Aline Bertinatto Cruz

Interação entre luz, etileno e auxinas durante o amadurecimento e carotenogênese em frutos de tomateiro

Light, ethylene and auxin crosstalk during tomato fruit ripening and carotenogenesis

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Dedico:

Às pessoas que não desistem de seus sonhos.

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"A ciência se construiu não pela prudência dos que marcham, mas pela ousadia dos que sonham.

Todo conhecimento começa com o sonho.

O conhecimento nada mais é que a aventura pelo mar desconhecido, em busca da terra sonhada."

(Rubem Alves)

Resumo

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O amadurecimento de frutos é um processo altamente regulado que envolve várias mudanças estruturais, bioquímicas e fisiológicas, muitas das quais são influenciadas tanto por fatores endógenos quanto ambientais. O sinal luminoso, bem como os hormônios vegetais etileno e auxina têm se revelado importantes reguladores do amadurecimento de frutos. Porém, ainda não está totalmente esclarecido como as cascatas de sinalização luminosa e hormonal interagem a fim de controlar o desenvolvimento e a fisiologia dos frutos carnosos. O presente estudo teve como objetivo analisar as interações entre as cascatas de sinalização da luz, do etileno e das auxinas durante o amadurecimento e carotenogênese em frutos de tomateiro por meio do uso de mutantes fotomorfogênicos dessa espécie. As análises do metabolismo e sinalização do etileno e das auxinas em frutos do mutante *high-pigment 2* (*hp2*), o qual apresenta respostas exageradas à luz, revelaram que a perda da função do gene *HP2* resultou no aumento dos níveis de transcritos de genes que codificam os reguladores-chaves do processo de amadurecimento bem como um incremento na sinalização do etileno, sendo que essas mudanças estiveram atreladas ao maior acúmulo de carotenoides tipicamente encontrados neste mutante. Comparado ao genótipo selvagem, frutos do mutante *hp2* também apresentaram uma elevação considerável na sinalização das auxinas, incluindo incrementos na ativação do promotor *DR5*, regulação negativa da maioria dos genes *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) envolvidos no amadurecimento do tomate, bem como alterações na abundância de transcritos dos genes que codificam os fatores de transcrição AUXIN RESPONSE FACTOR (ARF). Indícios obtidos também sugerem uma maior responsividade dos frutos de *hp2* aos hormônios etileno e auxinas. Além disso, as análises do metabolismo e a sinalização das auxinas e do etileno realizadas em frutos do mutante *aurea* (*au*), deficiente na síntese do cromóforo dos fitocromos, indicaram que a interação entre esses fotorreceptores e fitormônios influencia o início do amadurecimento dos frutos de tomateiro. Os frutos deste mutante, quando comparados ao selvagem, exibiram um atraso no amadurecimento, o qual se mostrou temporalmente relacionado à indução tardia dos genes que controlam esse processo, ao atraso na produção climatérica do etileno, bem como associado a alterações nos níveis de transcritos de genes-chaves relacionados com a sinalização das auxinas. Além das mudanças temporais na sinalização hormonal associadas ao amadurecimento, os dados obtidos também sugerem que a deficiência em fitocromos funcionais reprime a ciclização do licopeno, levando a níveis reduzidos de β-caroteno e luteína nos tecidos dos frutos. Embora os mecanismos moleculares responsáveis pelas alterações nas respostas hormonais desencadeadas pela luz ainda precisem ser melhor elucidados em frutos de tomateiro, os dados obtidos neste estudo forneceram evidências de que uma complexa interação entre a sinalização luminosa, do etileno e das auxinas estaria envolvida no controle do amadurecimento e carotenogênese nessa espécie. Portanto, estas descobertas trazem consigo oportunidades de melhoria na regulação de eventos relacionados ao processo de amadurecimento por meio da manipulação combinada de genes relacionados à sinalização luminosa e hormonal.

Palavras-chave: Luz. Etileno. Auxinas. Amadurecimento de frutos.

Abstract

CRUZ, Aline Bertinatto. Light, ethylene and auxin crosstalk during tomato fruit ripening and carotenogenesis. 2017. 86p. Thesis (Ph.D in Science – Botany) –Bioscience Institute, University of Sao Paulo, Sao Paulo, 2017.

Fruit ripening is a highly coordinated process involving numerous structural, biochemical and physiological changes, many of which are influenced by both endogenous and environmental stimuli. Light signaling and plant hormones such as ethylene and auxins have been identified as important regulators of tomato fruit ripening. However, it is still not fully understood how light and hormonal signaling cascades interact to control the development and physiology of fleshy fruits. By applying a mutant-based approach, this study investigated the potential interconnection among light, auxin and ethylene signaling cascades during tomato fruit ripening and carotenogenesis. Analysis of ethylene and auxin metabolism and signaling in ripening fruits of the light-hyperresponsive *high-pigment 2* (*hp2*) mutant revealed that the loss of *HP2* function promotes the transcription of genes encoding key regulators of fruit ripening and increases ethylene signaling along with the increments in carotenoid synthesis and accumulation typically found in this mutant. Compared to the wild type (WT), significant changes in fruit auxin signaling were also observed in the *hp2* mutant, including significantly higher activation of the auxin-responsive promoter *DR5*, severe down-regulation of all *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes more closely associated with fruit ripening as well as disturbed transcript abundance of genes encoding AUXIN RESPONSE FACTOR (ARF) transcription factors. Evidence of increased tissue responsivity to ethylene and auxins in *hp2* ripening fruits is also provided. Moreover, comparing the auxin and ethylene metabolism and signaling in fruits of the phytochrome chromophore-deficient mutant *aurea* (*au*) in relation to the WT genotype provided new insights into the phytochrome-hormonal signaling crosstalk regulating the timing of fruit ripening. Compared to the WT, fruits of the *au* mutant exhibited a delayed-ripening phenotype, which was associated with the late induction of genes encoding master controllers of ripening, delayed ethylene climacteric production as well as coordinated changes in the expression of auxin signaling-related genes. Besides the temporal changes in hormonal signaling associated with ripening, the deficiency in functional phytochromes also seems to repress the cyclization of lycopene, leading to reduced levels of β-carotene and lutein in the fruit tissues. Although the exact molecular mechanisms behind the altered hormonal responses in tomato fruits triggered by changes in light signaling remain to be further elucidated, the data obtained in this study provide clear evidence that an intricate crosstalk among light, ethylene and auxin signaling may be involved in controlling tomato fruit ripening and carotenogenesis. Therefore, these findings open up a window of opportunity for further improvement in the regulation of ripening-associated processes through the combined manipulation of hormonal and light signaling-related genes.

Key-words: Light. Ethylene. Auxins. Fruit ripening.

List of abreviations

ABA – Abscisic acid

ACC – 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID

ACO – ACC oxidase

ACS – ACC synthase

AGPases – ADP-glucose pyrophosphorylases

ARF – AUXIN RESPONSE FACTOR

Aux/IAA - AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE

AUX/LAX – AUXIN1/LIKE-AUX1

Bk – Breaker stage

Bk1 – 1 days post-breaker

Bk12 – 12 days post-breaker

Bk3 – 3 days post-breaker

Bk6 – 6 days post-breaker

CNR – COLORLESS NON-RIPENING

COP1 – CONSTITUTIVE PHOTOMORPHOGENESIS 1

CRTISO – CAROTENOID ISOMERASE

CRY – cryptochromes

CTR – CONSTITUTIVE TRIPLE RESPONSE

CUL4 – CULLIN4

CYC-β – LYCOPENE β-CYCLASE

DDB1 – DAMAGE DNA BINDING1

DET1 – DETIOLATED1

DMAPP – dimethylallyl diphosphate

EIN – ETHYLENE INSENSITIVE

ERF – ETHYLENE RESPONSE FACTORS

ETR – ETHYLENE RESPONSES

flc – Tomato *flacca* mutant

GGPP – geranylgeranyl diphosphate

Gr – Tomato *Green-ripe* mutant

HFR1 - HYPOCOTYL IN FAR RED 1

HIR – high irradiance responses

hp – Tomato *high-pigment* mutant

HY5 – LONG HYPOCOTYL 5

IAA – Indole-3-acetic acid

IPP – isopentenyl diphosphate

IPyA – indole-3-pyruvic acid

JAs - Jasmonates

LCY-β – LYCOPENE β-CYCLASE

LFR – low fluences responses

LINs – cell-wall invertases

LYC-ε – LYCOPENE ε-CYCLASE

MAPKKK – Serine/threonine mitogen-activated protein kinase kinase

MEP – methylerythritol

MG – Mature green stage

NCED – 9-cis-epoxycarotenoid dioxygenase

NOR – NON-RIPENING

Nr – Tomato *Never-ripe* mutant

PAT – polar auxin transport

PDS – PHYTOENE DESATURASE

PHY – Phytochromes

PIFs - PHYTOCHROME INTERACTING FACTORS

PIN – PIN-FORMED

PSY – PHYTOENE SYNTHASE

RIN – RIPENING-INHIBITOR

SAM or AdoMet – S-ADENOSYL-METHIONINE

SAMS – L-METHIONINE-S-ADENOSYLTRANSFERASE

sit – Tomato *sitiens* mutant

SPBP – SQUAMOSA PROMOTER BINDING PROTEIN

SUTs – sucrose transporters

TAA1 – *TRANSLATION OF psaA1*

Trp – Tryptophan

VLFR – very low fluence responses

Z-ISO – ZETA-CAROTENE ISOMERASE

ZDS – ZETA-CAROTENE DESATURASE

Index

1. Introduction

1.1. Tomato: the genetic model for fleshy fruit biology

Fruits are unique to flowering plants and confer a selective advantage to these species by facilitating seed maturation and dispersal. Overall, fruit development can be divided into five stages: organogenesis, expansion, maturation, ripening and senescence (GILLASPY; BEN-DAVID; GRUISSEM, 1993). After pollination and fruit set, intense mitotic activity is observed in fleshy fruits, followed by a gradual reduction in cell division rate, maximal rates of cell enlargement and subsequent gain of competence to initiate ripening. In fleshy fruits, ripening is associated with numerous structural, biochemical and physiological changes, including modifications in the general appearance, texture, flavor and aroma, which ultimately convert immature fruits into a considerably more attractive and palatable structure for seed dispersal animals (GIOVANNONI, 2001; GUPTA et al., 2014; SEYMOUR et al., 2013). Therefore, fruit ripening represents a key reproductive strategy that maximizes the efficacy of seed dispersal, thus facilitating the survival of the next generation (KARLOVA et al., 2014).

Fleshy fruits are classically divided into two major ripening groups: (i) climacteric, characterized by an intermittent increase in ethylene production and a concomitant rise in respiration during the onset of ripening, and (ii) non-climacteric, in which ripening initiation and progression are not associated with dramatic changes in ethylene emission and respiration rates (ALEXANDER; GRIERSON, 2002; KLEE; GIOVANNONI, 2011). Although widely adopted in the literature, these two major ripening categories are clearly insufficient to accommodate the wide physiological variation found in fruit ripening within flowering species and, therefore, more accurate fruit ripening categories may arise in the near future.

Most of the current knowledge on the regulatory modules controlling climacteric fruit ripening is based on tomato (*Solanum lycopersicum* L.), the model species for fleshy fruit physiology (KLEE; GIOVANNONI, 2011). “Omics” (*i.e.* genomic, epigenomic, transcriptomic, proteomic and metabolomic) data, efficient stable transformation protocols and large germplasm collections, including many well-characterized mutants, are currently available for this species (CAMPOS et al., 2010; CARVALHO et al., 2011). Moreover, tomato is one of the most important horticultural crops and a relevant source of nutrients for human health (KLEE; GIOVANNONI, 2011). Antioxidants substances such as lycopene, β-carotene, lutein, flavonoids, phenylpropanoids, ascorbic acids (vitamin C) and tocopherols (Vitamin E) are accumulated in ripe tomato, thereby conferring important health-promoting

attributes to this fruits (FRASER; ENFISSI; BRAMLEY, 2009). Tomato plants also exhibit several traits not found in *Arabidopsis thaliana*, such as photoperiod-independent flowering, compound leaves, agronomically-relevant plant-pathogen interactions and sympodial growth, thus representing an alternative genetic model for investigating many other plant developmental processes besides climacteric fruit ripening (CAMPOS et al., 2010; CARVALHO et al., 2011).

During tomato ripening, fruit color changes associated with chlorophyll degradation, carotenoid accumulation and chloroplast-to-chromoplast differentiation mark the transition from mature green (MG) stage to the so-called breaker (Bk) stage. At MG stage, chlorophyll-containing chloroplasts confer the typical green color of unripe tomato fruits whereas the degradation of chlorophyll and accumulation of carotenoids such as β-carotene and lutein at Bk stage renders the yellowish coloration characteristic of this transitory ripening stage. Dismantlement of chloroplast grana and thylakoids, degradation of starch granules and chlorophylls, synthesis of new membranes structures, increase in the number and size of plastoglobules and the accumulation of large quantities of carotenoids is initiated in fruits at Bk stage, ultimately leading to the conversion of chloroplasts into chromoplasts (EGEA et al., 2010; KLEE; GIOVANNONI, 2011). From Bk onwards, chlorophyll progressively disappears whereas massive lycopene amounts are accumulated giving rise to the distinctive red coloration of fully ripe tomato fruits (FRASER et al., 1994, 1999). Therefore, carotenoid accumulation is one iconic ripening-associated process in tomato fruits, contributing to the color change of this organ, which greatly facilitates the zoochoric dispersal of mature viable seeds.

Carotenoid biosynthesis is strictly controlled throughout the plant life cycle both in vegetative (*e.g.* leaves and stems) and reproductive tissues (*e.g.* flowers and fruits) (CAZZONELLI; POGSON, 2010). In green tissues, carotenoids, such as lutein, β-carotene, violaxanthin and neoxanthin, play a major role in photosystem assembly, light harvesting and photoprotection (LADO; ZACARÍAS; RODRIGO, 2016). In contrast, carotenoids found in flowers and ripen fruits fulfill the critically important ecophysiological role of conferring pigmentation attractive for pollinators and seed dispersers, respectively (LADO; ZACARÍAS; RODRIGO, 2016). Carotenoids are derived from the methylerythritol (MEP) pathway and can be divided into two groups according to their chemical structures: (1) xanthophylls, which contain oxygen as functional group (*e.g.* lutein and zeaxanthin) and (2) carotenes, containing linear hydrocarbon without any functional group (*e.g.* α-carotene, β-carotene and lycopene) (ALMEIDA et al., 2015; BRAMLEY, 2002; SAINI; NILE; PARK, 2015).

The MEP pathway initiates with the combination of isopentenyl diphosphate (IPP)

and dimethylallyl diphosphate (DMAPP) to generate geranylgeranyl diphosphate (GGPP), which is the precursor of phytoene, the first carotenoid in the pathway (BRAMLEY, 2013). The conversion of two molecules of GGPP into phytoene is catalyzed by PHYTOENE SYNTHASE (PSY), the rate-limiting enzyme in the carotenoid biosynthetic pathway (Fig. 1). After phytoene formation, four enzymes catalyze the formation of lycopene: PHYTOENE DESATURASE (PDS), ZETA-CAROTENE DESATURASE (ZDS), CAROTENOID ISOMERASE (CRTISO) and ZETA-CAROTENE ISOMERASE (Z-ISO). After that, lycopene can be either cyclized by LYCOPENE β-CYCLASES (β-LCY or CYC-β) forming β-carotene, zeaxanthin, violaxanthin and neoxanthin or by the combined action of LYCOPENE ε-CYCLASE (ε-LYC) and β-LCY or CYC-β giving rise to δ-carotene, α-carotene and lutein (SAINI; NILE; PARK, 2015; SU et al., 2015). The progressive accumulation of carotenoids in ripening tomato fruits relies on the coordinated transcriptional regulation of carotenoid-related genes such as those encoding the PSY, PDS, β-LCY and CYC-β (LADO; ZACARÍAS; RODRIGO, 2016). As discussed below, the transcriptional levels of these genes are tightly controlled by ripening-associated transcription factors, hormonal signaling and several environmental stimuli, including light, drought and temperature (LADO; ZACARÍAS; RODRIGO, 2016; SU et al., 2015).

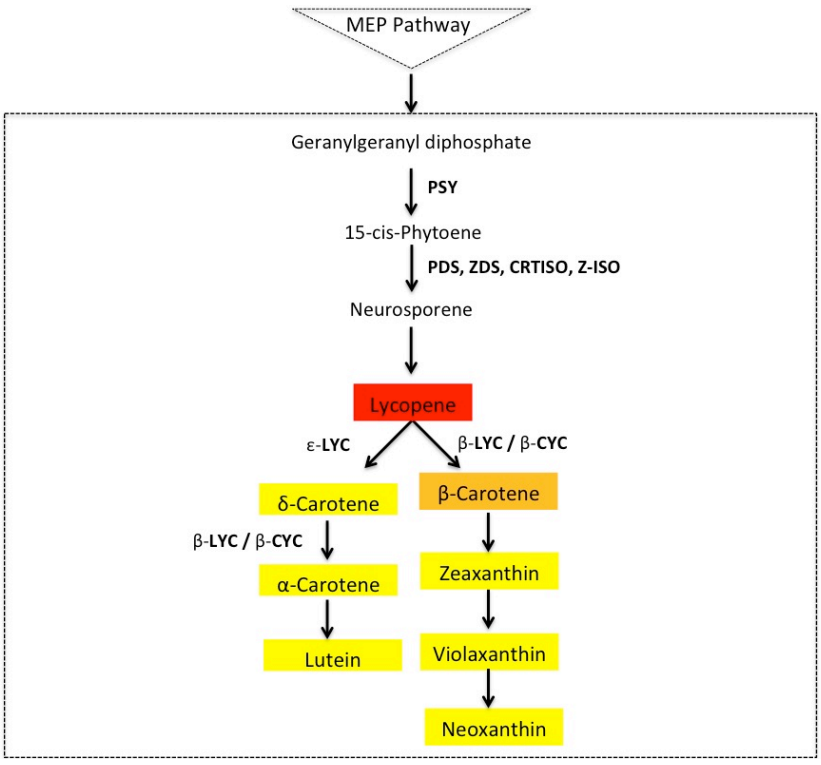

Figure 1. Simplified schematic representation of the carotenoid biosynthetic pathway in plants. The abbreviations indicate the following: PSY, PHYTOENE SYNTHASE; PDS, PHYTOENE DESATURASE; ZDS, ZETA-CAROTENE DESATURASE; CRTISO, CAROTENOID ISOMERASE; Z-ISO, ZETA-CAROTENE ISOMERASE, β-LCY or CYC-β, LYCOPENE β-CYCLASES; ε-LYC, LYCOPENE ε-CYCLASE (modified from SAINI; NILE; PARK, 2015).

1.2. Climacteric fruit ripening: molecular and hormonal regulation

In line with its complexity and reproductive relevance, fruit ripening onset and progression are intricately regulated by numerous endogenous signaling molecules (*e.g.* plant hormones, regulatory proteins) and external stimuli (*e.g.* temperature, light, water availability) (GIOVANNONI, 2004; MCATEE et al., 2013; ZHU et al., 2014). Over the last decades, significant progress has been achieved in identifying the regulatory modules controlling the ripening-associated genetic reprogramming responsible for transcriptionally controlling genes encoding proteins related to several metabolic pathways, including those related to fruit color modification (*i.e.* chlorophyll degradation, carotenoids and flavonoids accumulation), fruit flavor and aroma changes (*i.e.* metabolism of sugars, acids and volatile compounds), cell turgor and fruit texture alterations (KARLOVA et al., 2014; KLEE; GIOVANNONI, 2011).

Ripening-deficient tomato mutants, such as *ripening inhibitor* (*rin*; ROBINSON; TOMES, 1968), *nonripening* (nor; GIOVANNONI, 2004) and *Colorless nonripening (Cnr; THOMPSON et al., 1999)*, have been critically important for dissecting the regulatory networks controlling climacteric fruit ripening (ZHU et al., 2014).

Acting as a master controller of climacteric ripening, the MADS-box transcription factor RIN is induced at the onset of ripening, directly regulating several key ripening-associated genes (VREBALOV, 2002). Fruits from this mutant display deficient ethylene production as well as impaired carotenoid accumulation and fruit softening. As the ripening-deficient phenotype found in *rin* cannot be complemented by supplemental ethylene treatment, RIN has long been described to act upstream to this hormone during fruit ripening (VREBALOV, 2002). Interestingly, *rin* mutations are frequently used in the heterozygous form to create long shelf life fruits, despite the fact that lycopene production remains partially compromised in *rin* heterozygous lines (MARTEL et al., 2011).

Compelling genetic evidence suggests that RIN acts together with the NAC domain transcription factor NOR to regulate both ethylene-dependent and -independent processes during climacteric fruit ripening (KLEE; GIOVANNONI, 2011; ZHU et al., 2014). More extensive transcriptional changes in ripening-related gene are triggered by NOR than RIN; therefore, NOR is believed to act upstream of RIN in the tomato ripening regulatory cascade (OSORIO et al., 2011; ZHU et al., 2014).

Whereas *rin* and *nor* are mutations in coding sequences of their respective genes, the *cnr* mutation consists of an epigenetic change in promoter methylation of a member of the SQUAMOSA PROMOTER BINDING PROTEIN family (SPBP) (MANNING et al., 2006).

Ethylene production is considerably reduced in this mutant, whose fruits exhibit impaired softening, yellowish skin and a poorly pigmented pericarp. Synthesis of carotenoid precursor GGPP is severely limited in *cnr* fruits, explaining their extremely reduced levels of β-carotene, lycopene and other carotenoids (KLEE; GIOVANNONI, 2011; MANNING et al., 2006). Other ripening-impaired mutants continue to be discovered over the years (*e.g. Sl-NAC4* loss-of-function mutant) thus facilitating advances in elucidating even further the regulatory networks controlling ripening-related processes (ZHU et al., 2014).

Among the signaling molecules acting downstream of RIN and NOR and other master controllers of ripening, the gaseous plant hormone ethylene is responsible for regulating many ripening-related physiological, biochemical, and molecular processes. Accordingly, the deficiency in the biosynthesis, perception or signal transduction of this plant hormone directly impacts fruit ripening initiation and progression (LIU et al., 2015b).

Ethylene biosynthesis in higher plants is a relatively simple metabolic pathway, involving the rate-limiting enzymes 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE (ACS) and ACC OXIDASE (ACO). Ethylene biosynthesis initiates with the conversion of L-methionine to S-adenosylmethionine (AdoMet or SAM) in a reaction catalyzed by the enzyme L-METHIONINE-S-ADENOSYLTRANSFERASE (SAMS) (Fig. 2). SAM is converted in ACC by ACS and ACO converts ACC into ethylene (CARA; GIOVANNONI, 2008; VAN DE POEL et al., 2012).

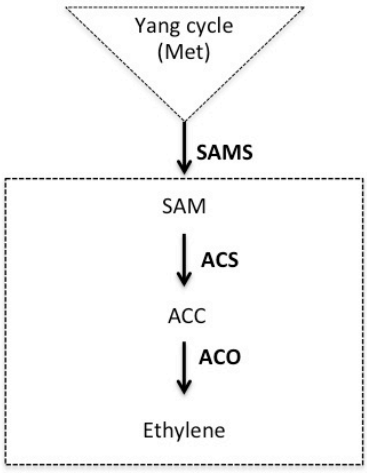

Figure 2. Simplified schematic representation of ethylene biosynthetic pathway in plants. The abbreviations indicate the following: Met, methionine; SAMS, S-ADENOSYLMETHIONINE; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC SYNTHASE; ACO, ACC OXIDASE.

ACS and ACO are encoded by multigene families whose members have been well characterized during the ripening of tomato and various other fruits (Fig. 2) (KLEE; GIOVANNONI, 2011). At least nine *ACS* (*Sl-ACS1a, Sl-ACS1b* and *Sl-ACS2* to *Sl-ACS8*) and

five *ACO* (*Sl-ACO1* to *Sl-ACO5*) genes are responsible for ethylene production in tomato and they are differentially expressed during fruit development and ripening (CARA; GIOVANNONI, 2008). The coordinated expression of distinct *ACS* and *ACO* genes facilitates the occurrence of two systems of ethylene biosynthesis *in planta*: (1) System 1, which predominates during normal vegetative growth and continues until the onset of ripening (pre-climacteric fruit development) is characterized by auto-inhibitory ethylene production, and (2) System 2, which operates during ripening of climacteric fruits when ethylene production is autocatalytic (ALEXANDER; GRIERSON, 2002; BARRY; LLOP-TOUS; GRIERSON, 2000; CARA; GIOVANNONI, 2008).

During pre-climacteric fruit development, *Sl-ACS6* and *Sl-ACS1A* genes are the main responsible for ethylene production. Subsequently, expression of *Sl-ACS1A* increases and *Sl-ACS4* is induced, initiating the climacteric ethylene production. The up-regulation of *Sl-ACS4* leads to the down-regulation of *Sl-ACS6* and *Sl-ACS1A* genes via the negative feedback of ethylene biosynthesis typically observed in System 1. At this moment, *Sl-ACS2* expression is induced to maintain the autocatalytic ethylene production characteristic of the System 2 (ALEXANDER; GRIERSON, 2002; BARRY; LLOP-TOUS; GRIERSON, 2000; CARA; GIOVANNONI, 2008). Three *ACO* genes are typically expressed in tomato fruits, *Sl-ACO1, Sl-ACO3* and *Sl-ACO4*. Whereas *Sl-ACO3* expression predominates from early fruit development through Bk stage, *Sl-ACO1* and *Sl-ACO4* genes are expressed from fruit set through the end of ripening (ALEXANDER; GRIERSON, 2002; CARA; GIOVANNONI, 2008).

Once produced, ethylene perception starts when ethylene binds to its specific receptors located in the endoplasmic reticulum membrane, which activates a signal transduction cascade leading to the transcriptional regulation of ethylene-responsive genes (Fig. 3). In tomato, seven ethylene receptors have been identified – ETHYLENE RESPONSES 1 (Sl-ETR1 to Sl-ETR7) – which can be divided into the subfamilies 1 and 2 according to their transmembrane domains (LIU et al., 2015b). Sl-ETR1, Sl-ETR2, and Sl-ETR3/NR belong to subfamily 1, whose members have a histidine kinases domain. Sl-ETR4 to Sl-ETR7 belong to subfamily 2, as both exhibits a serine kinase domain (CARA; GIOVANNONI, 2008; KLEE; GIOVANNONI, 2011; LIU et al., 2015b). Among tomato mutants defective for ethylene receptors, conspicuous fruit ripening phenotype is only observed in *Never-ripe* (*Nr*), in which an amino acid change in the N-terminus of the Sl-ETR3/NR ethylene receptor confers ethylene insensitivity. Therefore, *Nr* fruits do not fully ripe even upon treatment with exogenous ethylene (KLEE; GIOVANNONI, 2011; LANAHAN et al., 1994).

Interacting with ETRs, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a putative MAP-kinase kinase kinase (MAPKKK), acts as a negative regulator of ethylene response by forming a signaling complex that suppresses the ethylene response via the inactivation of ETHYLENE INSENSITIVE2 (EIN2) (LIU et al., 2015b). When ethylene is perceived by the receptor, a signaling cascade initiates by releasing the repression caused by CTR1 on EIN2, subsequently leading to the transcriptional activation of ETHYLENE INSENSITIVE 3 (EIN3) and EIL (EIN3-like), which in turn results in the activation of ETHYLENE RESPONSIVE FACTORs (ERFs) via binding to PRIMARY ETHYLENE RESPONSE ELEMENTS (PERE) (Fig. 3). Ethylene signaling cascade ends when ERF bind to 'GCC' box motif to promote ethylene responsive genes (CARA; GIOVANNONI, 2008; LIU et al., 2015b).

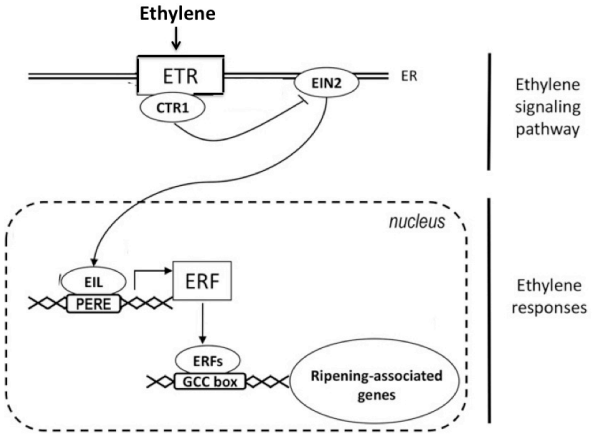

Figure 3. Simplified schematic representation of ethylene perception and signaling transduction. ETR, ETHYLENE RESPONSES; CTR1, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1); EIN2, ETHYLENE INSENSITIVE2; EIL, ETHYLENE INSENSITIVE 3-like; ERFs, ETHYLENE RESPONSIVE FACTORs (modified from LIU et al., 2015b).

ERFs are part of a multigene family, which in tomato encompasses 77 members, grouped into nine subclasses considering their structural features (LIU et al., 2016; PIRRELLO et al., 2012). Due to their ripening-related expression pattern and high transcript abundance in fruit tissues, some members of subclasses E, particularly *Sl-ERF.E1*, *Sl-ERF.E2* and *Sl-ERF.E4*, have recently been designated as priority targets for further functional characterization (LIU et al., 2016). Among them, Sl-ERF.E4 has been reported to play a major role in fruit ripening by integrating ethylene and carotenoid pathways (LEE et al., 2012).

Without undermining the role of ethylene, it has become clear that an integrated, multi-hormonal network controls climacteric ripening (AMPOPHO et al., 2013;

GIOVANNONI, 2004; KARLOVA et al., 2014; KLEE; GIOVANNONI, 2011; KUMAR; KHURANA; SHARMA, 2014; LIU et al., 2015b; MCATEE et al., 2013), which seems to involve hormones as diverse as abscisic acid (ABA), jasmonates (JAs) and auxins.

ABA is regarded as a promoter of tomato fruit ripening, as a peak in the endogenous levels of this hormone precedes the climacteric rise in ethylene production at the Bk stage (KUMAR; KHURANA; SHARMA, 2014; SUN et al., 2012). Further implicating ABA as promoter signal for the climacteric rise in ethylene production, *Sl-ACS2*, *Sl-ACS4*, and *Sl-ACO1* transcript levels in ripening tomato fruits were up- and down-regulated upon treatment with ABA and its biosynthetic inhibitor fluridone, respectively (ZHANG; YUAN; LENG, 2009). Also, application of ABA promotes starch hydrolysis, consequently leading to soluble sugar accumulation and fruit softening (SUN et al., 2012). Moreover, knockdown of genes encoding 9-cis-epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA synthesis, resulted in the down-regulation of many genes related to cell wall modification (SUN et al., 2012). Furthermore, red fruits of ABA-deficient mutants such as *flacca* (*flc*), *sitiens* (*sit*) and *high pigment 3* (*hp3*) display increased levels of carotenoids, particularly lycopene, compared to their wild-type counterparts (GALPAZ et al., 2008).

Besides their prominent signaling role in plant responses against herbivore attack and pathogen infection, JAs have also been implicated in accelerating climacteric fruit ripening (FAN; MATTHEIS, 1999). In tomato, JA application has been shown to promote ethylene production, chlorophyll degradation, β-carotene accumulation and ripening-related aroma compounds (ALMEIDA et al., 2015; PEÑA-CORTÉS et al., 2004; ZIOSI et al., 2008).

Compelling evidence also indicates that auxins intensively crosstalk with ethylene during fruit ripening. Exogenous application of indole-3-acetic acid (IAA) has been demonstrated to promote ripening-associated processes such fruit softening and anthocyanin formation in peach (PAYASI; SANWAL, 2010). Moreover, delayed ripening and slow starch degradation and soluble sugar accumulation have been observed in IAA-treated banana fruits (PURGATTO et al., 2001). In tomato, Su et al. (2015) demonstrated that auxin application delays ripening and interferes with carotenoid accumulation. IAA treatment repressed carotenoid biosynthesis-related genes such as *Sl-PSY, Sl-ZISO, Sl-PDS, Sl-CAROTENOID ISOMERASE* (*Sl-CRTISO*) and promoted others such as *β-LCY1* and *β-CAROTENE HYDROXYLASE* (*CRTR-β1*), leading to increased neoxanthin and violaxanthin levels and reduced accumulation of lycopene as well as α-, δ- and β-carotene (SU et al., 2015).

IAA, the most abundant auxin in plants, is mainly derived from tryptophan (Trp). This amino acid is initially converted to indole-3-pyruvic acid (IPyA) by members of the TRANSLATION OF psaA1 (TAA1) protein family of aminotransferases, and IPyA is

subsequently converted to IAA by flavin-containing monooxygenases encoded by *YUCCA* genes. YUC-catalyzed reaction is believed to represent the rate-limiting step in auxin biosynthesis (COOK; ROSS, 2016; DAI et al., 2013). Once produced, auxins are distributed within plant cells via a highly-coordinated transport mechanism known as polar auxin transport (PAT), which is mediated by PIN-FORMED (PIN) and AUXIN1/LIKE-AUX1 (AUX/LAX) proteins that control cellular auxin efflux and influx, respectively. The asymmetric distribution of these proteins across cells and tissues leads to the directional auxin flow and the establishment of auxin gradients (PATTISON; CATALÁ, 2012).

In the auxin-signaling cascade, hormone perception leads to targeting Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE) proteins for degradation via the 26S proteasome. Aux/IAA proteins act as repressors of auxin response by a constant physical inhibition of AUXIN RESPONSE FACTORS (ARFs), which are transcription factors that directly control auxin responsive genes through the binding to AuxRE motifs within their promoters (QUINT; GRAY, 2006; SANTNER; ESTELLE, 2009). Therefore, at low auxin levels, Aux/IAA proteins form dimers with ARFs, inhibiting their activity. In contrast, at higher auxin levels, Aux/IAAs are marked for proteasomal degradation, releasing ARFs to transcriptionally regulate auxin-responsive genes (LI et al., 2016a). Importantly, whereas IAAs always act by repressing the ARF binding to the promoters of auxin-responsive genes repressors, different members of the ARF family can either act as transcriptional repressor or activator of auxin-responsive genes (ZOUINE et al., 2014).

Tomato *Aux/IAA* and *ARF* gene families comprise 25 and 22 members, respectively (AUDRAN-DELALANDE et al., 2012; HAO et al., 2015; SANTNER; ESTELLE, 2009). *Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9* and *Sl-IAA15* are expressed at high levels from flower to ripe fruit, *Sl-IAA27* and *Sl-IAA36* transcripts are also expressed at significant levels all over the ripening phase whereas the transcripts of other *Sl-IAA*s remain at low levels during fruit ripening (HAO, 2014). Among tomato *ARF* genes, *Sl-ARF3*, *Sl-ARF5*, *Sl-ARF6*, *Sl-ARF13*, *Sl-ARF15* and *Sl-ARF17* are clearly up-regulated at MG stage, potentially playing a role in fruit development (ZOUINE et al., 2014), whereas *Sl-ARF2a* and *Sl-ARF2b* have been demonstrated to regulate fruit ripening and carotenogenesis (HAO et al., 2015). Data also reveal that whereas *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4* and *Sl-ARF10* act as repressors of auxin-responsive genes, *Sl-ARF5*, *Sl-ARF8a* and *Sl-ARF8b* are promoters of auxin responses (SAGAR et al., 2013; ZOUINE et al., 2014).

Sl-ARF- and *Sl-Aux/IAA*-suppressed lines have been instrumental in elucidating the roles played by these auxin signaling-related components. Under-expression of *Sl-IAA9* disturbed the expression of numerous ethylene-related genes leading to early fruit initiation

and parthenocarpy (WANG et al., 2005) whereas altered fruit morphology and size has been observed in *Sl-IAA27*-knockdown lines (BASSA et al., 2012). Conversely, *Sl-ARF4*-silenced plants exhibited increased sugar accumulation and plastid development in tomato fruits (SAGAR et al., 2013). In contrast, *Sl-ARF3* silencing revealed no obvious changes in fruit biology and quality traits but instead implicated this ARF as a key regulator of epidermal cell and trichome formation in vegetative tissues (ZHANG et al., 2015). *Sl-ARF9* was implicated in the regulation of cell division during early tomato fruit development (DE JONG et al., 2015) whereas *Sl-ARF7* was shown to act as a negative regulator of fruit set (DE JONG et al., 2009). Moreover, suppression of *Sl-ARF2a* or *Sl-ARF2b* altered ripening and the double repression of these genes dramatic inhibited ripening progression (HAO et al., 2015). Accordingly, ethylene synthesis and perception as well as pigment accumulation and transcript levels of *Sl-RIN*, *Sl-NOR* and *Sl-CNR* were markedly altered in *Sl-ARF2*-deficient lines (HAO et al., 2015).

Alongside with ERF, Aux/IAA and ARF apparently play a critical role in mediating auxin-ethylene crosstalk in both vegetative and reproductive tissues (CHAABOUNI et al., 2009; DRUEGE et al., 2014; MEIR et al., 2010; RUZICKA et al., 2007). Many tomato genes encoding these signaling proteins are differentially expressed in response to both auxin and ethylene, which suggest their action as the connection points between the signaling cascades initiate by these two hormones (JONES et al., 2002; LI et al., 2016b; TRAINOTTI; TADIELLO; CASADORO, 2007; ZOUINE et al., 2014). Moreover, auxin-ethylene crosstalk often involves reciprocal regulation at biosynthetic level (STEPANOVA et al., 2007).

1.3. Light influence on fruit development and ripening

Light not only provides energy for photosynthesis but also represents a crucial environmental signal responsible for adjusting plant growth, development and reproduction. Processes as diverse as seed germination, seedling deetiolation, phototropism, flowering, fruit pigmentation and entrainment of circadian rhythms are intrinsically regulated by light stimuli (AZARI et al., 2010a; LLORENTE; D'ANDREA; RODRÍGUEZ-CONCEPCIÓN, 2016). Light quality (spectral composition), intensity (irradiance), duration (including day length) and/or direction are perceived by a complex array of plant photoreceptors, which includes phytochromes (red and far-red light receptors), cryptochromes (blue and UV-A light receptors), phototropins (blue light receptors) and UVR-8 photoreceptors (GUPTA et al., 2014; LIU; COHEN; GARDNER, 2011). These photoreceptors can operate in concert or independently to regulate plant development (SMITH, 2000).

Due to their profound effects on plant physiology and development, phytochromes (PHY) have been intensively studied over the last 70 years. Therefore, many fundamental aspects of PHY structure, action mechanism and signaling cascades are currently known. In land plants and green algae, phytochromes are dimeric chromoproteins formed when PHY apoproteins – encoded by a small nuclear gene family – become covalently linked to the invariant linear tetrapyrrole chromophore phytochromobilin. Therefore, phytochromobilin-deficient mutants fail to produce functional phytochromes, resulting in pleiotropic phenotypic alterations such as increased stem, hypocotyl and petiole elongation and pale-green leaves and fruits (KENDRICK et al., 1997; MURAMOTO et al., 2005).

Photoactive holophytochromes perceive both red (R, 665 nm) and far-red (FR, 730 nm) light and exist in two distinctive photoconvertible forms, PHY_{Pr} and PHY_{Pfr}. R light can convert PHY from PHY_{Pr} to PHY_{Pfr} form, which is the biologically active state. This change is completely reversible, as FR light can convert PHY_{Pfr} back to the PHY_{Pr} form (INOUE; NISHIHAMA; KOHCHI, 2017). PHY photoconversion results in the translocation of holophytochromes from the cytoplasm to the nucleus, where the active PHY molecules initiate downstream transcriptional cascades (AZARI et al., 2010b).

Once in the nucleus, PHY bind to PHYTOCHROME INTERACTING FACTORS (PIFs), which are negative regulators of light response, targeting these basic helix-loop-helix (bHLH) transcription factors for degradation (FRANKLIN; QUAIL, 2010; LEIVAR; QUAIL, 2011). Active PHY also down-regulates the protein complexes formed by CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), DETIOLATED1 (DET1), DAMAGE DNA BINDING1 (DDB1) and CULLIN4 (CUL4), which are well-known negative regulators of light signaling in plants (CHORY et al., 1989; CHORY; PETO, 1990; DENG; QUAIL, 1991; INOUE; NISHIHAMA; KOHCHI, 2017). Protein complexes formed by COP, DET1, DDB1, CUL4 and some other light-regulated proteins give rise to the so-called COP9 signalosome, which targets photomorphogenesis-promoting factors, such as LONG HYPOCOTYL 5 (HY5), for proteasomal degradation (WEI; SERINO; DENG, 2008). Therefore, light-evoked degradation of COP9 signalosome components promotes HY5 accumulation and consequently activates the expression of photomorphogenesis-related genes such as those involved in chloroplast development and cell elongation and proliferation (LIU et al., 2004; WEI; SERINO; DENG, 2008).

Three distinct types of responses are triggered by PHYs depending on the photon flux density: (1) very low fluence responses (VLFR), which is triggered by fluences as low as 100 pmol m^{-2} (*e.g.* transcription of some photosynthetic genes), (2) low fluences responses (LFR), which requires at least 1 mmol m^{-2} (*e.g.* germination of some seeds), and (3) high

irradiance responses (HIR), which requires long periods of exposure to high fluence rate superior than 10 mmol m^{-2} (*e.g.* inhibition of hypocotyl elongation) (FANKHAUSER; CHORY, 1997; INOUE; NISHIHAMA; KOHCHI, 2017). Although VLFR, LFR and HIR converge to regulate plant development, compelling genetic evidence suggests that distinct PHYs are differently implicated in each of these three types of responses. Among the five PHY found in *Arabidopsis thaliana* (*i.e.*, PHYA, B, C, D and E), PHYA is particularly involved in mediating VLFR and HIR whereas PHYB, and sometimes PHYC, D or E, mediate LFR.

PHYA and PHYB displays marked differences in light stimulation and action mechanisms. PHYA can be activated either by R and FR light and this phytochrome does not present photoreversibility (QUAIL, 2002). In contrast, PHYB is essentially activated by R light, exhibits R/FR photoreversibility, and is significantly more photostable than PHYA. Whereas $PHYA_{Pfr}$ promotes the accumulation of FAR RED ELONGATED HYPOCOTYL (FHR) and FHR-LIKE (FHL) in the nucleus, PHYB mainly acts by regulating PIF and HY5 cellular abundance (KIRCHER et al., 2002; PFEIFFER et al., 2012).

Tomato genome harbors five *PHY* genes, *Sl-PHYA*, *Sl-PHYB1*, *Sl-PHYB2*, *Sl-PHYE* and *Sl-PHYF*. The impacts of loss of *Sl-PHYA*, *PHYB1* and/or *PHYB2* function on tomato vegetative growth have been extensively studied (KERCKHOFFS et al., 1997; VAN TUINEN et al., 1995a, 1995b; WELLER et al., 2000), revealing both exclusive and overlapping roles for these three PHYs. All the five PHY-encoding genes are expressed in tomato fruits; however, the role played by these photoreceptors during tomato fruit development and ripening has received relatively little attention.

The first set of evidence indicating that PHY-dependent light perception can impact tomato fruit biology and quality traits dates back to 1954 (PIRINGER; HEINZE, 1954), when the accumulation of a “flavonoid-like” pigment in pericarp tissues was shown to be regulated by R/FR light in a reversible manner. Further studies have also identified R, but not FR, radiation as a promotive signal controlling tomato fruit carotenogenesis (JEN; NORRIS; WATADA, 1977; KHUDAIRI; ARBOLEDA, 1971; THOMAS; JEN, 1975). A subsequent study showed that R-light-induced lycopene accumulation is not associated with changes in ethylene emission in ripening tomato fruits (ALBA; CORDONNIER-PRATT; PRATT, 2000). Moreover, reduced pigmentation is also evident in immature green fruits of either the *phyA,B1,B2* triple mutant (WELLER et al., 2000) or the phytochrome chromophore-deficient mutants *aurea* (*au*) and *yellow green-2* (*yg2*).

Data recently obtained by our research group reveals that the distinctive pale-green phenotype observed in the phytochrome chromophore-deficient mutant *au* primarily result

from a significant reduction in chloroplast abundance and size at pre-climacteric fruit development rather than changes in chloroplast ultrastructure (BIANCHETTI et al., submitted). Genes encoding sink and starch biosynthesis-related enzymes, such as cell-wall invertases (LINs), sucrose transporters (SUTs) and ADP-glucose pyrophosphorylases (AGPases), were down-regulated in *au* fruits (BIANCHETTI et al., submitted) limiting the accumulation of sugar in fruits of this mutant. Therefore, functional phytochromes apparently play a fundamental role in regulating chloroplast biogenesis and sugar import and accumulation in developing tomato fruits.

Eight *PIF*-encoding genes were identified in tomato, *Sl-PIF1a*, *Sl-PIF1b*, *Sl-PIF3*, *Sl-PIF4*, *Sl-PIF7a*, *Sl-PIF7b*, *Sl-PIF8a* and *Sl-PIF8b* (ROSADO et al., 2016). Suppression of *Sl-PIF1a* has been shown to promote carotenoid accumulation by alleviating the repression exerted by this transcription factor on *Sl-PSY1* expression (LLORENTE; D'ANDREA; RODRÍGUEZ-CONCEPCIÓN, 2016). Moreover, changes in the R/FR ratio triggered by fruit pigmentation also impacted *Sl-PSY1 (LLORENTE; D'ANDREA; RODRÍGUEZ-CONCEPCIÓN, 2016)*, which according to the authors may represent a mechanism responsible for synchronizing carotenoid production in tissues at different depths inside tomato fruits.

Alongside with PHY, other photoreceptors may also participate in fine-tuning tomato fruit biology and quality traits. Four tomato genes encode cryptochromes (*i.e. Sl-CRY1a*, *Sl-CRY1b*, *Sl-CRY2* and *Sl-CRY3*), and the overexpression of *Sl-CRY2* has been shown to promote the accumulation of flavonoids and lycopene in the fruit tissues (GILIBERTO et al., 2005). Moreover, overexpression or knockout/knockdown of genes encoding light signaling intermediates such as Sl-HY5, Sl-COP1, Sl-DET1/HP2, Sl-DDB1/HP1 or Sl-CUL4, which are involved in both PHY and CRY signaling cascades, also significantly impact tomato fruit physiology and nutritional composition (DAVULURI et al., 2005; LIU et al., 2004; WANG et al., 2008). For example, mutations in *HIGH PIGMENT 1* (*HP1*) and *HP2*, which encode the orthologs of *AtDDB1* and *AtDET1*, respectively, lead to exaggerated photoresponsiveness, thus promoting increased fruit chloroplast number and size and significant increments in carotenoid accumulation (AZARI et al., 2010a; COOKSON et al., 2003; KOLOTILIN et al., 2007; LEVIN et al., 2003, 2006; MUSTILLI et al., 1999). Constitutive deficiency in *Sl-DET1/HP2*, *i.e.* constitutive *Sl-DET1/HP2* silencing or the *hp2* mutation, greatly promotes β-carotene and lycopene accumulation in fruits tissues but also results in severe developmental defects, including the reduced plant stature and bushiness, thus limiting the *hp2* mutation in breeding programs targeting to improve tomato fruit nutritional value (DAVULURI et al., 2004, 2005). In line with these findings, fruit-specific down-regulation of *Sl-DET1/HP2* has

recently arisen as a successfully alternative to promote the accumulation of health-promoting substances in the fruits without carrying over the collateral adverse effects of *hp2* mutation on plant development and productivity (DAVULURI et al., 2005).

1.4. Phytochrome, auxin and ethylene signaling crosstalk

Intricated phytochrome-hormonal signaling networks control plant development and metabolism. PHY-dependent light perception is known to regulate the metabolism, transport, signal recognition and transduction of several plant hormone classes (reviewed by LAU; DENG, 2010; ZDARSKA et al., 2015). Conversely, hormone-signaling cascades also influence PHY signaling in plants via multiple mechanisms (ZDARSKA et al., 2015). Among the huge diversity of light-hormonal signaling crosstalk already described in the literature, those involving ethylene and auxins will be the main focus of this Thesis.

Phytochrome-dependent light perception has been extensively described as an inhibitory signal controlling ethylene production both in seedlings and in vegetative tissues of adult plants (BOURS et al., 2015; PIERIK, 2004; VANDENBUSSCHE et al., 2003). Overall, the inhibitory effect of PHY-dependent light perception on ethylene emission depends on duration and radiation fluence of the R light treatment (IMASEKI; PJON; FURUYA, 1971; KUREPIN et al., 2010; PIERIK et al., 2004).

Multiple mechanisms are involved in PHY_{Pfr}-dependent down-regulation of ethylene production in plants. For example, $PHYB_{Pfr}$ is known to promote the rapid conjugation of ACC into MACC in de-etiolation seedlings, thus reducing the cellular abundance of ACC available for ethylene synthesis (JIAO; YIP; YANG, 1987). Genetic evidence also shows that both PHYA and PHYB repress ethylene emission by down-regulating *ACS* mRNA levels (BOURS et al., 2015; FOO et al., 2006). Accordingly, either the loss of *PHYB* function or the overexpression of *PIF5* rendered increased *ACS* transcript abundance in *Arabidopsis* plants (BOURS et al., 2015; KHANNA et al., 2007). Light impacts on ethylene signaling cascades have also been reported. For example, COP1-mediated light signal transduction has been suggested to promote EIN3 protein accumulation (ZHONG et al., 2009), thus affecting the transcript levels of EIN3-downstream genes such as *ERF1* in *Arabidopsis* (LIANG et al., 2012; SOLANO et al., 1998).

Genetic and physiological data also suggest a significant influence of PHYs on auxin metabolism, transport and signaling. During seedling deetiolation and shade avoidance responses, PHY is believed to inhibit both TAA- ou YUC-mediated auxin synthesis (TAO et al., 2008). For example, loss of *HYPOCOTYL IN FAR RED 1* (*HFR1*) function, a protein that

closely interacts with PIFs, has been shown to promote *YUC2*, *8* and *9* in *Arabidopsis (HERSCH et al., 2014)*. Moreover, PHY-mediate light perception also promotes *GRETCHEN HAGEN3* (*GH3*) genes, which encodes IAA-amide synthetases known to be associated with IAA conjugation (KORASICK; ENDERS; STRADER, 2013; PARK et al., 2007; TANAKA et al., 2002). PHY-dependent light perception has also been demonstrated to regulate auxin transport in tomato seedlings through mechanisms that remain to be elucidated (LIU; COHEN; GARDNER, 2011).

According to data recently obtained by our research group, the deficiency in functional PHY results in marked changes in the fruit transcript abundances of several *ARF* and *Aux/IAA* tomato genes, along with alterations in the activation of the auxin-responsible promotor *DR5* (BIANCHETTI et al., submitted). Interestingly, very limited differences in auxin content were observed between phytochrome-deficient and wild type tomato fruits, thus suggesting that auxin responsiveness rather than its biosynthesis or transport may represent the primary phytochrome-auxin link during early developing tomato fruits. Data also revealed that functional PHYs stimulate fruit ripening by accelerating the climacteric rise in ethylene production and signaling (BIANCHETTI et al., submitted).

Aside from this study performed by Bianchetti et al. (submitted), additional data on whether and how the signaling cascades triggered by light and hormonal stimuli crosstalk during fruit development and ripening are currently missing. Therefore, deciphering the potential interconnection between light and hormone signaling controlling tomato fruit physiology represents a promising venue for generating relevant information for improving fruit ripening and quality traits.

We hypothesized that detailed analysis of ethylene and auxin metabolism and signaling in ripening fruits from tomato photomorphogenic mutants can be a useful strategy for identifying potential light-hormonal crosstalk mechanisms regulating tomato fruit biology.

2. Objectives

General Objectives

The overall aim of this Thesis was to explore the potential interconnection among light, auxin and ethylene signaling cascades during tomato fruit ripening and carotenogenesis.

Specific Objectives

To get insight into the general objective of this Thesis, the following specific goals were set:

1) By comparing ethylene and auxin signaling in ripening fruits from tomato *high pigment-2* mutant plants in relation to their near isogenic counterparts, investigate whether and how these plant hormones participate in the molecular mechanisms leading to the over-accumulation of carotenoids typically found in this light-hypersensitive mutant. This specific goal was addressed in Section 1 of this Thesis.

2) Explore the relevance of the phytochrome-dependent light perception for tomato fruit ripening and carotenogenesis and investigate whether the deficiency in functional phytochromes results in significant changes in ethylene and auxin metabolism and signaling. This specific goal was addressed in Section 2 of this Thesis.

3. Material and methods

3.1. Plant material

Wild type (WT) *Solanum lycopersicum* L. (cv. Micro-Tom) and the near-isogenic lines (NILs) harboring the mutation *aurea* (*au*) and *high pigment 2* (*hp2*) were obtained as previously described in Carvalho et al. (2011). All these genotypes, as well as transgenic plants carrying the synthetic auxin-responsive (*DR5*) or ethylene-responsive (*EBS*) promoters fused to the reporter gene *uid* (encoding a β-glucuronidase, GUS) were obtained from the tomato mutant collection maintained at ESALQ, University of São Paulo (USP), Brazil (http://www.esalq.usp.br/tomato/). Additionally, crosses and successive screening were performed to generate the double mutants *au-DR5::GUS, au-EBS::GUS, hp2-DR5::GUS* and *hp2-EBS::GUS.*

3.2. Growth conditions and treatments

Plants were grown in 6 L rectangular pots containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, São Paulo, Brazil) and expanded vermiculite, supplemented with 1 g L^{-1} of NPK 10:10:10, 4 g L^{-1} of dolomite limestone ($MgCO_3 + CaCO_3$) and 2 g L^{-1} thermophosphate (Yoorin Master®, Yoorin Fertilizantes, Brazil) in greenhouse under automatic irrigation at an average mean temperature of 25°C, 11.5 h/13 h (winter/summer) photoperiod and approximately 250-350 μmol m^{-2} s^{-1} PAR irradiance. Fruits at mature green (MG, ca. 30 dpa) stage were harvested about 30 days after anthesis (dpa) and were transferred to continuous white light or maintained under absolute darkness (D) until reaching distinct ripening stages. White light was delivered at around 50 μmol m^{-2} s^{-1} and supplied by an array of SMD5050 Samsung LEDs mounted in a temperature-controlled growth chamber maintained at 25±1°C and air relative humidity at 80±5%. Top and bottom illumination was applied to homogenize the light environment surrounding the fruits. Since the beginning of the treatments, fruits were placed into a 0.5-L sealed transparent vessel and continuously flushed with ET-free, humidified air (1 L min^{-1}) to avoid accumulation of ET inside the containers. Samples from light- or dark-incubated fruits were harvested under white light or dim green light, respectively. Pericarp samples (without seeds, columella, placental tissues and locule walls) were harvested as soon as the fruits have reached the following ripening stages: MG (displaying jelly placental tissues, 2 days after harvesting), Bk (breaker, displaying the first external yellow color signals) and Bk1, Bk3, Bk6, and Bk12, corresponding 1, 3, 6 and 12 days after Bk, respectively. Fruits at distinct treatments achieved

each ripening stage at a different number of days of treatment. All biological samples were harvested at the same daytime to avoid possible fluctuations in the parameters due to circadian rhythm. Four biological samples composed of at least five fruits each were harvested at each sampling time. Ethylene emission analysis and quantitative *in vitro* GUS activity assays were performed immediately after harvesting time. For all other analysis, samples were frozen in liquid N_2, powdered and stored at -80°C until use.

3.3. Fruit nutritional profile

3.3.1 Carotenoid profile

Carotenoids (namely lycopene, β-carotene, lutein and neurosporene) were extracted and analyzed by high-pressure liquid chromatography (HPLC) with photodiode array detector (PDA) as described by Lira et al. (2016). Approximately 200 mg of fresh weight (FW) pericarp samples grounded in liquid nitrogen were sequentially homogenized with a solution of saturated 100 µL of NaCl, 200 µL of dichloromethane and 1 mL of hexane:diethyl ether (1:1, v/v). The supernatant was collected after centrifugation at 4°C, 5000*g*, 10 min. The remaining carotenoids in the pellet were extracted twice more with 500 µL of hexane:diethyl ether (1:1, v/v). All supernatant fractions were combined, completely dried by vacuum and suspended with 150 µl of ethyl acetate. Chromatography was carried out on Agilent Technologies series 1100 HPLC system on a normal-phase column Phenomenex (Luna C18; 250 x 4.6 mm; 5 µm particle diameter) with a flow rate of 1 mL min^{-1} and temperature 25ºC. The mobile phase was a gradient of ethyl acetate (A) and acetonitrile:water 9:1 (v/v) (B): 0-4 min: 20% A; 4-30 min: 20-65% A; 30-35 min: 65% A; 35-40 min: 65-20% A. Eluted compounds were detected between 340-700 nm and quantified at 450 nm. The endogenous metabolite concentration was obtained by comparing the peak areas of the chromatograms with commercial standards.

3.3.2 Chlorophyll quantification

Chlorophyll extraction and quantification were carried out as described in Lira et al., (2016). Frozen pericarp samples (approximately 200 mg FW) were grounded in liquid nitrogen and homogenized with 1 ml of dimethylformamide (DMF). Samples were incubated for 24 hours at room temperature, under agitation and complete darkness. After centrifugation at 13.000*g* for 5 min, the supernatant was collected. Spectrophotometer measurements were performed at 664 and 647 nm. Total chlorophyll (Chl) content was estimated according to the formula: Total Chl = Chl *a* + Chl *b*, were Chl *a* = (12 x A664) – (3.11 x A647) and Chl *b* = (20.78 x A647) – (4.88 x A664) (PORRA; THOMPSON; KRIEDEMANN, 1989).

3.3.3 Fruit surface color measurement

Fruit surface color was assessed with a using Konica Minolta CR-400 colorimeter, using the D65 illuminant and the L*, a*, b* space, and the data were processed to obtain Hue and chroma values. Three measures were taken at the equator of each fruit and average values were calculated. 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 (ECARNOT et al., 2013).

3.3.4 Antioxidant activity

Antioxidant activity was measured using the method of Trolox equivalent antioxidant capacity (TEAC) as described by Lira et al. (2016). Frozen pericarp samples (approximately 200 mg FW) grounded in liquid nitrogen were homogenized with 1 mL of 100 mM sodium acetate buffer (pH 5) and kept shaking at 4°C for 30 min. After centrifugation (4°C, 5000*g*, 10 min), the supernatant was discarded, and 0.5 mL of hexane was added to the pellet that was kept shaking in 4°C for 30 min. After centrifugation (4°C, 5000*g*, 10 min), the supernatant was collected, and the same process was repeated twice. The lipophilic antioxidant extract was concentrated and suspended in 150 µL of hexane. The activity of the extract was spectrophotometrically determined by deactivation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+\bullet}$) and compared to a standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Absorbance was read at 734 nm after 2 h of incubation under darkness, and the results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

3.4. Hormonal profile

3.4.1. Auxin measurements

Endogenous levels of indole acetic acid (IAA) were determined by gas chromatography tandem mass spectrometry-selecting ion monitoring (GC-MS-SIM) as described by Santana-Vieira et al. (2016). Frozen pericarp samples (approximately 100 mg FW) were grounded in liquid nitrogen and homogenized with 1 mL of isopropanol:acetic acid (95:5, v/v). Exactly 0.5 µg [$^{13}C_6$]-IAA (Cambridge Isotopes, Inc.) was added to each sample as internal standards. Samples were incubated at 4°C for approximately 2 hours. After centrifugation (4°C, 16.000*g*, 20 min), the supernatant was collected and was added of 100 µL of ultrapure water and 500 µl of ethyl acetate. After centrifugation (4°C, 16.000*g*, 5 min) the

supernatant was collected, and this step was repeated once more. The extract was completely dried by vacuum and suspended in 50 µL methanol followed by a 30-min derivatization step at room temperature using 40 µL (trimethylsilyl)diazomethane.

The analysis was performed on a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) in selective ion monitoring mode. One microliter of each sample was automatically injected (model AOC-20i, Shimadzu) on splitless mode, using helium as the carrier gas at a flow rate of 4.5 mL min^{-1} through a fused-silica capillary column (30 m, 0.25 mm ID, 0.50-µm-thick internal film) DB-5 MS stationary phase in the following programme: 2 min at 100°C, followed by gradients of 10°C min^{-1} to 140°C, 25°C min^{-1} to 160°C, 35°C min^{-1} to 250°C, 20°C min^{-1} to 270°C and 30°C min^{-1} to 300°C. The injector temperature was 250°C, and the following MS operating parameters were used: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 230°C; and interface temperature, 260°C. Ions with a mass ratio/charge (m/z) of 130 and 189 (corresponding to endogenous IAA); 136 and 195 (corresponding to $[_{13}C^{6}]$-IAA) were monitored. Endogenous concentrations were calculated based on extracted chromatograms at m/z 130 and 136.

3.4.2. Ethylene emission

Ethylene emission was analyzed by gas chromatography with a flame-ionization detector (GC-FID) as described in Melo et al. (2016). Intact tomato fruits (typically 4 individuals) were enclosed in sealed transparent tube for 1 h under specific experimental conditions. After incubation, 9-mL gas samples were collected from tubes and injected into a glass vial headspace previously flushed with ethylene-free air (1 L min^{-1}) for 1 min. At least three 1-mL aliquots of each sample were injected in a headspace coupled to a Trace GC Ultra gas chromatography (Thermo Electron) fitted with a flame ionization detector (GC-FID) using an RT-alumina Plot column (Restek Corporation). Nitrogen was used as the carrier gas at a flow rate of 3 mL min^{-1}, and commercial standard mixtures of ethylene were used for the calibration curves. Column, injector and detector temperatures were 34ºC, 250ºC and 250ºC, respectively.

3.4.3. ACC measurement

ACC content were extracted and subsequently quantified as described by Bulens et al. (2011). Frozen pericarp samples (approximately 1 g FW) were grounded in liquid nitrogen and homogenized with 4 mL of a 5% (w/v) sulfosalicylic acid aqueous solution. Extracts were kept shaking for 30 min in 4°C at 180 rpm in the dark. The supernatant was collected after centrifugation at 4°C, 5000*g*, 10 min. Reaction was performed by adding 1.4 mL of extract to

a reaction mixture composed of 0.4 mL of 10 mM $HgCl_2$ and 0.2 mL of a 2:1 (v/v) solution of NaOCl 5%:NaOH 6 M. The final product of this reaction, ethylene, were measured by CG-FID as described above (item 3.4.2).

3.4.4. ACO activity

ACO extraction and activity assay were performed according to Bulens et al. (2011). Frozen pericarp samples (approximately 100 mg FW) were grounded in liquid nitrogen and homogenized with extraction buffer composed of 300 mM Tris-HCl (pH 8.0), 15 mg mL^{-1} insoluble polyvinylpolypyrrolidone (PVPP), 10% (v/v) glycerol and 30 mM ascorbic acid. After centrifugation (4°C, 20000 *g*, 20 min), 200 µL of the supernatant was added to 1.8 mL of reaction medium composed of 100 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 30 mM ascorbic acid, 100 µM $FeSO_4$, 50 mM $NaHCO_3$, 5 mM DTT and 2 mM ACC. ACO activity was determined by measuring the ability of the extract to convert exogenous ACC to ethylene after incubation at 30°C for 60 min. The ethylene formed during the reactions was measured by GC-FID as described above (item 3.4.2).

3.5. Hormone-responsive promoter analysis

3.5.1. Quantitative GUS activity assay

GUS activity was assayed according to Melo et al. (2016). Frozen pericarp samples (approximately 500 mg FW) were grounded in liquid nitrogen and homogenized in 1 mL extraction buffer composed of 50 mM Hepes-KOH (pH 7.0), 5 mM DTT and 0.5% (w/v) PVP. After centrifugation (4°C, 20.000g, 20 min), 200 µL aliquots of the supernatant were mixed with 200 µL of an assay buffer composed of 50 mM HEPES-KOH (pH 7.0), 5 mM DTT, 10 mM EDTA and 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) and incubated at 37°C for 30 min. Subsequently, aliquots of 100 µL were taken from each tube and the reactions were stopped with 2.9 mL of 0.2 M Na_2CO_3 (pH 9.5). Fluorescence was determined using a spectrofluorometer (LS55, Perkin Elmer) with 365 nm excitation and 460 nm emission wavelength (5 nm bandwidth). Fluorescence was measured at the same instrument settings in all experiments.

3.6. RNA isolation and quantitative RT-PCR analyses

Total RNA extraction was performed as described by Quadrana et al. (2013) with modifications. Frozen pericarp samples (approximately 100 mg FW) were grounded in liquid nitrogen and extracted using 1 mL of TRIzol® Reagent (Invitrogen). Then, 0.2 mL of

chloroform was added to sample. After centrifugation (4°C, 16.000*g*, 15 min), the organic phase was collected and this process was repeated once more. Both organic phases were combined and 0.5 mL of isopropyl alcohol was added to the mixture. After incubation for 30 min at room temperature, the extracts were centrifuged at 4°C, 16.000g, 10 min and the supernatant was discarded. To wash the samples, 1 mL ethanol 70% (v/v) was added to the remaining pellet, samples were centrifuged (4°C, 16000*g*, 5 min) and the supernatant was discarded. This washing process was repeated once more. Samples were dried at room temperature and resuspend with 30 µL RNase-free ultrapure water. Total RNA and integrity of samples were determined using spectrophotometer (NanoDrop - Thermo Scientific) and agarose gel 1%. Only RNA samples with 260/280 and 230/260 ratio values within 1.7-2.2 and 1.8-2.2, respectively, were used for the subsequent steps.

Subsequently, 1 µg of total RNA was treated with DNase (DNase I Amplification Grade, Invitrogen) for 60 min at room temperature and complementary DNA (cDNA) was synthesized using SuperScript® III Reverse Transcriptase kit (Invitrogen) according to manufacturer's instructions. Contamination with genomic DNA and efficiency of reverse transcriptase were checked via PCR reaction using Taq polymerase [buffer 1x, 0.2mM dNTP, 50 ng of cDNA (sample or control) and 1U of *Taq* DNA polymerase (Invitrogen)]. cDNA was diluted 10 times with ultrapure water and PCR reaction was performed using 2 µL cDNA sample and 300 nM of forward and reverse primers of *Sl-ACTIN*, whose sequences are described in Table 1. The amplification program consisted of 10 min initial step at 95°C, followed by 35 cycles with 15 sec 95°C, 30 sec 58°C and 1 min 72°C, and a final extension step of 10 min 72°C. Only cDNA samples free of DNA contamination were used in the subsequent steps.

Quantitative reverse-transcriptase PCR (qPCR) reactions were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) using 10 µl mix reaction composed of 5 µL Power SYBR green (Applied Biosystems), 2 µL cDNA sample and 200 nM of forward and 200 nM of reverse primer. The amplification program consisted of 10 min initial step at 95°C, followed by 40 cycles with 15 sec 95°C, 30 sec 55/60°C and 30 sec 72°C. Melting curve was analyzed to detect unspecific amplifications and primer dimerization. The primer sequences used in this study are listed in Table 1. Fluorescence data were analyzed used LingReg PCR software, and expression values were normalized against media mean two references genes: *SlEXPRESSED* and *SlCAC*, which have been already successfully used to normalize data from fruit development and ripening experiments (EXPÓSITO-RODRÍGUEZ et al., 2008).

Table 1. List of primers used for PCR and qPCR.

Gene	Primers for qRT-PCR[1]	Locus[2]
Sl-RIN	F:5′- TCAAACATCATGGCATTGTGGTG- 3′ R:5′- TGCATTTTCGGGTTGTACATTATCG- 3′	Solyc05g012020
Sl-NOR	F:5′- TAATGATGGGGTCGTCTTTCG- 3′ R:5′- ATTTTACAGGGCTAACTATTTTTTGC- 3′	Solyc10g006880
Sl-NAC4	F:5′- CCATCTTCGACGTCTTATCACG - 3′ R:5′- TTCGGCTTCGGCTCACTCT- 3′	Solyc11g017470
Sl-PSY1	F:5′- CGATGGTGCTTTGTCCGATAC- 3′ R:5′- CTCATCAACCCAACCGTACC- 3′	Solyc03g031860
Sl-PSY2	F:5′- GCATCACACATAACTCCACAAGC - 3′ R:5′- CGCATTCCTTCAACCATATCTCTG - 3′	Solyc02g081330
Sl-PDS	F:5′- CGTTCCGTGCTTCTCCGC- 3′ R:5′- CTAGAACATCCCTTGCCTCCAG - 3′	Solyc03g123760
Sl-CYCβ	F:5′- GCACCCACATCAAAGCCAGAG - 3′ R:5′- GCCACATGGAGAGTGGTGAAG - 3′	Solyc06g074240
Sl-LCYβ	F:5′- TTGACTTAGAACCTCGTTATTGG - 3′ R:5′- AACAGTTCCCTTTGTCATTATCT - 3′	Solyc04g040190
Sl-ERF.E4	F:5′- AGGCCAAGGAAGAACAAGTACAGA- 3′ R:5′- CCAAGCCAAACGCGTACAC- 3′	Solyc01g065980
Sl-ACS2	F:5′- GGCTACTAATGAAGAGCATGGC- 3′ R:5′- GACCCATTTGGATAACTCCGTTG- 3′	Solyc01g095080
Sl-ARF2a	F:5′- GCAAGGTCAAGAGTTATCGA- 3′ R:5CATTGGTTTCTCAGACAAGTC- 3′	Solyc03g118290
Sl-ARF2b	F:5′- CTGGGTTAAGCGACAAGCTC- 3′ R:5CCCCGCATTTGATACAGAG- 3′	Solyc12g042070
Sl-ARF3	F:5′- GATTGTTTTGCTCCCTTGGA- 3′ R:5GTGGCTGACCCCGATAGATA- 3′	Solyc02g077560
Sl-ARF4	F:5′- TGAAAGCCATCAACTCTCGG- 3′ R:5ATCCCATCTGACCATCAAGCATC- 3′	Solyc11g069190
Sl-ARF5	F:5′- TTCCGAGCCAAGAAAAGAAA- 3′ R:5 -CACTCGCATCAGTTGGAAGA- 3′	Solyc04g081240
Sl-ARF8a	F:5′- AGCCCGTCCAATATGTTCAG- 3′ R:5 -TTTGATGGTTGCTTCTGCTG- 3′	Solyc03g031970
Sl-ARF8b	F:5′- CATCTCCTTCCGACCACAGT- 3′ R:5 -TGGTGGATCAATTTGTCCTGC- 3′	Solyc02g037530
Sl-IAA3	F:5′- GCCACCAGTTCGATCATACA- 3′ R:5 -ATAAGGTGCTCCATCCATGC- 3′	Solyc09g065850
Sl-IAA4	F:5′- ACTCCACCTGTTGCCAAGAC- 3′ R:5 -AGATAAGGGGCTCCATCCAT- 3′	Solyc06g053840
Sl-IAA9	F:5′- CAGAGGGGAAGTTTCTGTCG- 3′ R:5 -CAACCTGTGCCTTTGTAGCA- 3′	Solyc04g076850
Sl-IAA15	F:5′- ATCGGAGACAGCCAAATCAG- 3′ R:5 -TTTGCTGGAGGTTTGTTTCC- 3′	Solyc03g120390
Sl-IAA27	F:5′- GCAAGAGAAGCTCAGTGA- 3′ R:5 -ACATCTCCCAAGGAACATCG- 3′	Solyc03g120500

Sl-CAC (constitutive)	F:5′- CCTCCGTTGTGATGTAACTGG- 3′ R:5 -ATTGGTGGAAAGTAACATCATCG- 3′	Solyc08g006960
SlEXPRESSED (constitutive)	F:5′- GCTAAGAACGCTGGACCTAATG - 3′ R:5′- TGGGTGTGCCTTTCTGAATG - 3′	Solyc07g025390
Sl-ACTIN	F: 5′ TCCGGGCATCTGAACCTCT 3′ R: 5′ TTGACATTTTCTTGATTGCCC 3′	Solyc01g104770

[1] F, forward; R, reverse.
[1] Locus according to the Sol Genomics Network database (http://solgenomics.net/).

3.7 Genes promoter analyses

Promoter sequences were retrieved from Sol Genomics Network (https://solgenomics.net/) and analyzed using PlantPAN 2.0 platform (http://plantpan2.itps.ncku.edu.tw/ CHOW et al., 2016) to identify the regulatory motifs. Fragments of 3 kb upstream from the initial codon ATG were analyzed for the presence of PBE-box (CACATG), G-box (CACGTG), CA-hybrid (GACGTA) and CG-hybrid (GACGTG) motifs, which are recognized by HY5 and/or PIFs (MARTÍNEZ-GARCÍA; HUQ; QUAIL, 2000; SONG et al., 2008).

3.8 Statistical analysis

Analysis of variance (ANOVA) followed by the Student's t-test were using the JMP software package (14th edition), with significance defined as $P \leq 0.05$. Standard errors of light/dark ratios were calculated by multiplying the mean light/dark ratio values by the sum of the standard error/mean value ratios of each treatment. Likewise, standard errors of *au*/WT ratios were calculated by multiplying the mean *au*/WT ratio values by the sum of the standard error/mean value ratios of each genotype.

4. Results

4.1 Section 1

The role of light and hormones in fruit carotenogenesis has been assessed in several studies (EPSTEIN; COHEN; SLOVIN, 2002; LLORENTE; D'ANDREA; RODRÍGUEZ-CONCEPCIÓN, 2016; ZDARSKA et al., 2015); however, whether and how the signaling cascades triggered by these stimuli crosstalk remains elusive. In this Section, the potential interconnection among light, auxin and ethylene signaling and its impact on tomato fruit carotenogenesis were investigated by monitoring fruit color, carotenoid metabolism as well as ethylene and auxin metabolism and signaling during the off-the-vine ripening of wild-type (WT) and *high pigment 2* (*hp2*) fruits under two contrasting light conditions: continuous darkness or white light.

In agreement with the promotive role of light in plastid biogenesis and differentiation (GALPAZ et al., 2008; WANG et al., 2008), mature green (MG) fruits from the light-hyperresponsive mutant *hp2* exhibited a distinctive dark-green coloration, which is consistent with the increased total chlorophyll levels and higher color saturation (chroma, which is indicative of color intensity) compared to the WT (Fig. 4). Despite these differences in chlorophyll content in WT and *hp2* fruits at MG stage, both genotypes exhibited undetectable chlorophyll levels from Bk stage onwards (data not shown). Chroma values were significantly higher in *hp2* than in the WT fruits during most of the dark treatment and specifically at MG and Bk stages in the light conditions (Fig. 4C). As indicated by the Hue angle values, light-incubated fruits acquired the distinctive red coloration faster and more intensively than those maintained under complete darkness (Fig. 4C). Regardless of the light condition, Hue values for mature green and ripe stages were within the expected values for tomato, *i.e.* 90º to 110º and 40º to 55º on mature green and red ripe stages, respectively (ECARNOT et al., 2013).

To investigate whether these light-triggered differences in fruit coloration were associated with changes in fruit carotenoid composition, the levels of these compounds were profiled. Regardless of the genotype or light conditions, lycopene was the carotenoid most abundantly accumulated, with levels exceeding in more than ten times those detected for lutein, β-carotene or neurosporene (Fig. 5A). In dark-incubated fruits, cyclized (*i.e.*, β-carotene, lutein) and acyclic (*i.e.*, lycopene, neurosporene) carotenoid levels were significantly higher in *hp2* than in the WT (Fig. 5A). However, when fruits were ripened in the presence of light, β-carotene, lutein and neurosporene levels were higher in *hp2* compared to the WT (Fig. 5A). Light treatment significantly promoted β-carotene accumulation in *hp2*

fruits all over the ripening phase (Fig. 5B). The other carotenoids analyzed were also promoted by light in *hp2* fruits at intermediate ripening stages (Fig. 5B).

Lipophilic extracts obtained from either dark- or light-incubated *hp2* fruits exhibited higher values of antioxidant capacity than WT counterparts (Fig. 5A). Light treatment increased lipophilic antioxidant capacity in *hp2* fruits at Bk1, Bk3 and Bk12 stages (Fig. 5B). No marked differences in lipophilic antioxidant capacity were observed in WT fruits ripened under light or dark conditions (Fig. 5B).

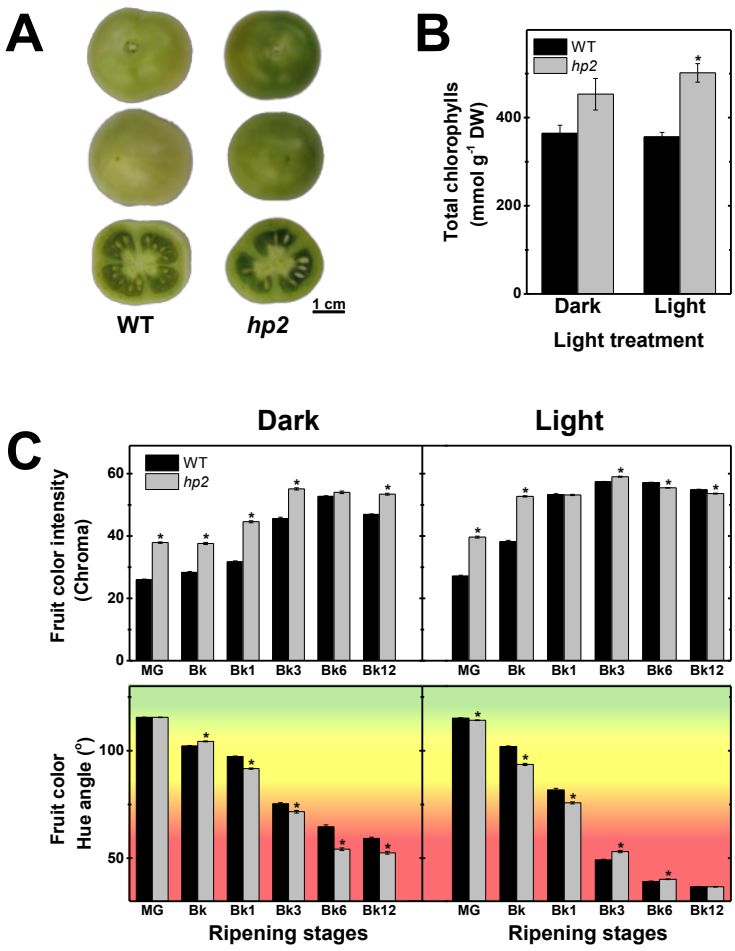

Figure 4. Light impact on tomato fruit color and chlorophyll levels. (**A**) Representative images of wild-type (WT) and *high pigment 2* (*hp2*) fruits at mature green (MG) stages. (**B**) Chlorophyll content at MG stage. (**C**) Ripening-related changes in fruit color intensity (Chroma) and fruit color (Hue angle). Fruits harvested at MG stage were left to ripen under constant light or dark conditions. Analysis were performed at MG (two days after the beginning of the treatment), breaker (Bk), Bk1 (1 day after Bk), Bk3, Bk6 and Bk12 stages. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes at each sampling time.

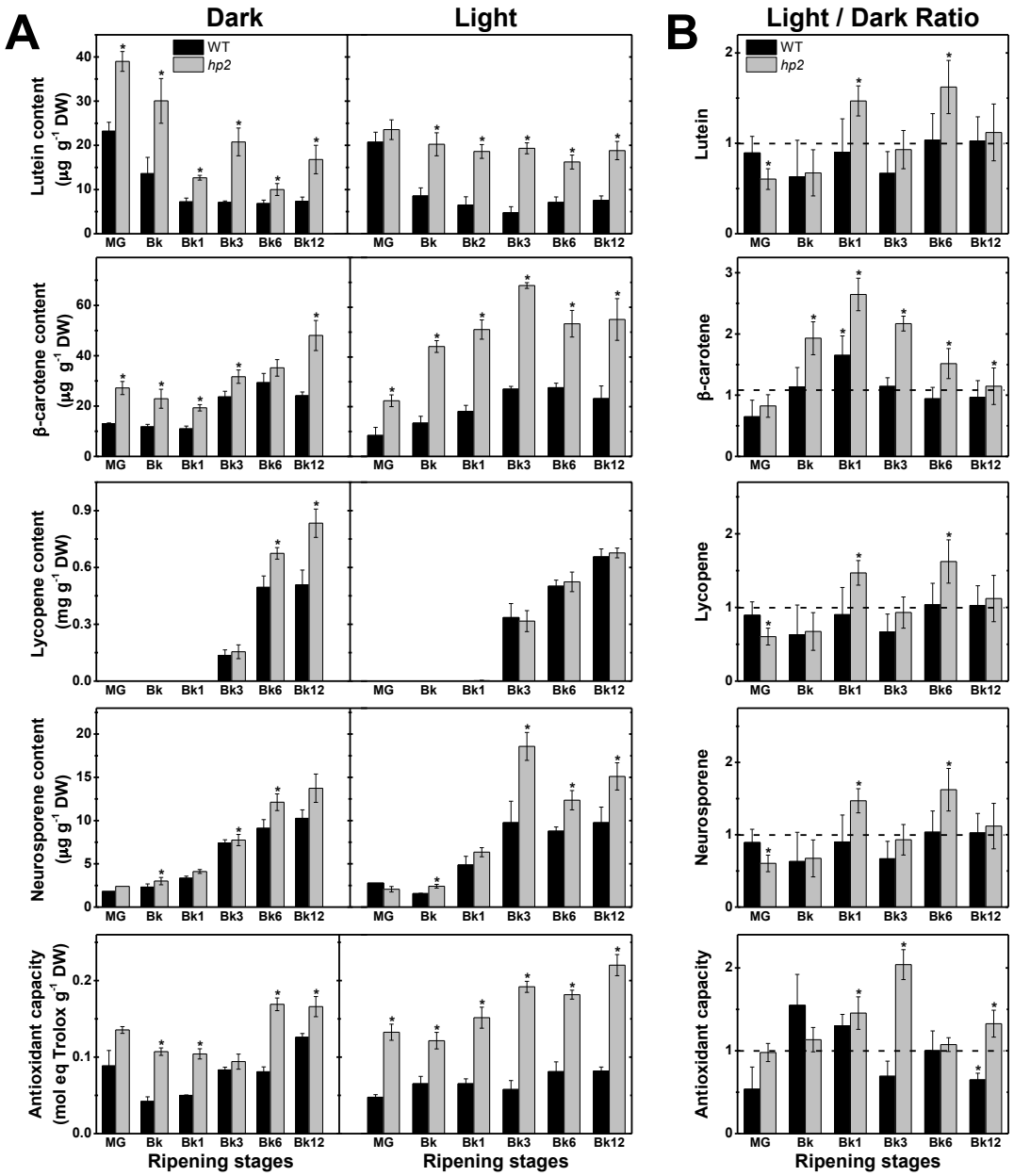

Figure 5. Light promotes tomato fruit carotenoid profile and antioxidant capacity. Wild-type (WT) and *high pigment 2* (*hp2*) fruits harvested at mature green (MG) stage were left to ripen under constant light or dark conditions. Pericarp samples were harvested at MG (two days after the beginning of treatment), breaker (Bk), Bk1 (1 day after Bk), Bk3, Bk6 and Bk12 stages. (**A**) Lutein, β-carotene, lycopene and neurosporene levels and Trolox equivalent antioxidant capacity (TEAC) content in lipophilic extracts. (**B**) Data expressed as the ratio between values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes (in **A**) or light treatments (in **B**) at each sampling time.

To investigate whether the changes in carotenoids profile observed between genotypes can be explained by the transcriptional regulation of carotenoid biosynthetic enzyme-encoding genes, transcripts of these genes were profiled in WT and *hp2* fruits incubated under white light (Fig. 6). Tomato genome harbors three *PHYTOENE SYNTHASE* genes, *i.e. Sl-PSY1* to *Sl-PSY3*, but only *Sl-PSY1* and *Sl-PSY2* are expressed in aerial plant

tissues (BRAMLEY, 2013; LIU et al., 2015a). In line with previous (BRAMLEY, 2013; LIU et al., 2015a), *Sl-PSY1* was significantly more expressed than *Sl-PSY2* in ripening fruits (Table 2). *Sl-PSY1*, Sl-*PSY2* and *Sl-PDS* mRNA levels were significantly higher in *hp2* than in WT fruits all over the ripening period (Fig. 6A). In both genotypes, the highest mRNA levels of *Sl-PSY1, Sl-PSY2* and *Sl-PDS* were detected from Bk1 to Bk6 stages.

CHLOROPLAST-SPECIFIC β-LYCOPENE CYCLASE (*Sl-CYCβ*) was significantly less expressed than *CHROMOPLAST-SPECIFIC β-LYCOPENE CYCLASE* (*Sl-LYCβ*) in both genotypes and at all ripening stages (Table 2). Compared to the WT, *hp2* fruits exhibited increased *Sl-CYCβ* mRNA levels at all sampling times, except for the Bk stage, and higher *Sl-LYCβ* transcript abundance particularly at Bk and Bk3 stages (Fig. 6). Therefore, the data indicates that the increments in fruit carotenoid levels in the *hp2* are associated with the up-regulation of genes encoding key carotenoid biosynthetic enzymes in tomato fruits.

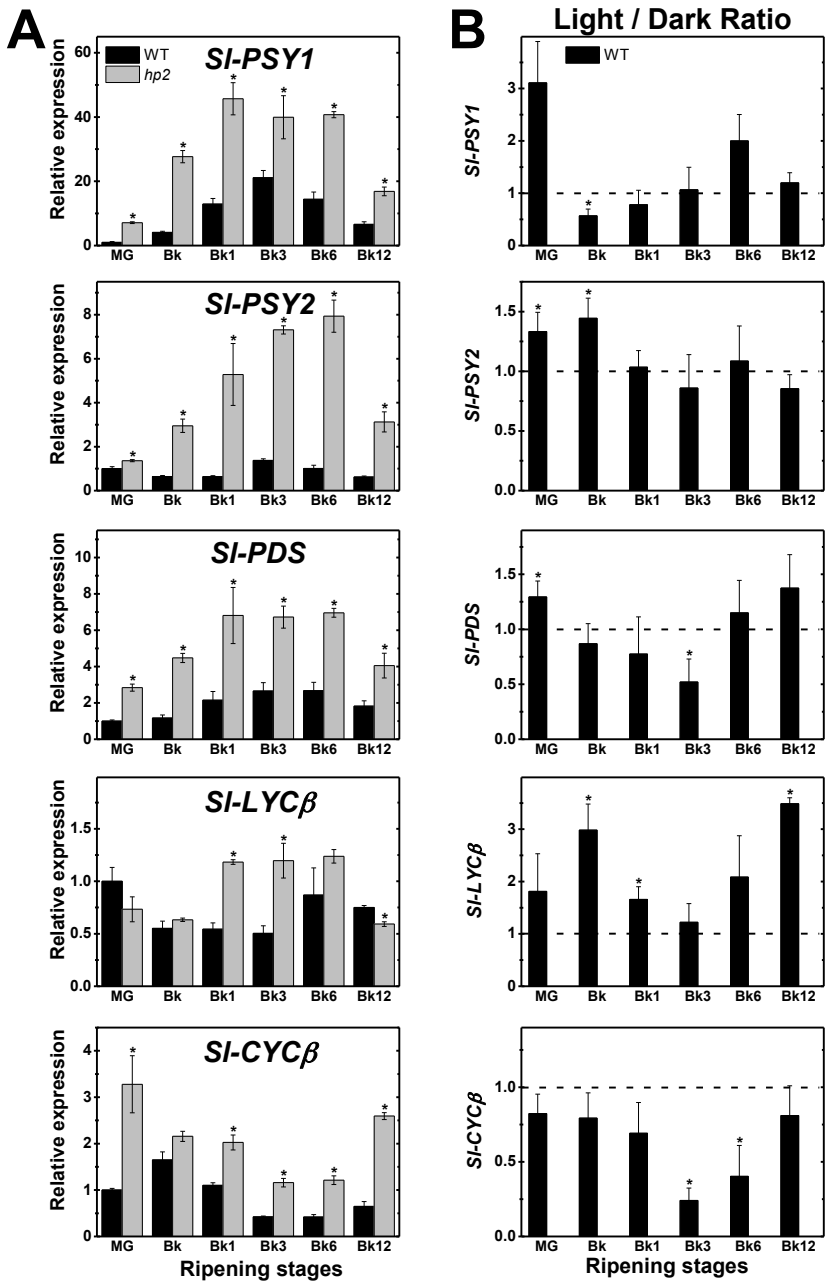

Figure 6. Loss of *Sl-DET1/HP2* function up-regulates carotenoid biosynthesis-related genes during tomato fruit ripening. Treatment details as described in Fig. 5. (**A**) Relative mRNA levels of tomato genes encoding carotenoid biosynthetic enzymes. Mean relative expression was normalized against WT samples at MG stage. (**B**) Data expressed as the ratio between qPCR values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes (in **A**) or light treatments (in **B**) at each sampling time. In **B**, mRNA levels of carotenoid biosynthesis-related genes were not determined. The abbreviations indicate the following: WT, wild-type; *hp2*, *high pigment 2*; MG, mature green; Bk, breaker; *Sl-PSY1*, *PHYTOENE SYNTHASE 1*; *Sl-PSY2*, *PHYTOENE SYNTHASE 2*; *Sl-PDS*, *PHYTOENE DESATURASE Sl-LYCβ*, *CHLOROPLAST-SPECIFIC β-LYCOPENE CYCLASE; Sl-CYCβ*, *CHROMOPLAST-SPECIFIC β-LYCOPENE CYCLASE*.

Table 2. Relative transcript ratio of *PHYTOENE SYNTHASE* and *β-LYCOPENE CYCLASE* genes.

		MG	Bk	Bk1	Bk3	Bk6	Bk12
Sl-PSY1	***WT***	1.00 ± 0.46	1.00 ± 0.21	1.00 ± 0.24	1.00 ± 0.10	1.00 ± 0.44	1.00 ± 0.35
	hp2	1.00 ± 0.37	1.00 ± 0.13	1.00 ± 0.17	1.00 ± 0.12	1.00 ± 0.34	1.00 ± 0.27
Sl-PSY2	***WT***	**0.22 ± 0.10**	**0.02 ± 0.01**	**0.01 ± 0.01**	**0.01 ± 0.00**	**0.03 ± 0.01**	**0.02 ± 0.01**
	hp2	**0.04 ± 0.02**	**0.02 ± 0.00**	**0.02 ± 0.00**	**0.03 ± 0.00**	**0.03 ± 0.01**	**0.03 ± 0.01**
Sl-LYCβ	***WT***	1.00 ± 0.13	1.00 ± 0.01	1.00 ± 0.28	1.00 ± 0.20	1.00 ± 0.35	1.00 ± 0.44
	hp2	1.00 ± 0.22	1.00 ± 0.14	1.00 ± 0.18	1.00 ± 0.06	1.00 ± 0.27	1.00 ± 0.20
Sl-CYCβ	***WT***	**0.03 ± 0.00**	**0.06 ± 0.01**	**0.06 ± 0.01**	**0.02 ± 0.00**	**0.01 ± 0.00**	**0.01 ± 0.01**
	hp2	**0.10 ± 0.02**	**0.08 ± 0.01**	**0.04 ± 0.01**	**0.02 ± 0.00**	**0.03 ± 0.01**	**0.10 ± 0.01**

Statistically significant differences in the transcript ratio between the two *PHYTOENE SYNTHASE* genes or between the two *β-LYCOPENE CYCLASE* genes at each ripening stage are indicated in bold terms (T-test, $P<0.05$). Values represent means ± SD from at least three biological replicates and are expressed as the relative transcript amount compared to *Sl-PSY1* and *Sl-LCYβ,* correspondingly. *Sl-PSY1*, *PHYTOENE SYNTHASE 1*; *Sl-PSY2*, *PHYTOENE SYNTHASE 2*; *Sl-LYCβ*, *CHLOROPLAST-SPECIFIC β-LYCOPENE CYCLASE; Sl-CYCβ*, *CHROMOPLAST-SPECIFIC β-LYCOPENE CYCLASE*.

To gain insight into the potential influence of light signaling on fruit ethylene metabolism, we next monitored ethylene emission, ACC content, ACO activity and the relative mRNA levels of *Sl-ACS2* in ripening WT and *hp2* fruits. Regardless of the genotype or light conditions, maximal values of ethylene emission, ACO activity and *Sl-ACS2* mRNA levels were detected from Bk to Bk3, whereas ACC was clearly accumulated at the end of the ripening (Bk12) (Fig. 7A). ACO activity was at undetectable levels from Bk6 onwards. Compared to the WT, *hp2* fruits exhibited significantly reduced ethylene emission rates, ACC content and ACO activity regardless the light treatment (Fig. 7A). In contrast, less marked differences in *Sl-ACS2* transcript abundance were observed between WT and *hp2* ripening fruits. Therefore, an overall down-regulation in fruit ethylene metabolism was triggered by the *hp2* mutation. Light exposure negatively impacted both ethylene emission and ACC content in both genotypes particularly from the Bk to Bk6 (Fig. 7B). A tendency of lower *Sl-ACS2* transcript abundance in light- rather than dark-incubated fruits was also observed. Collectively, the data obtained implicated light as a negative signal controlling climacteric ethylene production in tomato fruits.

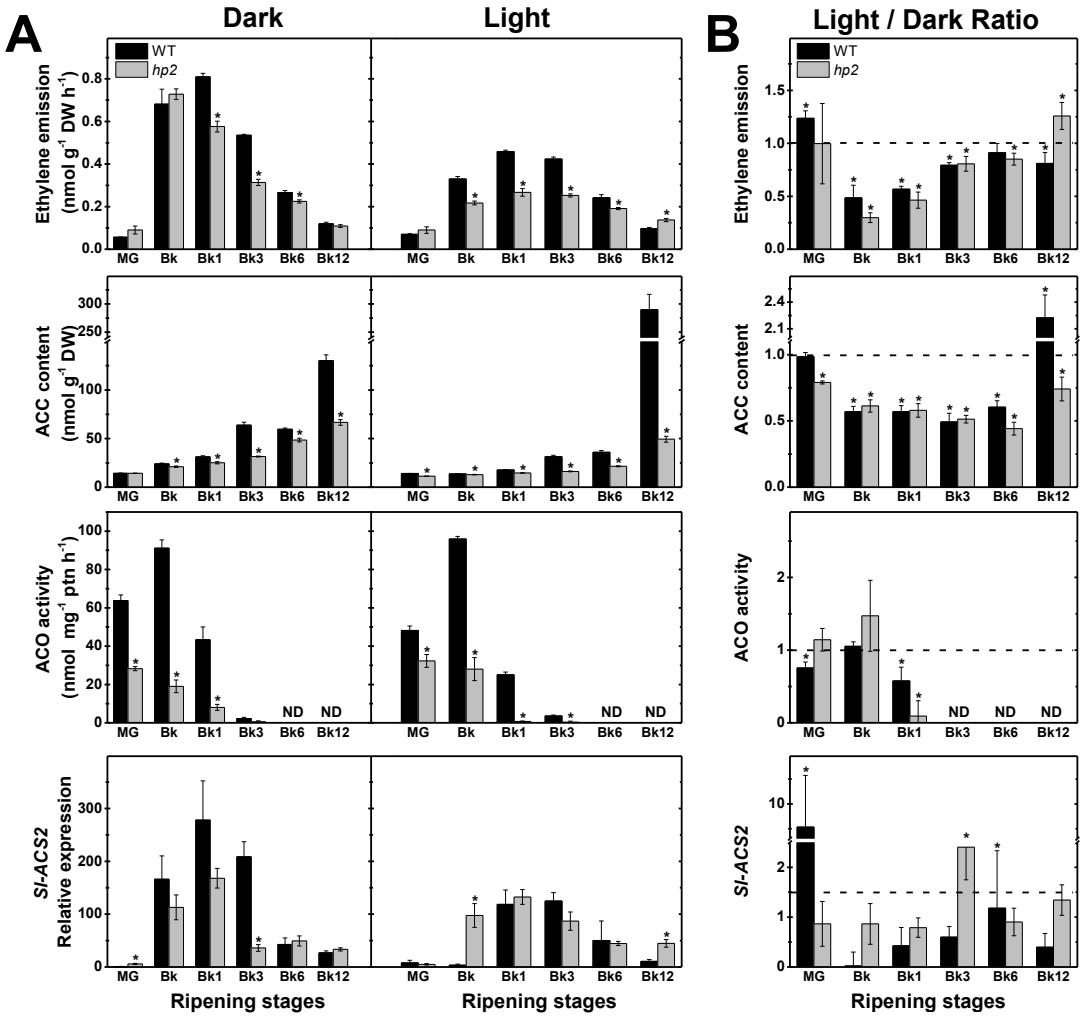

Figure 7. Light represses ethylene metabolism in ripening tomato fruits. Treatment details as described in Fig. 5. (**A**) Ethylene emission, ACC content, *in vitro* ACO activity and relative mRNA levels of *Sl-ACS2*. Mean *Sl-ACS2* relative expression was normalized against WT samples at MG stage under dark conditions. (**B**) Data expressed as the ratio between values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes (in **A**) or light treatments (in **B**) at each sampling time. The abbreviations indicate the following: WT, wild-type; *hp2*, *high pigment 2*; MG, mature green; Bk, breaker; ND, not detected; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC OXIDASE; *Sl-ACS2*, *ACC SYNTHASE2*.

Given the marked differences in ethylene metabolism observed between *hp2* and WT ripening fruits, potential light-triggered changes in the ethylene signaling output were also investigated by monitoring the activity of the reporter protein GUS expressed under the control of the *EBS* ethylene-responsive promoter in *EBS::GUS* and *hp2-EBS::GUS* genotypes. Interestingly, the loss of *Sl-DET1/HP2* function resulted in higher *EBS* promoter activation, and this phenomenon was clearly intensified by the presence of light (Fig. 8A). Moreover, transcript levels of *Sl-ERF.E4*, a critical repressor of tomato carotenogenesis (LEE et al., 2012), were significantly reduced in both dark- and light-incubated *hp2* fruits compared to the WT (Fig. 8B). Further analysis revealed that genes encoding ripening master controllers such as Sl-RIN, Sl-NOR and Sl-NAC4 were also up-regulated in the mutant compared to the WT

(Fig. 8C). The presence of PBE-box, G-box, CA-hybrid and/or CG-hybrid motifs within the 3-kb promoter sequence of *Sl-RIN*, *Sl-NOR* and *Sl-NAC4* (Fig. 9) is consistent with the potential transcriptional regulation of these genes directly by light-responsive transcription factors such as HY5 and PIF. Together, these data suggest that the loss of *Sl-DET1*/*HP2* function promotes genes encoding master regulators and ethylene signaling in tomato ripening fruits.

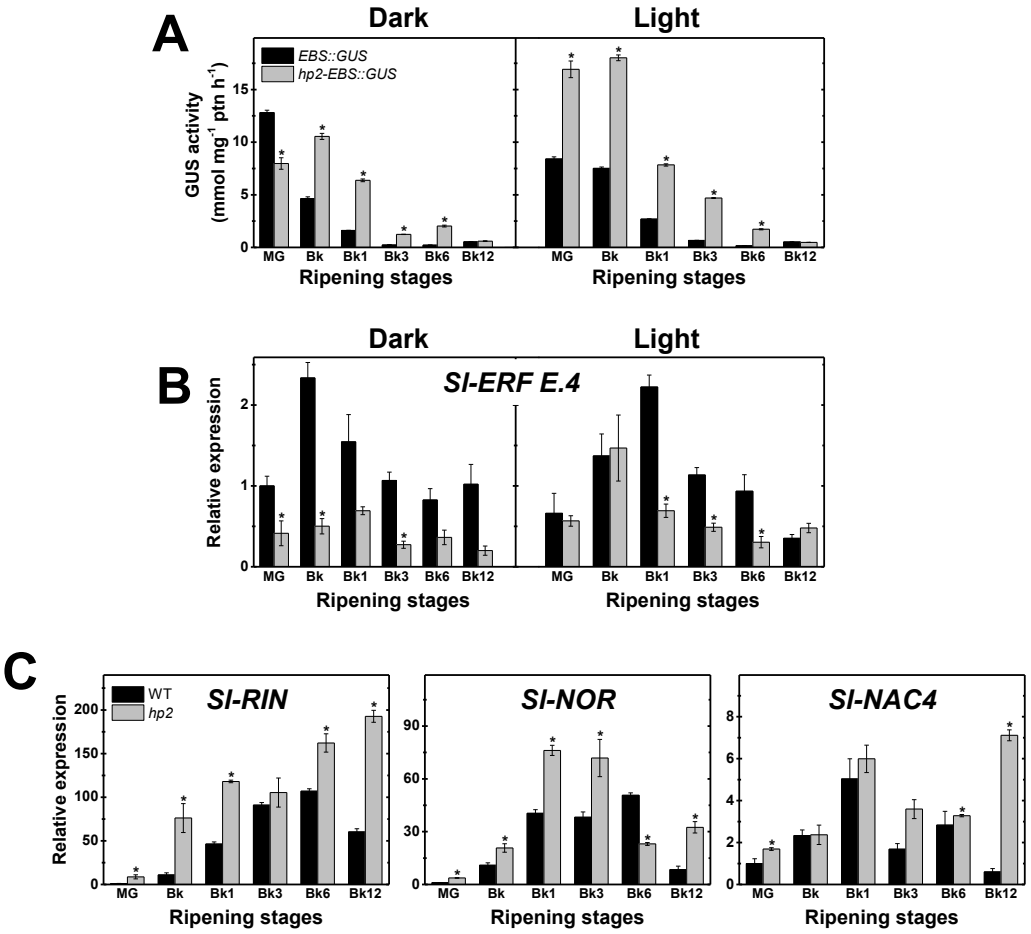

Figure 8. Loss of *Sl-DET1/HP2* function alters ethylene signaling output and induces genes encoding master controllers of ripening. Treatment details as described in Fig. 5. (**A**) *In vitro* GUS activity assayed in WT and *hp2* fruits carrying the ethylene-responsive promoter *EBS* fused to the GUS reporter protein (*DR5::GUS* and *hp2-EBS::GUS*). (**B**) Relative mRNA levels of *Sl-ERF.E4* in WT and *hp2* fruits ripening under dark or light conditions. (**C**) Relative mRNA levels of ripening-related genes in WT and *hp2* fruits ripening under light conditions. Mean relative expression was normalized against WT samples at MG stage. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes at each sampling time. The abbreviations indicate the following: WT, wild-type; *hp2*, *high pigment 2*; MG, mature green; Bk, breaker; *Sl-RIN*, *RIPENING INHIBITOR*; *Sl-NOR, NONRIPENING*; *Sl-NAC4, NAC (NAM/ATAF1/2/CUC2) 4.*

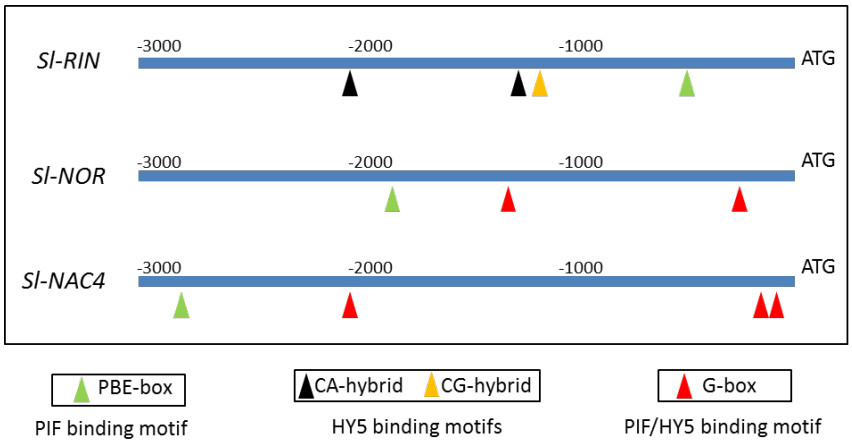

Figure 9. Binding motifs of known light-responsive transcription factors are abundantly found in the promoter regions of *Sl-RIN*, *Sl-NOR* and *Sl-NAC4* tomato genes. Fragments of 3 kb upstream ATG initiation site of these genes are represented by a blue line. Motif positions are indicated by triangles: PBE-box (green), recognized by PHYTOCHROME-INTERACTING FACTORs (PIFs). CA-hybrid (black) and CG-hybrid (yellow), recognized by LONG HYPOCOTYL 5 (HY5). G-box (red), recognized by both PIFs and HY5.

In concert with ethylene, auxin is also part of the regulatory network controlling tomato fruit ripening and carotenoid synthesis (GOMES, 2016; SU et al., 2015). To evaluate whether the carotenoid overaccumulation and disturbed ethylene metabolism and signaling found in *hp2* ripening fruits are associated with changes in auxin levels and signaling, we next compared the endogenous IAA content, the *DR5* promoter activation and the transcriptional profile of genes encoding auxin-related signaling elements in ripening fruits from *hp2* mutant plants in relation to their near isogenic counterparts.

Endogenous IAA levels were remarkably similar in WT and *hp2* ripening fruits (Fig. 10A). In contrast, the activity of the reporter protein GUS expressed under the control of the auxin-responsive *DR5* promoter was considerably higher in either light or dark-incubated fruits of *hp2-DR5::GUS* compared to the *DR5::GUS* (Fig. 10A). In both genotypes, a progressive reduction in auxin signaling output, as indicated by the *DR5* promoter activation, was observed during fruit ripening. The ripening-associated reduction in auxin signaling was significantly accelerated by light in *hp2* fruits (Fig. 10A); consequently, GUS activity driven by the *DR5* promoter in this mutant was found to be lower under light than under dark conditions (Fig. 10B). Auxin signaling output remained higher in *hp2-DR5::GUS* than in the *DR5::GUS* fruits from MG to Bk6 and from MG to Bk stage in dark- and light-incubated fruits, respectively.

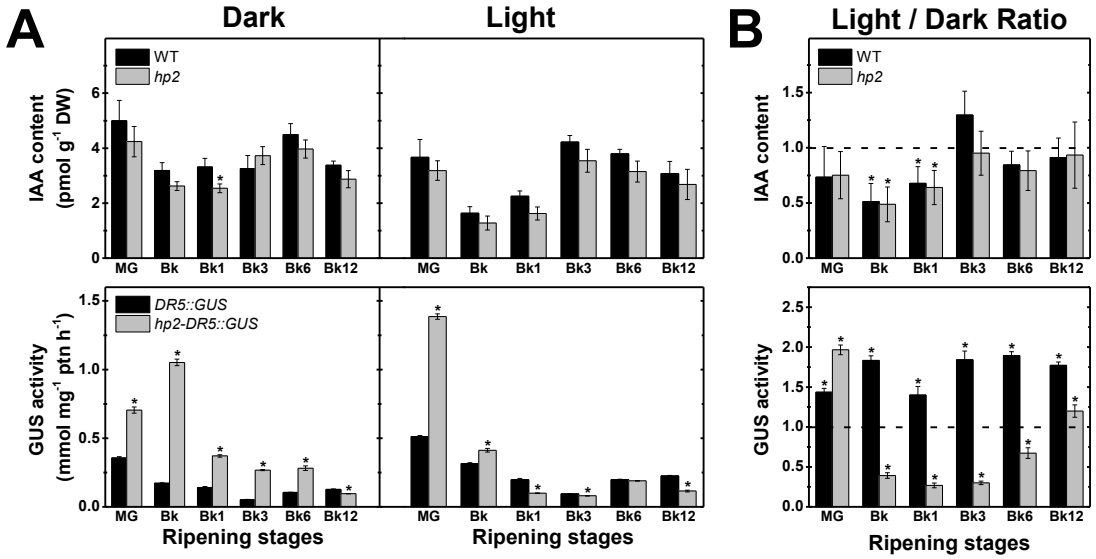

Figure 10. Light-hypersensitivity promotes auxin signaling output without altering endogenous IAA levels. Treatment details as described in Fig. 5. (**A**) Endogenous IAA levels in WT and *hp2* ripening fruits and *in vitro* GUS activity assayed in WT and *hp2* fruits carrying the auxin-responsive promoter *DR5* fused to the GUS reporter protein (*DR5::GUS* and *hp2-DR5::GUS*) (**B**) Data expressed as the ratio between values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes (in **A**) or light treatments (in **B**) at each sampling time. The abbreviations indicate the following: IAA, indole-3-acetic acid; WT, wild-type; *hp2*, *high pigment 2*; MG, mature green; Bk, breaker.

As the higher auxin signaling output detected in *hp2* fruits were not associated with differences in endogenous IAA content between the genotypes (Fig. 10), it seems plausible to suggest that *hp2* fruit tissues display an increased sensitivity to this hormone compared to the WT. Corroborating these findings, the *hp2* mutation was found to trigger marked changes in the transcriptional profile of genes encoding auxin-associated signaling proteins such as Aux/IAA and ARFs.

Among the five *Aux/IAA* tomato genes closely associated with fruit ripening *Sl-IAA3, Sl-IAA4, Sl-IAA9, Sl-IAA15* and *Sl-IAA27* (AUDRAN-DELALANDE et al., 2012), a dramatic reduction in *Sl-IAA4, Sl-IAA9* and *Sl-IAA27* mRNA levels in *hp2* compared to WT fruits was observed (Fig. 11A). *Sl-IAA3* and *Sl-IAA15* mRNA levels were also reduced in *hp2* compared to the WT at certain ripening stages (Fig. 11A), which indicates an overall down-regulation of *Sl-IAA* genes in fruits of this mutant.

In both genotypes, *Sl-IAA3* transcript abundance was significantly lower in light- than in dark-incubated fruits (Fig. 11B). The impact of light treatment on *Sl-IAA4, Sl-IAA9, Sl-IAA15* and *Sl-IAA27* mRNA levels was considerably more variable as these genes were either up- or down-regulated by light depending on the genotype and ripening stage considered (Fig. 11B).

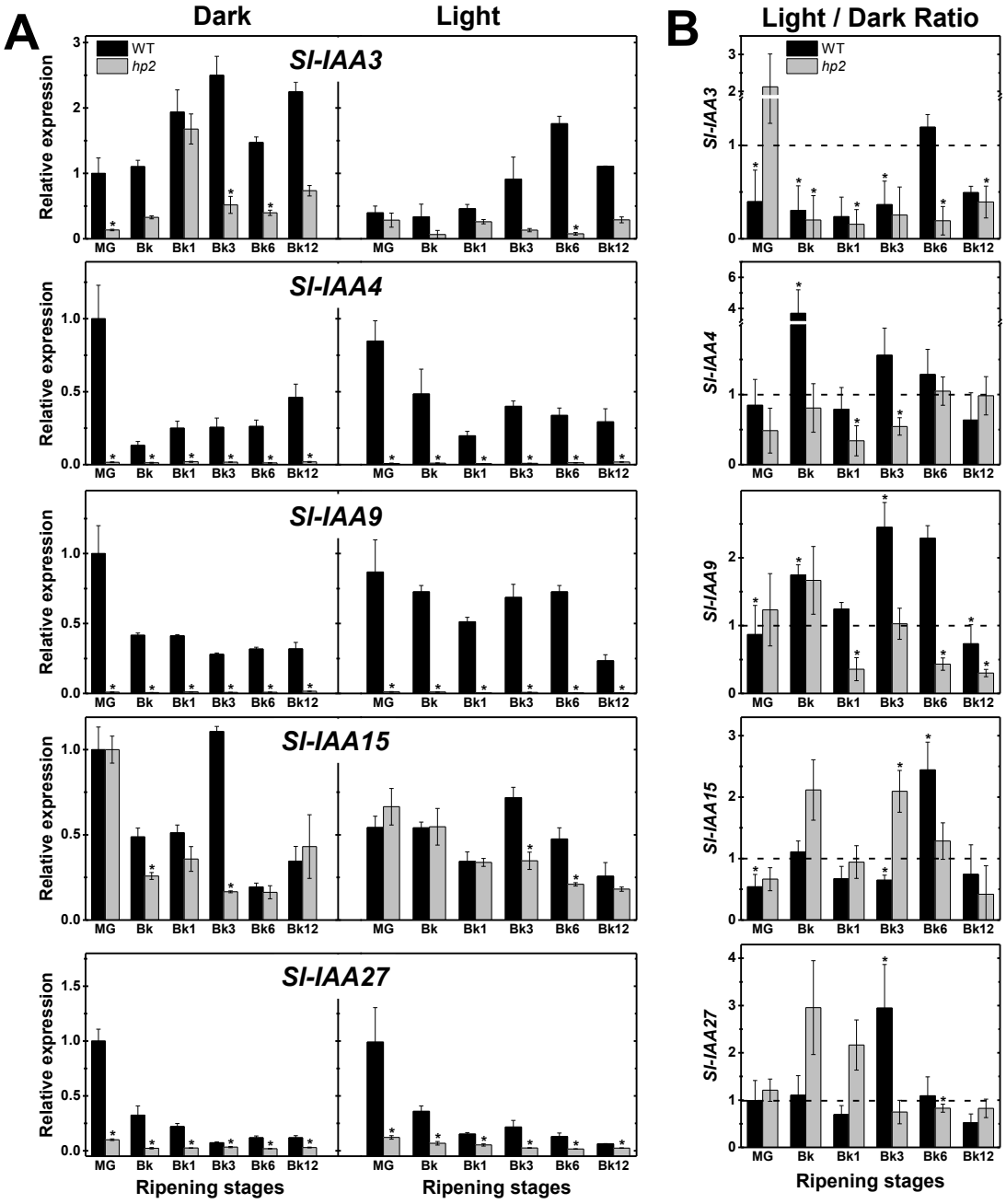

Figura 11. Light signaling represses ripening-related tomato *Aux/IAA* genes. Treatment details as described in Fig. 5. (**A**) Relative mRNA levels of *Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9*, *Sl-IAA15* and *Sl-IAA27*. Mean relative expression was normalized against WT samples at MG stage under dark conditions. (**B**) Data expressed as the ratio between qPCR values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes (in **A**) or light treatments (in **B**) at each sampling time. The abbreviations indicate the following: WT, wild-type; *hp2*, *high pigment 2*; MG, mature green; Bk, breaker; *Aux/IAA*, *AUXIN/INDOLE-3-ACETIC ACID*.

The marked impact of loss of *Sl-DET1*/*HP2* function on auxin signaling output and *Aux/IAA* mRNA levels (Figs. 10 and 11), prompted us to investigate whether changes in light signaling lead to significant alterations in the transcript abundance of seven *Sl-ARF* genes highly expressed in fruits, *i.e. Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4*, *Sl-ARF5*, *Sl-ARF8a* and *Sl-ARF8b*.

Transcript levels of *Sl-ARF2a* and *Sl-ARF2b*, which are considered key convergence points of auxin and ethylene signaling and important promoters of tomato fruit carotenogenesis and ripening (BREITEL et al., 2016; HAO et al., 2015), were higher in light-incubated *hp2* fruits compared to the WT counterparts (Fig. 12A). Similarly, mRNA levels of *Sl-ARF8b*, a known activator of auxin-dependent gene transcription (KUMAR; KHURANA; SHARMA, 2014), were considerably higher in *hp2* than in WT fruits; a response also exacerbated under light conditions. Conversely, transcript abundance of the repressor of auxin-dependent gene transcription *Sl-ARF3* (ZOUINE et al., 2014) was dramatically reduced in *hp2* than in WT fruits regardless of the light treatment (Fig. 12A). Dark-incubated *hp2* fruits also exhibited Sl-*ARF8a* mRNA levels significantly lower than WT fruits ripening under similar circumstances. Less noticeable impact of the loss of *Sl-DET1*/*HP2* function was observed on *Sl-ARF4* and *Sl-ARF5* transcript levels (Fig. 12A). Light treatment triggered increments of up to 3-fold in *Sl-ARF2a*, *Sl-ARF2b* and *Sl-ARF8b* mRNA levels and reductions of up to 70% in *Sl-ARF3* and *Sl-ARF5* transcript abundance during tomato fruit ripening (Fig. 12B). Overall, light-triggered impacts on these *ARF* genes were most prominently found in the *hp2* than in the WT fruits. Among all the light-triggered alterations in the transcriptional profile of *Sl-ARF* genes, it is important to highlight that *Sl-ARF3* mRNA levels were down-regulated in response to both light exposure and the loss of *Sl-DET1*/*HP2* function, the opposite being observed for *Sl-ARF2a*, *Sl-ARF2b* and *Sl-ARF8b*.

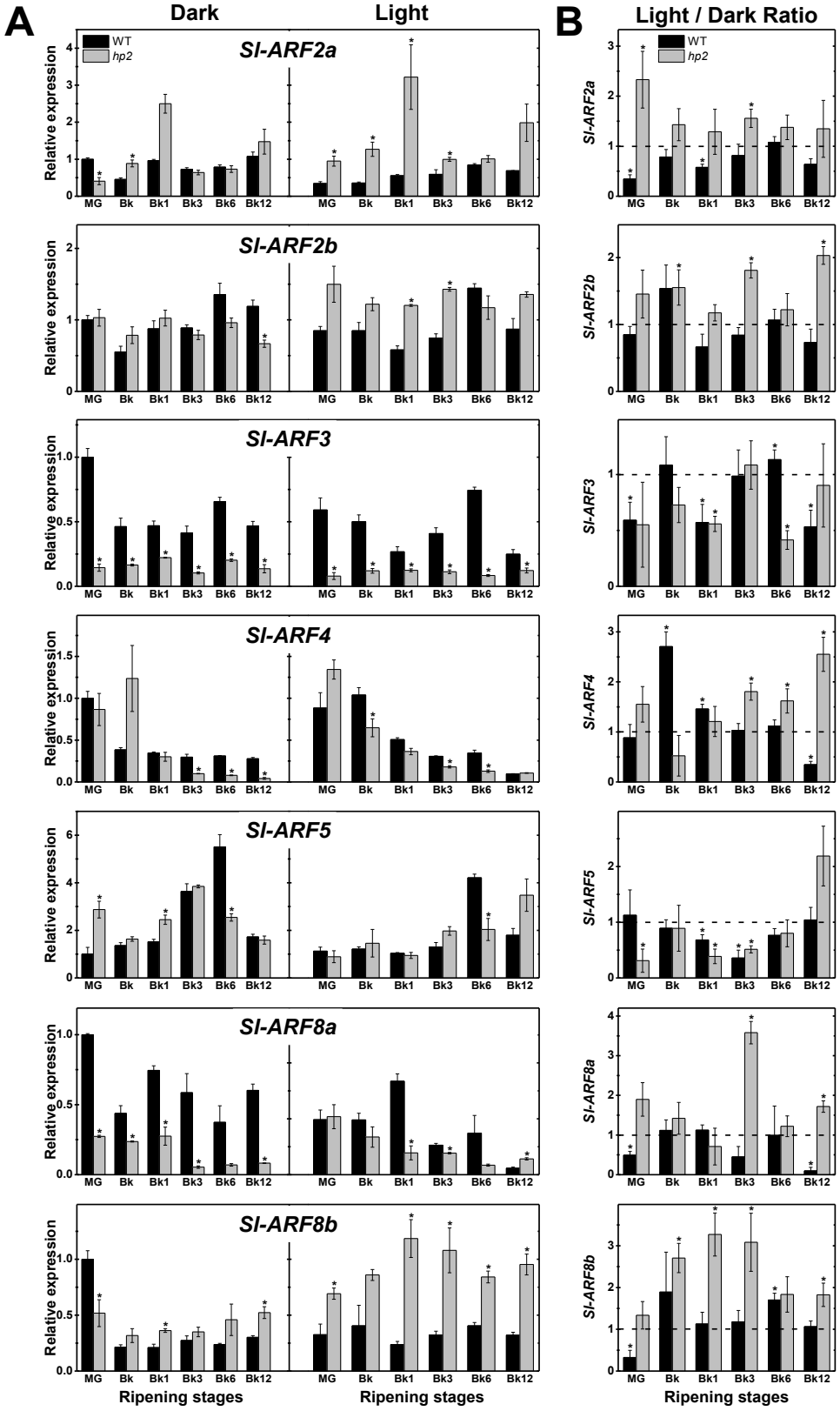

Figura 12. Tomato *ARF* genes are differentially expressed in ripening fruits of the *hp2* mutant. Treatment details as described in Fig. 5. (**A**) Relative mRNA levels of *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4*, *Sl-ARF5*, *Sl-ARF8a* and *Sl-ARF8b*. Mean relative expression was normalized against WT samples at MG stage under dark conditions. (**B**) Data expressed as the ratio between qPCR values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes (in **A**) or light treatments (in **B**) at each sampling time. The abbreviations indicate the following: WT, wild-type; *hp2*, *high pigment 2*; MG, mature green; Bk, breaker; *ARF*, *AUXIN RESPONSE FACTOR*.

4.2 Section 2

Phytochrome(PHY)-mediated light perception has been suggested to modulate tomato fruit ripening; however, whether and how PHY signaling crosstalk with ripening-related hormonal signals remain elusive. In this Section, the impact of PHY-mediated light perception on the metabolism and signaling of two key ripening-associated hormones, ethylene and auxins, was monitored in fruits from the wild-type (WT) and the phytochrome chromophore-deficient mutant *aurea* (*au*) ripening under constant darkness or white light.

Fruit ripening was initiated later in *au* than in WT fruits regardless of the light treatment (Fig. 13A). Whereas the time required for the transition from mature green (MG) to breaker (Bk) stage was exactely the same in *au* fruits incubated under either light or dark treatments, the ripening in WT fruits was initiated earlier in light than in dark conditions. The light-induced acceleration of ripening in WT fruits, as well as the delayed ripening phenotype of the *au* fruits, were confirmed by the late transcript accumulation of genes encoding master controllers of ripening (*i.e. Sl-RIN*, *Sl-NOR* and *Sl-NAC4*), which were maximum within the days after Bk in all cases (Fig. 13B). Interestingly, maximum transcript levels of *Sl-RIN* and *Sl-NOR* were up to 4-fold higher in *au* fruits than in the WT counterparts (Fig. 13B), which suggest a compensatory mechanism possibly linked to the delayed ripening phenotype caused by the deficiency in functional phytochromes.

In line with the pale-green phenotype of *au* fruits (MURAMOTO et al., 2005), the Chroma values, which indicates color saturation, were lower in *au* than in the WT fruits at early ripening stages (Fig. 13C). Ripening-associated changes in fruit color saturation were conspicuously delayed in the mutant compared to the WT fruits (Fig. 13C). Time course analysis of Hue angle further confirmed the accelerated progression in ripening-related fruit color change in WT compared to *au* fruits, being this difference particularly evident in the light treatment (Fig. 13C). Under light conditions, *au* fruits attained the red ripe stage approximately 5 days later in the WT genotype (Fig. 13C). In both genotypes, the final Hue angle values were lower in light- than dark-incubated fruits, indicating a light-triggered increase in the external red color pigmentation.

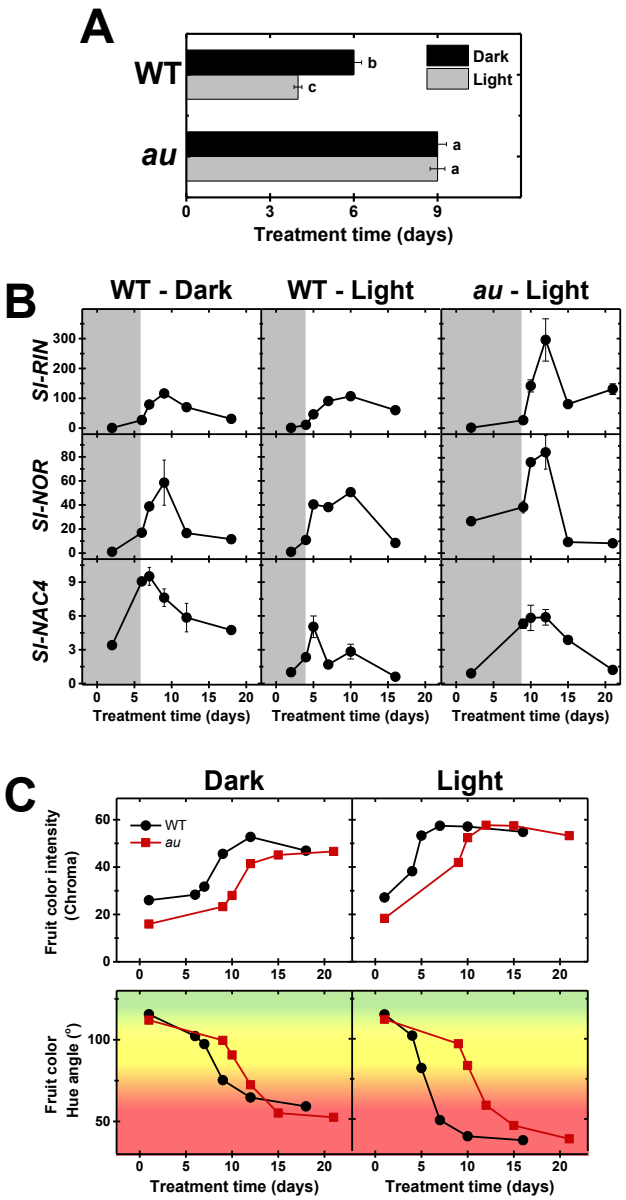

Figure 13. Deficiency in functional phytochromes delays tomato fruit ripening and color change. Fruits of wild-type (WT) and *aurea* (*au*) mutant were left to ripen under constant light or dark conditions. Samples were harvested at mature green (MG, two days after the beginning of the treatment), breaker (Bk), Bk1 (1 day after Bk), Bk3, Bk6 and Bk12 stages. (**A**) Time required for the transition from MG to Bk stage. (**B**) qRT-PCR analysis of ripening-associated genes. Mean relative expression was normalized against WT samples at mature green (MG) stage under light conditions. (**C**) Ripening-related changes in fruit color intensity (Chroma) and fruit color (Hue angle). Values shown are mean ± SE. In **A**, different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within genotypes and light treatments. The abbreviations indicate: *Sl-RIN, RIPENING INHIBITOR*; *Sl-NOR*, *NON RIPENING*; *SlNAC4*, *NAM/ATAF1/2/CUC2*.

To investigate whether the light-triggered temporal and quantitative differences in external fruit pigmentation were associated with differences in pericarp carotenoid composition, cyclized (β-carotene, lutein) and linear (lycopene and neurosporene) carotenoids were quantified (Fig. 14A). Regardless of the light treatment or genotype, lycopene was the carotenoid most abundantly accumulated at the end of the ripening process (Bk12), followed by β-carotene and subsequently neurosporene and lutein. Lutein levels were progressively

reduced soon after ripening started, remaining at low and stable levels thereafter. In contrast, lycopene, β-carotene and neurosporene were progressively accumulated from Bk stage onwards, achieving the highest levels when ripening was completed (Bk12). Compared to the WT, *au* fruits exhibited delayed carotenoid accumulation; a difference that was intensified in the presence of light.

Stage-based comparison between the genotypes during early ripening (*i.e.* MG to Bk3) revealed reduced lutein, β-carotene and total carotenoid levels in dark-treated *au* compared to WT counterparts (Fig. 14B). Among the carotenoid biosynthesis-related genes analyzed, mRNA levels of *Sl-PHYTOENE SYNTHASE1* (*Sl-PSY1*), *Sl-PSY2* and *Sl-PHYTOENE DESATURASE* (*Sl-PDS*), which encode enzymes associated with lycopene synthesis, were higher in *au* than in WT fruits at the early stages of ripening (Fig. 15). In contrast, both genotypes exhibited relatively similar transcript abundances of genes encoding enzymes related to the conversion of lycopene into cyclized carotenoids (*i.e. Sl-LYCβ* and *Sl-CYCβ*) (Fig. 15). Collectively, these data indicate the deficiency in functional PHYs not only significant delays carotenoid accumulation but may also inhibit lycopene cyclization leading to lower β-carotene and lutein accumulation in early ripening fruits.

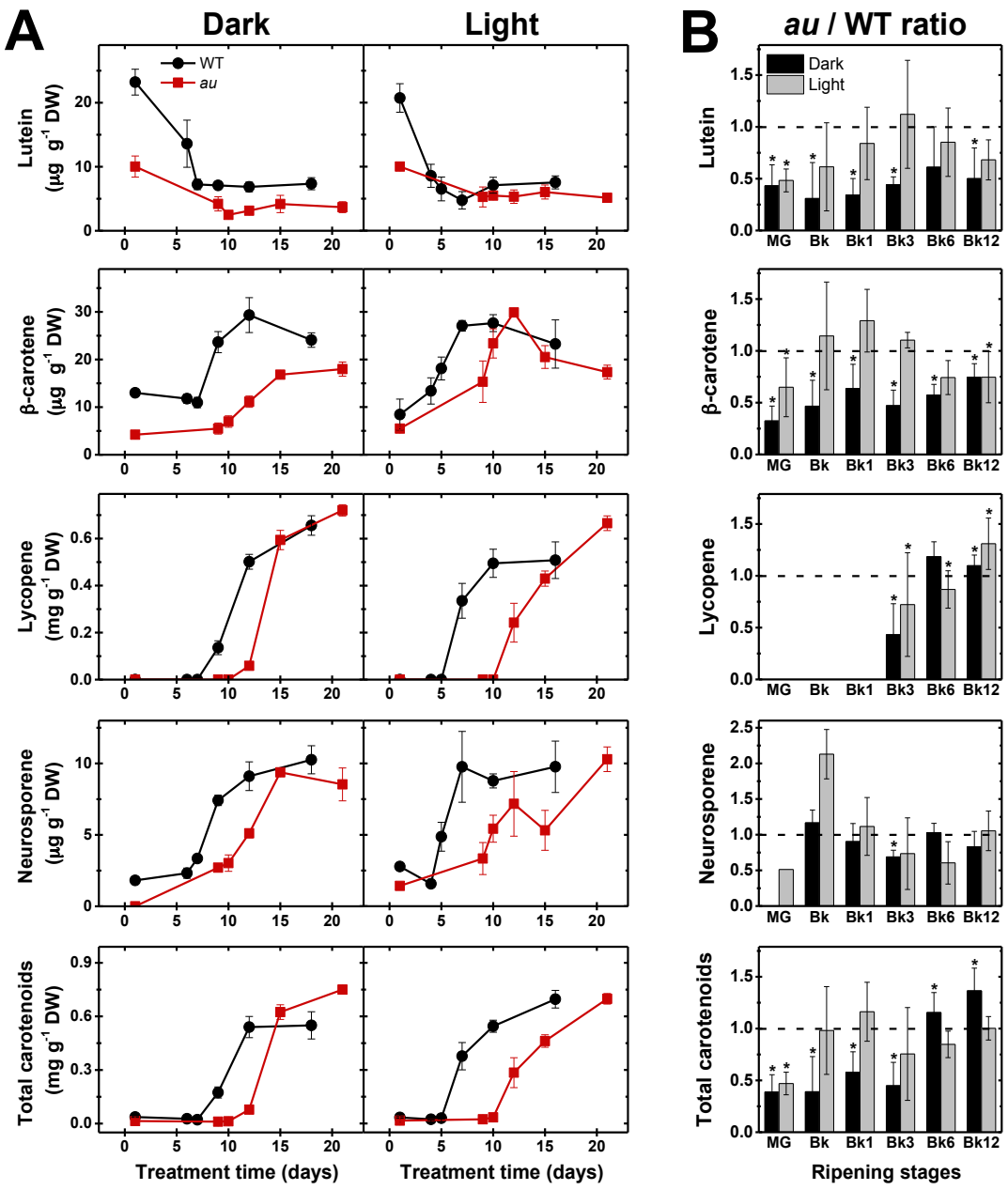

Figure 14. Phytochrome-dependent light perception accelerates tomato fruit carotenoid accumulation. Treatment details as described in Fig. 13. (**A**) Lutein, β-carotene, lycopene, neurosporene and total carotenoids content. (**B**) Stage-based comparison between genotypes, in which data are expressed as the ratio between WT and *au* values obtained in light and dark samples at each sampling time. Means ± SE. In **B**, asterisks represent significant (Student's t-test, $P<0.05$) differences among the genotypes at each sampling time. The abbreviations indicate the following: WT, wild-type; *au*, *aurea*; MG, mature green; Bk, breaker.

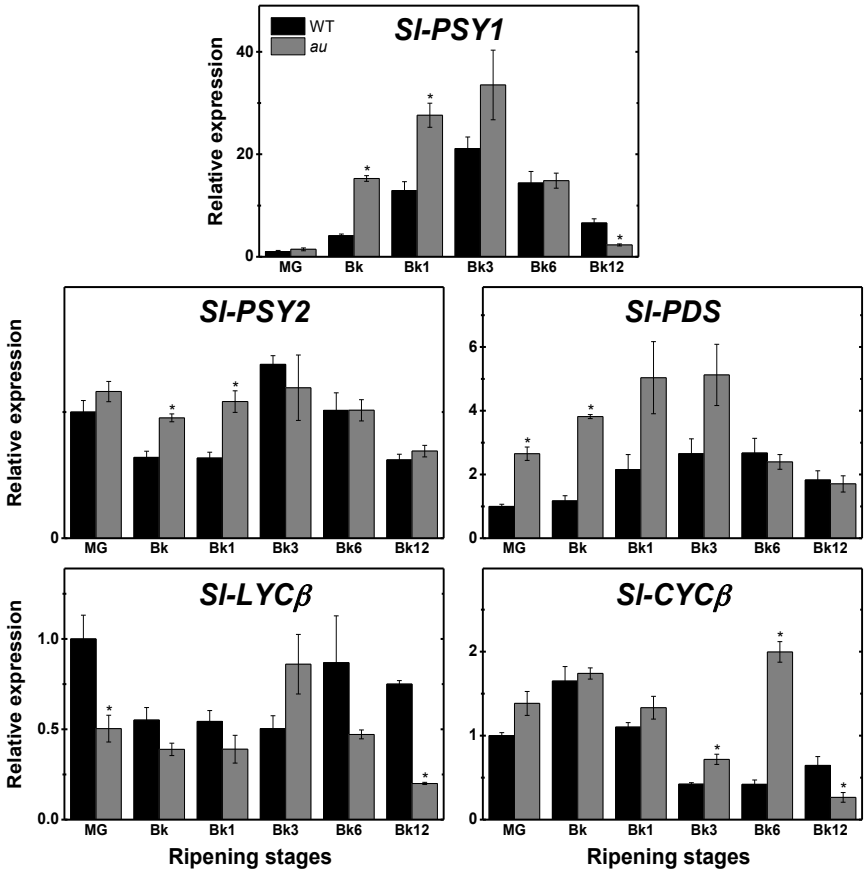

Figure 15. Impacts of the deficiency in functional phytochromes on the transcript abundance of carotenoid biosynthesis-related genes. Treatment details as described in Fig. 13. Relative mRNA levels of tomato genes encoding carotenoid biosynthetic enzymes in fruits ripening under light conditions. Mean relative expression was normalized against WT samples at MG stage. Values shown are mean ± SE. Asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes at each sampling time. The abbreviations indicate the following: WT, wild-type; *au*, *aurea*; Bk, breaker; MG, mature green; *Sl-PSY1*, *PHYTOENE SYNTHASE 1*; *Sl-PSY2*, *PHYTOENE SYNTHASE 2*; *Sl-PDS*, *PHYTOENE DESATURASE Sl-LYCβ*, *CHLOROPLAST-SPECIFIC β-LYCOPENE CYCLASE; Sl-CYCβ*, *CHROMOPLAST-SPECIFIC β-LYCOPENE CYCLASE*.

To investigate whether the delay in fruit color pigmentation and carotenoid accumulation caused by the deficiency in functional PHYs is associated with temporal and quantitive changes in ethylene metabolism, we next monitored the ethylene emission, ACC content, ACO activity and relative mRNA levels of *Sl-ACS2* in dark- and light-incubated *au* and WT ripening fruits. The climacteric rise in ethylene production was delayed in *au* fruits compared to the WT, and this difference was intensified in the presence of light (Fig. 16A). Maximum ethylene emission only marginally differed between *au* and WT fruits in the same treatment conditions, and light significantly reduced ethylene production in both genotypes (Fig. 16A). Dark- or light-incubated WT fruits gradually accumulated ACC after the climacteric peak of ethylene emission, whereas no marked changes in the content of this ethylene precursor were observed through the ripening of *au* fruits under either light treatment. Consequently, the stage-based comparison between the genotypes revealed

progressively lower ACC levels in *au* compared to the WT fruits throughout ripening. This difference between genotypes become evident earlier in the dark than in the light conditions (Fig. 16B).

The rise in ACO activity was not only delayed but also attenuated in *au* compared to the WT fruits ripening under similar light conditions (Fig. 16A and B). Overall, the highest activity levels of this enzyme were observed at the early ripening preceding the climacteric peak of ethylene emission and virtually disappearing from Bk3 stage onwards. *Sl-ACS2* transcript accumulation was also delayed in *au* fruits regardless of the light treatment, but the ripening-associated induction of this gene was more prominent in *au* than in WT fruits (Fig. 16A and B).

As these findings suggest that PHY-dependent light perception significantly influences ethylene metabolism, we next evaluated whether the deficiency in this photoreceptor also leads to changes in ethylene signaling output during fruit ripening. The activity of the reporter GUS expressed under the control of the *EBS* promoter in ripening fruits of *EBS::GUS* and *au-EBS::GUS* indicated that PHY deficiency results in the up-regulation of ethylene signaling output regardless the light treatment (Fig. 16). In both genotypes, the highest levels of *EBS* promoter activation were observed at the early ripening stages, thus coinciding with the maximum ethylene production in each genotype. Therefore, besides disturbing ethylene metabolism, the deficiency in functional phytochromes also alter the signaling output of this hormone in ripening fruits.

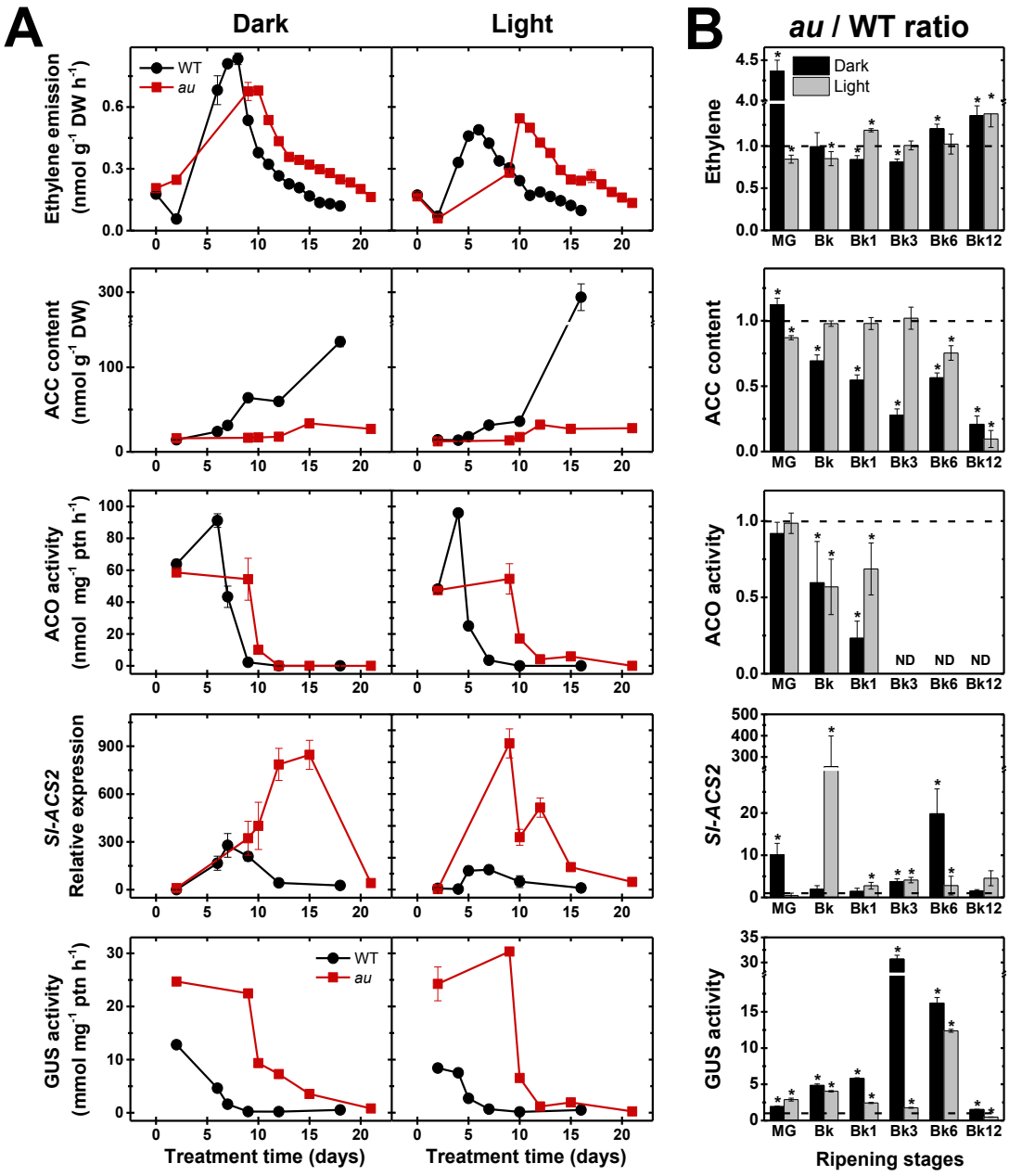

Figure 16. Phytochrome-dependent light perception influences ethylene metabolism and signaling during tomato fruit ripening. Treatment details as described in Fig. 13. (**A**) Ethylene emission rates, ACC content, *in vitro* ACO activity, *Sl-ACO2* mRNA levels in WT and *au* fruits and *in vitro* GUS activity in fruits carrying the ethylene-responsive promoter *EBS* fused to GUS reporter (*EBS::GUS* and *au-EBS::GUS*). Mean *Sl-ACO2* relative expression was normalized against WT samples at MG stage under dark conditions. (**B**) Stage-based comparison between genotypes, in which data are expressed as the ratio between WT (or *EBS::GUS*) and *au* (or *au-EBS::GUS*) values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. In **B**, asterisks represent significant (Student's t-test, $P<0.05$) differences between genotypes at each sampling time. The abbreviations indicate the following: WT, wild-type; *au*, *aurea*; MG, mature green; Bk, breaker; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC OXIDASE; *Sl-ACS2*, *ACC SYNTHASE2*.

Without minimizing the importance of ethylene, auxins have increasingly been implicated as key regulators of tomato fruit ripening (SU et al., 2015). To investigate whether this plant hormone is also involved in the delayed ripening phenotype detected in *au* fruits, we first compared the endogenous IAA content and the activation of the auxin-responsive

promoter *DR5::GUS* in ripening fruits of WT and phytochrome-deficient genotypes. Whereas IAA levels did not differ between the genotypes, the GUS activity in *au-DR5::GUS* fruits was higher and lower than in the *DR5::GUS* fruits ripening under dark and light conditions, respectively (Fig. 17). In both genotypes, a progressive reduction in auxin signaling output, as indicated by the GUS activity driven by the *DR5* promoter, was observed through fruit ripening.

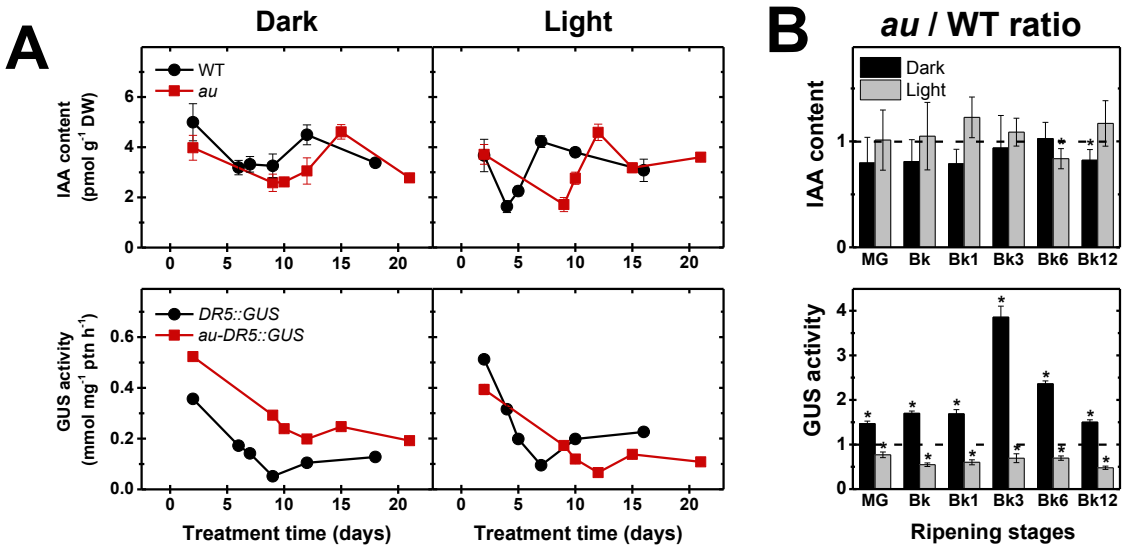

Figure 17. Phytochrome-dependent light perception influence on tomato fruit auxin signaling output is not associated with changes in endogenous IAA levels. Treatment details as described in Fig. 13. (**A**) Endogenous IAA levels in wild-type (WT) and *aurea* (*au*) ripening fruits and *in vitro* GUS activity assayed in WT and *au* fruits carrying the auxin-responsive promoter *DR5* fused to the GUS reporter protein (*DR5::GUS* and *au-DR5::GUS*). (**B**) Stage-based comparison between genotypes, in which data is expressed as the ratio between WT and *au* values obtained in light and dark samples at each sampling time. Means ± SE. In **B**, asterisks indicate statistically significant differences compared with the WT at each ripening stage (Student's t-test, $P<0.05$). The abbreviations indicate the following: WT, wild-type; *au*, *aurea*; IAA, indole-3-acetic acid; MG, mature green; Bk, breaker.

As the analysis of the *DR5* promoter activation suggest that PHY deficiency disturbs auxin signaling output, we also performed a transcript profile of key auxin signaling-related genes in the *au* and WT samples. First, the transcript abundance of five *Aux/IAA* tomato genes closely associated with fruit ripening (*i.e.*, *Sl-IAA3, Sl-IAA4, Sl-IAA9, Sl-IAA15* and *Sl-IAA27*) was determined, revealing that *Sl-IAA3, Sl-IAA4* and *Sl-IAA9* mRNA levels in *au* were less than 10% those observed in WT fruits (Fig 18). A general tendency of reduction in *Sl-IAA15* and *Sl-IAA27* mRNA levels was also detected in *au* compared to the WT fruits (Fig 18). In the WT, *Sl-IAA3* was the only *Aux/IAA* gene clearly induced through ripening as the *Sl-IAA4, Sl-IAA9, Sl-IAA15* and *Sl-IAA27* transcript levels were progressively reduced at early ripening stages, mostly from MG to Bk3. The ripening-related fluctuations in transcript abundances of

these *Aux/IAA* gene in WT fruits was only marginally influenced by light (Fig. 18B).

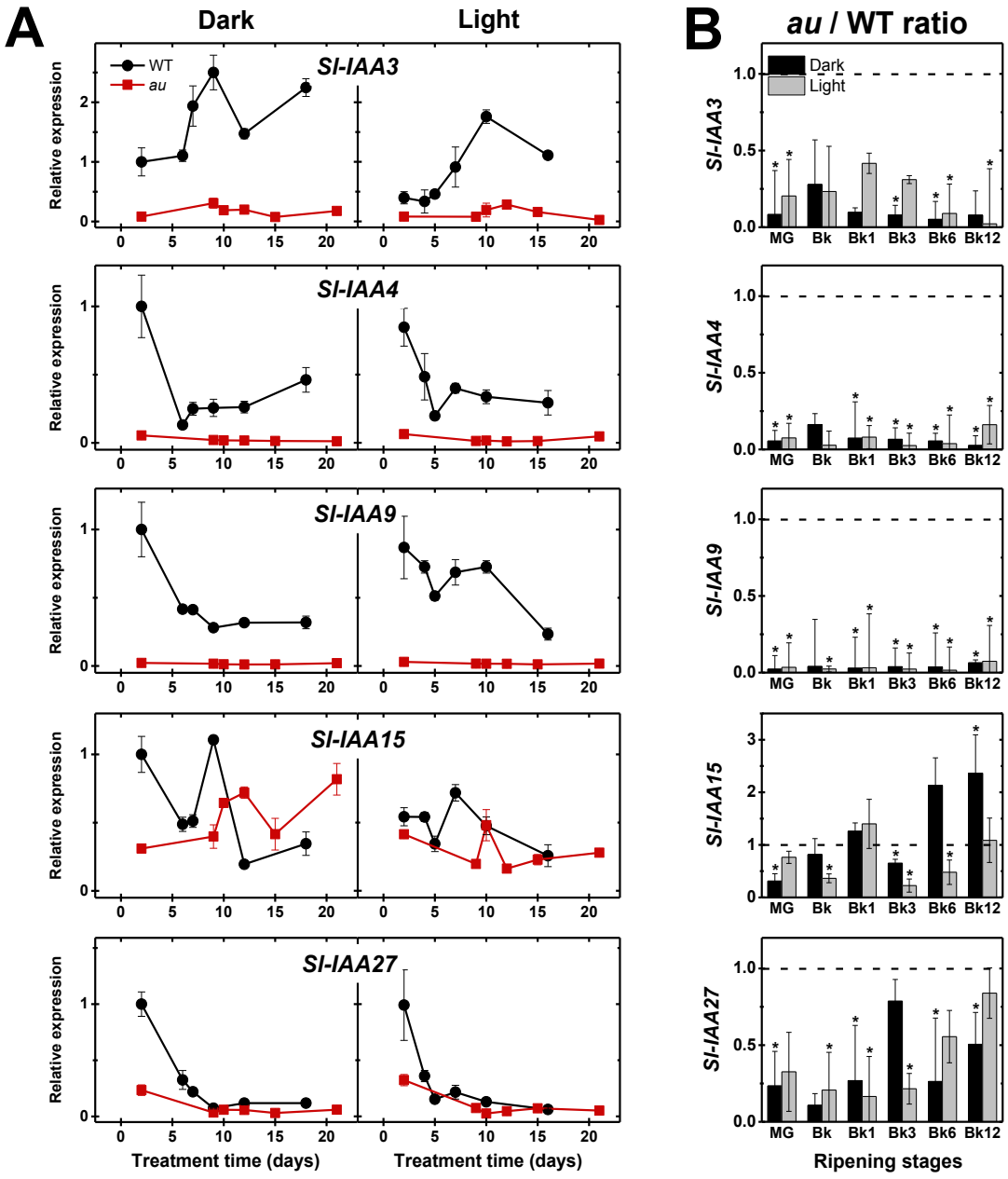

Figure 18. Deficiency in functional phytochromes down-regulates tomato *Aux/IAA* genes closely associated with fruit ripening. Treatment details as described in Fig. 13. (A) Relative mRNA levels *Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9*, *Sl-IAA15* and *Sl-IAA27*. Mean relative expression was normalized against WT samples at MG stage under dark conditions. (**B**) Stage-based comparison between genotypes, in which data are expressed as the ratio between WT and *au* values obtained in light and dark samples at each sampling time. Values shown are mean ± SE. In **B**, asterisks indicate statistically significant differences compared with the WT at each ripening stage (Student's t-test, $P<0.05$). The abbreviations indicate the following: WT, wild-type; *au*, *aurea*; *Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID*; MG, mature green; Bk, breaker.

To gain further insight on the potential PHY-auxin connection links during tomato fruit ripening, the transcript accumulation of seven *ARF* genes highly expressed in fruit tissues (*i.e. SlARF2a, SlARF2b, SlARF3, SlARF4, SlARF5, SlARF8a* and *SlARF8b*) was also determined. Among these, *SlARF2a, SlARF2b, SlARF4* and *SlARF5* were up-regulated whereas *SlARF3, SlARF8a* and *SlARF8b* were down-regulated in light-incubated *au* fruits compared to the WT counterparts (Fig. 19). The same general pattern was also observed in the dark treatment, with the exception of *SlARF2a*, whose transcript levels were similar in dark-incubated *au* and WT fruits.

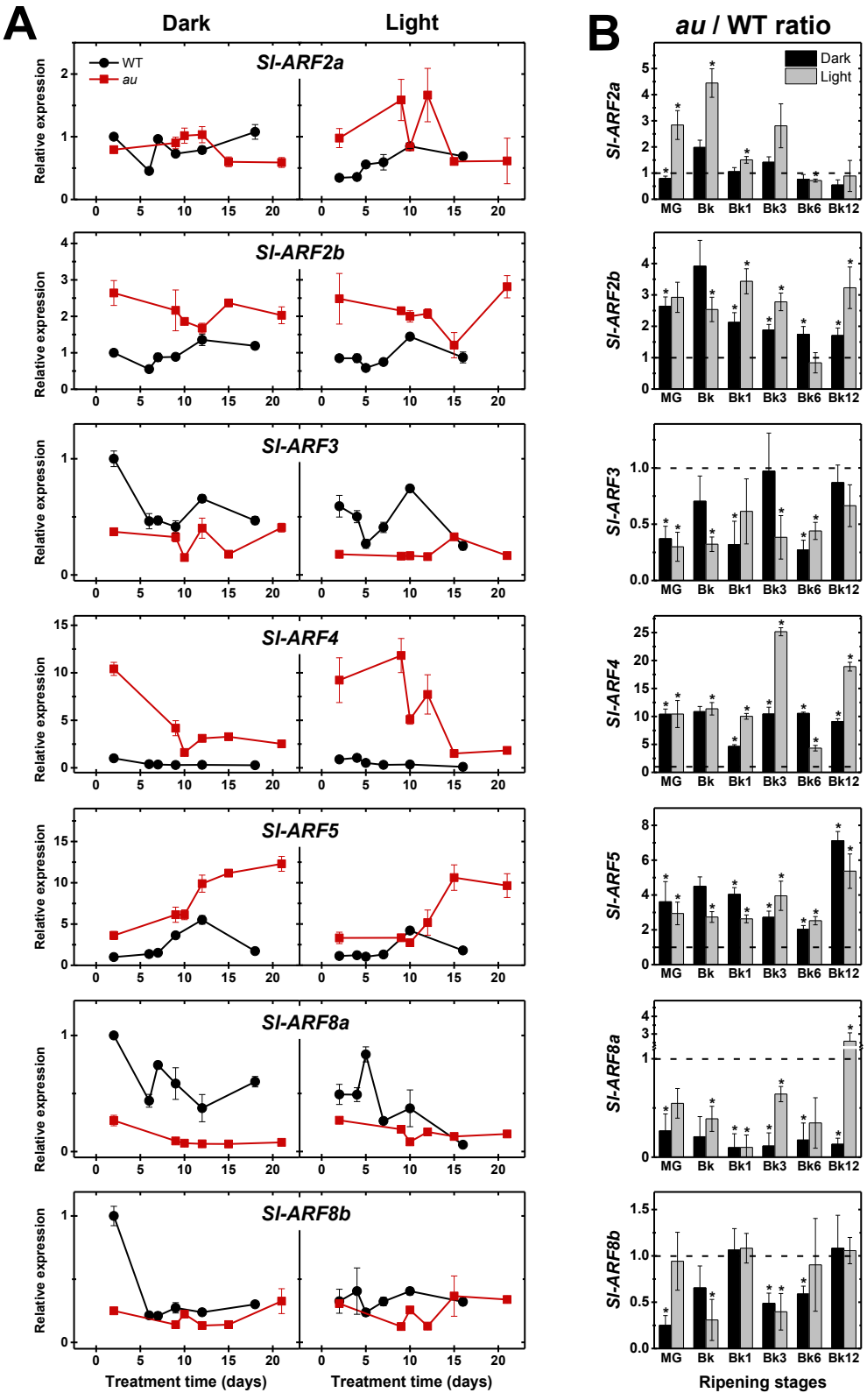

Figure 19. Tomato *ARF* genes are differentially expressed in phytochrome-deficient fruits. Treatment details as described in Fig. 13. (**A**) Relative mRNA levels of *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4*, *Sl-ARF5*, *Sl-ARF8a* and *Sl-ARF8b*. Mean relative expression was normalized against WT samples at MG stage under dark conditions. (**B**) Stage-based comparison between genotypes, in which data are expressed as the ratio between WT and *aurea* (*au*) values obtained in light and dark samples at each sampling time. Means ± SE. In **B**, asterisks indicate statistically significant differences compared with the WT at each ripening stage (Student's t-test, $P<0.05$). The abbreviations indicate the following: WT, wild-type; *au*, *aurea*; MG, mature green; Bk, breaker; *ARF*, *AUXIN RESPONSE FACTOR*.

5. Discussion

5.1 Section 1

Photomorphogenic mutants have been instrumental in elucidating the influence of light signaling on fruit biology and quality traits (AZARI et al., 2010b; LEVIN et al., 2006). In tomato, mutants carrying the monogenic recessive *high pigment* (*hp1* or *hp2*) have long been characterized by their exaggerated light responsiveness, over-accumulation of chlorophyll and chloroplasts in leaves and immature fruits and intense red fruit pigmentation (LEVIN et al., 2003, 2006; MUSTILLI et al., 1999). Compared to their WT counterparts, ripe fruits of these mutants display higher levels of antioxidants and other health-promoting substances, including carotenoids, flavonoids, tocopherol (vitamin E) and ascorbic acid (vitamin C) (FRASER; ENFISSI; BRAMLEY, 2009; LIU et al., 2004; SAINI; NILE; PARK, 2015). Fruit carotenogenesis is particularly up-regulated in *hp* mutants, which agrees with the positive influence of light on this metabolic route (ALBA; CORDONNIER-PRATT; PRATT, 2000; PIRINGER; HEINZE, 1954; SCHOFIELD; PALIYATH, 2005). Although light signaling and plant hormones, such as ethylene and auxins, are important regulators of tomato fruit carotenogenesis, whether and how the light and hormonal signaling cascades crosstalk to control this metabolic route remains poorly elucidated. In this Section, the potential involvement of ethylene and auxins in the light-mediated regulation of tomato fruit carotenogenesis was investigated by comparing the impact of light and dark treatments combined with the loss of *Sl-DET1*/*HP2* function on both carotenoid synthesis and hormonal signaling.

To the best of our knowledge, the impacts of *Sl-DET1*/*HP2* knockout or knockdown on tomato fruit carotenogenesis have been exclusively investigated in fruits ripening on-the-vine under greenhouse conditions (AZARI et al., 2010a; DAVULURI et al., 2004; ENFISSI et al., 2010; KOLOTILIN et al., 2007; SESTARI et al., 2014). However, after achieving the mature green stage, tomato fruits are also able to ripen off-the-vine, *i.e.* isolated from the plant, and this is a usual commercial practice in harvesting tomato fruit for human consumption (SORREQUIETA et al., 2013). Our findings indicate that loss of *Sl-DET1*/*HP2* function up-regulates all the major carotenoid-related genes, *i.e. Sl-PSY*, *Sl-PDS* and *Sl-LYCβ* and *Sl-LYCβ*, even when tomato ripening occurs separated from the plant (Fig. 6). Among these genes, *Sl-PSY* and *Sl-PDS* were the most highly differentially expressed between WT and *hp2* fruits, which is consistent with previous reports in *Sl-DET1/HP2*-deficient fruits ripening on-the-vine under greenhouse conditions (ENFISSI et al., 2010; KILAMBI et al.,

2013; KOLOTILIN et al., 2007) and implicates these carotenoid-related genes as important targets of light signal transduction. Findings obtained via the manipulation of other light signaling-related genes such as *Sl-DDB1*/*HP1*, *Sl-HY5* and *Sl-PIF1a* (LLORENTE, 2016; TOLEDO-ORTIZ et al., 2014; WANG et al., 2008) or via exposure of WT fruits to contrasting light conditions (TOLEDO-ORTIZ et al., 2014) further confirms the importance of the light-dependent regulation of *Sl-PSY* for tomato fruit carotenogenesis.

As dark-incubated *hp2* fruits exhibited carotenoid levels and lipophilic antioxidant capacity higher than dark- or even light-incubated WT fruits (Fig. 5), this mutation seems to represent a valid strategy to promote fruit nutritional quality even when the light stimulus is not present during ripening. In fact, lycopene over-accumulation in *hp2* was evident under dark rather than light conditions, suggesting that excessive light signaling generated by the combination of light-hypersensitivity and continuous light exposure may compromise the synthesis or accelerate the degradation of lycopene in tomato fruits. A possible explanation for this apparently contradictory finding is that light-induced generation of oxidative stress in the more abundant and developed chloroplasts and chromoplasts typically found in this mutant (EGEA et al., 2011; WANG et al., 2008; YEN et al., 1997) may have disturbed the delicate balance between light absorption and redox protection required for the synthesis and accumulation of lycopene in tomato fruits (COCALIADIS et al., 2014; LIU et al., 2015a). Nevertheless, other carotenoids, including lutein, neurosperene and particularly β-carotene, were considerably more accumulated in light-incubated *hp2* fruits compared to WT counterparts (Fig. 5), which is consistent with the increased mRNA levels of key carotenoid biosynthesis-related genes observed under these circumstances.

Assumptions that light-hormonal crosstalk may be involved in controlling tomato fruit ripening and carotenoid metabolism have been formulated for a long time in the literature (LIEBERMAN, 1979; YANG; HOFFMAN, 1984), albeit unequivocal genetic or physiological evidence supporting this hypothesis remained lacking. As a major regulator of numerous ripening-associated processes, ethylene was the first hormones suggested as part of the regulatory mechanisms behind the light-triggered up-regulation of tomato fruit carotenogenesis (ALBA; CORDONNIER-PRATT; PRATT, 2000). However, due to experimental limitations ranging from the lack of dark treatment controls (SCHOFIELD; PALIYATH, 2005) to excessive irradiation (JEN; NORRIS; WATADA, 1977), the involvement of ethylene as an intermediate in the light-promoted accumulation of fruit carotenoids remains controversial (ALBA; CORDONNIER-PRATT; PRATT, 2000).

In vegetative tissues, ethylene biosynthesis is highly regulated by light quality, intensity and duration. Overall, light perception via photoreceptors such as phytochromes and

cryptochromes acts inhibiting ethylene emission (CORBINEAU et al., 1995; GILIBERTO et al., 2005; MELO et al., 2016; PIERIK, 2004; VANDENBUSSCHE et al., 2003), ACC production (JIAO; YIP; YANG, 1987; MELO et al., 2016), ACO activity (MELO et al., 2016), *ACS* transcript levels (KHANNA et al., 2007) and ethylene signaling output (MELO et al., 2016). Our data revealed that the negative influence of light on ethylene metabolism typically found in vegetative tissues is also observed in ripening tomato fruits as indicated by the light-triggered reduction in ACC content, ACO activity and ethylene emission; a response that was further intensified in fruits of the light-hyperresponsive *hp2* mutant (Fig. 7). As the *Sl-ACS2* mRNA levels were clearly down-regulated by light exposure but only marginally affected by the *hp2* mutation, the potential impact of this mutation on the transcript abundance of other ripening-associated *ACS* and *ACO* tomato genes remains to be investigated.

As the marked reduction in climacteric ethylene production in *hp2* fruits was associated with significantly higher ethylene signaling output (as indicated by analysis of the *EBS* promoter) and this response was intensified by light exposure (Fig. 8), we are left to speculate that increments in light signaling promote tomato fruit tissue sensitivity to ethylene. The exact mechanisms behind the light-promoted increment in tomato fruit responsiveness to ethylene is not clear yet, but it might involve light-triggered changes in ethylene signaling-related components, as described in *Arabidopsis* vegetative tissues (GREFEN et al., 2008; ZDARSKA et al., 2015). In *Arabidopsis* seedlings, light exposure disturbs the expression of genes encoding ethylene receptors, *i.e. ETR2* and *ERS2* are down-regulated whereas *ETR1* and *EIN4* are induced by this environmental signal (GREFEN et al., 2008). Moreover, the EIN3 protein levels have been shown to be up- and down-regulated by COP1 and light exposure, respectively (ZHONG et al., 2009). Alterations in the abundance of ethylene receptors and downstream signaling transduction elements are supposed to alter the tissue sensitivity to this hormone. Therefore, evaluating the impact of *hp2* mutation on the transcript abundance of key ethylene signaling-related genes such as the *Sl-ETR* (*Sl-ETR1* to *Sl-ETR6*) as well as *Sl-EIN2* and *Sl-EIN3*-like genes (*Sl-EIL2* and *Sl-EIL3*) may shed more light on the mechanisms behind the increased ethylene sensitivity observed in fruit tissues of this mutant. Supporting this assumption, the disturbed expression of *Sl-ETR*, *Sl-EIN2* and *Sl-EIL* genes is generally associated with the changes in fruit tissue sensitivity to ethylene as reveled by several tomato ripening-impaired mutants or transgenic lines (HAO et al., 2015; KEVANY; TAYLOR; KLEE, 2008; LANAHAN et al., 1994). Moreover, whether the increased ethylene signaling in *hp2* ripening fruits is linked to the downregulation of the production of this hormone production in this mutant via negative feedback regulation remains to be determined.

Besides altering ethylene signaling output, the *hp2* mutation also promoted the

transcription of genes encoding key regulators of ripening (*i.e. Sl-RIN*, *Sl-NOR* and *Sl-NAC4*), thus suggesting a regulatory role of DET1/HP-mediated light transduction on the abundance of these master controllers of tomato ripening. The presence of PBE-box, G-box, CA-hybrid and/or CG-hybrid motifs within the 3-kb promoter sequence of these genes (Fig. 9) supports the hypothesis that light signaling-related transcription factors (*i.e.* PIFs and HY5) may directly regulate components upstream ethylene in the signaling cascades controlling tomato fruit ripening. As RIN promotes carotenogenesis by directly interacting with the *Sl-PSY1* promoter (MARTEL et al., 2011), the up-regulation of this transcription factor in *hp2* ripening fruits is consistent with the increased expression of *Sl-PSY1* and over-accumulation of fruit carotenoids in this mutant (ENFISSI et al., 2010; KOLOTILIN et al., 2007). In this context, it is also worth mentioning that *Sl-ERF.E4* mRNA levels were severely reduced in *hp2* fruits and this ERF has been proposed as a central integrator of ethylene and carotenoid biosynthesis and repressor of carotenoid synthesis in tomato as *Sl-ERF.E4*-knockdown lines over-accumulate carotenoids compared to WT counterparts (LEE et al., 2012).

Tomato fruit carotenogenesis is undeniably regulated by ethylene-related signaling components, but other plant hormones have been increasingly implicated in controlling this metabolic pathway (KUMAR; KHURANA; SHARMA, 2014; LIU et al., 2015a). Auxins, for instance, have been demonstrated to counteract the promotive influence of ethylene on tomato fruit ripening and carotenogenesis (GOMES, 2016; PIRRELLO et al., 2012; SU et al., 2015). Here, we provide several lines of evidence indicating that the loss of *Sl-DET1*/*HP2* function promotes auxin responsiveness in fruit tissues via changes in the transcript abundance of auxin signaling-related genes. The increased activation of *DR5* promoter in *hp2* fruits was not associated with significant differences in the endogenous IAA levels between the mutant and WT genotypes but instead was accompanied by a clear down-regulation of the *Sl-IAA* genes most highly expressed in tomato fruits (*i.e. Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9* and *Sl-IAA27*). Accordingly, functional characterization studies have revealed that the down-regulation of *Sl-IAA3*, *Sl-IAA9* or *Sl-IAA27* disturbs auxin responsiveness in tomato plants. Whereas *Sl-IAA3* knockdown resulted in lower auxin sensitivity, *Sl-IAA9*- or *Sl-IAA*27-silenced lines exhibited increased auxin responsiveness (BASSA et al., 2012; CHAABOUNI et al., 2009; WANG et al., 2005). Therefore, the progressive reduction in *DR5* promoter activity observed from MG to Bk12 stage in either dark- or light-incubated fruits may be linked to the gradual increment in transcripts of the repressor of auxin responsiveness *Sl-IAA3* (CHAABOUNI et al., 2009), and the progressive reduction in transcripts of *Sl-IAA9* and *Sl-IAA*27, two promoters of tissue responsivity to auxins (BASSA et al., 2012; WANG et al., 2005).

Among the *Sl-IAA* genes differently expressed in *hp2* fruits, the additive effect of

light treatment and loss of *Sl-DET1*/*HP2* function was only observed for *Sl-IAA3*, as the *hp2*-triggered down-regulation of *Sl-IAA4*, *Sl-IAA9* and *Sl-IAA27* was as drastic in the dark as in the light condition (Fig. 11). Interestingly, *Sl-IAA3* has been suggested to represent a crossroad of auxin and ethylene signaling in tomato; *i.e. Sl-IAA3* expression is highly regulated by both these hormones and contrasting alterations in ethylene sensitivity are observed in *Sl-IAA3*-knockdown tomato seedlings grown under light or dark conditions (CHAABOUNI et al., 2009). Therefore, it seems tempting to speculate that the light-dependent regulation of this particular *Sl-IAA* gene may be implicated in the increased responsivity to ethylene observed in *hp2* fruits compared to the WT (Fig. 11).

Aux/IAA proteins are known to inhibit the activity of ARF, and ARFs can either act as transcriptional repressors or activators of auxin-responsive genes (ZOUINE et al., 2014). Therefore, changes in the abundances of ARF transcription factors also significantly impact plant tissue responsiveness to auxins (HAO et al., 2015; (SAGAR et al., 2013; ZOUINE et al., 2014). Accordingly, the increased auxin responsiveness observed in *hp2* fruits was associated with a marked down- and up-regulation of the repressor of auxin-dependent gene transcription *Sl-ARF3* and the activator of auxin responses *Sl-ARF8b*, respectively (Fig. 12). In both cases, the impact of the *hp2* mutation was intensified by light exposure, which suggests that the light-dependent transcriptional regulation of these two *ARF*s may be associated with the increased auxin responsiveness observed in *hp2* ripening fruits.

The up-regulation of *Sl-ARF2a* and *Sl-ARF2b* caused the loss of *Sl-DET1*/*HP2* function (Fig. 12) is also consistent with the proposed role suggested for these two *ARF*s on tomato fruit ripening and carotenogenesis (BREITEL et al., 2016; HAO et al., 2015). Both *Sl-ARF2* paralogs are known to cooperate in promoting the expression of master controllers of ripening such as *Sl-RIN*, *Sl-NOR* and *Sl-CNR*, stimulating ethylene biosynthesis and signaling and inducing fruit carotenogenesis (BREITEL et al., 2016; HAO et al., 2015). Therefore, the up-regulation of both *Sl-ARF2a* and *Sl-ARF2b* genes observed in light-incubated *hp2* fruits agrees with the higher expression of ripening-related (*e.g. Sl-RIN*, *Sl-NOR* and *Sl-NAC4*) and carotenogenesis-related genes (*e.g. Sl-PSY*, *Sl-PDS*, *Sl-LYCβ* and Sl-*LYCβ*) and increased ethylene signaling and carotenoid content detected in this light-hyperresponsive mutant.

Here, we put forward the hypothesis that light-triggered changes in auxin and ethylene signaling are involved in the positive influence of this environmental stimulus on tomato fruit carotenogenesis. In the proposed working model of light-hormonal crosstalk controlling tomato carotenogenesis (Fig. 20), the positive and negative influence of light on ethylene biosynthesis and signaling, respectively, are supported by both genetic (*i.e. hp2* mutation versus WT genotype) and physiological evidence (*i.e.* light versus dark treatment).

The hypothesis of light-triggered changes in auxin signaling is corroborated by the marked down-regulation of *Aux/IAA* tomato genes and significant changes in transcript abundance of many of the *ARF* genes in *hp2* fruits compared to WT counterparts. The two *ARF* genes most closely associated with tomato fruit ripening and carotenogenesis (*i.e. Sl-ARF2a* and *Sl-ARF2b*) and the genes encoding the master regulators of ripening Sl-RIN and Sl-NOR were up-regulated, whereas *Sl-ERF.E4*, a major repressor of tomato fruit carotenogenesis, was repressed in *hp2* fruits compared to the WT counterparts. All these changes in the central fruit ripening modules are consistent with the increased transcript abundance of carotenogenesis-related genes such as *Sl-PSY*, *Sl-PDS* and *Sl-LYCβ* and *Sl-CYCβ* and the over-accumulation of carotenoids typically observed in the *hp2* mutant.

The intriguing question of what determines the increased fruit tissue sensitivity to ethylene in the light-hypersensitive mutant *hp2* remains open. According to the current knowledge on ethylene perception and signaling in tomato ripening fruits (KLEE; GIOVANNONI, 2011; LANAHAN et al., 1994) and deetiolating *Arabidopsis* seedlings (TOLEDO-ORTIZ; HUQ; RODRÍGUEZ-CONCEPCIÓN, 2010), it seems tempting to propose that light-triggered changes in the abundance of ethylene receptors (*i.e.* ETRs) and signal transduction molecules (*i.e.* EIN and EILs) may be at least partially responsible for this differential hormonal responsiveness in the mutant.

Although the exact mechanisms behind the altered hormonal responses triggered by changes in light signaling in tomato fruits remain to be elucidated, the data obtained in this study provide clear evidence that an intricate crosstalk among light, ethylene and auxin signaling may be involved in controlling tomato fruit carotenogenesis. Therefore, these findings open up a window of opportunity for further improvement in tomato fruit carotenogenesis through the combined manipulation of auxin, ethylene and light signaling-related genes.

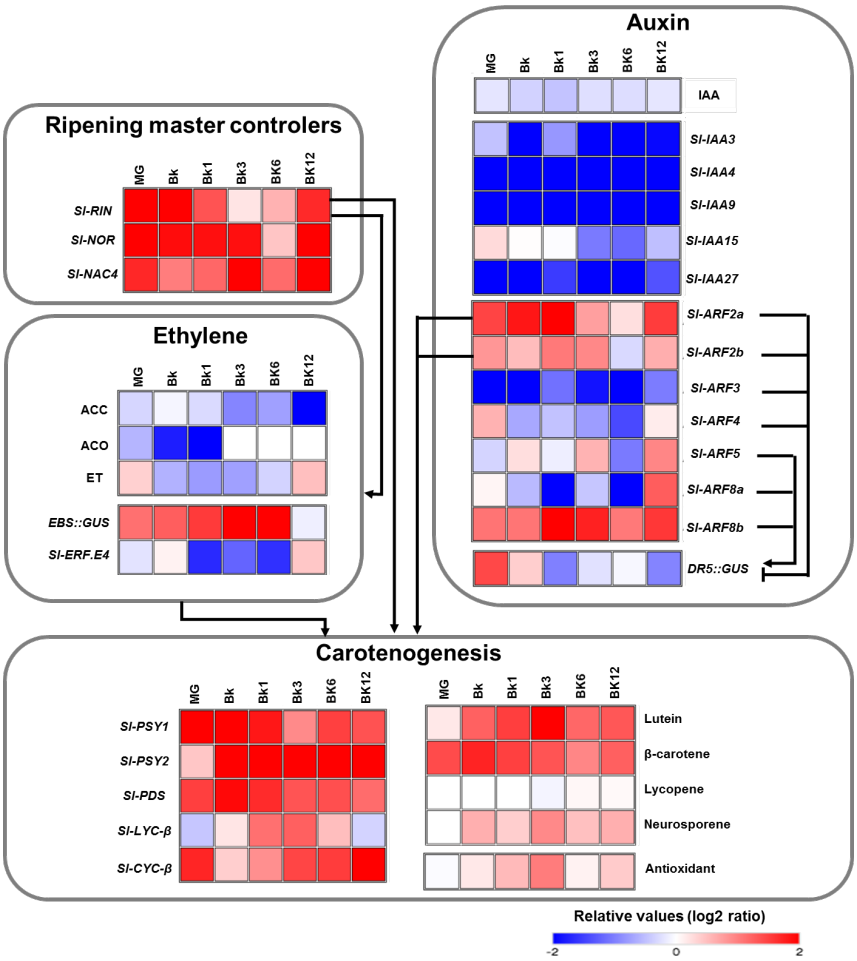

Figure 20. Heatmap representation of the light-triggered changes in auxin and ethylene metabolism and signaling and its impacts on carotenoid metabolism during tomato fruit ripening. In the light-hyperresponsive mutant *high pigment 2* (*hp2*), light exposure promotes significant increments in genes encoding master regulators of ripening (*i.e. Sl-RIN*, *Sl-NOR*, *Sl-NAC4*) as well as in the ethylene signaling output (as indicated by the activation of *EBS* promoter). Whereas light down-regulates ethylene metabolism and emission, an increased ethylene responsiveness is observed in light-ripened fruits. Light also down-regulates all *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes analyzed, promotes certain *AUXIN RESPONSE FACTOR* (*ARF*) genes such as *Sl-ARF2a*, *Sl-ARF2b* and *Sl-ARF8b* and represses others *Sl-ARF*s, including *Sl-ARF3*. All major carotenoid-related genes are up-regulated by light, which is consistent with the increment in carotenoid content and antioxidant activity and the up- and down-regulation of promoter (*i.e. Sl-RIN* and *Sl-ARF2a*, *Sl-ARF2b*) and repressor elements (*i.e. Sl-ERF.E4*) regulating tomato fruit carotenoid biosynthesis, respectively. Arrows at the end of lines indicate stimulatory effects, whereas bars indicate inhibitory effects. Transcript abundance data were expressed as the ratio between qPCR values obtained in light- and dark-incubated *hp2* fruits. The abbreviations indicate the following: MG, mature green (two days after the beginning of the treatment); Bk, breaker; Bk1, 1 day after Bk; *Sl-RIN*, *RIPENING INHIBITOR*; *Sl-NOR, NONRIPENING*; *Sl-NAC4, NAC (NAM/ATAF1/2/CUC2) 4,* ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC OXIDASE; *Sl-ACS2*, *ACC SYNTHASE2*; *EBS::GUS,* ethylene-responsive promoter *EBS* fused to the GUS reporter protein; *DR5::GUS,* auxin-responsive promoter *DR5* fused to the GUS reporter protein; *Sl-PSY1*, *PHYTOENE SYNTHASE 1*; *Sl-PSY2*, *PHYTOENE SYNTHASE 2*; *Sl-PDS*, *PHYTOENE DESATURASE Sl-LYCβ*, *CHLOROPLAST-SPECIFIC β-LYCOPENE CYCLASE; Sl-CYCβ*, *CHROMOPLAST-SPECIFIC β-LYCOPENE CYCLASE*.

5.2 Section 2

Although light perception and signal transduction have long been implicated as instrumental in regulating fruit biology and quality traits (GONG et al., 2015; PEACOCK, 1972), the mechanisms responsible for interconnecting light perception and the regulatory modules controlling fruit ripening remain poorly understood.

A recent study using the single phytochrome tomato mutants *phyA*, *phyB1* and *phyB2* as well as the combinations *phyAB1*, *phyB1B2* and *phyAB1B2*, revealed that *phyB1*, *phyAB1*, *phyB1B2* and *phyAB1B2* mutations promote ripening, with the triple mutant having the most accelerated fruit ripening phenotype compared to WT counterparts (GUPTA et al., 2014). Here, we demonstrate that the deficiency in the phytochrome chromophore leads to an opposite phenotype as fruits of the *au* mutant exhibited significantly delayed fruit ripening compared to the WT (Fig. 13). Alongside with the temporal differences in fruit pigmentation (Fig. 13C), the delayed ripening phenotype observed in *au* was confirmed by the late transcript accumulation of genes encoding master regulators of ripening such as *Sl-RIN*, *Sl-NOR* and *Sl-NAC4* (Fig. 13B), as well as by the delayed synthesis of ripening-related fruit carotenoids and late climacteric rise in ethylene production (Figs. 14 and 16). Before comparing our findings with those obtained by Gupta et al. (2014), some critical differences must be highlighted. First, the distinct nature and physiological consequences of single and multiple *phy* mutations in relation to the phytochrome chromophore-deficient mutants must be considered. Second, the study using the single and multiple *phy* mutants were conducted in the commercial tomato varieties MoneyMaker and GT, whereas the present study was performed using near-isogenic lines (NILs) in the Micro-Tom genetic background. Third, Gupta et al. (2014) monitored the ripening on-the-vine under greenhouse conditions and the data presented here was obtained in fruits ripening off-the-vine under strictly controlled light conditions. The implications of the first two differences highlighted here are difficult to estimate. However, the use of different ripening strategies seems unlikely to account for the opposite ripening phenotype observed between the *phyAB1B2* and *au* mutants since the delayed ripening phenotype was also confirmed when *au* fruits ripened attached to the plant under photoperiodic conditions (BIANCHETTI et al., submitted).

Further suggesting that PHY-mediated light perception promotes tomato fruit ripening, light treatment accelerated fruit ripening in the WT but not in the *au* mutant (Fig. 13A). This particular finding also reveals that blue light perception via other photoreceptors, *i.e.* cryptochromes and phototropins, cannot rescue the delayed ripening phenotype observed in the *au* mutant, which corroborates the lack of significant alterations in ripening detected in

Sl-CRY2-knockdown or overexpressing transgenic lines (GILIBERTO et al., 2005). The reasons behind the contrasting fruit ripening phenotypes caused by the deficiency in functional PHY due to the lack of the chromophore (Fig. 13) compared to that triggered by *phyB1*, *phyAB1*, *phyB1B2* and particularly *phyAB1B2* mutations (GUPTA et al., 2014) are far from elucidated. However, the presence of functional Sl-PHYE and Sl-PHYF in the *phyAB1B2* triple mutant and the deficiency in all phytochromes in the *au* mutant is a significant difference that must be taken into consideration when comparing these genotypes. Moreover, differently from single and multiple *phy* mutants, chromophore-deficient mutants such as *au* fail to produce functional holoproteins of all PHY types; therefore, they are not susceptible to the potential masking effects associated with the functional redundancies frequently found within members of the *PHY* family.

Although the first indication of the impact of PHY-mediated light perception on tomato fruit pigmentation dates back to 1954 (PIRINGER; HEINZE, 1954), the knowledge concerning PHY-dependent regulation of fruit carotenogenesis is still very limited (LIU et al., 2015a). Physiological evidence indicates that R, but not FR, radiation promotes lycopene accumulation in tomato fruits (ALBA; CORDONNIER-PRATT; PRATT, 2000; JEN; NORRIS; WATADA, 1977; KHUDAIRI; ARBOLEDA, 1971; THOMAS; JEN, 1975). Intriguingly, however, accumulating evidence also shows that light is not an essential requirement for the ripening-related accumulation of this particular carotenoid (ALBA; CORDONNIER-PRATT; PRATT, 2000; CHEUNG; MCNELLIS; PIEKOS, 1993; GUPTA et al., 2014). In line with this later assumption, β-carotene and lutein rather than lycopene levels were most prominently altered in ripening fruits of the *au* mutant.

As transcripts involved in lycopene synthesis (*i.e. Sl-PSY*s and *Sl-PDS*) rather than those related to the conversion of lycopene into cyclized carotenoids (*i.e. Sl-LYCβ* and *Sl-CYCβ*) were up-regulated in *au* during early fruit ripening (Fig. 15), it seems plausible to suggest that the accumulation of lycopene in *au* at levels as higher as those detected in the WT may be associated with a reduction in the cyclization of this carotenoid (LIU et al., 2015a). Genetic evidence obtained using *phy* mutants indicated that the conversion of lycopene to β-carotene is stimulated by PHYA, PHYB1 and PHYB2, and PHYA- and PHYB1-mediated light perception promotes and inhibits lycopene accumulation in tomato fruits, respectively (GUPTA et al., 2014). Therefore, both specificity and redundancy seem to occur in the PHY-mediated regulation of tomato fruit carotenogenesis. By affecting all PHY at once, the *au* mutation revealed that the deficiency in functional PHYs represses lycopene cyclization, thus leading to lower β-carotene and lutein accumulation in *au* at early ripening stages (Fig. 14).

Studies on the PHY-hormonal signaling crosstalk have largely focused on the vegetative development of tomato plants (CARVALHO; QUECINI; PERES, 2010; CHEN; XIONG, 2008; LIU; COHEN; GARDNER, 2011; MELO et al., 2016), overlooking its potential role in regulating fruit biology and quality traits. Here, we provide a series of evidence indicating that PHY-dependent light perception influences the metabolism of ethylene, which indisputably is the major hormonal signal controlling ripening in climacteric fruits. First, the climacteric rise in ethylene emission was shown to be delayed in *au* fruits, a response intensified by the presence of light (Fig. 16). This delay in the ethylene climacteric peak coincided with the late induction of genes encoding master controllers of ripening in *au* fruits (Fig. 13B). Second, ACC drastically accumulated in the WT fruit at late ripening stages probably as a consequence of the reduced conversion of this compound into ethylene, whereas in *au* fruits ACC remained at remarkably low levels through ripening. This finding combined with the low ACO activity and high *Sl-ACS2* mRNA levels detected in *au* suggest that ACC production may be restricted in this mutant. The drastically increased *Sl-ACS2* transcript abundance in *au* suggests a compensatory mechanism possibly linked to the reduced ACC levels in this mutant.

PHY-dependent light perception has been shown to down-regulation ethylene emission in plant vegetative tissues once PHY-deficient mutants usually display enhanced production of this plant hormone (CORBINEAU et al., 1995; PIERIK et al., 2004; VANDENBUSSCHE et al., 2003). Therefore, it seems tempting to speculate that in the absence of limitations in ACC production, the climacteric production of ethylene in the *au* fruits could be higher than in the WT. The restriction in ACC synthesis appears to represent a specific response of *au* fruits ripening off-the-vine as the accumulation of this molecule was virtually indistinguishable in *au* and WT fruits ripening on-the-vine under photoperiodic conditions (BIANCHETTI et al. submitted).

Interestingly, at red ripe stage, on-the-vine ripened fruits of the *phyB1*, *phyAB1* and *phyAB1B2* tomato mutants exhibited higher ethylene production than WT counterparts (GUPTA et al., 2014). However, as only MG and red ripe fruits were analyzed by Gupta et al (2014), it is impossible to predict whether the climacteric ethylene production is affected in these *phy* mutants. Monitoring ethylene metabolism and emission throughout the ripening of tomato *phy* mutants may be instrumental in shedding further light on PHY-ethylene interaction during fruit ripening. In *Arabidopsis* and sorghum, PHYB has been demonstrated to down-regulate ethylene levels in vegetative tissues (FINLAYSON; LEE; MORGAN, 1998; VANDENBUSSCHE et al., 2003). As tomato genome harbors two *PHYB* paralogs, which exhibit different expression patterns during tomato fruit ripening (BIANCHETTI et al.

submitted), we are left to speculate whether *Sl-PHYB1* and *Sl-PHYB2* may differentially contribute to the phytochrome-mediated regulation of ethylene metabolism in tomato fruits.

Analysis of the activation of EBS promoter suggested that PHY-dependent light perception negatively impacts ethylene-signaling output in ripening tomato fruits (Fig. 16). Compared to the WT, *au* fruits exhibited an increased ethylene signaling output, which together with the higher transcript levels of *Sl-RIN* and *Sl-NOR* in this mutant, suggest that ripening-related responses are intensified when ripening is initiated in *au* fruits. Whether the increment in ethylene signaling output observed in *au* relies on altered fruit tissue responsiveness to this hormone remains to be investigated. As PHY and ethylene signaling networks crosstalk in *Arabidopsis* seedlings via the cooperative action of PHYTOCHROME-INTERACTING FACTORs (PIFs) and ETHYLENE INSENSITIVE 3 (EIN3)/EIN3-LIKE transcription factors (JEONG et al., 2016); therefore, a similar mechanism may also occur during tomato fruit ripening.

Auxins counteract the promotive influence of ethylene in tomato fruit ripening (JONES et al., 2002; KUMAR; KHURANA; SHARMA, 2014; TRAINOTTI; TADIELLO; CASADORO, 2007). Moreover, auxin crosstalk with PHY signaling cascades at multiple levels (HALLIDAY; FANKHAUSER, 2003; HALLIDAY; MARTINEZ-GARCIA; JOSSE, 2009; MELO et al., 2016). For example, PHY-mediated light perception has been shown to regulate auxin biosynthesis, conjugation and catabolism in vegetative tissues, which ultimately leads to changes in the endogenous levels of this hormone. According to our findings, neither PHY- or light-mediate regulation of tomato fruit auxin metabolism seems likely. However, the auxin signaling output, estimated by the activation of the auxin-responsive *DR5* promoter, was significantly altered in ripening fruits of the *au* mutant (Fig 17). These results are consistent with the PHY-dependent alterations in auxin signaling described in vegetative tissues of other species (HALLIDAY; FANKHAUSER, 2003; SALISBURY et al., 2007). In *Arabidopsis*, for example, PHY-mediated light perception is proposed to down-regulate auxin signaling as these photoreceptors stabilize AUX/IAA proteins (CLUIS; MOUCHEL; HARDTKE, 2004; CÓLON-CARMONA et al., 2000), which in turn repress ARF proteins and consequently alter auxin signaling cascade (LI et al., 2016b).

Accordingly, the increased *DR5* promoter activation detected in *au* fruits was associated with a drastic reduction in the transcript abundance of most ripening-associated *Aux/IAA* tomato genes, except *Sl-IAA15*. Intriguingly, the depletion in *Sl-IAA3, Sl-IAA4, Sl-IAA9* and *Sl-IAA27* mRNA levels observed in *au* fruits was not dependent on the light conditions and was observed from the first to the last sampling time (Fig. 18). Consequently, it seems plausible to suggest that these genes may be differentially expressed between *au* and

WT fruits even before the start of the light treatments. Alternatively, the transcript abundance of these *Sl-IAA* may suffer rapid reduction particularly in *au* fruits within the first 48h of treatment. As previous reports indicate that *Sl-IAA3, Sl-IAA4, Sl-IAA9* and *Sl-IAA27* mRNA levels in *au* fruits ripening on-the-vine are actually higher than in the WT counterparts (BIANCHETTI et al. submitted), transcriptional profiling these *Aux/IAA* in samples harvested within the first 48h of off-the-vine ripening seems critical to increase our understanding regarding the regulation of these key auxin signaling-related genes.

Contrary to the depletion observed for all *Aux/IAA* genes in *au* fruits, both up- and down-regulation of *Sl-ARF* genes were found in this mutant compared to the WT (Fig. 19). *Sl-ARF2a* and *Sl-ARF2b*, which are known to cooperate in promoting tomato fruit ripening and carotenogenesis (HAO et al., 2015), were significantly more expressed in *au* compared to the WT fruits (Fig. 19). Between all *Sl-ARF* analyzed, *Sl-ARF2a* was the only gene differently expressed in *au* exclusively under light conditions. This ARF is known to act as a repressor of the *DR5* promoter (HAO et al., 2015); therefore, the higher expression *Sl-ARF2a* in *au* exclusively under light conditions may explain the reduced *DR5* promoter activity observed in light- but not in dark-incubated *au* fruits (Fig. 19).

Sl-ARF4 transcripts were conspicuously more abundant in *au* than in the WT ripening fruits (Fig. 19). This ARF is known to display multiple functions during fruit development and ripening such as controlling fruit chloroplast formation, sugar metabolism and accumulation, cell wall architecture, shelf-life as well as water loss during ripening (SAGAR et al., 2013). More recently, studies performed in columella tissues of early developing fruits also implicated *Sl-ARF4* as a PHY-auxin connection point during the regulation of sugar import, metabolism and accumulation in tomato fruits (BIANCHETTI et al. submitted).

As summarized in Fig. 21, the stage-based comparison between WT and *au* fruits revealed that light perception via PHY down-regulates *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF4*, *Sl-RIN*, *Sl-NOR* and *Sl-NAC4*, which are important promoters of tomato fruit ripening. In contrast, PHY-dependent light perception up-regulates all *Sl-IAA* genes most closely associated with tomato ripening (*i.e. Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9*, *Sl-IAA15* and *Sl-IAA27*) and certain *Sl-ARF* genes such as *Sl-ARF3*, *Sl-ARF8a* and *Sl-ARF8b*. The PHY-mediated changes in ethylene metabolism and auxin signaling-related genes were accompanied by significant alterations in ethylene and auxin signaling output, as indicated by the changes in the activation of the *EBS* and *DR5* promoters, respectively. Altogether, the data uncover the involvement of PHY in regulating the timing of tomato fruit ripening and provides the first set of evidence implicating PHY, auxin and ethylene crosstalk in the regulatory network responsible for controlling ripening in fleshy fruits.

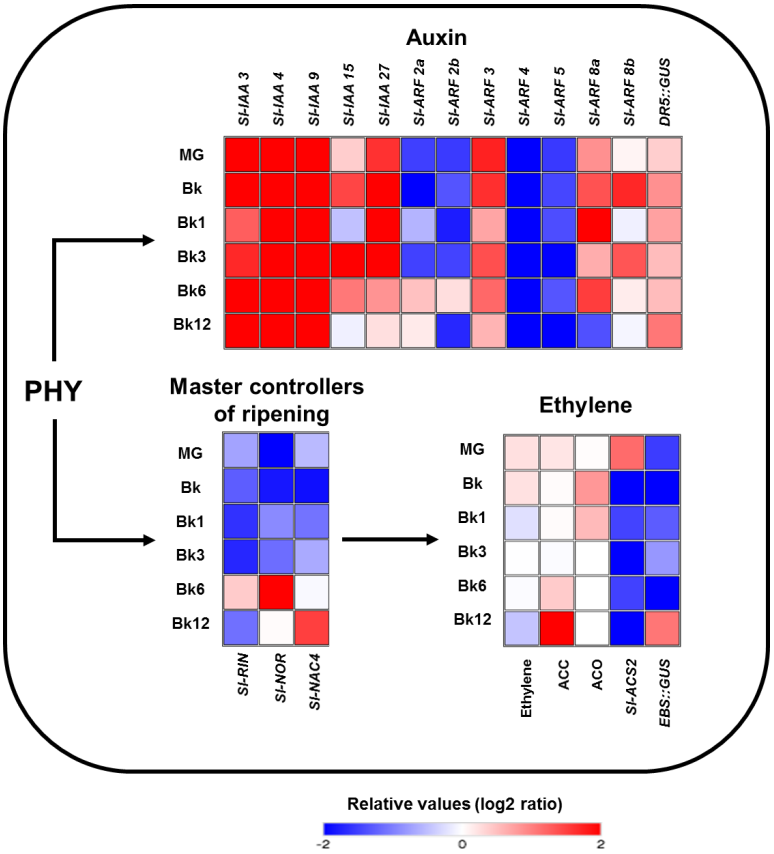

Figure 21. Heatmap representation of the phytochrome-dependent light perception impact on fruit ethylene metabolism and transcriptional profile of auxin signaling- and ripening-associated genes. Data were expressed as the ratio between values obtained in samples from wild-type and *aurea* at each ripening stage under light treatment. The abbreviations indicate the following: MG, mature green (two days after the beginning of the treatment); Bk, breaker; Bk1, 1 day after breaker; PHY, phytochromes; *Sl-IAA*, AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA); *Sl-ARF*, AUXIN RESPONSE FACTOR (ARF); *Sl-RIN*, RIPENING INHIBITOR; *Sl-NOR*, NONRIPENING; *Sl-NAC4*, NAC (NAM/ATAF1/2/CUC2) 4; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC OXIDASE; *Sl-ACS2*, *ACC SYNTHASE2*.

6. Conclusion and Future Remarks

Despite the extensive knowledge concerning the metabolic processes responsible for the synthesis and accumulation of phytonutrient in tomato fruits, how these metabolic routes are regulated by light and hormonal signals is comparatively less understood. In this Thesis, we provide a comprehensive set of genetic and physiological evidence indicating an intricate interplay among light, auxin and ethylene signaling during tomato fruit ripening and carotenogenesis.

Several lines of evidence discussed along this Thesis strongly suggest that the overaccumulation of carotenoids caused by the loss of *HP2* function is associated with multiple changes in the central signaling cascade controlling ripening, including the regulation of master regulators of ripening, ethylene metabolism and many auxin signaling-related components. Phytochrome-dependent light perception was also shown to critically regulate the timing of fruit ripening via coordinated temporal and quantitative changes in ethylene and auxin metabolism and signaling. Evidence of phytochrome-dependent impact on carotenoid metabolism was also obtained, providing further support for the relevance of this class of photoreceptors in determining tomato fruit quality traits. Taking into consideration, however, the inherent complexity of plant signaling mechanisms, this study also raises important questions for future investigation, such as:

What are the molecular mechanisms behind the altered tissue responsiveness to ethylene and auxins found in ripening fruits of the light-hypersensitive mutant hp2?

Answering this question will require further experiments dissecting the impact of the loss of *HP* function on the numerous components involved in ethylene and auxin signaling during tomato fruit ripening. Moreover, verifying whether other tomato light-hypersensitive mutants also exhibits altered hormone responsiveness may also help to clarify the molecular mechanisms behind this intriguing light-hormonal interaction.

Which are the tomato phytochrome genes more closely associated with the phytochrome-dependent changes in fruit hormonal signaling?

Overexpression and knockout/knockdown of distinct tomato phytochrome-encoding genes may be instrumental for answering this question. Dissecting which tomato *PHY* genes are more directly associated with the PHY-dependent changes in tomato fruit hormonal signaling may provide important targets for the future manipulation of light-dependent responses in this fruit crop species.

We hope the findings presented in this Thesis substantiate further advances in fruit improvement via intragenesis, synthetic biology and other biotechnological tools. The coordinated manipulation of auxin, ethylene and light signaling-related genes in tomato and other fruit crops seems to represent a promising venue for adjusting fruit ripening and quality traits.

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