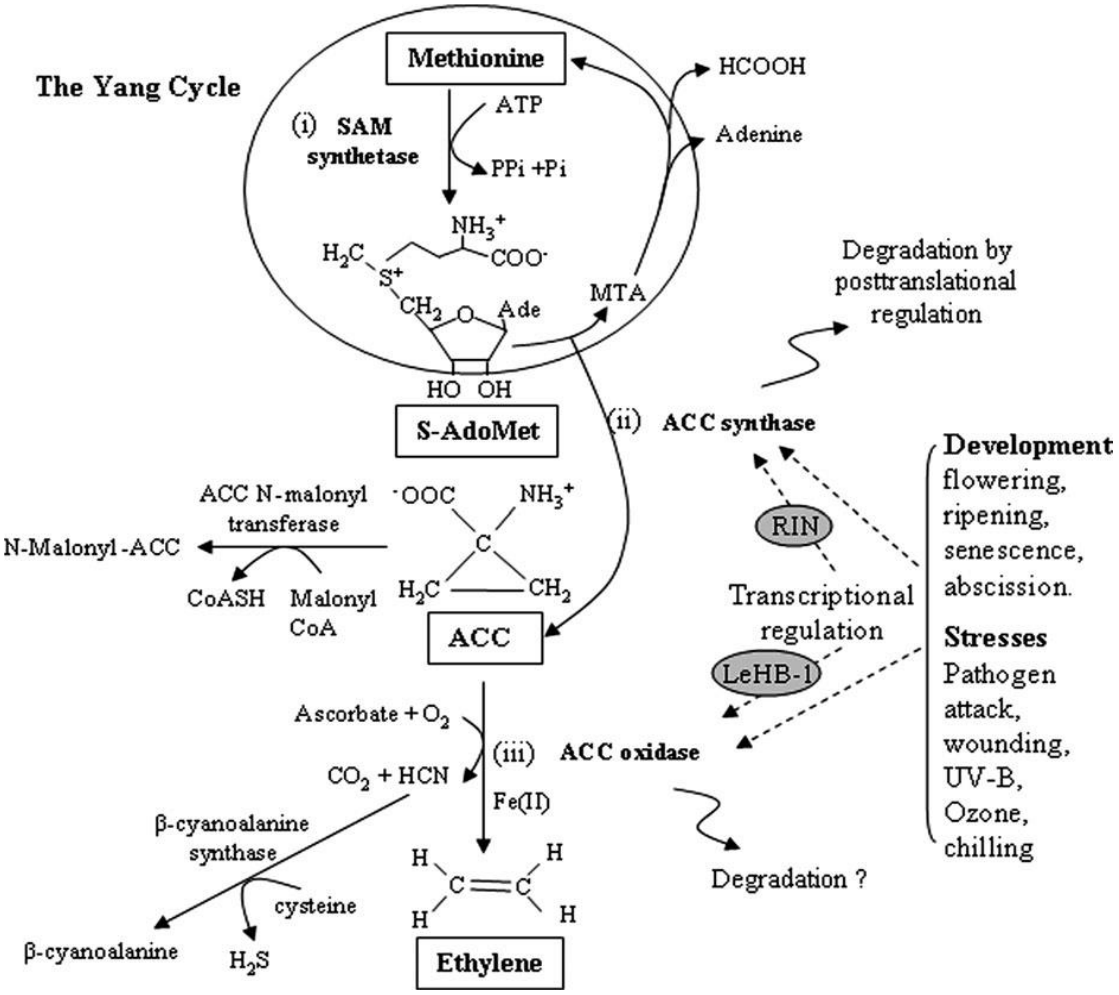

Fig.4: Ethylene biosynthetic pathway. (i). Methionine converted to S-AdoMet (S-adenosyl methionine) by SAM synthetase (ii). S-AdoMet converted to ACC by ACC (iii). ACC converted to Ethylene is catalysed by ACC oxidase (Reprinted from Lin et al. 2009)

2.2 Ethylene response factors (ERFs)

ERFs are last downstream components in the ethylene signal transduction pathway. ERFs are part of AP2 (APETALA2)/ERF super-family which also contains AP2 and RAV family genes (Riechmann et al., 2000). ERF family proteins contain only one AP2/ERF domain, whereas, AP2 family genes have two such domains. RAV family proteins contain an additional B3 DNA binding domain along with AP2/ERF domain. First, EIN3 enters the

nucleus and binds to ERE (Ethylene Response Elements) and activate transcription of ERFs, then ERFs bind the GCC-box and activate the genes required for certain ethylene functions such as fruit ripening and others (Fig. 5). In *Arabidopsis*, it was shown that the ERF subfamily included 65 members which be divided into 5 subclasses (Nakano et al., 2006). Recently, 28 clone were isolated from tomato base on the functional analysis and be divided into 9 subclasses, in which only a few *ERF* genes have been characterized functionally so far (Pirrello et al., 2012).

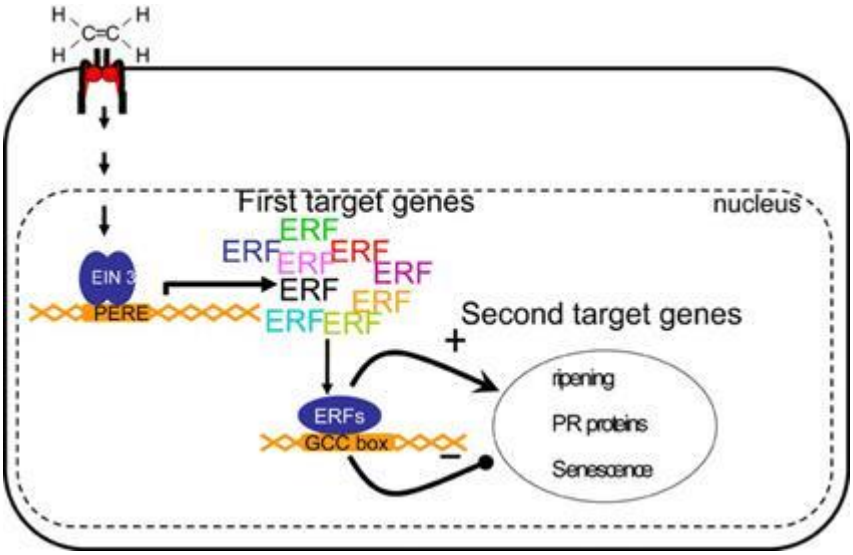

Fig. 5: ERFs are the last actors of ethylene transduction pathway. (From GBF website *http://gbf.inp-toulouse.fr/en/research-1/ethylene-signalling.html*)

Recent studies have shown that ERFs involved in a wide range of processes during the plant development, such as hormonal signal transduction (Ohme-Takagi et al., 1995), response to biotic and abiotic stresses (Yamamoto et al., 1998, 1999; Dubouzet et al., 2003). In tomato, most of ERFs have been shown to participate in stress and/or hormonal responses (Gu et al., 2002; Pirrello et al., 2006; Li et al., 2007; Zhang et al., 2009; Lee et al., 2012; Pan et al., 2012), like *ERF1* could be induced by both ethylene and NaCl treatment (Huang et al., 2004), and *Sl-ERF6* has been shown play an important role in fruit ripening by integrating the ethylene and carotenoid synthesis pathways in tomato (Lee et al., 2012), and more recently,

it’s has been shown that *Sl-ERF.B3* involved in red pigment accumulation via impacts carotenoid biosynthesis genes through mechanisms beyond the influence of ethylene (Liu et al., 2014)

3. Auxin

3.1 Auxin perception and signalling

Auxin is the first plant hormone discovered by Charles Darwin presented in his book "The Power of Movement in Plants" in 1880. Indole Acetic Acid (IAA) is the major auxin involved in many of the physiological processes in plants (Arteca, 1996). During the past decades, more and more functions of auxin have been discovered including regulation of numerous developmental responses like apical dominance, tropism, vascular patterning, embryos patterning and fruit set. Auxin can be synthesized in many different tissues, such as young leaf (the most important source), stem apical meristem, root tips, cotyledon, and seeds in the developing fruits, then auxin be transported to different tissues via polar transport model, Auxin signal is perceived by plant cells and activates a set of transduction cascades (Fig. 6)

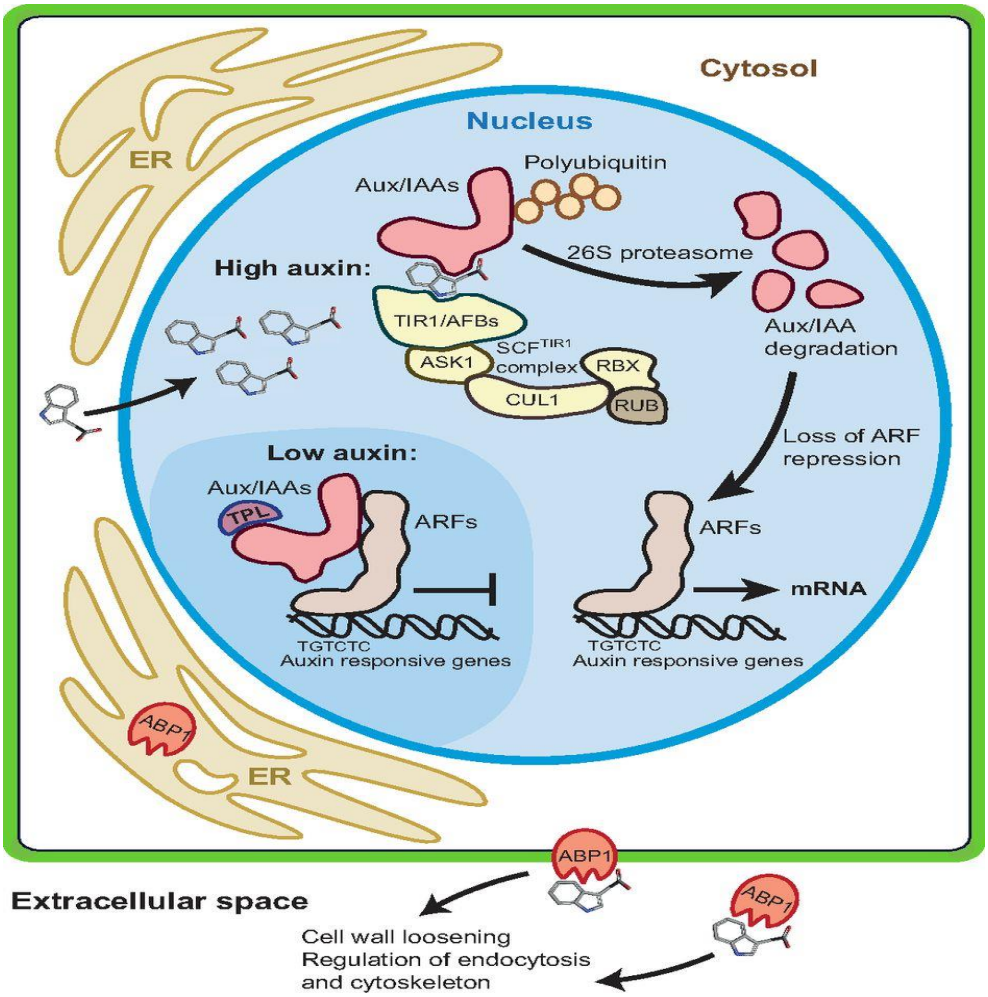

Fig. 6: The key components in auxin perception and signalling. Auxin can modulate both transcriptional regulation and transcription-independent responses. In the nucleus, IAA binds to its receptors, the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALLING F-BOX proteins (TIR1/AFBs) and to the auxin/indole-3-acetic acid (Aux/IAA) proteins. TIR1/AFBs are F-box proteins that form part of the SCF/TIR1 complex, which consists of four subunits (TIR1/AFB, ASK1, CUL1 and RBX). An additional protein, RUB, binds to the SCF/TIR1 complex to regulate its function. The TIR1/AFB and Aux/IAA proteins function as co-receptors for IAA, binding IAA with high affinity. When IAA levels are low (darker blue background), the Aux/IAA proteins form heterodimers with auxin response factors (ARFs) to repress gene transcription. The TOPLESS (TPL) protein functions as a transcriptional co-repressor for Aux/IAAs. However, when IAA levels are high, the binding of IAA to its co-receptors targets the Aux/IAA proteins for degradation by the 26S proteasome, which leads to derepression of ARF transcriptional regulation and the expression of auxin responsive genes. AUXIN BINDING PROTEIN 1 (ABP1), which is located in the endoplasmic reticulum (ER) or at the plasma membrane and/or in the apoplast (extracellular space), is also believed to function as an IAA receptor, mediating rapid auxin responses such as cell wall loosening, cytoskeleton rearrangement and regulation of endocytosis, leading to cell expansion. (Reprinted from Ljung, 2013).

Auxin signalling has been extensively over the past decade (Bargmann and Estelle, 2014). Auxin-responsive genes have a very quick response for auxin concentration, 5 to 15 min after an auxin application, the change of protein level could be detected (Oeller and Theologis, 1995; Bargmann and Estelle, 2014). The response is due to the interaction of several nucleus proteins: Aux/IAAs. Aux/IAAs can be found in many plants including *Arabidopsis*, maize, rice, poplar, sorghum and tomato, but not in bacteria, animal or fungi.

In tomato, Aux/IAA family has four conserved domains (I-IV). Domain I is responsible for the repressing activity of the protein (Tiwari et al., 2004), whereas domain II confers instability to the Aux/IAA proteins (Worley et al., 2000; Ouellet et al., 2001; Audran-Delalande et al., 2012). Domains III and IV are involved in homo- and hetero-dimerization with other Aux／IAA proteins (Kim et al., 1997; Ouellet et al., 2001, Dharmasiri and Estelle, 2004) and with ARFs (Ulmasov et al., 1997b; Ouellet et al., 2001, Hayashi, 2012). ARFs are auxin response factors, are transcription factors activating specific genes of the response to auxin. Recent studies showed that Sl-IAA7, Sl-IAA14, Sl-IAA16 and Sl-IAA17 display an additional DLxLxL motif between conserved domains I and II, but the presence of two conserved repression motifs (LxLxLx and DLxLxL) did not result in stronger repression activity (Audran-Delalande et al., 2012).

3.2 Roles of auxin in plants

Most of Aux/IAA functions has been identified in *Arabidopsis* (Paul et al., 2005), But phenotypes associated with loss of function are scarce probably due to important functional redundancy among Aux/IAA family members (Overvoorde et al., 2005). In contrast, down-regulation of various Aux/IAA genes in the *Solanaceae* species results in visible and distinct phenotypes (Wang et al., 2005; Bassa et al., 2012; Deng et al., 2012). So, tomato as an important model plant has been used by more and more researchers. Chaabouni et al. (2009) found that down-regulation *Sl-IAA3* results in both auxin- and ethylene- associated phenotypes in tomato. Wang et al. (2005) showed that down-regulation *Sl-IAA9* resulted in pleiotropic phenotypes, consistent with its ubiquitous expression pattern. Deng et al. (2012) found *Sl-IAA15* is involved in trichome development in tomato. Likewise, down-regulation of

Sl-IAA27 leads to reduced fruit size (Bassa et al., 2012). Taken together, these data declared specialized roles of Aux/IAAs in plant developmental processes, clearly indicating that members of the Aux/IAA gene family in tomato perform both overlapping and specific functions.

IAA17 belongs to clade C with *IAA7, IAA14* and *IAA16*. *IAA17* mutants (called *AXR3*) have been described in *Arabidopsis*, which exhibited a stronger apical dominance, a reduction in root elongation, an increased adventitious root formation, and a lack of root gravitropism, and these phenotypes suggest an increased response to auxin in the *IAA17* mutants. On the other hand, *AXR3* mutation refers to resistance to the ACC (Leyser et al., 1998). More recently, it has been proved that IAA17 may interact with the protein TOPLESS (TPL) at the domain I. But IAA17 may also interact with the TPL protein at the second repressor domain between domain I and domain II. A modification in the second motif LxLxLx prevents binding domain of the protein TPL on IAA17 (Hao et al., 2014). In tomato, expression of the gene *IAA17* is induced by auxin and inhibited by ethylene (Audran-Delalande et al., 2012), but there no more research about the function of IAA17 during the fruit development.

4. Crosstalk between auxin and ethylene

4.1 Mechanism of the crosstalk

There are many plant hormones regulating the plant growth and development, the crosstalk between the hormones is the key point to understand the function of hormones in this process (Achard et al., 2007; Su et al., 2011; Muday et al., 2012;). One set of the plant hormones with complex interactions is auxin and ethylene (Muday et al., 2012). Over the past decade, study of the crosstalk between those two hormones never stopped. A brief summary about the model of auxin–ethylene crosstalk was raised in the article by Muday et al (2012). In *Arabidopsis*, ethylene is initiated by binding the ETR1, and then inhibits ETR1 activity, which in turn leads to inhibition of the CTR kinase. CTR kinase is a negative regulator of

EIN2 activity. In the end, EIN2 activates the EIN3 and EIN3- like (EIL) family of transcription factors, which in turn promote transcription of genes containing an ethylene-responsive element (ERE) in their promoter region. For auxin, which started by binding TIR1 receptors, Auxin signalling is mediated by proteasome-dependent degradation of AUX/IAA transcriptional repressors, which release ARF transcription factors to activate transcription of genes with auxin responsive elements (AUXRE) in their regulatory region. Primary crosstalk occurs by activation of genes that contain both AUXRE and ERE in their promoter region, allowing both signalling pathways to directly regulate transcription. Secondary crosstalk occurs through expression of genes that are either auxin or ethylene responsive, but the activities of which control expression of genes that regulate the other hormones' synthesis, signalling, or response.

4.2 The crosstalk in the hormones synthesis level

In the very early stage, Morgan et al. has presented that elevated the level of auxin lead to increased ethylene synthesis (1962), and then Abel et al. (1995) and Stepanova et al. (2007) found that the reason for this effect is mediated by increased transcription of the ACC synthase (ACS) family genes, which is the rate-limiting enzyme in ethylene synthesis pathway. Moreover, ethylene may also positively regulate auxin synthesis also. Firstly, after treated the *Arabidopsis* seedlings with 100 mM ACC, the level of free IAA has been increased in the root tip (Swarup et al., 2007), and this response was disappeared in etr1 or after treatment with ACC synthesis inhibitor 1-aminoethoxyvinyl-glycine (AVG), but the effect of AVG on IAA synthesis may be direct, rather than working through altered ethylene levels (Ruzicka et al., 2007). Secondly, compare to wild type, the concentration of auxin was increased five times as a result of constitutive ethylene signalling in the *ctr1* root apex of *Arabidopsis*, which means ethylene affected the auxin biosynthesis partly or auxin transport in that organ (Ikeda et al., 2009).

4.3 The crosstalk in the seedling development process

In plants, auxin and ethylene act synergistically and antagonistically to control the

seedling growth. **Firstly,** root elongation. Studies have proved that both ACC (ethylene precursor 1-aminocyclopropane carboxylic acid) and auxin can inhibited root elongation via decrease the expansion rate of the cells in the central elongation zone (Swarup et al., 2007; Rahman et al., 2007), which has been demonstrated in plant mutants. In the early stage, Guzman et al. (1990) have proved that enhanced ethylene signalling or synthesis could reduce root elongation, moreover, both auxin overproducing mutant and treated wild-type with exogenous auxin shown extreme reduction in root elongation (Delarue et al., 1998; Zhao et al., 2001; Rahman et al., 2007). Although auxin and ethylene have similar effects on root elongation, but nobody knows whether there are any convergence in the signalling pathways during this process. **Secondly,** lateral root development. Auxin and ethylene worked antagonistically on lateral root initiation. Auxin increased lateral root formation and elongation (Ivanchenko et al., 2008), and the mutants or inhibitors reduced auxin transport, and then reduced lateral root initiation and emergence (Reed et al., 1998; Casimiro et al., 2001; Peret et al., 2009). It's has been proved that the root tip is the best place for auxin play a role (De Smet et al., 2007; Moreno-Risueno et al., 2010), but other reports have argued of that, in regions of roots close to the apex, auxin inhibits lateral root initiation (Ivanchenko et al., 2010). So, from now, what is clear from all these studies is that the function of auxin in lateral root initiation is complex, especially near the tip, but most of the reports supported that auxin is a positive regulator of lateral root development (Casimiro et al., 2001; Laskowski et al., 2008; Dubrovsky et al., 2008; Peret et al., 2009). Many reports has presented that treated the plant with ethylene or ACC both reduces lateral root initiation in *Arabidopsis* and tomato (Strader et al., 2010; Negi et al., 2008), what is more, it has been proved that ethylene play negatively in regulating auxin transport and accumulation patterns, particularly in shoot tissues (Suttle et al., 1988; Lewis et al., 2011). **Thirdly,** recent evidence suggests that ethylene-induced inhibition of the root gravity response might be tied to the synthesis of flavonoids, which act as endogenous negative regulators of auxin transport (Buer et al., 2006; Lewis et al., 2011), but the role of ethylene in regulating the shoot gravitropic response is more complex. Lastly, auxin and ethylene may act both synergistically and independently in controlling the initiation, elongation, and cellular positioning of root hairs (Tanimoto et al.,

1995; Rahman et al., 2002; Muday et al., 2012 and herein), and other studies also suggest that ethylene causes enhanced apical hook formation by both increasing the levels of components important for auxin signalling and increasing auxin levels on the concave side of the apical hook (Muday et al., 2012 and herein).

4.4 The crosstalk in the fruit development process

Compare to related reports about the crosstalk of auxin and ethylene during the in the seeding growth, there are poorly understood of the interaction of these two hormones during fruit growth and ripening.

Ethylene act as a crucial role in the ripening of climacteric fruit, such as tomato, or banana, and there is a sharp increase of ethylene production and activity during onset of ripening (Tucker, 1993), but the application of natural or synthetic auxin has been found to lead to a ripening delay during the pre-ripening stage of fruit (Böttcher and Davies, 2012) and therefore auxins have been widely viewed as ripening inhibitors (Frenkel, 1972; Buta et al., 1994; Purgatto et al., 2002; Böttcher et al., 2010). The first presentation was raised by Morgan and Hall (1962), who reported 2,4-dichlorophenoxyacetic acid application led to an increase in ethylene production in cotton plants, and then Yoshii and Imaseki (1982) present exogenous auxins induce the transcription of ACS genes in mung beans (*Vigna radiata (L.) Wilczek*). Later, in 2006, Raghavan et al. conformed that low concentration of 2,4- D (0.001 mM) applied to the roots of *Arabidopsis* induced an increase in transcript of gene encoding ACC oxidase, and exogenous auxins also led to an increase in transcript levels for one member of the ACC synthase family gene-*AtACS6.* Also, Kondo et al. (2006) present that *ACS4* was induced by the synthetic auxin 2,4-D and *ACS4* transcript accumulation may contribute to the increase of ACC synthase activities in ‘La France’ pear, this point has been found in many fruits also, such as apple (Li and Yuan, 2008), peach (Trainotti et al., 2007) and grape (Ziliotto et al., 2012). The reciprocal effect of auxin on ethylene biosynthesis through the activation of several ACC synthases has been also described in tomato, Coenen et al. (2003) have shown that auxin induction of a gene encoding ACC synthase *LE-ACS3* and *LE-ACS5*. However, results suggest that there are target genes that are regulated by both

hormones through distinct signalling pathways, yet the functional significance of many of these transcriptional changes in controlling growth and development has not been determined. Although a dependence of ethylene action on IAA biosynthesis, perception, signalling and transport has been illustrated in studies using *Arabidopsis* mutants and auxin measurements (Růžička et al., 2007; Swarup et al., 2007), but the complementary effect of ethylene on auxin biosynthesis has long remained elusive during the fruit development process.

Tomato ripening is a complex and highly coordinated developmental process. The main changes associated with ripening is color change (loss of green color and increase in non-photosynthetic pigments that vary depending on species and cultivar). As we mentioned in last paragraph, many results have provided that auxin affect ethylene biosynthesis through the activation of several ACC synthases, but the role of auxin and ethylene crosstalk during the tomato ripening, especially during the color change process, is still poorly understood.

Chapter II: Modulation of carotenoid accumulation in ripening tomatoes is modulated by the ethylene-auxin balance

Chapter II: Carotenoid accumulation in ripening tomatoes is modulated by the ethylene-auxin balance

1. Introduction

Auxins and ethylene are hormones known to impact plant development, often with antagonistic roles. Auxins exert pleiotropic effects, including the development of roots, shoots, flowers and fruits (Tromas and Perrot-Rechenmann, 2010). Ethylene is one of the plant hormones regulating the ripening of fruit, the opening of flowers, and the abscission of leaves. Tomato is a model plant for the study of climacteric fruit development, which is promoted by ethylene (Giovannoni, 2007). Observations of tomato fruits and some non-climacteric fruits, like grape berry and strawberry, have suggested that ripening is also regulated by auxins, since they can delay ripening and regulate gene expression (Cohen, 1996; Davies et al., 1997; Given et al., 1988; Vendrell, 1985). However, the impact of auxin on tomato ripening has not been extensively studied and, most importantly, the crosstalk between auxin and ethylene in the tomato ripening progress is not yet clear (Muday et al., 2012).

ABA is an important regulator of plant development and stress responses, such as seed dormancy, germination, growth, and stomatal movements (Finkelstein et al., 2002). A cross-talk between ABA and auxin has been found in root growth (Wilson et al., 1990; Nagpal et al., 2000), seedling germination (Wang et al. 2011). Also, ABA has a cross-talk with ethylene not only in developmental processes but also in adaptive stress responses of plants (Yang et al., 2005; Cutler et al., 2010). Based on the timing of ABA accumulation, ethylene production and the expression of ABA and ethylene biosynthesis genes, Zhang et al. (2009) has concluded that ABA and ethylene may play a coordinating role in tomato ripening. Color change from green to red is a very important indicator of tomato ripening and can easily be measured by chromametry (McGuire, 1992). This change is associated with the degradation of chlorophylls and the shift of the carotenoid composition from leaf-like xanthophylls (mainly lutein and neoxanthin) to carotenes (mainly lycopene, phytoene and -carotene)

(Fraser et al., 1994). The carotenoid biosynthetic pathway in tomato is well described in Fig. 3.

For the purpose of this article, the pathway will be divided into two parts, comprising respectively the steps upstream and downstream of lycopene (Fig. 7). In the upstream part, the key rate-limiting steps are catalyzed by PSY1, PDS, ZDS, ZISO and CRTISO (Giuliano et al, 1993; Fraser et al., 1994; Fantini et al, 2013). The expression of *psy1, ziso, crtiso is* directly regulated by the Ripening Inhibitor (RIN) protein, which is a member of the MADS-box family of transcription factors (Martel et al., 2011; Fujisawa et al., 2013). In the downstream part, lycopene cyclases (ε-LCY/β-LCY) are also key enzymes, catalyzing the transformation of lycopene to δ- and β- carotene (Ronen et al., 1999; Ronen et al., 2000; Rosati et al., 2000; Ma et al., 2011). Additionally, it is believed that ABA biosynthesis occurs indirectly through the production of carotenoids (Milborrow, 2001). The carotenes are transformed to lutein and zeaxanthin by heme and non-heme β-carotene hydroxylases (CYP97 and CRTR β). Zeaxanthin is converted to violaxanthin and neoxanthin by the actions of zeaxanthin epoxidase (ZEP) and neoxanthin synthase (NXD). Zeaxanthin accumulation was not observed in our experiments. ABA4 protein has been shown to be necessary for neoxanthin accumulation in *Arabidopsis* leaves (North et al., 2007). Violaxanthin and neoxanthin are precursors for the synthesis of ABA. NCED1 encodes 9-cis-Epoxycarotenoid Dioxygenase (NCED), a key enzyme in the biosynthesis of ABA (Ji et al., 2014). The 9-cis-forms of neoxanthin and violaxanthin are the precursors of ABA (Parry et al., 1990), a phytohormone known to control ripening of many fruits, including tomato, in which it is able to trigger ethylene biosynthesis and thus accelerate ripening (Zhang et al., 2009).

To study the involvement of crosstalk between auxin and ethylene in the accumulation of carotenoid pigments in tomato fruits, we treated mature green fruits with the auxin indole acetic acid (IAA) and the ethylene precursor aminocyclopropane carboxylic acid (ACC), alone or in combination, and also p-chlorophenoxy isobutyric acid (PCIB). This latter is an antagonist of auxin action, although its mechanism of action is not characterized very well at the molecular level (Oono et al., 2003). The effects of these treatments have been investigated on color change, pigment content and on the levels of transcripts involved in carotenoid

biosynthesis.

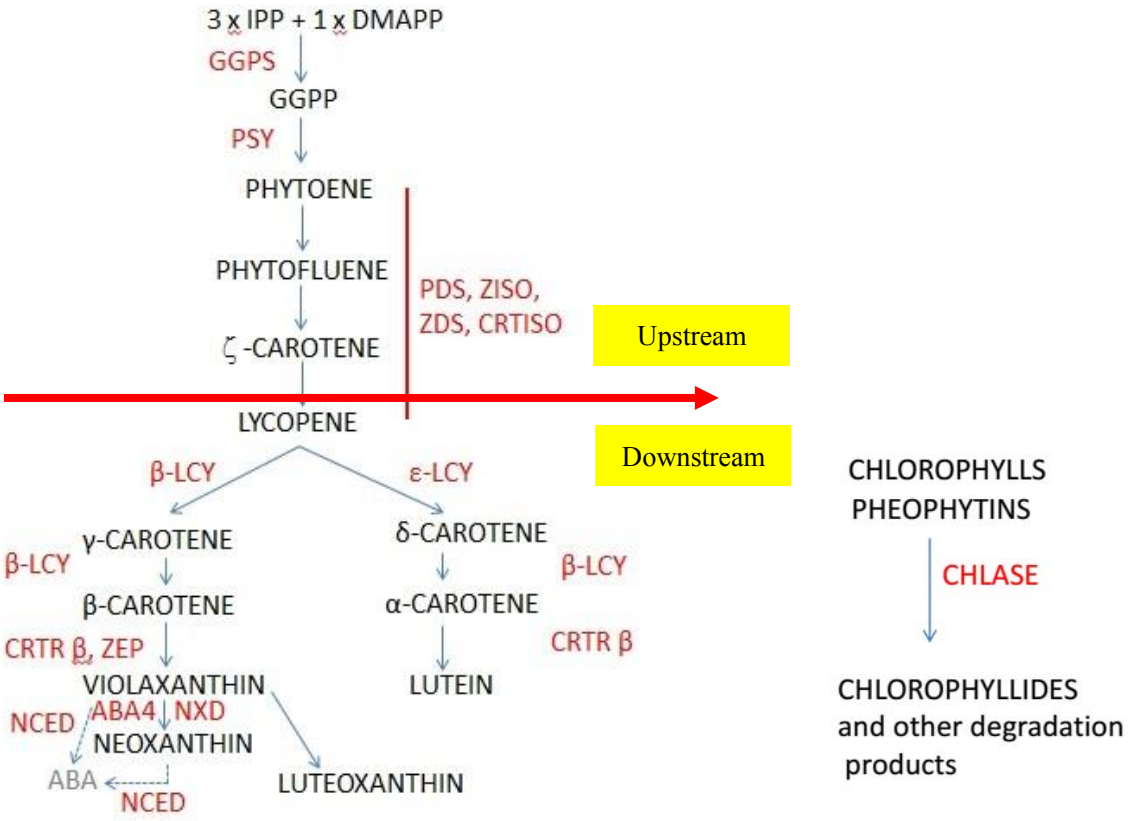

Fig. 7: The carotenoid biosynthetic pathway based on Giuliano et al. (2008). Names of intermediate compounds are in black and names of enzymes are in red. IPP = isopentenyl diphosphate, GGPS = GGPP synthase, GGPP = geranyl-geranyl pyrophosphate, PSY = phytoene synthase, PDS = phytoene desaturase, ZISO = ζ -carotene isomerase ZDS = ζ -carotene desaturase, CRTISO = carotenoid isomerase, ε-LCY = lycopene ε-cyclase, *β*-LCY = lycopene *β*-cyclase, CRTR-β = *β*-carotene hydroxylase, ZEP = zeaxanthin epoxidase, NCED = Nine-cis-epoxy carotenoid dioxygenase, NXD = neoxanthin synthase, CHLASE = chlorophyllases, ABA = abscisic acid, ABA4 = ABA - deficient 4.

2. Materials and Methods

2.1 Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum* cv. MicroTom) were grown under standard greenhouse conditions. The culture chamber room was set as follows: 14-h day/10-h night cycle, 25/20 ℃ day/night temperature, 80% relative humidity, 250 μM m-2 s-1 light intensity. Tomato seeds were first sterilized 5 min in sterile water and sown in Magenta vessels containing 50 ml 50% Murashige and Skoog (MS) culture medium and 0.8% (w/v) agar, pH 5.9 (Egea et al., 2010).

2.2 Treatments of tomato fruits

Tomato fruits were harvested at the mature green stage of development and injected with a buffer solution contained 10 mM MES, pH 5.6, sorbitol (3% w/v) and 100 μM of ACC, or IAA, or IAA+ACC, or PCIB (Sigma). Buffer injection was performed as described previously (Orzaez et al., 2006). Briefly, tomato fruits were infiltrated using a 1ml syringe with a 0.5 mm needle. Needle was inserted 3 to 4 mm into the fruit tissue through the stylar apex, and the infiltration solution was gently injected into the fruit until the solution ran off the stylar apex and the hydathodes at the tip of the sepals. Only completely infiltrated fruits were used in the experiments. Controls were treated with buffer only. After the treatment, fruits were incubated for 6, 24, 48, 96 h in a culture room at 26°C, under 16h light/8h dark cycle with a light intensity of 100 μM s-1 m-2. After incubation, pericarp was collected and frozen at -80°C until further analysis. For each condition, 27 fruits were treated arising from different plants.

2.3 Color and pigment measurement

Surface color was assessed with a Chromameter (CR400, Konica Minolta), using the D65 illuminant and the L*, a*, b* space, and the data were processed to obtain Hue as previously described (McGuire, 1992). The measurements were taken on 6 independent batches of 9 fruit. The Hue angle (in degrees) was calculated according to the following

equations: Hue = tan-1 (b*/a*) if a>0 and 180 + tan-1 (b/a) if a<0. For pigment analysis, fruit samples were chosen at 96 h after treatment with IAA, ACC, IAA+ACC, PCIB and ground to a fine powder in liquid nitrogen. The samples were pooled in 3 biological replicates, each one arising from 9 tomatoes. Pigments (chlorophylls/carotenoids) were extracted from freeze-dried tissues and analyzed as described previously (Fiore et al., 2012; Liu et al., 2014) using an Accela U-HPLC system coupled to an Orbitrap high-resolution mass spectrometer (HRMS) operating in positive mode-atmospheric pressure chemical ionization (APCI) (Thermo Fischer Scientific, Waltham, MA).

2.4 Ethylene production analysis

The fruit after treatments 0h was incubated in opened 100 ml jars for 2 hours after injection, and then jars were sealed and incubated at room temperature for 1h in the 100 ml closed jars at room temperature. Then 1 ml of headspace gas was injected into an Agilent 7820A gas chromatograph equipped with an alumina column and a flame ionization detector (Agilent, Santa Clara, CA, USA). Samples were compared with 1ppm ethylene standard and normalized for fruit weight.

2.5 ABA assay

The assays were performed as described before (Forcat et al. 2008). Briefly, 110 mg of frozen tissue were extracted at 4°C for 30 min with 400 µl of H_2O with 10% methanol + 1% acetic acid. The internal standard was 2H6 ABA. The extract was centrifuged at 13,000 g for 10 min at 4°C. The supernatant was carefully removed and the pellet re-incubated for 30 min with 400 µl of methanol-acetic acid mix. Following the centrifugation, the supernatants were pooled. Extracts were then analyzed by LC-MS using an Acquity UPLC coupled to a XevoQtof (Waters, Massachusetts, USA). Analysis parameters were described in Jaulneau et al. (2010).

2.6 RNA isolation and quantitative PCR (qPCR)

Total fruit RNA was extracted using the PureLinkTM Plant RNA Reagent (Invitrogen)

according to the manufacturer's instructions. Total RNA was treated by DNase I to remove any genomic DNA contamination. First-strand cDNA was reverse transcribed from 2 μg of total RNA using an Omniscript kit (Qiagen). qPCR analyses were performed as previously described (Pirrello et al., 2006). The primer sequences are listed in Table 1. Relative fold changes were calculated using *Sl-Actin*as housekeeping gene. As for pigment analyses, three independent RNA isolations were used for cDNA synthesis.

2.7 Tobacco protoplast and fluorescence assay

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, the protoplast was divided into 96 well plate (Greiner 96, Bio-One) with 100μL each well. And treated with Buffer (control) or IAA or IAA+PCIB, the using concentration of 10 μM. GFP/YFP expression was analyzed and quantified by Luminometer "Tristar LB 941" base on the operating introduction (Berthold, LRSV). All transient expression assays were repeated at least three times with similar results.

Table 1: qPCR primer list

Gene Name	AccessionSoly No.in (SGN)data libraries	Primer Sequence for Q-PCR
ggps	Solyc09g008920.2.1	F 5'-GCTGTTGGTGTCTTATATCGTG-3'
		R 5'-CTTCTCAATGCCATAAACGCTG-3'
psy1	Solyc03g031860.1.1	F 5'-GGAAAGCAAACTAATAATGGACGG-3'
		R 5'-CCACATCATAGACCATCTGTTCC-3'
psy2	Solyc02g081330.2.1	F 5'-GAGGTGACAAGGTAACATAAAGG-3'
		R 5'-CCCACAACAAAGCAACAGAC-3'
psy3	Solyc01g005940.2.1	F 5'-CAAGTTTCCTTTAGACATCAAGCC-3'
		R 5'-TTCCTCTCAATGCATCTTCTCCA-3'
pds	Solyc03g123760.2.1	F 5'-GGTCACAAACCGATACTGCT-3'
		R 5'-AAACCAGTCTCGTACCAATCTC-3'
zds	Solyc01g097810.2.1	F 5'-AGTGGTTTCTGTCTAAAGGTGG-3'
		R 5'-ACCGAGCACTCATGTTATCAC-3'
crtiso	Solyc10g081650.1.1	F 5'-AAGACCCACAGACGATACCT-3'
		R 5'-ATCGCCAACACAATATAGACCA-3'
ziso	Solyc12g098710.1.1	F 5'-CCTTCTTCTTCCTATACCCGTCG-3'
		F 5'-AGCGTGTGAGCTAAGCACCA-3'
ε lcy	Solyc12g008980.1.1	F 5'-CTTACCAGTTCAAGTATCCCGAG-3'
		R 5'-GCAATATCAGAGCCAGTCCA-3'
β lcy1	Solyc04g040190.1.1	F 5'-GTCCACTTCCAGTATTACCTCAG-3'
		R 5'-TGTCCTTGCCACCATATAACC-3'
β lcy2	Solyc10g079480.1.1	F 5'-CGGGTTATATGGTAGCAAGGA-3'
		R 5'-CAGATGCCGATAACTCATTACC-3'
β lcy3/cyc	Solyc06g074240.1	F 5'-TGTTATTGAGGAAGAGAAATGTGTGAT-3'
		R 5'-TCCCACCAATAGCCATAACATTTT-3'
crtr β1	Solyc06g036260.2.1	F 5'-CTGCTCATTCGCTTCATCAC-3'
		R 5'-CGTCCCTCCTACTTCTTCCA-3'
crtr β2	Solyc03g007960.2.1	F 5'-CTTCTTTCCTACGGTTTCTTCCA-3'
		R 5'-CTCTTATGAACCAGTCCATCGT-3'
rin	Solyc05g012020.2.1	F 5'-CCCAAACTTCATCAGATTCACAG-3'
		R 5'-AATTGTCCCAAATCCTCACCT-3'
zep	Solyc02g090890.2.1	F 5'-ATGATAGACCGCCAACCTTTAGTT-3'
		R 5'-CCATGCATCCCCCTTGAC-3'
nced	Solyc07g056570.1.1	F 5'-CATGAACTTGAACACCCTTTGC-3'
		R 5'-CGTTTCGAACGTAAACGCCT-3'
aba4	Solyc02g086050.2.1	F 5'-TGTGCTCGGACTTCTGTACG-3'
		R 5'-ACTTGCCTTGCAGCAAAAAG-3'
nxd	Solyc12g041880.1.1	F 5'-CGATGAGCTTGTGGTGATTG-3'
		R 5'-CTTTCCGGTTTCTGTGGAAG-3'

chlas1	Solyc09g065620.2.1	F 5'-AGTTCAACCGGACCTGGAGA-3'
		R 5'-GAATGCCGTGTTACCGCCT-3'
chlas2	Solyc06g053980.2.1	F 5'-CAGATGCAACCGAGGATATCAAA-3'
		R 5'-TGGAAGGTGATGCTGCAATC-3'
chlas3	Solyc09g082600.1.1	F 5'-AATCTGGATATGGCGGTTATGG-3'
		R 5'-CGGAGCACAAGCAGGAAATAG-3'
actin	Solyc03g078400.2	F 5'-TGTCCCTATCTACGAGGGTTATGC-3'
		R 5'-AGTTAAATCACGACCAGCAAGAT-3'

2.8 Factorial analyses of correspondence

We used transcript accumulation relative to controls under the Ct format to get only positive values, and the carotenoid accumulation levels were calculated relative to controls. The factorial analyses of correspondence were calculated with the explore.xla Excel macro developed previously (Vaillé, 2010).

3. Results and discussion

3.1 Contrasting effects of ethylene and auxin on tomato fruit color

The hormonal treatments induced significant color changes within 96h (Fig. 8). Treatment with ACC accelerated the transition from green to orange/red compared to controls. On the contrary, treatment with IAA induced a delay in the transition from green to orange/red compared to controls. After 96 h, IAA-treated fruits began to turn orange and then never became red (data not shown). In fruits treated with a combination of ACC and IAA, the evolution of the color was slower than in controls, close to that of the fruits treated by IAA alone, indicating that the IAA treatment is epistatic over ACC treatment. In the presence of the auxin antagonist PCIB, fruits turned red faster than control ones and the color change kinetics were very similar to those treated with ACC (Fig. 8A). These results confirmed the previous studies showing that IAA slows down ripening of tomato fruits (Cohen, 1996; Vendrell, 1985), and that ACC accelerates it (Giovannoni, 2007).

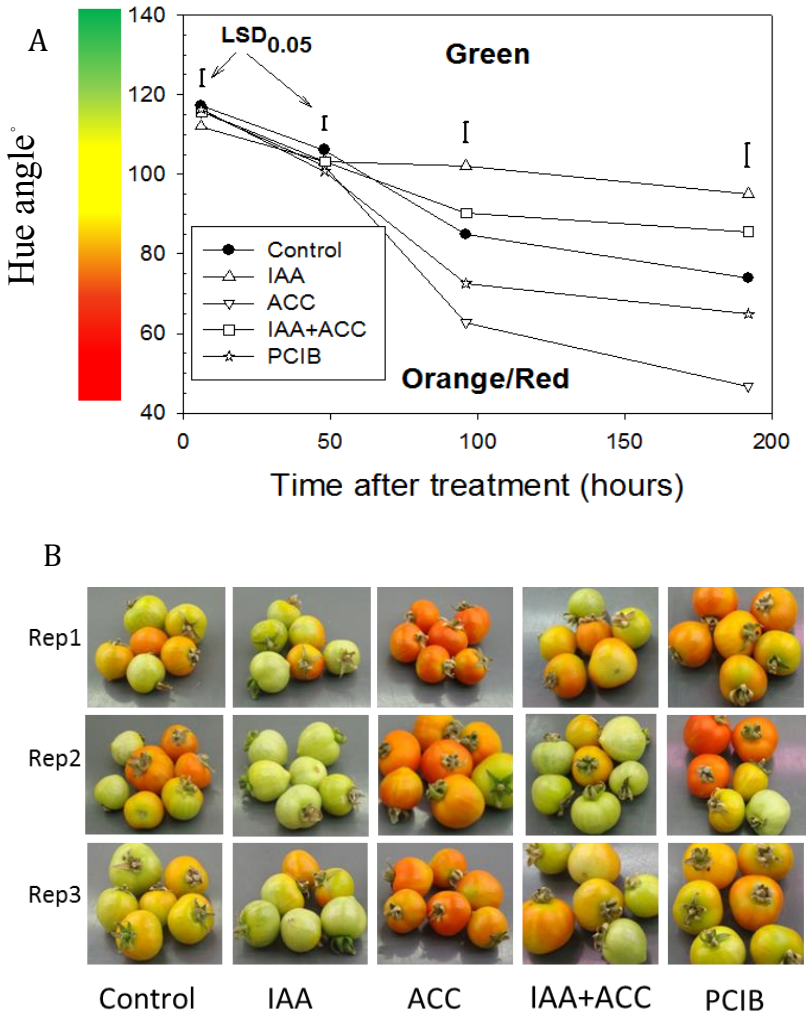

Fig. 8: A): Changes of tomato color as a function of time after hormonal treatments, n = 6. IAA stands for indole acetic acid, ACC stands for aminocyclopropane carboxylic acid (ethylene precursor), IAA+ACC is the combination of both and PCIB stands for p-chlorophenoxy isobutyric acid (auxin antagonist). The color bar next to the Y axis gives an indication of the relation between Hue angle and fruit color, but it is not the exact color of the fruit of the CIELab scale. LSD bars calculated at 0.05 comparison within one sampling date. **B): Pictures of tomatoes arising from the three biological replicates, 96 h after hormonal treatments.**

3.2 Effects of hormonal treatments on carotenoid, chlorophyll and ABA accumulation

For further investigation on the influence of hormonal treatments on fruit pigment composition, fruit extracts were analyzed by high pressure liquid chromatography coupled with photodiode array and high resolution mass spectrometry (LC-PDA-HRMS). At 96 hours, the main carotenoids in control fruits were lutein and β-carotene (Fig. 9). Significant amounts of chlorophylls a and b were observed, together with trace amounts of lycopene, violaxanthin, neoxanthin, luteoxanthin ζ -, δ- and α-carotene. The early compounds phytoene and phytofluene were not detectable. This composition is diagnostic of a ripening stage between the Breaker and Orange stages of ripening.

The ACC and PCIB treatments induced large changes in carotenoid composition at 96 hours (Fig. 9). Lycopene was greatly induced, becoming a major pigment, together with β-carotene which was also induced and lutein which was unaffected. The early compounds phytoene, phytofluene and ζ-carotene and the late compounds δ- and α-carotene were also induced, while the β-xanthophylls neoxanthin and, to a lesser extent, violaxanthin were reduced.

The IAA treatment reduced significantly lycopene accumulation compared to controls while it did not affect α-, β- or δ-carotene accumulation. It also led to higher levels of neoxanthin, violaxanthin and chlorophyll a than in the controls (Fig. 9).

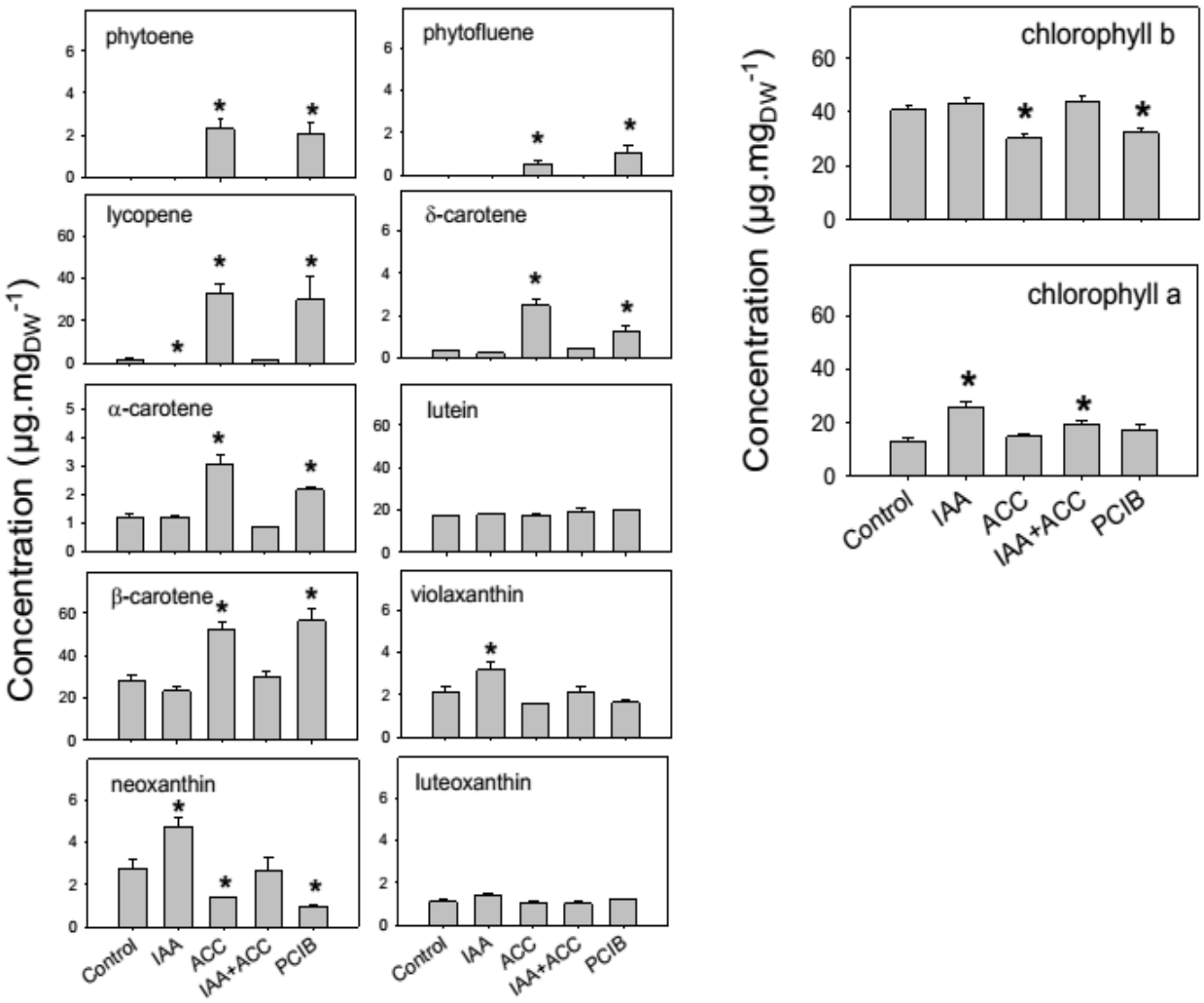

Fig. 9: Carotenoid contents, 96h after treating the fruits with different hormones, HPLC results, n = 3. Abbreviations of hormonal treatments are same as in Figure 8. Error bars are standard errors. An asterix (*) shows a significant difference at 0.05 level using t-test between control and the corresponding treatment (for treatment abbreviations see legend of Fig. 8).

ABA levels were decreased by the ACC and PCIB treatments and increased by the IAA treatment (Fig. 10), implying that its levels were directly correlated to those of neoxanthin/violaxanthin. Sun et al. (2012) and Galpaz et al. (2008) suggested that the NCED dioxygenase and its xanthophyll substrates are rate limiting in the ABA accumulation, but our results suggest that in the response to ethylene/auxin treatments the regulation may be upstream the violaxanthin. Finally, the ACC and PCIB treatments led to an increased degradation of chlorophyll b (Fig. 9).

These results confirmed observations performed in previous studies, showing that ethylene treatments accelerated chlorophyll degradation, the appearance of orange color (Saltveit, 1998), and the accumulation of lycopene (Frenkel and Garrison, 1976 and refs herein). To the opposite, auxin treatment delayed the accumulation of lycopene and carotenes, also confirming previous observations (Vendrell, 1985). PCIB acted as an auxin antagonist, inducing the same effects as ACC.

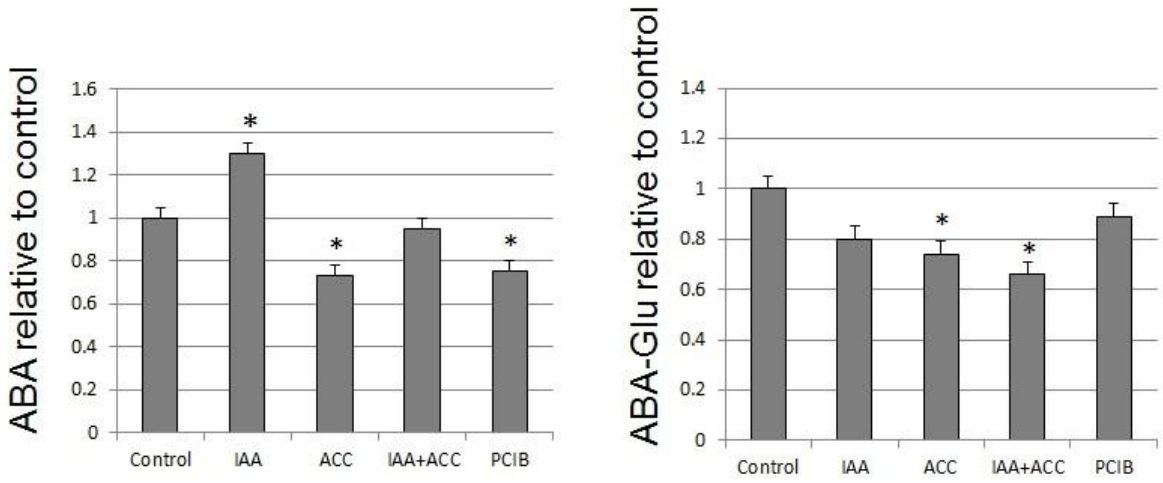

Fig. 10: Variations of free ABA levels and ABA glucoside in MicroTom tomatoes 96h after hormonal treatments. n=5 biological replicates. The figures above bars are *P* values for t-test between control and the corresponding treatment. The asterix (*) shows a significant difference at 0.05 level using t-test between control and the corresponding treatment (for treatment abbreviations see legend of Fig. 8).

3.3 Effects of the various hormonal treatments on fruit ethylene production

Ethylene is assumed to be a “master switch” controlling tomato fruit ripening. Therefore, it is interesting to verify if the hormonal treatments described above alter ethylene production.

We measured ethylene production in hormone-treated fruits at various times after the treatment (Fig. 11).

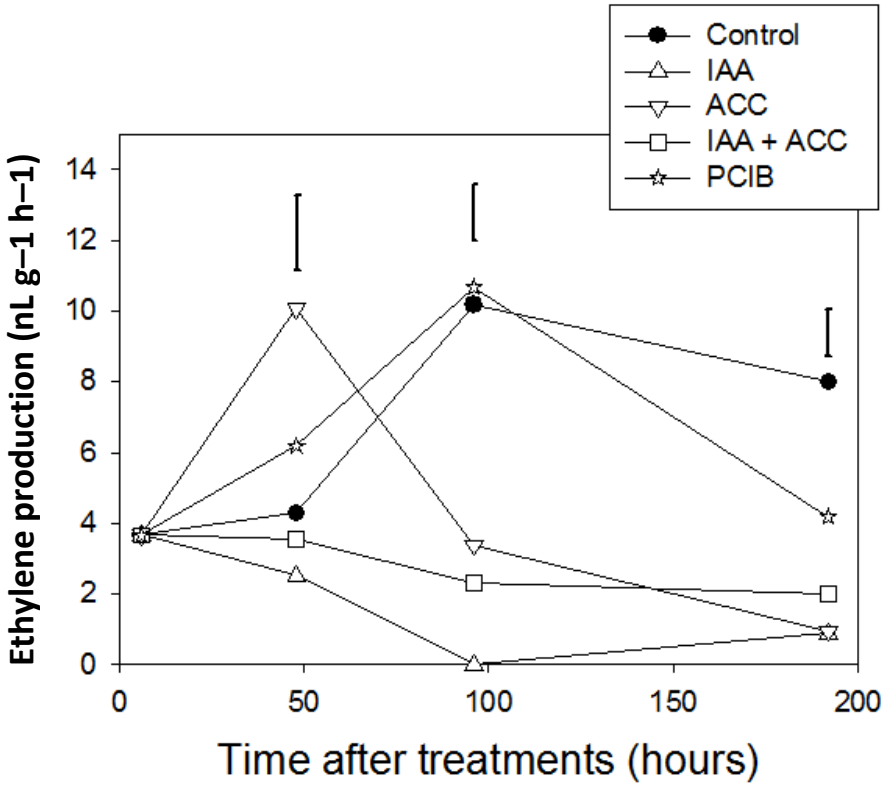

Fig. 11: Variations in ethylene production after hormonal treatments. n=3 biological replicates, error bars are LSD at 0.05 (for treatment abbreviations see legend of Fig. 8).

ACC treatment accelerated the appearance of the climacteric ethylene peak by about 2 days, IAA treatment completely repressed ethylene production during all the store stages, and this repression was only partially reversed by combined IAA+ACC treatments, also indicating that the IAA treatment is stronger than ACC treatment, which match well with fruit color change showing in Fig. 8. PCIB treatment had little, if any, effect up to 100 hours after treatment, while it slightly decreased ethylene production between 100 and 200 hours after treatment.

3.4 Effects of hormonal treatments on gene expression

In order to investigate if the above hormone-induced phenotypes were controlled at least

partially at the gene expression level, we determined the levels of all transcripts involved in carotenoid biosynthesis by quantitative Real Time PCR (qPCR) at two different times after the hormonal treatments (Fig. 12A and 12B).

As observed in Figure 12A the IAA treatment resulted in lower transcript levels for most of the genes upstream of lycopene (*psy1, psy3, pds* and *crtiso*). With the exception of *psy3*, which is mainly expressed in roots, all these genes are rate-limiting for lycopene accumulation (Fantini et al., 2013). Thus, these changes in transcript levels match well the slower color change and the decreased accumulation of lycopene after treatment with IAA (Fig. 8 and 9). Regarding the downstream part of carotenoid pathway (Figure 12B), the transcript levels of *β-lcy1* and *crtrβ1* genes were increased by IAA, which could explain the higher amounts of violaxanthin, neoxanthin and ABA observed in figures 9 and 10. The other gene transcripts of the "bottom" of the pathway: *zep, aba4, nxd* and *nced* did not show variations correlating with accumulation of the compounds cited above.

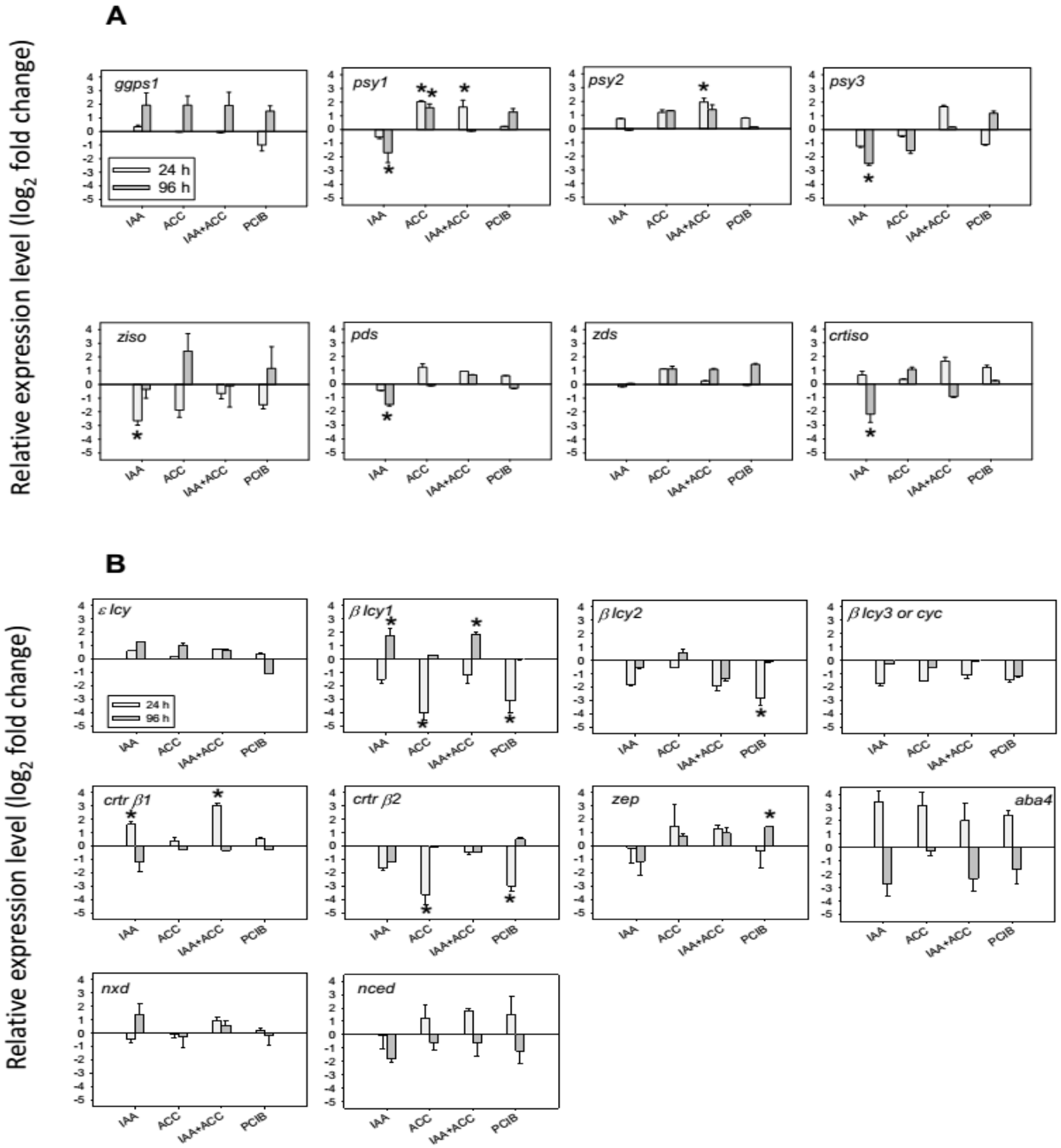

Fig. 12: Modulation of transcript accumulation related to carotenoid pathway by various hormonal treatments, 24h or 96h after treatment A): Upstream of lycopene; **B):** Downstream of lycopene (Fig. 7 and see abbreviations in legend of Fig. 8), n=3 biological replicates, bars = std errors. Expression relative to controls at 24h and 96h (set at 0). *showing significant difference at 0.05 with controls (t-test).

ACC treatment led to higher levels of the *psy1* transcripts, and also, to a lesser extent, of the *ziso, pds, zds* and *crtiso* ones compare to control (Fig. 12A). All these genes encode rate-limiting steps for lycopene biosynthesis (Fantini et al., 2013) and thus the observed changes in gene expression are in agreement with the faster color change and accelerated lycopene accumulation (Fig. 8 and 9). Moreover, ACC treatment decreased *β-lcy1* transcript levels (Fig. 12B), which logically should lead to lower conversion of lycopene to α-/β-carotenes (Fig. 7). Surprisingly, these compounds are increased, and not decreased by the ACC treatment. This phenotype might be partially explained by the simultaneous repression of the *crtrβ2* transcript, which converts β-carotene to zeaxanthin, the first β-xanthophyll upstream of violaxanthin, neoxanthin and ABA. A possible hypothesis is that β-carotene hydroxylation, and not lycopene cyclization, is the main reaction controlling flux through the β-branch, leading ultimately to ABA biosynthesis. These results explain the faster accumulation of lycopene and also the lower accumulation of β-xanthophylls and ABA in ACC treated fruits than in controls.

Similar changes in transcript levels occurred in PCIB-treated fruits (Fig. 12), which showed an additional repression of *β-lcy2* and a very similar carotenoid profile (Fig. 9) with respect to ACC-treated ones. There was no significant effect of any treatment on any of the *ggps* expression (Fig. 12A)

Chlorophyll degradation in Citrus fruits is an active process mediated by chlorophyllase (Chlase) (Jakob-Wilk et al, 1999). In tomato, chlorophyll degradation was affected by hormonal treatments, with IAA treatment retarding chlorophyll a degradation, both alone and in combination with ACC treatment, while chlorophyll b degradation was accelerated by both ACC and PCIB treatments (Fig. 9). We measured the levels of the three Chlase transcripts identified in the tomato genome. Repression of all three transcripts was obvious 96 h after the IAA treatment (Fig. 13). This correlates well with the higher levels of chlorophyll a and to a lesser extent of chlorophyll b, in both treatments with IAA (Fig. 9). However the marked decrease of chlorophyll b in ACC and PCIB treatments does not correlate with increased transcript accumulation of chlorophyllases (Fig.13). This suggests that, in contrast to Citrus (Jakob-Wilk et al, 1999), tomato Chlase gene expression is not under ethylene control and

that, as observed in citrus (Harpaz-Saad et al., 2007), posttranscriptional mechanisms may also regulate Chlase activity in tomato.

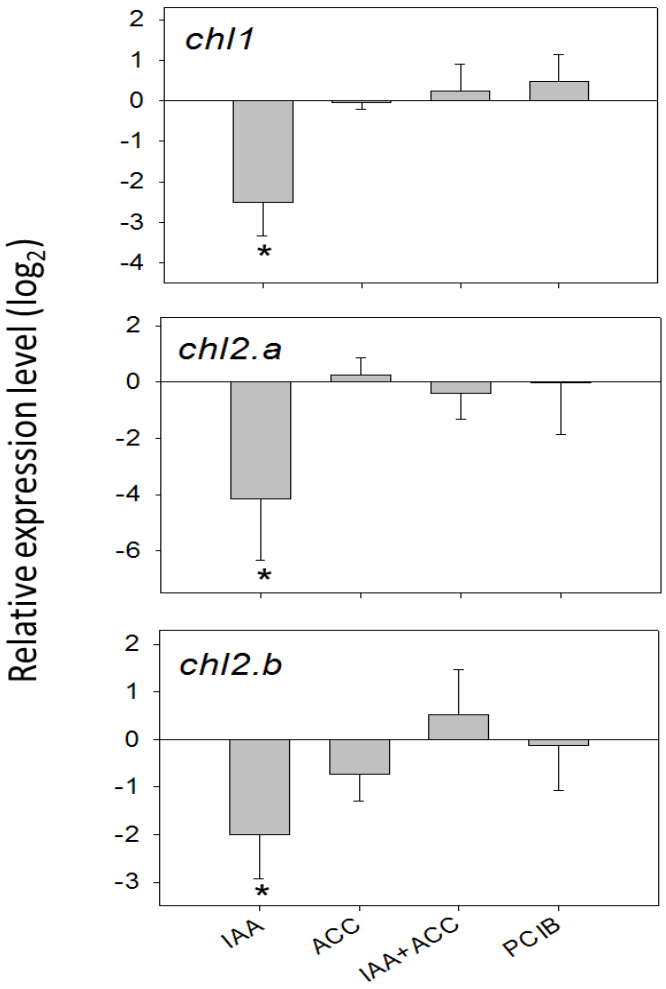

Fig. 13: Modulation of transcript accumulation, related to chlorophyll degradation pathway, by various hormonal treatments, 96h after treatment (see abbreviations in legend of Fig. 8). n=3 biological replicates. Expression relative to controls (set at 0). Error bars are standard errors. *showing significant difference at 0.05 with controls (t-test).

3.5 Effects of ACC and IAA on the *rin* gene

Several genes in the carotenoid pathway are regulated by the RIN transcription factor (Martel et al., 2011; Fujisawa et al., 2013): *psy1, ziso and crtiso* are directly, positively regulated, *zds* is indirectly, positively regulated, and *ε-lcy* and *β-lcy* are indirectly, negatively regulated (Martel et al., 2011; Fujisawa et al., 2013). Q-PCR analyses (Fig. 14A) showed that

the transcript levels of *rin* were substantially stimulated by ACC but inhibited by IAA. The qPCR profiles of *rin* (Fig. 14A) and *psy1* (Fig. 12) seemed to match well under IAA or ACC treatment, and indeed the correlation between *rin* and *psy1* transcript accumulation was positive and significant (Fig. 14B). This confirms a previous study (Martel et al., 2011), determining that RIN is also involved in PSY1 regulation.

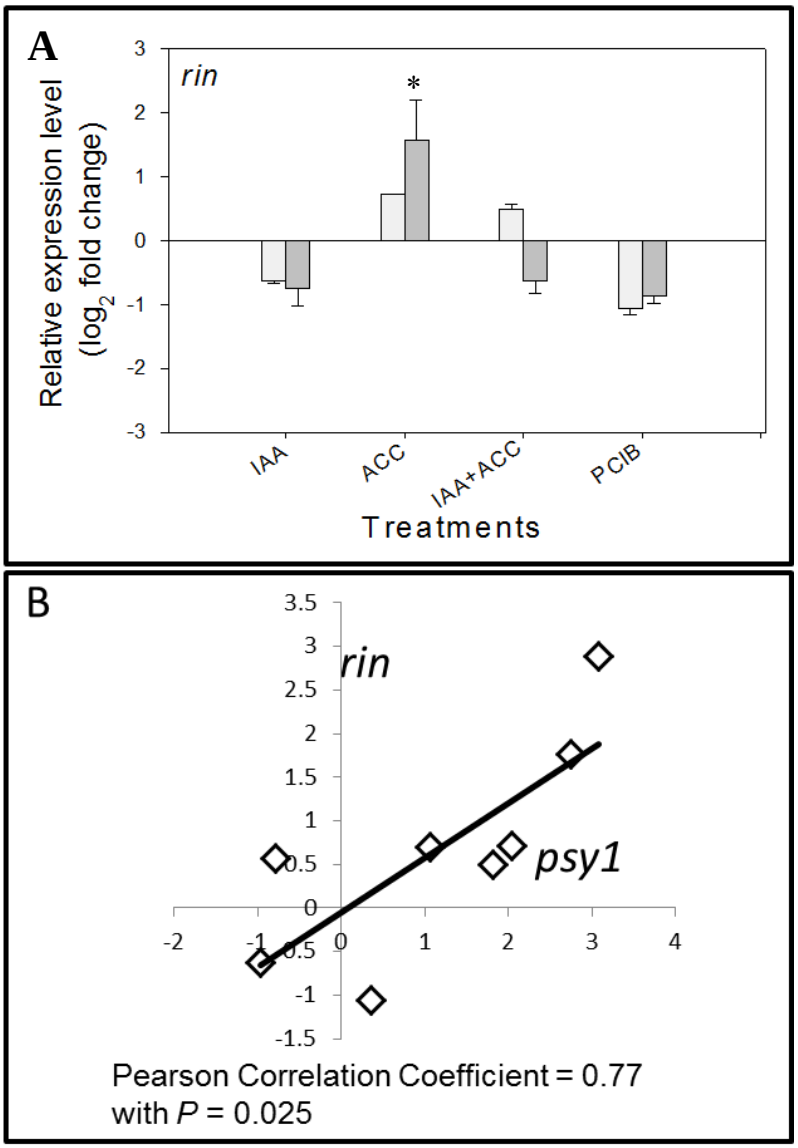

Fig. 14: A): Modulation of transcript accumulation of *rin* by various hormonal treatments, 24h or 96h after treatment, n=3 biological replicates expression relative to controls (set at 0). Error bars are standard errors. **B):** Correlation between *rin* and *psy1* expression. n=3 biological replicates, bars = std errors. Expression relative to controls (set at 0). *showing significant difference at 0.05 with controls (t-test).

3.6 Factorial analyses show clear associations between hormonal treatments, carotenoid content and/or transcript levels

Factorial analyses, are used to determine and describe the dependencies within sets of variables, here the treatments, many observed variations, and the transcript levels or the carotenoid levels. A factorial correspondence analysis (Fig. 15), clearly shows a strong positive correlation between the effects of ACC and PCIB, and their negative correlation to the effects of IAA treatment, whatever the regulatory level measured: transcript accumulation (Fig. 15A) or carotenoid accumulation (Fig. 15B). It is noticeable that, at the transcript level, the IAA+ACC treatment is strongly correlated to the ACC and PCIB ones (Fig. 15A), while at the carotenoid composition level – which matches the fruit phenotype more closely - it is correlated with the IAA treatment (Fig. 15B). This may be due to the fact that changes transcript accumulation occur ahead of those in metabolite accumulation or to the fact that some of the latter changes are due to post-transcriptional events, or to both.

The transcripts correlating well with the ripening delay associated to IAA treatment are the lycopene cyclases (*ε-lcy* and *β-lcy*) and the carotene hydroxylases (crtr) to a lesser extend (Fig. 15A). These results confirm previous studies (Ronen et al., 1999; Ronen et al., 2000; Rosati et al., 2000; Ma et al., 2011). The transcripts correlating well with the accelerated ripening induced by ACC or PCIB are phytoene synthases (psy1 and 3), and a carotene isomerase (ziso) to a lesser extent. The ACC and PCIB treatments are also correlated with accumulation of *pds, zds,* and *crtiso* transcripts. It worth noting that the left position of the chlorophyllases (chlases) in Fig. 15A, is mainly due to the strong inhibition induced by IAA, rather than to a stimulation of chlases by ACC.

Regarding carotenoids, the accumulation of early intermediates and lycopene and, to a lesser extent, of α-, β-, and δ-carotene is inversely correlated to that of late xanthophylls, especially violaxanthin and neoxanthin (Fig. 15B). This confirms that tomato fruit ripening is associated with the accumulation of early compounds and of cyclic carotenes and the decrease in xanthophylls (Fraser et al., 1994).

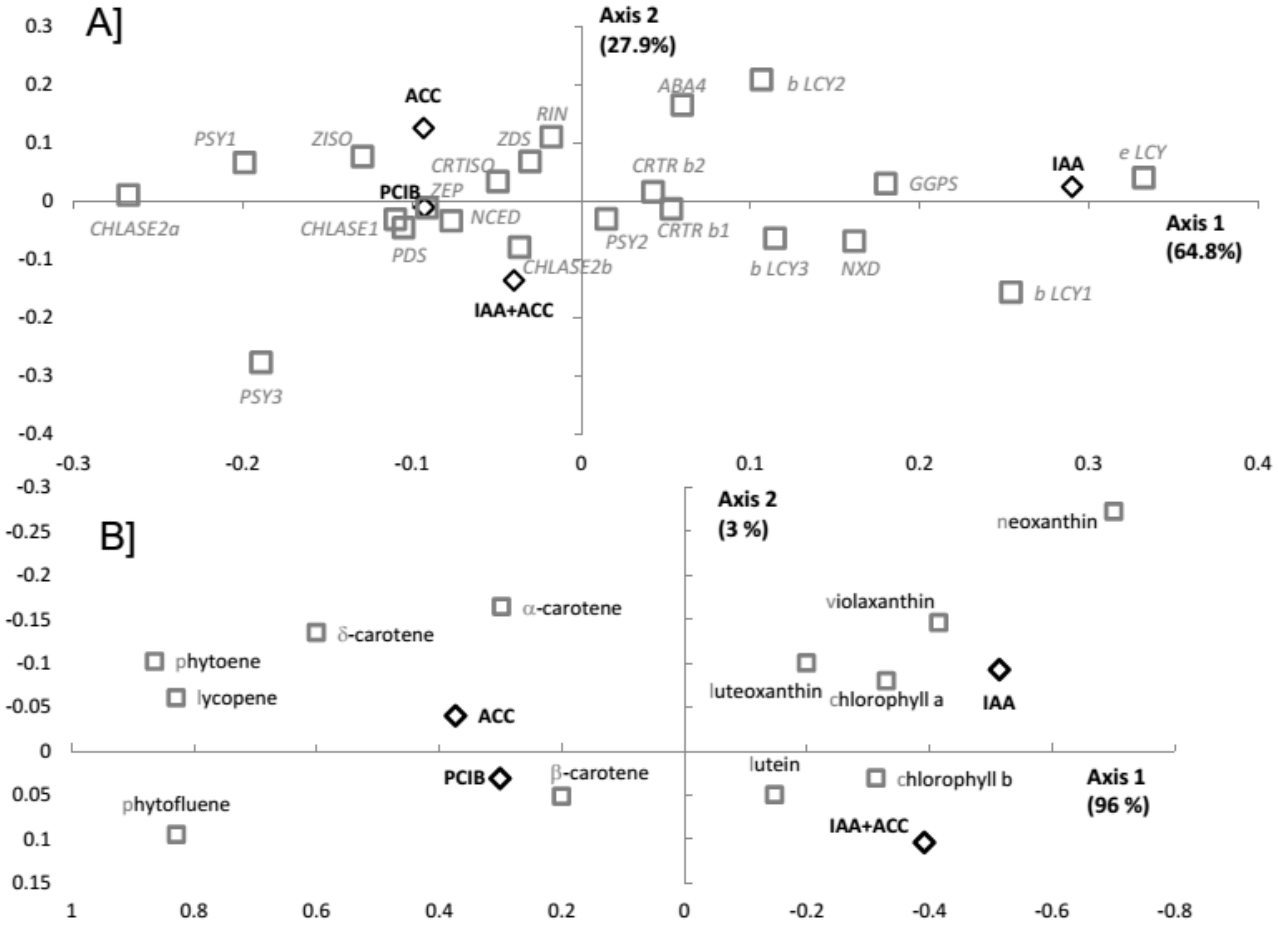

Fig. 15: Factorial correspondence analysis at 96 h of A): transcript accumulation and **B):** carotenoid content, modulated by hormonal treatments (abbreviations as in Fig. 7 and Fig. 8)

3.7 Role of PCIB

One question is: "How PCIB affects the color change?" We checked the production of ethylene after hormonal treatments (Fig. 11). PCIB did not change the ethylene production compared to controls. Oono et al. (2003) suggested that PCIB affects Aux/IAA stability, thus we checked it in tobacco protoplasts. We confirmed in our experimental conditions that IAA is decreasing Aux/IAA stability, as described previously (Gray et al., 2001; Dos Santos et al., 2009), as the fluorescent signal of the fusion proteins: Aux/IAAs-GFP or Aux/IAAs-YFP is decreased compared to control when IAA is added at 10µM (Fig. 16). This method can be convenient to check the stability or accumulation of several Aux/IAA proteins in plant cells.

However in our conditions the addition of PCIB concomitantly to IAA did not prevent the signal decrease, suggesting that PCIB does not prevent Aux/IAA degradation mediated by IAA.

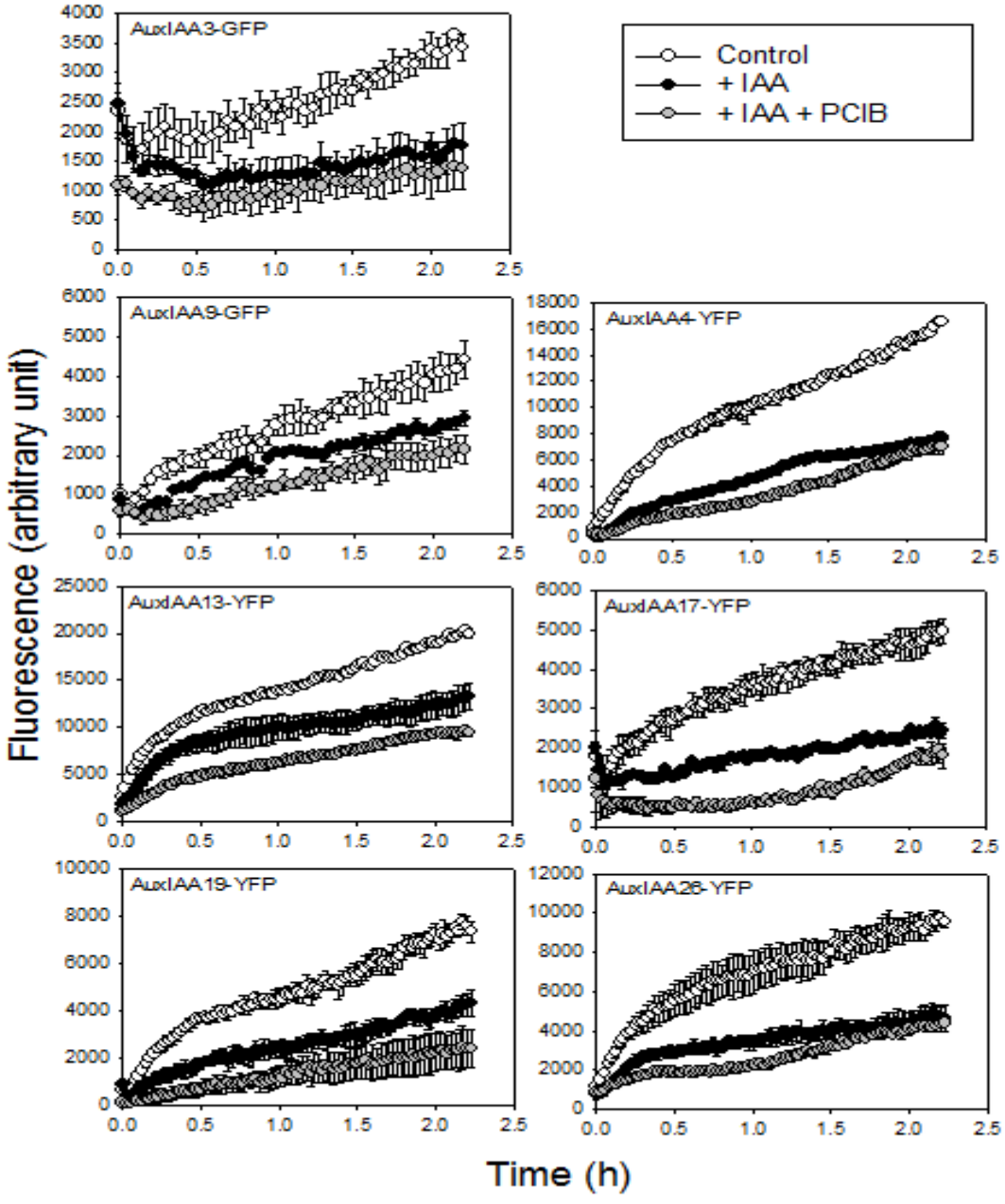

Fig. 16: Variations of fluorescent markers associated with the stability of Aux/IAA proteins transfected in tobacco protoplasts IAA stands for indole-3-acetic acid, PCIB stands for p-chlorophenoxy isobutyric acid; n=3 biological replicates.

4. Conclusion

Our results suggest that IAA inhibits lycopene accumulation by inhibiting the transcript accumulation of several genes upstream of this metabolite in the carotenoid pathway. At transcript level, IAA also activates lycopene β-cyclase1 and β-carotene hydroxylase 2, leading to a higher accumulation of neoxanthin and violaxanthin. The ABA content matches neoxanthin and, limited to IAA-treated fruits, violaxanthin contents.

Our results also suggest that ACC treatments favors lycopene and α-, β- and δ-carotene accumulation by increasing the transcript accumulation of several genes leading to their synthesis, and decreasing lycopene β-cyclase1 and β-carotene hydroxylase 2. We also showed that PCIB (an auxin antagonist) led to similar results than those obtained after ACC treatment, confirming the antagonism between ethylene and auxins, although our understanding of the PCIB role still needs further investigation. Indeed PCIB neither did not increased ethylene in comparison to controls, a result that would be consistent with anti-auxin effects.

Finally, we identified several homologs of *psy*, *lcy* and *β-crtr* genes that may be important in the fruit response to the auxin-ethylene crosstalk.

Chapter III: The auxin response *Sl-IAA17* transcriptional repressor controls fruit size via the regulation of the endoreduplication-related cell expansion

Chapter III: The auxin response *Sl-IAA17* transcriptional repressor controls fruit size via the regulation of the endoreduplication-related cell expansion

1. Introduction

Ethylene and auxin are two main hormones during the plant growth and development (Su et al., 2011; Muday et al., 2012). The crosstalk between these two hormones has been studied during the past decade, in organs such as roots (root elongation and root hair formation), and in seedling growth and development, but little refers to fruit ripening (Muday et al., 2012 and here in). Tomato as a model plant has been widely used for studying fruit development process (Giovannoni, 2001). In order to understand more interactions between ethylene and auxin during the fruit ripening, a preliminary experiment has been run to create a RNAseq data library in order to identify differentially expressed genes that related to fruit ripening 48 h the treatments that have been described in the previous chapter (Control, IAA, ACC, IAA+ACC). Based on these preliminary results (Mohamed Zouine, personal communication), we found that *Sl-IAA17* was decreased by ACC and increased by IAA (Fig. 17), while when treated the fruit with IAA+ACC, the expression of *Sl-IAA17* has the same trend with IAA alone, which means IAA is the main effector of the two hormones.

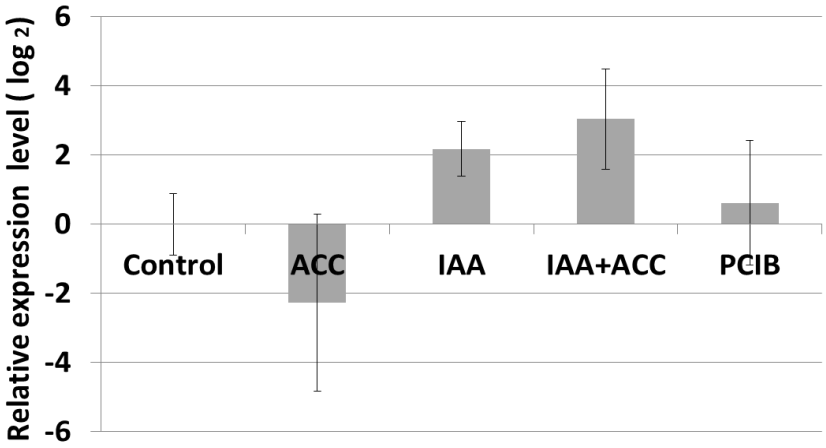

Fig. 17: The expression of *IAA17* in tomato fruit after treated by different hormones (Control, IAA, ACC, IAA+ACC) 48h based on the results of RNAseq. Error bars are standard errors. (Mohamed Zouine, unpublished results).

In *Arabidopsis*, Nakamura et al. (2006) have proved that *axr3* (*IAA17* homologous gene) is related to root elongation, adventitious root development, and leaf expansion, but there are no more studies about fruit development. In our laboratory, one of the main research objectives is Aux/IAA family members function study during tomato growth and development, and the phenotype of tomato fruit quality is one of the major targets. Actually, we have got numerous results such as down-regulation of *Sl-IAA27* affecting fruit size (Bassa et al., 2012), down-regulation of *Sl-IAA9* has an impact on fruit set and development (Wang et al., 2005).

Initially, a set of preliminary experiments showed that down-regulation of *Sl-IAA17* may affects fruit development. So, in order to get more information of the function of *Sl-IAA17* gene in tomato, we decided to do further studies about this Aux/IAA, which will be developed in the following chapter.

Auxin is largely involved in all the stages of fruit development (Pattison et al., 2014 and references herein). A recent study has highlighted some of its roles in controlling fruit size in

apple (Devoghalaere et al., 2012). In tomato, the final fruit size is largely influenced by cell expansion which itself is dependent upon endopolyploidy occurring via the endoreduplication process (Cheniclet et al., 2005). Indeed, modifying endoreduplication during fruit development greatly impacts fruit growth and final fruit size (Chevalier et al., 2014). In *Arabidopsis* tissues, the link between endopolyploidy and auxin has been established by Ishida et al. (2010) demonstrating that low levels of the auxin signalling complex lead to increased endopolyploidy. However, the link between tomato fruit size, endopolyploidy and auxin is yet to be shown.

The understanding of the molecular mechanisms of auxin metabolism and perception is now well advanced in the plant model *Arabidopsis*, as reviewed by Ljung (2013) and Bargmann and Estelle (2014). As far as signalling level is concerned, auxin promotes the degradation of Aux/IAA proteins by stimulating their interaction with the SCFTIR1 E3 ubiquitin ligase complex, where TIR1 acts as the auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The 26S proteasome degrades the ubiquitinated Aux/IAA transcriptional repressors, allowing auxin-responsive factors (ARFs) to regulate the expression of their target genes. In recent years, building on the achievement of the whole genome sequence (Sato et al., 2012), the complete set of Aux/IAA and ARF genes have been isolated in the tomato, a reference plant for both *Solanaceae* and fleshy fruit species, thus providing the ground for functional characterization and spatio-temporal expression studies of the two gene family members (Audran-Delalande et al., 2012; Zouine et al. 2014; Hao et al. 2014). Furthermore, the physiological significance of several Aux/IAA and ARF proteins has been addressed through reverse genetics approaches demonstrating their participation in the control of fruit development in tomato, such as *Sl-IAA9* (Wang et al., 2005), *Sl-IAA27* (Bassa et al., 2012), *Sl-ARF4* (Sagar et al., 2013 and references herein), *Sl-ARF7* (De Jong et al., 2009), and an ortholog of *At-ARF8* (Goetz et al., 2006).

This study aimed at unraveling the function of *Sl-IAA17* during tomato fruit development. The identification of gain-of-function mutations in the cluster of Aux/IAA genes closely

related to *IAA17* in *Arabidopsis* (*IAA16, IAA7/AXR2, IAA14/SLR and IAA17/AXR3*), suggested that these proteins play important roles in inhibiting auxin responses in a variety of tissues and developmental programs (Rinaldi et al., 2012). On the other hand, the suppression of a closely related *IAA17* gene in potato, *St-IAA2*, resulted in clear phenotypes including increased plant height, petiole hyponasty and curvature of growing leaf primordial in the shoot apex without affecting tuber formation (Kloosterman et al., 2006). To address the potential role of *IAA17* in fruit development, RNA interference (RNAi) transgenic lines were generated resulting in the down-regulation of the *Sl-IAA17* gene in tomato fruits. Fruit phenotyping, histological analyses of fruit tissues revealed the involvement of this Aux/IAA in the control of fruit size and ploidy levels.

2. Materials and Methods

2.1 Plant material and growth conditions

Tomato seeds (*Solanum lycopersicum* cv. MicroTom) were sterilized for 5 min in bleach, rinsed in sterile water and sown in recipient Magenta vessels containing 50 mL of 50% MS culture medium and 0.8% (w/v) agar, pH 5.9. Plants were grown in culture rooms as follows: 14 h/10 h day/night cycle; 25/20℃ day/night temperature; 80% relative humidity; 250 μM m-2 s-1 for light intensity. The number of fruits per plant was restricted to 12, i.e. 3 bunches of 4 fruit, left after fecundation, in order to limit the fruit size variability due to the variability in the number of fruit per plant.

2.2 Sequence data and analysis

Sequence data for the *Arabidopsis* genes used in this article can be found in the *Arabidopsis* Genome Initiative data library under the following accession numbers: *At-IAA17* (AT1G04250), *At-IAA7* (AT3G23050), *At-IAA14* (AT4G14550) and *At-IAA16* (AT3G04730).

Sequence data for the tomato genes used in this article can be found in Genbank/EMBL

data libraries under the following accession numbers: *Sl-IAA17* (JN379444), *Sl-IAA7* (JN379435), *Sl-IAA14* (JN379441) and *Sl-IAA16* (JN379443).

2.3. Transient expression using a single-cell system

For nuclear localization of the Sl-IAA17 protein, the *Sl-IAA17* open reading frame was cloned by Gateway technology (using the combination of 5'ATGAGTAGTAATAAGTTG3' forward and 5'GAACAGAAATGGATGA3' reverse primer) in-frame with YFP into the pEarlyGate104 vector, and expressed under the control of the 35S CaMV promoter. The empty vector pEarleyGate104 was used as a control. Protoplasts were obtained from suspension-cultured tobacco (Nicotiana tabacum) BY-2 cells, transfected according to the method described previously and YFP localization was monitored using confocal microscopy as described previously (Audran-Delalande et al. 2012).

2.4 Plant transformation

The forward primer P1 (5'ATGAGTAGTAATAAGTTG3') and reverse primer P2 (5'CCTTGGCAGCTGGGGTTTTGTGTG 3') were used to amplify a 196 bp-long *Sl-IAA17* sequence. This fragment was cloned into the pHellsgate12 vector using Gateway technology. RNAi transgenic plants were generated via Agrobacterium tumefaciens mediated transformation according to Wang et al. (2005). All experiments were carried out using homozygous lines from F3 or later generations.

2.5 RNA extraction and gene expression analysis qPCR

Total RNA was extracted using a Plant RNeasy Mini kit (Qiagen, http://www.qiagen.com) according to the manufacturer's instructions. Total RNA was treated by *DNase I* to remove any genomic DNA contamination. First-strand cDNA was reverse transcribed from 2 µg of total RNA using the Omniscript kit (Qiagen) according to the manufacturer's instructions. qPCR analyses was performed as described previously (Chervin and Deluc, 2010). Gene-specific primers were designed using the Primer Express 1.0 software (PE-Applied

Bio-systems, Foster, CA, USA). The sequences of primers used in this study are listed in Table 2.

Table 2: qPCR primer list

Gene Name	Accession No. in Genbank /EMBLdata libraries	Primer Sequence for Q-PCR
actin	Q96483	F 5'-TGTCCCTATCTACGAGGGTTATGC-3'
		R 5'-AGTTAAATCACGACCAGCAAGAT-3'
Sl-IAA17	JN379444	F 5'-CAAGAATTATTTGATGCCTTAACCAA-3'
		R 5'-ACTATTCAAAAGGTCCATCAGTTTCC-3'
Sl-IAA7	JN379435	F 5'-ACTCAACCTCCATCATAATGATAATATTCC-3'
		R 5'-ACCCCACCACTTGAGCCTTA-3'
Sl-IAA14	JN379441	F 5'-GTTTACGCATAATGAAAGGATCAGAAG-3'
		R 5'-TTATCTATGGAGCTTGCACACCA-3'
Sl-IAA16	JN379443	F 5'-TTGTGAAAGTTAGTGTTGACGG-3'
		R 5'-GAAATCCTTAAATCCTTGAGTCCC-3'

2.6 Tomato fruit phenotyping and pericarp thickness analyses

Eighty fruits out of 24 different plants were chosen for each line at the breaker + 7 days stage (about 45 days post anthesis, dpa), and assessed for various fruit quality traits such as weight, volume (assessed by measuring the water displacement in a small measuring cylinder after plunging the fruit), diameter (assessed with a Harpenden Skinfold Caliper), number of locules, number of seeds and water content (assessed by measuring weight loss of a fruit section after dessication for 3 days at 60°C). For pericarp thickness a different culture was used: 20 fruits at the mature green stage (35 dpa) of an average volume (as shown in Fig. 22) were selected for each tomato line. Fruits at the mature green stage have reached almost their final size, but pericarp cells are more rigid than in red ripe fruits, giving less variable readings. Vertical sections of each fruit were scanned; pericarp thickness measurements were performed at the equator of the fruit somewhere between the two black lines as shown on Fig. 24B, and the images were analyzed using the ImageJ software.

2.7 Cytological analyses

Ten fruits at the mature green stage for each line were selected. Thin pericarp slices (80 µm thick) were cut using a vibratome (Vibratome LEICA VT 1000S), stained with Congo red for 2 min at room temperature, then rinsed briefly in water immediately before observation. Images were acquired with confocal laser scanning microscopy (TCS SP2 AOBS; Leica instruments Heidelberg, Germany) using a ×10 dry-objective-lens (numerical aperture 0.30; PL FLUOTAR). Fluorescence emission spectra were acquired using the 561 nm of a laser diode and recorded in one of the confocal channels in the 569–662nm emission range. Images were acquired using Leica LCS software (version 2.61). For each fruit, two zones of the pericarp were analyzed around each black line, as shown in Fig. 24A. To assess the number of cell layers from the outer epidermis to the limit of the locule, a straight line was drawn in the middle of each image, as shown in Fig. 25A, and the number of cells intersecting this line was counted. The mean pericarp cell size was estimated using a method as follows. In each image, we counted the number of cells appearing in a rectangle, as shown on Fig. 25A (width x height = 1500 x 1220 µm), the top of which was parallel to the outer epidermis, but 280 µm below the first outer epidermis cell layer, in order to avoid counting the small cells which create lots of variability and error. The average cell area (in mm2) was calculated from the ratio 1.5 x 1.22 / nb of cells.

2.8 Ploidy analyses

Nuclei were prepared from pericarp tissues of 5 fruits at the mature green stage of an average weight for the Wild-type line and three *Sl-IAA17* down-regulation lines (Rlin1, Rline2 and Rline3). The pericarp tissues (0.1 - 0.2g in fresh weight) were chopped with a razor blade in 0.5 mL of the Partec suspension solution, then 0.7 mL of the Cystain UV ploidy solution (Partec) was added. The suspension was filtered through a 100 µm nylon mesh. The combined filtrates were analyzed using the CyFlow® Space flow cytometer from Partec. Ploidy histograms were quantitatively analyzed using the DPAC software (Partec), after manual treatment to exclude noise. The mean ploidy level of each pericarp tissue was calculated as the sum of each C value class weighed by its frequency. The Endoreduplication

Index (EI) was calculated according to Barow and Meister (2003).

3. Results

3.1 Sl-IAA17, a canonical Aux/IAA protein, is exclusively localized within the nucleus

Sl-IAA17 has been identified previously as a member of the tomato Aux/IAA gene family which encompasses 25 members in tomato (Audran-Delalande et al., 2012). The phylogenetic analysis between *Arabidopsis* and tomato Aux/IAAs showed that *Sl-IAA17* and its closest tomato homologs *Sl-IAA7, Sl-IAA14, Sl-IAA16*, together with their closest *Arabidopsis* homologs *At-IAA17, At-IAA7, At-IAA14* and *At-IAA16* belong to a distinct clade, named clade C (Audran-Delalande et al., 2012). The *Sl-IAA17* open reading frame is 627 bp long encoding a putative protein of 209 amino acids. Sl-IAA17 harbors the four conserved amino acid sequence motifs known as domains I, II, III and IV found in Aux/IAA proteins (Fig. 18). As a common characteristics of clade C members, Sl-IAA17 contains a second putative repressor domain (DLxLxL) in the close vicinity of the repression domain I. Alike the majority of Aux/IAA proteins, Sl-IAA17 displays two conserved nuclear localization signal domains (NLSs): a bipartite structure of a conserved basic doublet KR between domains I and II associated with basic amino acids in domain II, and the SV40-type NLS located in domain IV (Fig. 18).

To analyze its subcellular localization, a translational fusion between Sl-IAA17 and the yellow fluorescent protein (YFP) under the control of the 35S promoter of Cauliflower mosaic virus (CaMV) was used to transfect tobacco protoplasts. Microscopy analysis showed that the Sl-IAA17-YFP fusion was exclusively localized in the nucleus, in contrast to control protoplasts transformed with YFP alone which displayed fluorescence throughout the cell (Fig. 19).

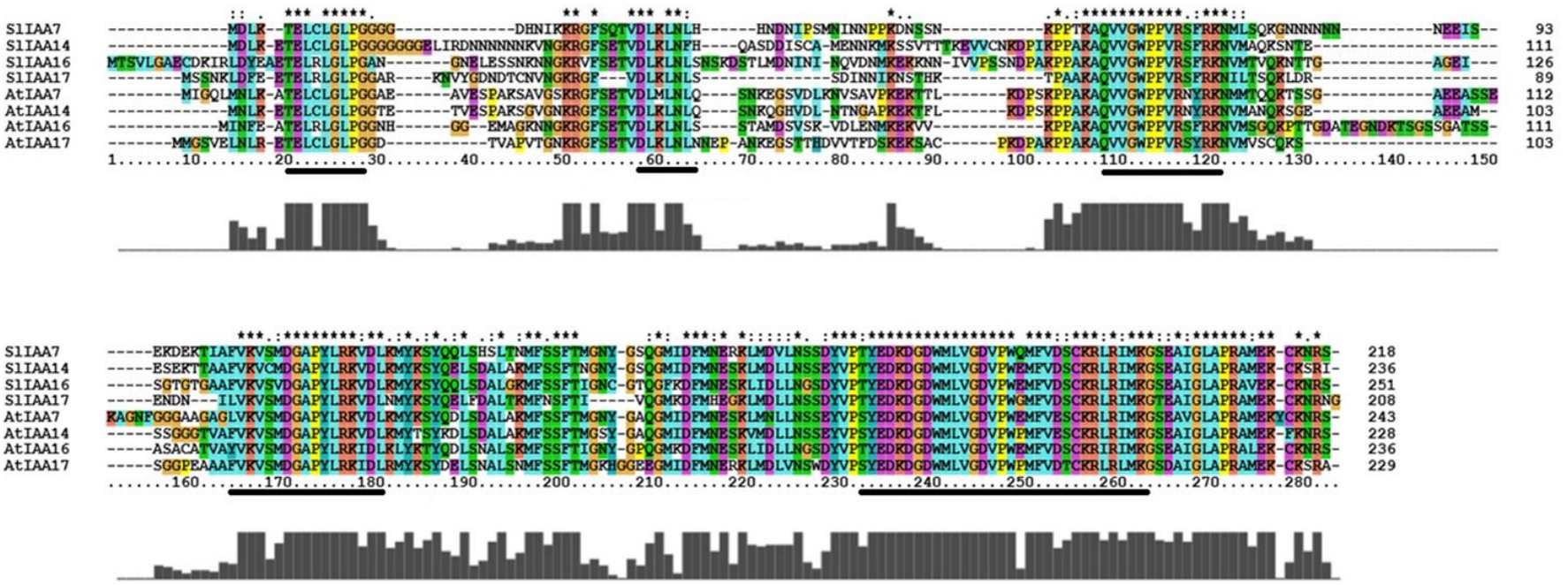

Fig. 18: Multiple sequence alignment of clade C Sl-IAA and At-IAA proteins obtained with ClustalX2. Conserved amino acid domains are underlined and NLSs are indicated by red dots. The amino acid position is given on the right of each sequence.

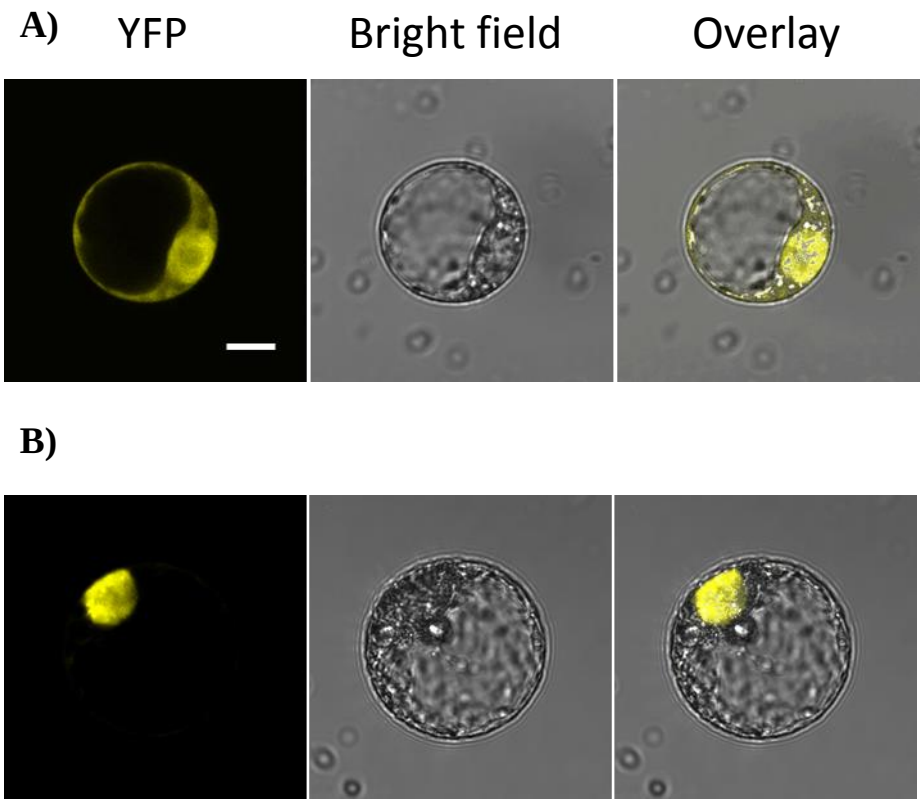

Fig. 19: Subcellular localization of Sl-IAA17 proteins A): Control cells expressing YFP alone. **B):** Cells expressing the Sl-IAA17-YFP fusion protein. The scale bar indicates 10 µm.

3.2 *Sl-IAA17* shows high transcript accumulation during tomato fruit development

The analysis of *Sl-IAA17* transcript levels in vegetative and reproductive organs indicated that the expression of *Sl-IAA17* was identical in leaf and flower (Fig. 20). By contrast, the accumulation of *Sl-IAA17* transcripts undergo up to a 10-fold increase in developing fruit when compared to the level in flower. This dramatic increase in expression starts as early at 10 day post-anthesis (dpa) corresponding to a developmental stage where cell division activities stopped to give way to cell expansion which accounts for fruit growth until ripening (Gillaspy et al., 1993; Joubès et al., 1999). Then, the level of *Sl-IAA17* mRNA declined gradually up to the breaker stage (45 dpa), when the fruit undergoes the color change.

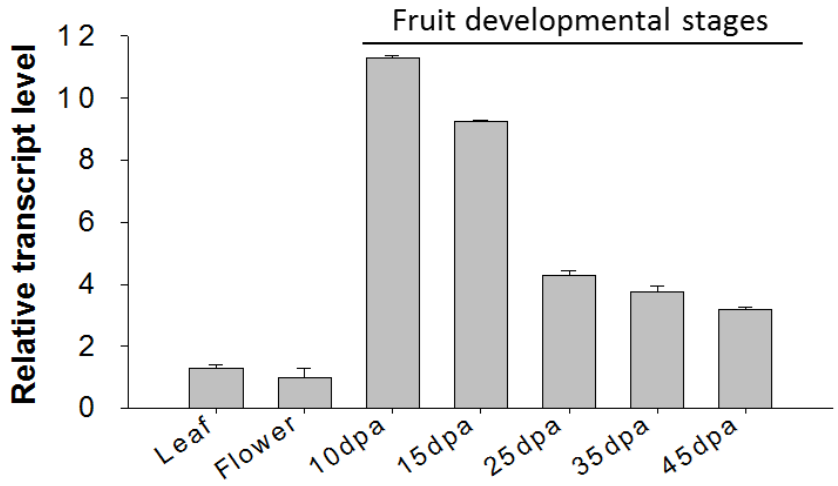

Fig. 20: Profiling of *Sl-IAA17* transcript accumulation in wild-type tomato plant tissues monitored by qPCR. The level for Flower was used as a reference. "F": flower; "R": root; "L": leaf; "dpa": days post anthesis. The data are mean values corresponding to three independent experiments. Error bars are standard errors.

3.3 The silencing of *Sl-IAA17* increases tomato fruit size

To gain better insight on the function of Sl-IAA17 in tomato fruit development, we generated RNA interference (RNAi) plants and obtained several independent homozygous lines. Three *Sl-IAA17* RNAi lines, named Rline1, Rline2 and Rline3, were selected for further studies. The analysis of gene expression by qPCR showed that the accumulation of *Sl-IAA17* transcripts was strongly reduced in the three RNAi lines when compared to wild type plants (Fig. 21A). Rline 2 and Rline 3 retained 25% of the control mRNA level whereas Rline 1 showed only 9% of the mRNA level displayed in the wild type. To check whether the inhibition by RNAi was specific to *Sl-IAA17*, we assessed the mRNA accumulation of three Aux/IAA members, namely *Sl-IAA7, Sl-IAA14*, and *Sl-IAA16*, belonging to the same clade as *Sl-IAA17*. Figure 21B shows that the RNAi construct did not significantly reduce the mRNA accumulation of these three close homologous genes, even though a slight increase in *Sl-IAA7* transcripts was noticeable in two lines (Rline1 and Rline3) without any statistical significance.

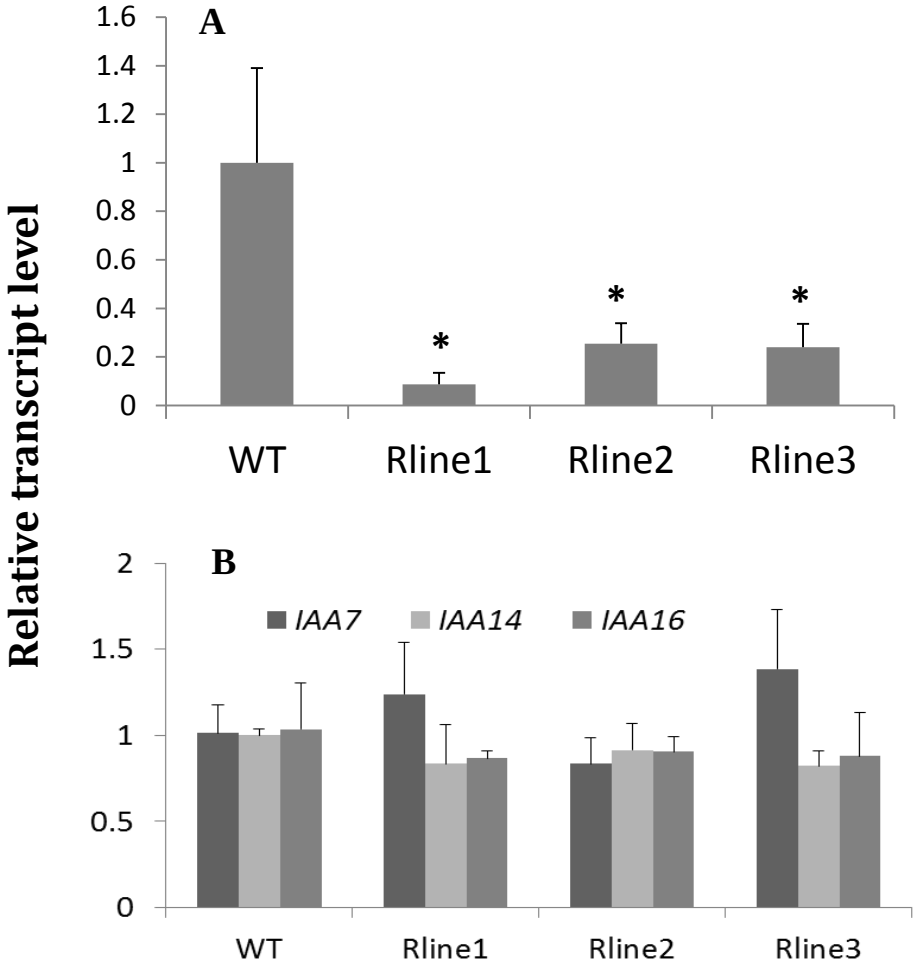

Fig. 21: Characterization of three independent *Sl-IAA17* RNAi lines A): Transcript levels of *Sl-IAA17* in fruit of three independent *Sl-IAA17* RNAi lines (Rline1, Rline2, Rline3) relative to WT, at the 35 dpa. Statistical analyses were performed using the t-test comparing WT with each line, *P <0.05. **B):** Transcript levels for *Sl-IAA7, Sl-IAA14* and *Sl-IAA16* in three independent *Sl-IAA17* RNAi lines (Rline1, Rline2 and Rline3) were assessed in 35 dpa fruit. All the data are mean values corresponding to three independent experiments. Error bars are standard errors.

The effects of Sl-IAA17 silencing on fruit development were then investigated in the three *Sl-IAA17* RNAi lines. A large screening was performed at a late stage of fruit development, namely breaker +7 days, aiming to assess fruit weight, fruit volume, fruit diameter, water content, the number of locules, the number of seeds, and other biochemical

parameters. Fruits from *Sl-IAA17* RNAi plants (Rline1, Rline2 and Rline3) displayed a larger size compared to control fruits, weighing up to 19% more than wild type fruit (Fig. 22A). This increase was even more noticeable when measuring the fruit volume (Fig. 22B), with RNAi fruit volumes reaching up to 18% more than the wild type. This fruit size enlargement was also observed at the fruit diameter level (Fig. 22C) with RNAi fruit displaying up to a 7% average increase in diameter when compared to wild type. It is noteworthy that the severety of the fruit phenotypic modifications are well correlated with the level of *Sl-IAA17* silencing (Fig. 21A), with Rline1 displaying indeed the most pronounced effects.

In all tomato RNAi lines tested, the down-regulation of *Sl-IAA17* had no significant effect on the water content (Fig. 23A). Finally, the locule number and seed number per fruit (Fig. 23B and 23C) were similar to that in wild type, ruling out a possible impact of these tissues on the observed differences in fruit size.

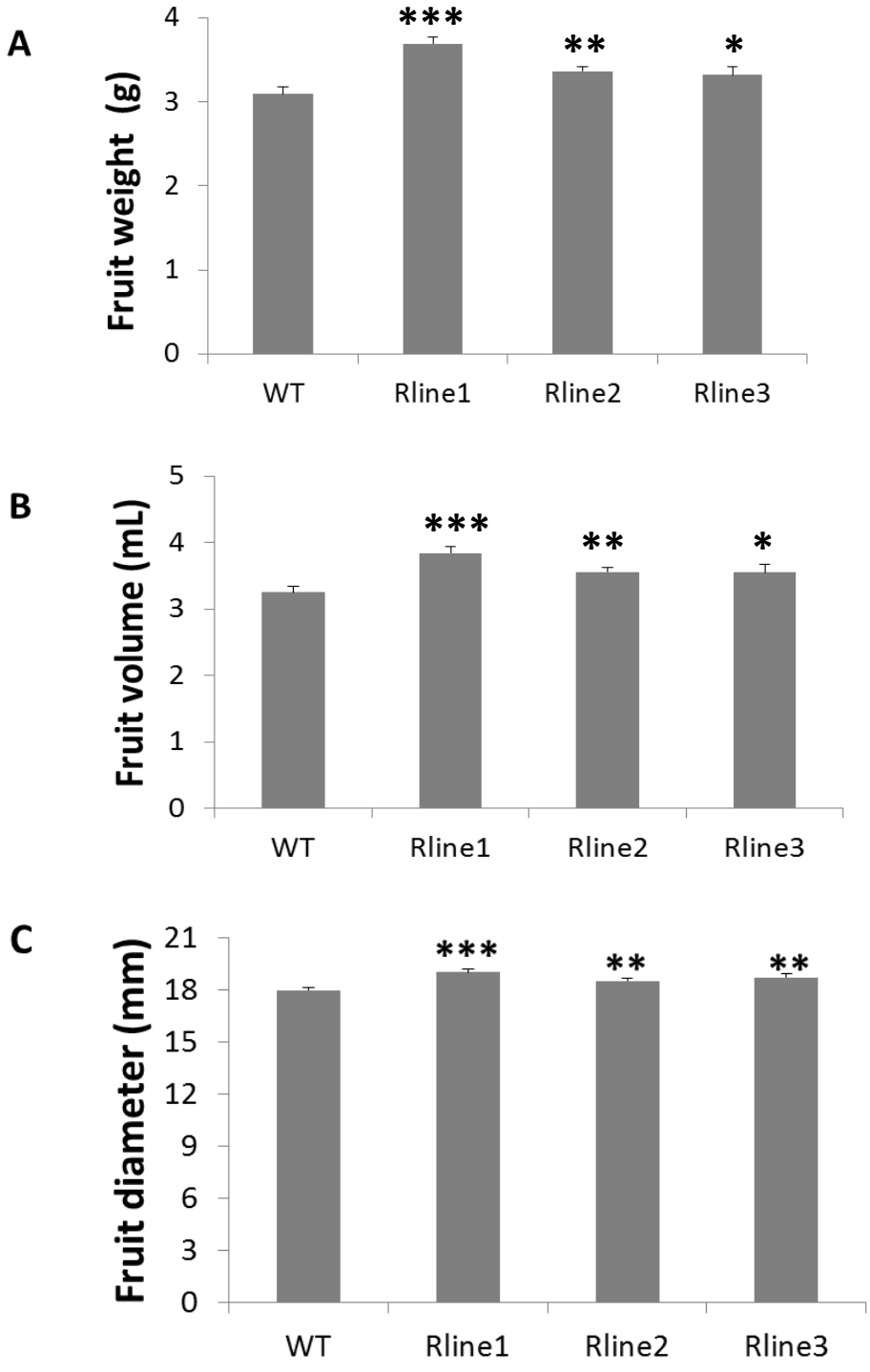

Fig. 22: Alteration of A): fruit weight, B): fruit volume and C): fruit diameter in *Sl-IAA17* RNAi plants (three independent lines: Rline1, Rline2, Rline3) determined at Br+7 stage (breaker + 7 dpa).The data represent mean values obtained from 50 to 80 fruits of each line. Statistical analyses were performed using the t-test comparing WT with each line, ***P <0.001, **P <0.01, *P <0.05. Error bars are standard errors.

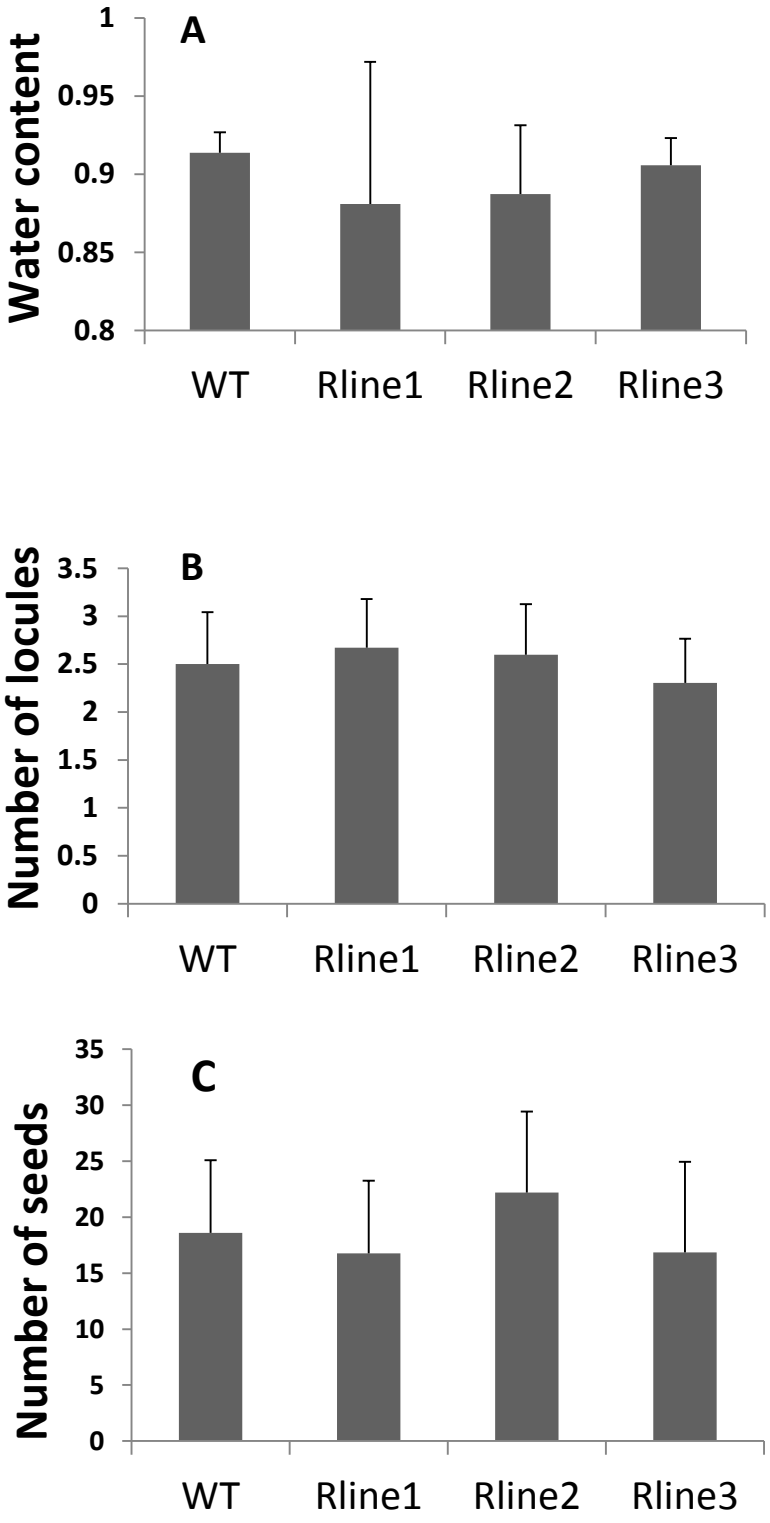

Fig. 23: A): Fruit water content (1=100%). B): Number of seed locules. C): Number of seeds per fruit. n = 80, error bars are standard errors.

Since the observed differences in fruit size are likely to originate from differences in tissue growth during the fruit development, the pericarp and locule thickness (Fig. 24A) were measured to further investigate the factors underlying the increase in fruit size and weight encountered in *Sl-IAA17* down-regulated lines. Fruits from the three *Sl-IAA17* RNAi lines showed thicker pericarp tissues than the wild type (Fig. 24B). This difference reached up to 28% in the Rline1, where the *Sl-IAA17* transcript accumulation was the most reduced (Fig. 21A). However there was no significant difference in the locule thickness between all the three lines (data not shown).

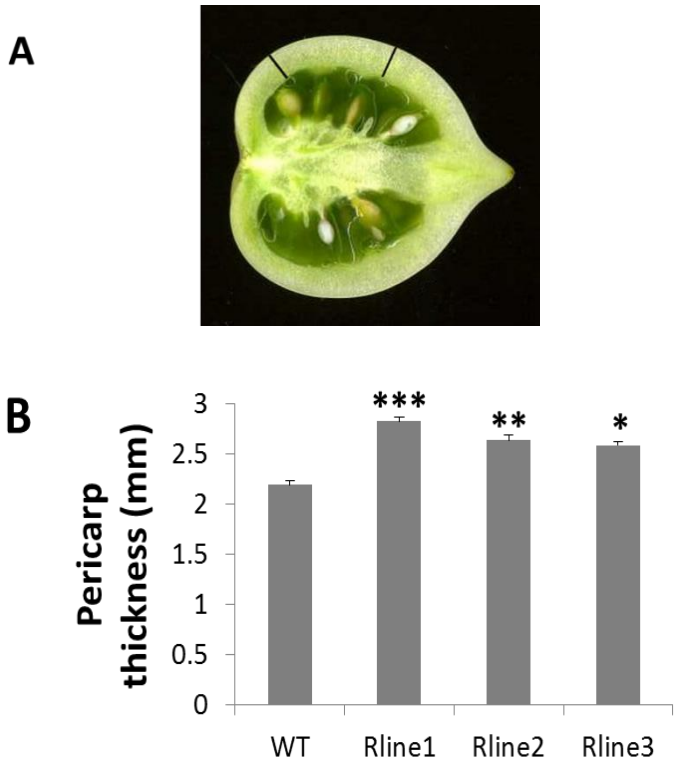

Fig. 24: A): The pericarp thickness was measured at the equator of the fruit between the two black lines, these latter were the spots where the microscopic images were taken, as shown in Fig. 25A and 25B and between which the tissues were extracted for ploidy analyses, as shown in Fig. 24. **B): Effect of RNAi on the fruit pericarp thickness**; the data represent mean values obtained from 20 fruits at 35 dpa stage. Statistical analyses were performed using the t-test comparing WT with each line, *** P <0.001, **P <0.01, *P <0.05. Error bars are standard errors.

3.4 Down regulated*Sl-IAA17* increased the pericarp cell size and nuclear ploidy levels

In order to analyze the pericarp at the cellular level, microscopic observations were performed showing that the cells in the pericarp of the RNAi lines were much larger than that in the wild type (Fig. 25A and B).

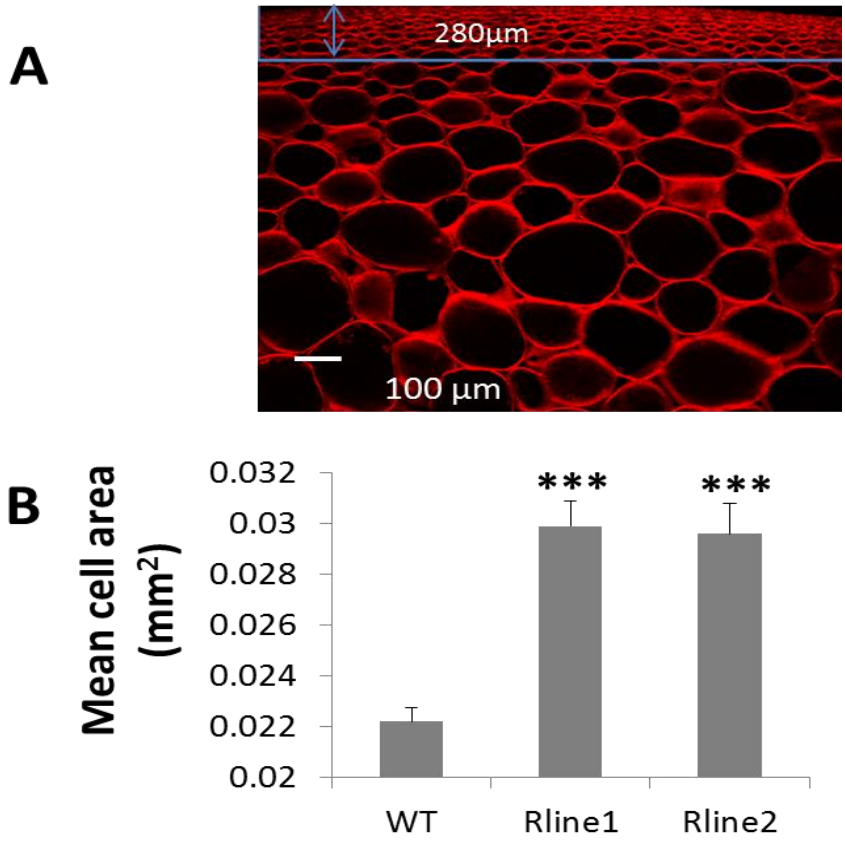

Fig. 25: A): Confocal image of the pericarp tissue used to measure mean cell area. To take into account the variation of cell size from the skin to the seed locule, the cell number was counted on the same rectangle (represented here), 280 µm away from the first epicuticular cell layer. **B): Effect of RNAi on the tomato pericarp cell mean area.** The data represent mean values obtained from 10 fruit at the 35 dpa stage, two measures per fruit, tissue taken at two different spots on the pericarp, around the black lines shown in Fig. 24A. Statistical analyses were performed using the t-test comparing WT with each line, *** P <0.001, **P <0.01, *P <0.05. Error bars are standard errors.

This increase in mean cell size reached up to a 36% difference. In the same set of microscopic observations, we did not notice any significant differences in the number of pericarp cell layers using fruits either at the immature green stage (25 dpa) or the mature green stage (35 dpa) (Fig. 26). Hence the difference in pericarp thickness observed in the RNAi lines is due to enhanced cell size rather than increased cell number.

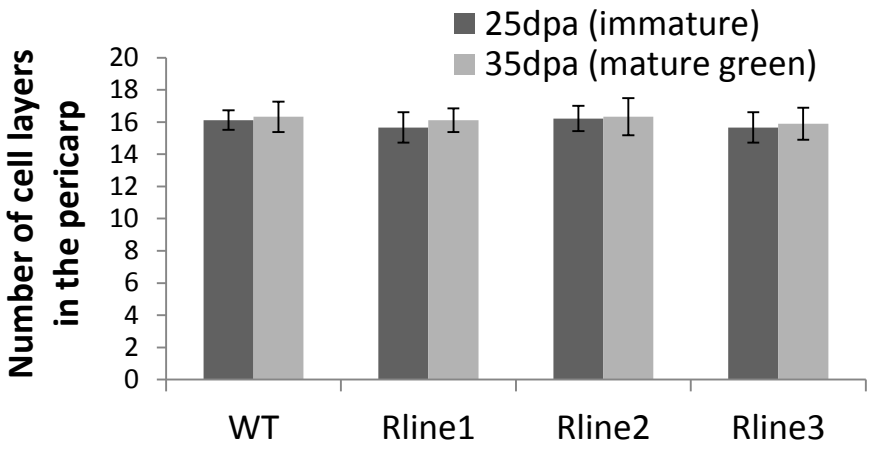

Fig. 26: Average number of cell layers in the pericarp (at 20 dpa and 35 dpa), n = 10, error bars are standard errors.

Since a correlation exist between cell size and the nuclear DNA ploidy level resulting from endoreduplication in tomato fruit (Cheniclet et al. 2005; Chevalier et al., 2011; Bourdon et al., 2012; Chevalier et al. 2014), we checked whether such a relationship existed between the increase in pericarp cell size in the RNAi *Sl-IAA17* transgenic fruit and the endoreduplication level of these cells. The nuclear DNA content (ploidy level) of pericarp cells from wild-type and the three independent *Sl-IAA17* RNAi fruits, harvested at 35 dpa, was determined by flow cytometry (Fig. 27).The analysis revealed that the nuclear DNA ploidy level of the pericarp cells from the three transgenic lines increased significantly: the mean ploidy level of the transgenic fruits was about 20% higher than that in wild-type fruit (Fig. 27A). The Endoreduplication Index (EI) was significantly increased in Rline1 (2.42),

Rline2 (2.47) and Rline3 (2.33) when compared to that in wild type (2.19). Furthermore, the nuclear DNA ploidy distribution in the three RNAi lines showed significant increases mostly for the 32C and 64C peaks and also in some cases for the 128C peak compared to the wild type, suggesting the promotion of successive endocycles in transgenic *Sl-IAA17* RNAi lines (Fig. 27B). For the most affected lines, Rline1 and Rline2, there was a signifiacnt decrease for the 2C and 4C DNA levels compared to the wild-type. These data show that the cell expansion in the down-regulated *Sl-IAA17* transgenic fruits is tightly coupled to the level of endoreduplication and suggest that Sl-IAA17 could be involved in the control of endoreduplication in tomato fruits.

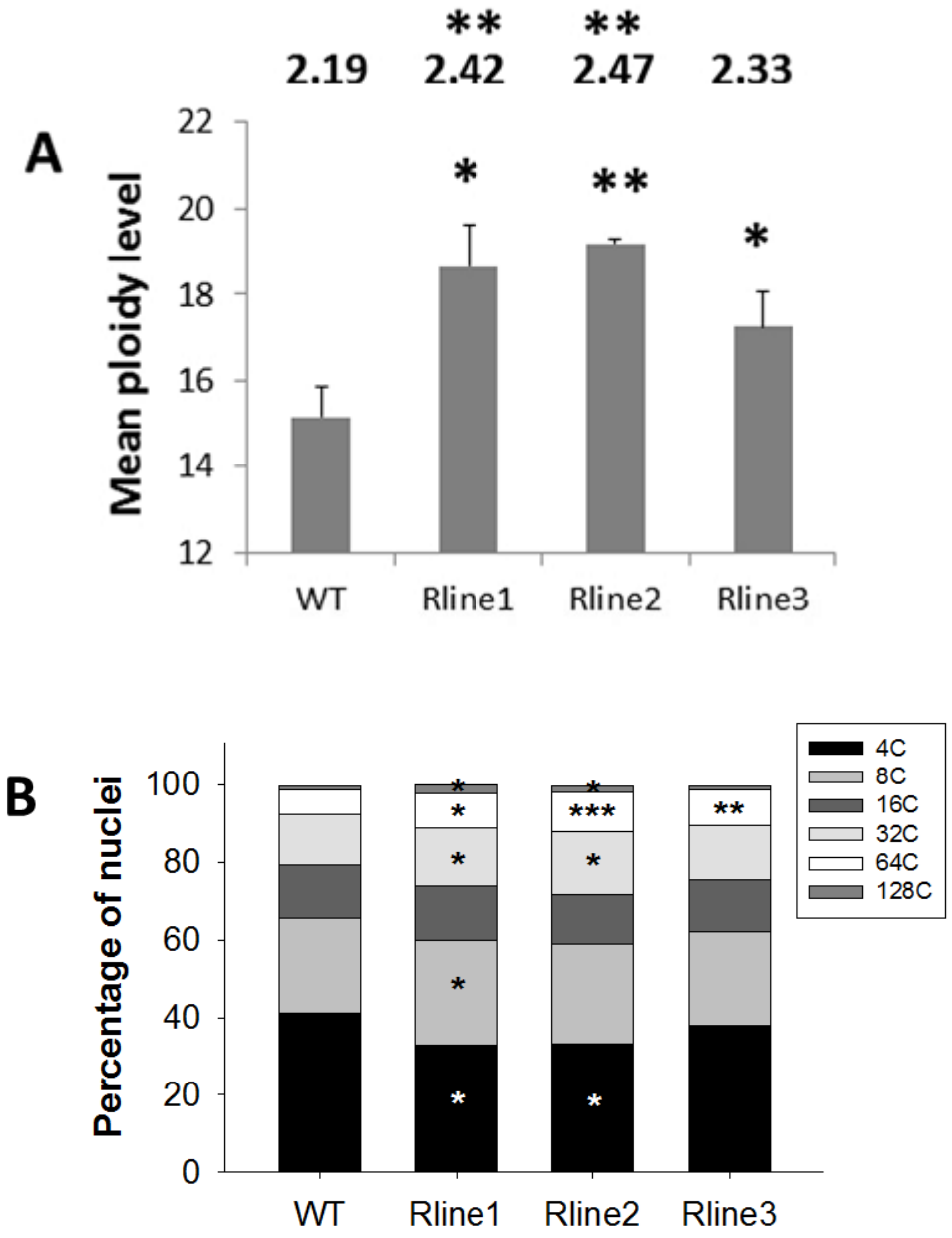

Fig. 27: Altered ploidy level in fruit pericarp cells of *Sl-IAA17* RNAi tomato lines. **A)**: Effect of *Sl-IAA17* RNAi transformation on the mean ploidy level in tomato fruit at the 35 dpa stage. E.I stands for Endoreduplication Index. **B)**: Proportion of each ploidy level of the wild-type (WT) and three RNAi lines. Ploidy has been analyzed by flow cytometry in 5 fruit pericarp of each line, tissue taken within the two black lines as shown in Fig. 24A. Statistical analyses were performed using the t-test comparing WT with each line, ***P <0.001, **P <0.01, *P <0.05. Error bars are standard errors.

4. Discussion

After successful flower pollination and ovule fertilization, the fruit and seed initiation (during the so-called fruit set) and subsequent development occur concomitantly according to a tightly genetically controlled process operated by phytohormones (Gillaspy et al. 1993). In the early fruit developmental stages, plant hormones exert a direct control on the cell division and cell expansion processes that determine respectively the cell number and cell size inside tomato fruit (Ariizumi et al., 2013; Gillaspy et al., 1993; Pattison et al., 2014; Ruan et al., 2012). As a result, the combination of cell number and cell size drives fruit growth, and finally influence the final fruit size.

In the present study, we describe a functional analysis of *Sl-IAA17* encoding a member of the tomato Aux/IAA gene family. At the level of its primary sequence, Sl-IAA17 displays all the characteristics of an Aux/IAA transcriptional repressor, in particular the presence of the canonical repressor domain I (Tiwari et al., 2004) and a putative second repressor domain of the DLxLxL type. The presence of these domains is in agreement with our previous demonstration that the Sl-IAA17 protein does function as an active repressor of auxin-dependent gene transcription (Audran-Delalande et al., 2012). In addition, the exclusive subcellular localization of Sl-IAA17 within the nucleus (Fig. 19) is fully consistent with a transcriptional regulatory function.

The expression profile of *Sl-IAA17* revealed a preferential accumulation of transcripts in the early developing fruit, with a maximum of expression at 10 dpa (Fig. 20). Interestingly, mining RNaseq data available for the development of tomato in the Heinz cultivar revealed also a strong up-regulation of *Sl-IAA17* after pollinisation within the developing fruits, and then a decline of expression at the mature green stage (http://ted.bti.cornell.edu/). These observations thus suggest a putative role for Sl-IAA17 in the early fruit development, as previously observed for other AUX/IAA genes (Wang et al., 2005; Wang et al., 2009; Bassa et al., 2012). Classically, a bimodal pattern of auxin flux during tomato fruit development is described in the literature (Gillaspy et al., 1993; Srivastava and Handa, 2005): a first peak in

activity occurs at about 10 days after anthesis and then a second one at about 30 days after anthesis in developing tomato fruits, which then suggests that auxin controls the initiation of the cell expansion phase (phase III) and initiation of the ripening process and final embryo development phase (phase IV), respectively corresponding to these two developmental timepoints. Remarkably, the peak of *Sl-IAA17* expression coincides with the first peak of auxin concentration at 10 dpa, associated to the promotion of cell elongation and thus accelerated fruit expansion (Pattison et al., 2014).

Down-regulating *Sl-IAA17* in tomato transgenic plants resulted in an increased fruit size (Fig. 22). The phenotypic analysis of fruits from the three generated RNAi lines revealed that this increase in fruit size was associated to a thicker pericarp, resulting from an enhanced cell expansion and not from a higher number of cells (Fig. 24 and Fig. 26). The effect on fruit growth is likely to be specific of the down-regulation of *Sl-IAA17*, since the expression of other Aux/IAA genes belonging to the same supposed functional clade is unaffected in the three RNAi lines (Fig. 21). In addition the effect on fruit size is only related to modifications within the pericarp and did not originate from a higher number of locules or seeds (Fig. 23). It is known that the number of locules greatly influences the final fruit size (Tanksley 2004), and that seeds promote fruit expansion through producing or delivering auxins to the surrounding tissues (Ariizumi et al., 2013, and references herein).

In various plant cell types, a correlation has often been found between cell size and the nuclear DNA ploidy level resulting from the endoreduplication process (Joubés and Chevalier 2000; Sugimoto-Shirasu and Roberts, 2003; Inze and De Veylder, 2006). Hence we could clearly demonstrate that down-regulating *Sl-IAA17* in tomato transgenic plants promotes endoreduplication inside the pericarp cells which enhances cell expansion (Fig. 27), thus resulting in the increase of fruit size. In tomato, endoreduplication plays a functional role in regulating the karyoplasmic homeostasy during fruit development (Chevalier et al., 2011; Bourdon et al., 2012; Chevalier et al. 2014), and fruit size is largely dependent upon the endoreduplication-associated cell expansion inside the pericarp tissue (Cheniclet et al., 2005).

Not only these data are in full agreement with the influence of endoreduplication on cell expansion and consequently on fruit growth/size, but they uncover an active role for auxin signalling in the transition from mitotic cycle to endocycle in tomato fruit cells. It has been reported that auxin modulates the switch from mitotic cycles to endocycle in the root meristem in *Arabidopsis* (Ishida et al., 2010). Furthermore, it was suggested that the mitotic–to-endocycle transition is mediated by the TIR1-AUX/IAA-ARF-dependent auxin signalling pathway. However, in contrast to the situation in *Arabidopsis* root meristem tissues where the ploidy distribution was significantly increased as a result of gain-of-function mutations in *IAA7/AXR2* and *IAA17/AXR3* genes, the herewith reported effect of *Sl-IAA17* down-regulation seems to lead to higher nuclear DNA ploidy level in tomato fruits. These seemingly contradictory data may be resolved by the different nature of the tissues and development processes involved, namely the fruit organ in tomato and the root tissue in *Arabidopsis*. Moreover, since ARF proteins, the natural partners of Aux/IAAs, can act either as repressors or activators of gene transcription, it cannot be ruled out that IAA17 may interact with different ARFs in the different tissues, leading to transcriptional repression of target genes in one case and to transcriptional activation in the other case.

In conclusion, our data suggest that the promotion of endoreduplication in the tomato fruit cells is under the control of auxin signalling pathway. The *Sl-IAA17* RNAi fruit series generated in this study offer a powerful tool to unravel better the mechanisms by which auxin signalling regulates the endoreduplication process. Bearing in mind that the endocycle is under the control of several key components (Chevalier et al., 2011), further studies are now required to decipher the network of genes and associated signalling pathways involved in this regulation.

Chapter IV: Roles of *Sl-IAA17* on the morphology and biochemical characteristics of tomato fruits

Chapter IV: Roles of *Sl-IAA17* on the morphology and biochemical characteristics of tomato fruits (Additional results to chapter III)

1. Introduction

In the chapter III, we present that down regulated *Sl-IAA17* affected the fruit size via regulating the endoreduplication-related cell expansion. Actually, in order to know more details about the fruit morphology, the fruit shapes and some physico-chemical traits have been assessed by using the same set of fruit material as in chapter III.

More results of *Sl-IAA17* RNAi lines fruit will be shown here, such as ethylene production, color measuring, soluble solids content (SSC), water content, fruit firmness, and more details about fruit shape. Additionally, we have seen that the *Sl-IAA17* expression could be decreased by ethylene (shown previously in chapter III). In order to check whetherSl-IAA17 is affecting the ethylene response in seedlings, an ethylene treatment was carried out with *Sl-IAA17* RNAi lines seedling, and the seedling shape was observed. Indeed, ethylene is known to enhance apical hook curvature in dark-grown seeding leading cotyledons to form a 270° bend relative to the hypocotyls (Ecker, 1995). This is the classical phenotype of ethylene triple response (Bleecker and Kende 2000).

2. Materials and Methods

2.1 Analysis of ethylene production in *Sl-IAA17* RNAi lines

The fruits used for experiment were harvested at the mature green stage and placed in open 100-ml jars for 2 h to minimize the effects of wound ethylene which produced after picking. Then the fruit was incubated for 1h in the 100ml closed jars at room temperate, and 1

ml of headspace gas was injected into an Agilent 7820A gas chromatograph equipped with a flame ionization detector. Samples were compared with ethylene standards and normalized for fruit weight. 10 fruits of each line were used for experiments.

2.2 Ethylene treatment

Seeds were sown on plates with MS medium and grown in dark 4-5days. 40 seeds used for analysis of each line, including *Sl-IAA17* RNAi lines and wild type, 20 seeds of each line treated by 10 ppm of ethylene16 h and 20 seeds were let it grown in normal conditions

2.3 Analysis the phonotypes of *Sl-IAA17* RNAi lines fruit

The fruit used for analysis were in Breaker+7 days stage. Soluble solids content (SSC) was assessed with a hand refractometer, water content was calculated based on weight loss of fruit quarters after 3 days at 60°C: ((initial weight - final weight) / initial weight), fruit firmness was assessed using Harpenden calipers (British Indicators Ltd) as described by Ecarnot et al. (2013), pH of the fruit juice was assessed with a micro pH probe (Hanna instruments HI8521) on fruit juice, vertical section analysis was assessed by Tomato Analyzer 3.0 using the same method described previously by Marin Talbot Brewer et al. (2006).

3. Results and discussion

3.1 Ethylene production and response

We checked the alteration of ethylene production after down regulated *Sl-IAA17*from Breaker to Breaker+8 days (Fig. 28), found that there is no significant difference between Rlines and wild type fruits at the same stage of fruit ripening.

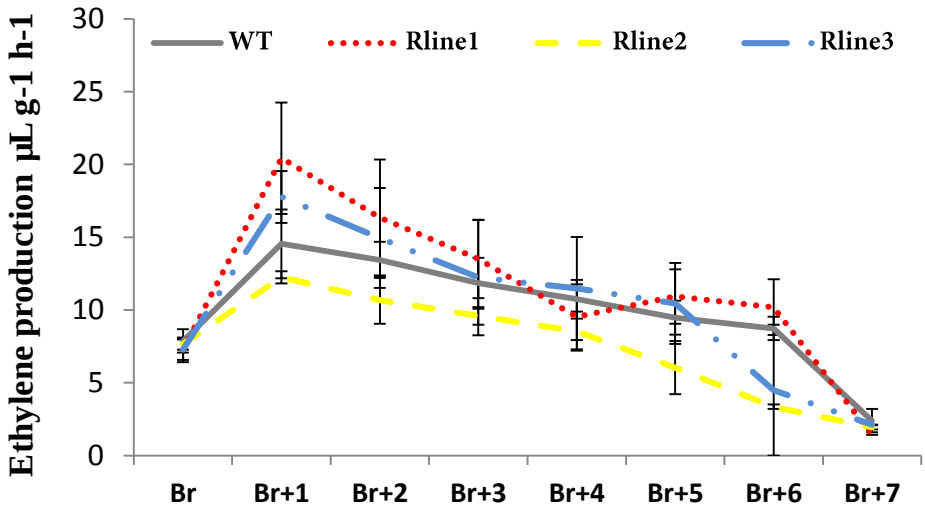

Fig. 28: Ethylene production of *Sl-IAA17* RNAi lines and in wild type fruit during ripening: Breaker (Br), Br+1, Br+2, Br+3, Br+4, Br+5, Br+6, Br+7. Error bars are standard errors.

3.2 Phenotypes of *Sl-IAA17* RNAi lines fruit

Soluble solids content (SSC), water content, fruit firmness, acid content were assessed for identify the fruit quality. The results showed that the soluble solids content was increased in the *Sl-IAA17* RNAi lines (Rline1 and Rline2) compared to wild type fruit (Fig. 29). 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, 1965; Dinar and Stevens, 1981).Sugar accumulation in tomato fruit is the consequence of various linked physiological processes (Bouzayen et al., 2010).Our results matched previous results showing that affecting the auxin signalling pathway may lead to higher SSC than WT (Sagar et al., 2013). The link between *Sl-IAA17* and sugar metabolism could be an interesting point for further study. There were no significant difference fruit firmness (Fig. 30A), and pH value (Fig. 30B).

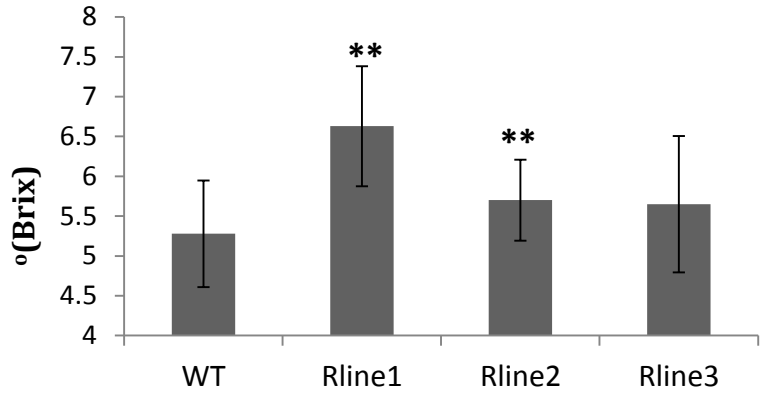

Fig. 29: The detection of *IAA17* RNAi lines and wild type fruit soluble solids content; Statistical analyses were performed using the t-test comparing WT with each line, **P <0.01. Error bars are standard errors.

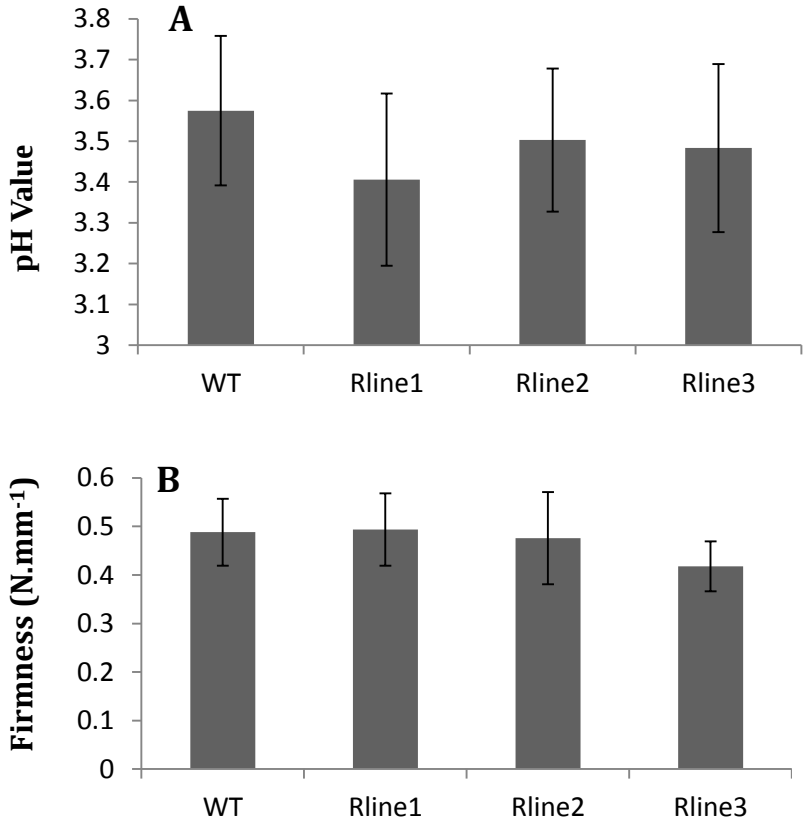

Fig. 30: The detection of *Sl-IAA17* RNAi lines and wild type fruit quality A) pH value. B) Fruit firmness. Statistical analyses were performed using the t-test comparing WT with each line, Error bars are standard errors.

The shape of tomato fruit is a key element of fruit development (Brewer et al., 2006). We were interested in identifying the alterations of fruit shape between down regulated *Sl-IAA17* lines and wild type fruit. Lots of variations were observed in Fruit Shape Index External I (H/W) (Fig. 31 A), Distal Fruit Blockiness (W_2/ W_m= Ratio of fruit width at the distal end / mid width) (Fig. 31 B), Proximal Fruit Blockiness (W_1 / W_m= Ratio of fruit width at the distal end / mid width) (Fig. 31 C), Fruit Shape Triangle (W_1/W_2) (Fig. 32 A), Distal Angle Micro (Fig. 32 B), Proximal Fruit Blackness (Fig. 32 C). The main differences are associated with variations in fruit diameter described in the article about *Sl-IAA17* role in fruit weight and endoreduplication.

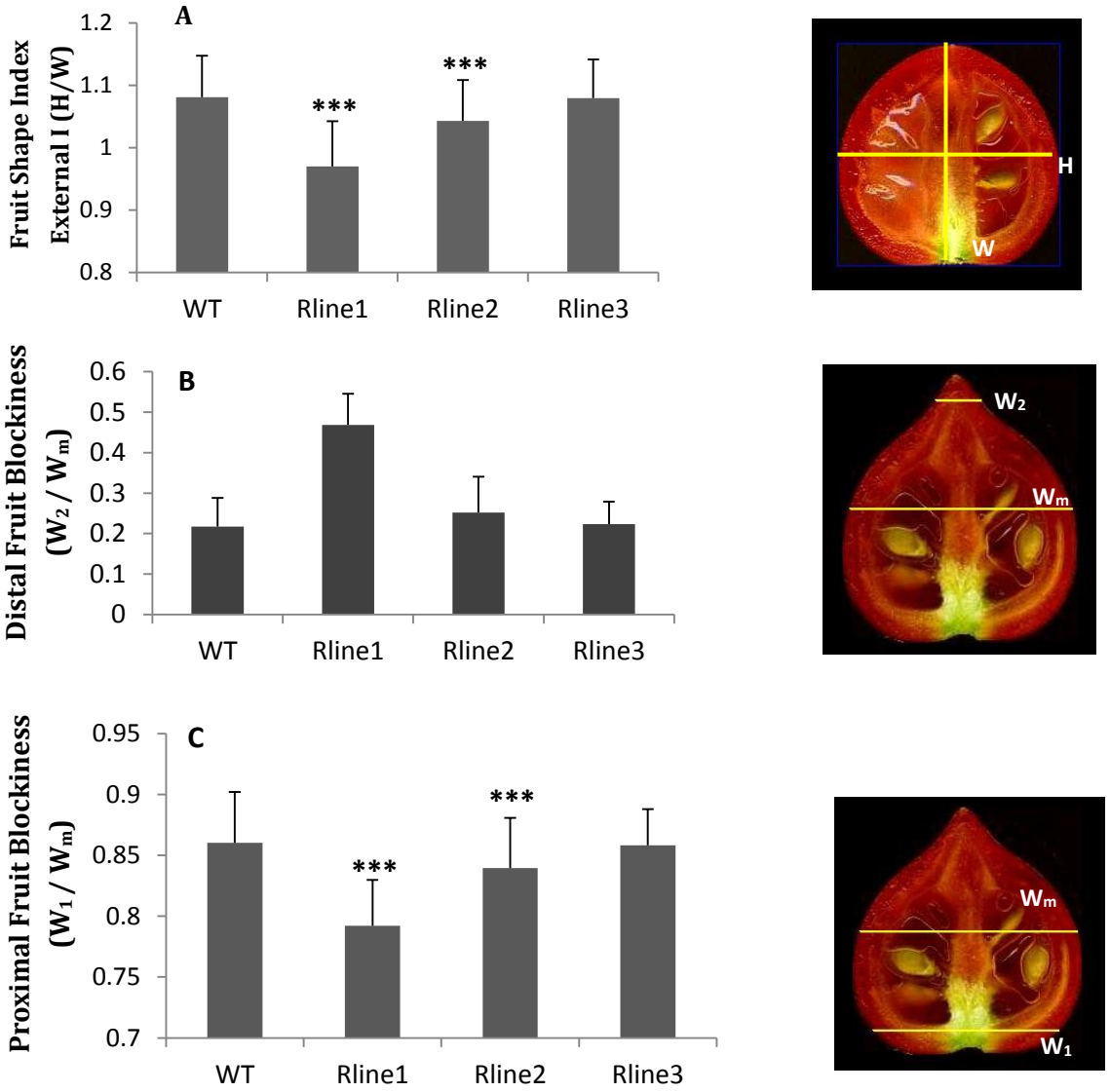

Fig. 31: Fruit morphology traits of *Sl-IAA17* RNAi lines and wild type. A): Fruit Shape Index External I. B): Distal Fruit Blockiness. C): Proximal Fruit Blockiness Statistical analyses were performed using the t-test comparing WT with each line, *** P <0.001. Error bars are standard errors.

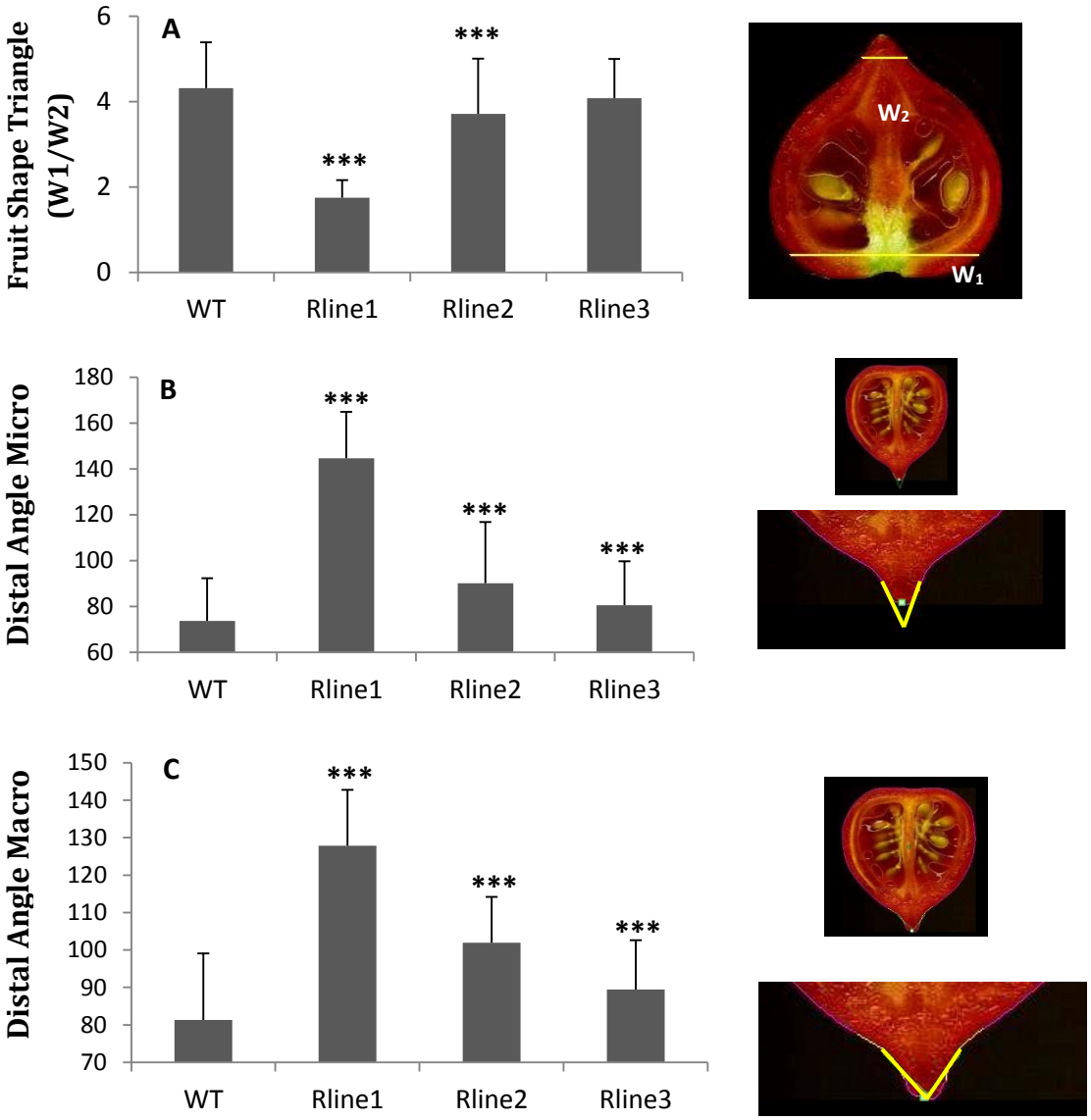

Fig. 32: Fruit morphology traits of *Sl-IAA17* RNAi lines and wild type. A) Fruit Shape Triangle. B) Distal Angle Micro. C) Distal Angle Macro. Statistical analyses were performed using the t-test comparing WT with each line, *** P <0.001. Error bars are standard errors.

4. Conclusion

Phenotypic analysis of *Sl-IAA17* down regulated plants shown that Sl-IAA17 affects many aspects of fruit shape, such as Fruit Shape Index External I, Proximal Fruit Blockiness, Fruit Shape Triangle, Distal Angle Micro, Distal Angle Macro, probably in relation with variations in endoreduplication in some pericarp areas. Compare to wild type, the *Sl-IAA17* RNAi lines fruit have more flat distal end, and more round of the fruit shape. Moreover, the silencing of *Sl-IAA17* lead to an increase in fruit soluble solids content but have no influence in the water content, acid content, fruit firmness. However these results seem disconnected from a direct involvement of the ethylene pathway.

General conclusions and perspectives

General conclusions and perspectives

As described in this thesis and previous works, the plant hormones auxin and ethylene act in crosstalk during the plant development, mostly studied in *Arabidopsis* (Swarup et al., 2007; Ikeda et al., 2009; Muday et al., 2012). However, considering the special characteristic of *Arabidopsis*, the study of the interaction between ethylene and auxin during the fruit development is limited. Tomato is a model plant that has been widely used for fruit study. During my work, the tomato fruit in mature green stage were treated with different hormones (IAA, ACC, IAA+ACC, PCIB), then the influence of the hormones on tomato fruit color change during the ripening process has been analyzed. Additionally, based on the RNAseq results (preliminary results), we knew that *Sl-IAA17* can be significantly affected by auxin and ethylene, and observed in a set of preliminary experiments that down-regulating *Sl-IAA17* gene in the tomato plant may affect fruit development. So, the second part of my work is focus on the functional characterization of one member of Aux/IAAs family, the *Sl-IAA17* gene.

The interaction of auxin and ethylene during tomato fruit color change process.

Firstly, the application of IAA induced a delay in the transition from green to orange/red compared to controls 96 h after hormone treatment, and the analysis by liquid chromatography shown that IAA treatment reduced significantly lycopene accumulation and led to higher levels of violaxanthin, neoxanthin and chlorophyll a than in controls. Secondly, IAA decreased the transcript accumulation of *psy1, psy3, pds, crtiso* genes in 24 h or 96 h, which alteration could inhibits lycopene accumulation. Thirdly, IAA treatment inhibited the transcript accumulation of the three chlorophyllases compared to control and ACC or PCIB, This correlates well with the sustained higher levels of chlorophyll a in treatments with IAA.

The application of ACC and PCIB, which accelerated the color transition from green to red compared to control and IAA treatment, lead to a higher accumulation of phytoene, lycopene, phytofluene, carotenes and a lower neoxanthin accumulation. But, ACC and PCIB

treatments did not lead to higher accumulation of the free abscisic acid (ABA), and those treatments also led to an increased degradation of the chlorophyll b. Secondly, ACC led to greater accumulation of *psy1* and *psy2*, and also *pds, zds*, *crtiso* transcripts compared to control, which is matching well the faster color turn and increased lycopene accumulation, and ACC treatment decreased the *β-lcy1* transcript accumulation which also lead to less conversion of lycopene to carotenes and more accumulation of lycopene. The decrease of *crtr2* transcript may also be linked to an accumulation of carotenes, and less conversion into violaxanthin. A similar set of changes occurred in PCIB treated fruit, which confirmed the antagonist role to auxin of this compound.

In the combination of ACC and IAA treatments, the evolution of the color was also slower than controls, close to the fruits that treated by IAA alone, suggesting that at equal dose the IAA leads to dominant effects over ACC and ethylene

The transcription factors RIN regulating *psy* transcription have been tested by qPCR, and the results shown that transcript levels of *rin* and *psy1* match quite well. This confirms the previous study (Martel et al., 2011), and proves that *rin* is involved in the *psy1* regulation.

In conclusion, IAA inhibits lycopene and carotene accumulation by inhibiting the transcript accumulation of several genes upstream of lycopene and carotene in the carotenoid pathway. ACC treatments favor lycopene and carotene accumulation by increasing the transcript accumulation of several genes leading to their synthesis, and decreasing lycopene cyclases and β-carotene hydroxylase which lead to their transformation in downstream products. We also showed that PCIB (an auxin antagonist) led to similar results than those obtained after ACC treatment, confirming the antagonism between ethylene and auxins in tomato ripening. We have identified several homologs of *psy, lcy, crtr* and *rin* genes that may be important in the fruit response to the auxin-ethylene crosstalk.

In perspective, further work about the roles of these genes (*psy, lcy, crtr* and *rin)* in carotenoid accumulation would be worth studying. For example, mutants affected in the various homologs could be either obtained by TILLING or transgenics. A particular attention could be given to the potential role of carotene hydroxylases (crtr) that looks to restrict the ABA accumulation.

Functional characterization of the Sl-IAA17 gene during the fruit developmental

Preliminary gene expression screenings showed that the *Sl-IAA17* gene can be regulated by both auxin and ethylene based on the RNAseq data and qPCR results. Information regarding IAA17 function was lacking either in *Solanaceae* or in *Arabidopsis*. In this study, we observed that：

Tomato transgenic plants with down regulation of *Sl-IAA17* gene presented an increased fruit size, weight and volume compare to wild type. The bigger size of *Sl-IAA17* down-regulated fruits associated with thicker pericarp. Then, it has been proved that the thicker pericarp was due to a larger cell size, but no change in cell number was observed, by microscopy analyses.

It is known that endoreduplication has a great impact on fruit growth and final fruit size (Chevalier et al., 2011; Bourdon et al., 2012; Chevalier et al. 2014). In our study, a correlation has been found between cell size and the nuclear DNA ploidy level in the RNAi lines of *Sl-IAA17* at the mature green stage. In transgenic fruit the mean ploidy level was about 20% higher than that in wild-type fruit.

Furthermore, compared to the wild type, in the three RNAi lines the ploidy levels I were predominantly the 32C and 64C peaks, and also in some cases of the 128C peak, compared to WT for which the major ploidy level was 4C. For the most affected lines, Rline1 and Rline2, there was a significant decrease for the 4C DNA levels compared to the wild-type. These results suggest there are promotions of successive endocycles in transgenic *Sl-IAA17* RNAi lines. So, those results provide a further insight into the roles of Sl-IAA17 in the control of fruit size and ploidy levels in tomato.

Further research, including a RNAseq analysis of transcripts at the fruit set, of at the mature green stage, after either IAA or ACC treatments will generate a large amounts of data from which hormone cross-talks could studied with more details. Plant hormones play a direct regulation on cell division and cell expansion processes. We know that Sl-IAA17 protein does function as an active repressor of auxin-dependent gene transcription (Audran-Delalande et al., 2012). In this study, all the results proved that *Sl-IAA17*

down-regulation lead to higher nuclear DNA ploidy level in tomato fruits. In order to know more about the role of Sl-IAA17 on the endocycle process, it will be necessary to test the expression of several genes (Chevalier et al., 2011) which control the process of endocycle. It would be interesting to check if the IAA17 has the same role in other cultivars (like ones of commercial interest), by looking at TILLING mutants.

Moreover, as partners of Aux/IAAs, ARF proteins play crucial roles during the tomato ripening process, and it has been reported that Sl-IAA17 has an interaction with ARF4, ARF5, ARF6, ARF7 and ARF8 in tomato (Wang et al., 2013). Further studies are required to understand the network of genes and associated signalling pathways involved in this regulation.

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