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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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Botânica.

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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).

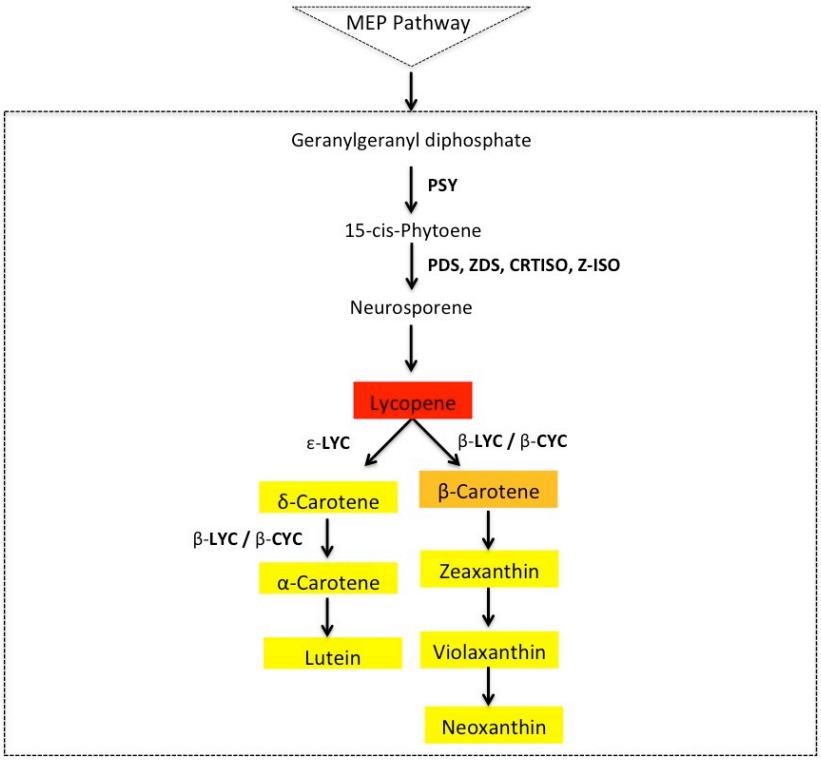


**Figure 1. Simplified schematic representation of the carotenoid biosynthetic pathway in plants**.

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).

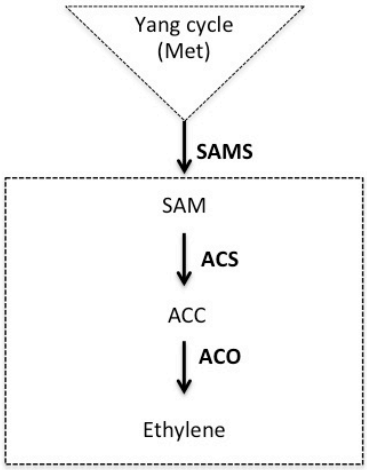


**Figure 2. Simplified schematic representation of ethylene biosynthetic pathway in plants.** The abbreviations indicate the following: Met, methionine; SAMS, S-ADENOSYLMETHIONINE; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC SYNTHASE; ACO, ACC OXIDASE.

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).

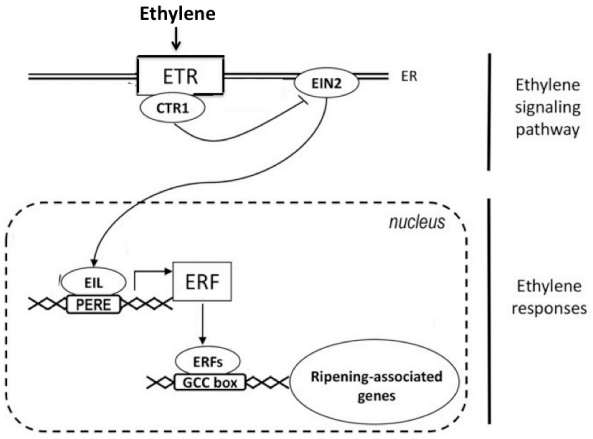


**Figure 3. Simplified schematic representation of ethylene perception and signaling transduction.** ETR, ETHYLENE RESPONSES; CTR1, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1); EIN2, ETHYLENE INSENSITIVE2; EIL, ETHYLENE INSENSITIVE 3-like; ERFs, ETHYLENE RESPONSIVE FACTORs (modified from LIU et al., 2015b).

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.

# 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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