Universidade de São Paulo
Instituto de Biociências

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Interações entre fitocromos e fitormônios em tomateiro: impactos na fisiologia e qualidade nutricional dos frutos

Phytochrome and phytohormone interplay in tomato: impacts on fruit physiology and quality traits

São Paulo

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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.
Orientador: Dr. Luciano Freschi
Co-orientadora: Dra. Magdalena Rossi

São Paulo
2017

Ficha Catalográfica

Bianchetti, Ricardo Ernesto

Interação entre fitocromos e fitormônios em tomateiro: impactos na fisiologia e qualidade nutricional dos frutos.

Número de páginas: 113

Tese (Doutorado em Ciências) – Programa de Pós-Graduação em Botânica, Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, 2017.

1. Sinalização luminosa. 2.Auxina. 3.Citocinina. 4.Cloroplasto. 5.Amido. Universidade de São Paulo. Instituto de Biociências. Departamento de Botânica.

Comissão Julgadora

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Prof. Dr. Luciano Freschi
Orientador

A quem faz do dia a dia o seu laboratório,
dedico

Agradecimentos

Tomo a palavra para expressar a imensa gratidão às pessoas que contribuiram com esta tese e com minha formação como pesquisador e como pessoa. Assim agradeço:

Ao Luciano Freschi, por confiar em mim tal trabalho, pela possibilidade de escolhas e sempre estar disponível. Por aceitar minhas ideias e por inserir as suas. Pela orientação, pela amizade e pelo incentivo. Por estar presente todos esses anos.

À Magdalena Rossi, pela orientação e amizade. Pelas inúmeras conversas que nos abrem a mente. Pelas intervenções metodológicas, cientítificas e teóricas, sem as quais não seria possível realizar este trabalho.

Ao Lázaro Peres, pelo suporte para obter as plantas mutantes e pelas sugestões que tornaram o Capítulo 1 possível. Ao Diego Demarco pela colaboração na parte estrutural (e pela paciência com um fisiologista "mão-de-ogro" para fazer bons cortes no material vegetal). Ao Eduardo Purgatto, por ceder seu laboratório e desenvolver a análise de açúcares (vital aos dois capítulos). Ao Christophe Rothan, por ceder os vetores que tornou possível a obtenção das linhagens transgênicas, além das valiosas contribuições conceituais para o Capítulo 2.

À Bruna Oliveira, por ter aceitado ser IC neste projeto e por ter me confiado várias questões metodológicas e teóricas. À Aline Bertinatto pela valiosa colaboração nas análises hormonais

presentes no Capítulo 1, ainda pelo apoio técnico inigualável que facilitou e permitiu a realização de todas as metodologias desta tese. Ainda aproveito para estender o agradecimento ao William, Aline Coelho, Camila, Silvia e Ana Maria, pela disponibilidade e apoio técnico, tornarndo a realização dos experimentos possível.

À Helenice Mercier e ao Gilberto Kerbauy pela genorisade e contribuições ao longo dos anos. Ainda ao Gilberto, ao Igor Cesarino e ao Norberto Palacios pelas resenhas de futebol sempre após as rodadas do Brasileirão!!!!

Aos tomateiros da fisiologia vegetal Rafael e Fred, pela troca de ideias, de experiências e pela amizade, que contribuiram muito para esta tese. À Dani Rosado, por toda amizade além da bancada, por sempre estar presente para ouvir, pela troca de expirências e pelas dicas. À Priscila Bittencourt, pelas longas conversas, amizade e confiança, à Paula Natália pela amizade e por estar presente sempre que se fez necessário, ao Perdigão, pela paciência para auxiliar nos "retratos" das plantas e dos frutos e pela inspiradora filosofia de vida, à Paula Elbl, pela lúcidez e pelos conselhos. Ademais, oos valiosos amigos do Laboratório de Fisiologia do Desenvolvimento Vegetal e Genética Molecular de Plantas: Alejandra, Alessandra, Ana Z, Antônio, Auri, Bruno Gobara, Bruno Lira, Carol, Dioceni, Filipe, Giovana, Nielda, Paulo, Renata, Valéria, que fizeram da convivência à melhor possível.

Ao Paulo Peixoto, por ter aberto as portas para os primeiros passos na Fisiologia Vegetal na UFJF.

Aos amigos lá de Minas Gerais, que, assim como eu, já estão perdidos mundo afora. Lívia, Paula, Elisa, Ítala, Samantha, Fernanda, Tamiry, Cintinha e Mary que da sala de aula, dos churrascos e bares fizeram uma amizade sólida com quem tenho oportunidade de falar, rir e reclamar das canseiras da vida diariamente. Ao Nícolas (*in memoriam*) por ser e estar presente ao longo da trajetória, pelo incentivo e pela amizade. Ao Guilherme (*in memoriam*), por ter acredito em mim antes mesmo de eu começar a acreditar. Ao Fred, que fez a rivalidade cômica-agressiva no futebol ser elevada ao nível de grande amizade com quem conto pra tudo e a qualquer momento (mas que me deve um whisky). À Luana pelas amizade e risadas. À Isadora pelas trocas de ideia madrugada afora, pela amizade e lucidez. Ao Andrey por toda amizade mesmo que apareça só quando passa o cometa halley. Ao Felipe Rust por ser o responsável por dar os "choques de realidade", pelo apoio e pela amizade.

Aos amigos da GaloSampa, que trouxeram a torcida do Galo para São Paulo, como não é

só futebol, junto trouxeram um pedaço de Minas Gerais para território paulista, o que já é muito. Aos caras do Pokerstars, por de dentro da Galosampa fazer bate-papo, zuação e boêmia nas noites de sexta. À Paulinha Rangel por toda amizade, preocupação e consideração, além dos papos cabeça e das boas músicas. Ao Henrique, por ter encarado o desafio de dividir a casa comigo no fim do doutorado, pelas demonstrações de amizade, companheirismo e confiança. Ao Rapha, pelas dicas, companheirismo e amizade, por estar presente sempre independente da situação, por toda confiança e por ser testemunha das histórias mais legendárias que tenho cá em SP.

Aos familiares (tios, tias, avôs e cunhado – são muitos para citar!!!!), que mesmo sem entender direito sempre estiveram presente e apoiaram essa tragetória. À minha avó Terezinha (*in memoriam*) pela dedicação na minha criação. À minha irmã, Jesiani, pelo apoio, torcida, companherismo e amizade, por ter me dado de presente minha sobrinha, Ana Luiza, a melhor pessoa das nossas vidas. Aos meus pais, Rita e Jésus, por serem e estarem sempre, pela dedicação, pelo amor, pela criação, pelo apoio, pelas confidencias, pela amizade. Por tudo.

Por fim, ao Departamento de Botânica do Instituto de Biociências da Universidade de São Paulo pelo apoio estrutural e ao CNPq e à FAPESP pelo pelo suporte acadêmico e financeiro sem o qual não seria possível realizar este projeto.

ObriGALO!

"O correr da vida embrulha tudo.
A vida é assim: esquenta e esfria,
aperta e daí afrouxa,
sossega e depois desinquieta.
O que ela quer da gente é coragem"
João Guimarães Rosa

ÍNDEX

BIANCHETTI, Ricardo Ernesto. Interação entre fitocromos e fitormônios em tomateiro: impactos na fisiologia e qualidade nutricional dos frutos. 2017. 113f. Tese (Doutorado em Ciências – Área Botânica) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2017.

Fitocromos (PHYs) e fitormônios têm sido caracterizados como importantes reguladores da fisiologia e qualidade de frutos carnosos; todavia, a importância de interações entre a sinalização hormonal e dos PHYs no controle do desenvolvimento e metabolismo de frutos ainda permanece pouco elucidada. Este trabalho de Tese avaliou o papel dos PHYs e das suas interações com as auxinas, as citocininas e o etileno sobre a regulação do desenvolvimento e amadurecimento de frutos de tomateiro (*Solanum lycopersicum*), particularmente no que tange ao controle da biogênese plastidial e metabolismos de açúcares e de carotenoides. No **Capítulo I** são apresentadas evidências de que a deficiência na produção de fitocromobilina (PΦB), a qual resulta numa deficiência global in PHYs funcionais, impacta negativamente a biogênese de cloroplastos em frutos imaturos e inibe o acúmulo de açúcares por meio da repressão transcricional de enzimas relacionadas a biossíntese de amido e força de dreno nos frutos. Evidências genéticas e fisiológicas indicaram o envolvimento tanto das auxinas quanto das citocininas como mediadoras do impacto negativo da deficiência de PΦB sobre a força de dreno dos frutos bem como na formação de cloroplastos. Durante a fase de amadurecimento, a deficiência em PΦB atrasou a produção climatérica de etileno, afetando o início do amadurecimento mas não a sua progressão. As interações entre PHYs e hormônios mostraram-se ativas não apenas nos tecidos posicionados mais externamente (*i.e.*, pericarpo) mas também nas regiões mais internas do fruto (*i.e.,* columela). Conclui-se, portanto, que a deficiência global em PHYs funcionais afeta drasticamente o metabolismo de açucares, formação de cloroplastos, bem como o tempo de amadurecimento através de uma interação complexa envolvendo fitocromos, auxinas, citocininas e etileno. No **Capítulo II** utilizamos o silenciamento fruto-específico de *PHY*s a fim de desvendar de que forma a fisiologia e parâmetros de qualidade do tomate seriam regulados por PHYs presentes no próprio fruto. Os dados obtidos revelaram que moléculas de SlPHYB2 presentes no próprio fruto regulam negativamente o acúmulo de clorofilas nos frutos imaturos, já as de SlPHYA influenciam positivamente a maquinaria de divisão plastidial, e tanto SlPHYA quanto SlPHYB2 desempenham papel sobrepostos, porém distintos, no controle do metabolismo de amido e acúmulo de carotenoides em frutos de tomateiro. Evidências sugerem que proteínas relacionadas à sinalização de citocininas atuariam como mediadoras do impacto de SlPHYA sobre a maquinaria de divisão plastidial, e que AUXIN RESPONSE FACTORs específicos seriam intermediários no controle dos PHYs sobre os metabolismos de açúcares e carotenoides. Conclui-se, dessa forma, que a percepção de luz mediada por moléculas de SlPHYA e SlPHYB2 presentes no próprio fruto regulam a biogênese plastidial e os metabolismos de açúcares e carotenoides por meio de alterações coordenadas em componentes chaves das cascatas de sinalização de auxinas e citocininas. Quando combinados, os dados obtidos neste estudo apresentam novidades importantes sobre a ação conjunta de PHYs e fitormônios no controle da biogênese plastidial e demonstram que a interação entre esses sinalizadores influencia características essenciais da qualidade de frutos de tomateiro, tais como o acúmulo de açúcares e de carotenoides.

Palavras-Chave: Sinalização luminosa. Auxina. Citocinina. Cloroplasto. Amido.

ABSTRACT

BIANCHETTI, Ricardo Ernesto. Phytochrome and phytohormone interplay in tomato: impacts on fruit physiology and quality traits. 2017. 113p. Thesis (Ph.D. in Science – Botany) – Bioscience Institute, University of Sao Paulo, Sao Paulo, 2017.

Phytochromes (PHYs) and plant hormones have been emerging as important regulators of fleshy fruit physiology and quality traits; however, the relevance of PHY-hormonal signaling crosstalk in controlling fruit development and metabolism remains elusive. This Thesis assesses the role of PHYs and their interplay with auxins, cytokinins and ethylene during the regulation of tomato (*Solanum lycopersicum*) fruit development and ripening, with a focus on the control of the plastid biogenesis, sugar metabolism and carotenoid accumulation. In **Chapter I**, we present evidence that the deficiency in PHY chromophore phytochromobilin (PΦB) biosynthesis, which leads to a global deficiency in functional PHYs, represses fruit chloroplast biogenesis in immature fruits and inhibits fruit sugar accumulation by transcriptionally downregulating sink- and starch biosynthesis-related enzymes. Genetic and physiological evidence suggested the involvement of both auxins and cytokinins as mediators of the negative impact of PΦB deficiency on fruit sink strength and chloroplast formation. During the ripening phase, PΦB deficiency was shown to delay the rise in climacteric ethylene production, affecting the ripening initiation rather than its progression. PHY-hormonal signaling crosstalk was shown to be active not only in the more externally positioned fruit tissues (*i.e.*, pericarp) but also in the most inner fruit regions (*i.e.*, columella). We, therefore, concluded that the global deficiency in functional PHY drastically affects fruit sugar metabolism, chloroplast formation as well as the timing of ripening via an intricate interplay involving phytochromes, auxins, cytokinins and ethylene. In **Chapter II**, we employed fruit-specific RNAi-mediated silencing of *PHY* genes to shed light on the specific role played by fruit-localized PHYs and their downstream signaling cascades on tomato fruit physiology and quality traits. Data revealed that fruit-localized SlPHYB2 negatively regulates chlorophyll accumulation in immature fruits whereas SlPHYA positively influences the plastid division machinery. Both SlPHYA and SlPHYB2 were shown to play overlapping, yet distinct, roles in controlling fruit starch metabolism and carotenoid biosynthesis. Our data implicated cytokinin signaling-related proteins as mediators of the SlPHYA-dependent regulation of plastid division machinery, and specific AUXIN RESPONSE FACTORs as intermediates in the PHY-mediated regulation of fruit sugar and carotenoid metabolisms. We concluded that fruit-localized SlPHYA- and SlPHYB2-mediated light perception regulate fruit plastid biogenesis as well as sugar and carotenoid metabolisms via coordinated changes in key components of both auxin and cytokinin signaling cascades. Altogether, this study brings important insights into the combined action of PHYs and hormones in the control of fruit plastid biogenesis and highlights that the interplay between PHY-hormonal signaling cascades influences essential features of tomato fruit quality, such as the sugar and carotenoid accumulation.

Key-words: Light signaling. Auxin. Cytokinin. Chloroplast. Starch.

GENERAL INTRODUCTION

1. *Solanum lycopersicum* as a genetic model for fleshy fruit biology

Tomato, *Solanum lycopersicum* L., has become an excellent model for investigating the regulatory mechanisms controlling fleshy fruit development and climacteric ripening. Genomic, epigenomic, transcriptomic, proteomic and metabolomic datasets as well as efficient stable transformation protocols and large germplasm collections, including many well-characterized mutants, are currently available for this species (Pino et al., 2010; The Tomato Genome Consortium, 2012; Lin et al., 2014).

Tomatoes are one of the most consumed vegetable crop worldwide (Bergougnoux, 2014). The global tomato production has more than doubled from 1994 to 2014, achieving approximately 171 millions of tons in 2014 (http://www.fao.org). Tomato fruits display high nutritional value due to the presence of many compounds with health-promoting properties, including carotenoids, tocopherol, flavonoids, ascorbate, aminoacids, organic acids and soluble sugars (Abushita et al., 2000). One of the most remarkable changes during tomato ripening is the progressive color shift resultant from the concomitant degradation of chlorophylls and accumulation of carotenoids, particularly lycopene (Klee and Giovanonni, 2011). Alongside with the visible color shift, drastic metabolic changes progressively alter tomato fruit flavor and nutritional properties (Giovanonni, 2004; Seymour et al., 2013). Most of these ripening-associated changes in fruit metabolism are intricately controlled by plant hormones (*e.g.*, auxin, ethylene), transcription factors (*e.g.*, RIPENING INHIBITOR, NONRIPENING, COLORLESS NONRIPENING) (Zhu et al., 2013) and environmental signals (*e.g.*, light, temperature).

Most attempts to improve the nutritional composition have focused on manipulating specific genes directly involved in the production of carotenoids, flavonoids and other health-promoting substances (Davuluri et al., 2005; Levin et al.; 2006, Luo et al., 2013). Alternatively, the manipulation of transcription factors regulating certain fruit metabolic routes has also resulted in significant increments in the content of target compounds (Fraser et al., 2002; Bovy

et al., 2007; Butelli et al., 2008). However, increasing attention has recently been devoted to the manipulation of multiple, independent fruit metabolic pathways as a strategy to simultaneously alter distinct classes of nutritional compounds (Luo et al., 2013; Nguyen et al., 2014). In this context, the manipulation of key components of the fruit light perception and signaling transduction pathway holds enormous promise as an alternative to improve tomato fruit nutritional composition by simultaneously altering the production of multiple nutraceutical compounds, including carotenoids, ascorbate, flavonoids, tocopherols, among others.

2. Light regulation of fruit development and quality traits

As sessile organisms, plants have evolved highly sophisticated mechanisms to perceive multiple abiotic stimuli, including water, nutrient, light availability and changes in temperature. Among these environmental factors, light not only provides energy to photosynthesis but also regulates numerous developmental and metabolic processes throughout the plant life cycle (Jiao et al., 2007; Chen and Chory, 2011). Accordingly, changes in light availability, quality, intensity and direction are continuously monitored by dedicated sensorial mechanisms, allowing plants to adjust their growth according to the light conditions (Gyulia et al., 2003; Seluzicki et al., 2017). Therefore, disturbances in central components of the light perception and signaling transduction networks frequently lead to drastic changes in plant development and metabolism, affecting processes as diverse as seed germination, plant architecture, photosynthesis, hormonal balance, circadian rhythms, flowering induction, fruit development and ripening, among others (Quail, 2002).

Four major photoreceptor families have been identified in plants (Mawphlang and Kharshiing, 2017), whose members, once activated by specific light wavelengths, will initiate highly complex and extensively interconnected signaling cascades that ultimately lead to the differential expression of photomorphogenesis-related genes (Yang et al., 2016). Early associated with red/far-red light wavelength perception, phytochromes (PHYs) regulate a wide range of photomorphogenesis processes throughout the plant life cycle, including seed germination, deetiolation, photoperiodic control of the floral transition, shade avoidance responses, among others (Quail, 2010). PHYs are encoded by a multigene family, which in tomato comprise *SlPHYA*, *SlPHYB1*, *SlPHYB2*, *SlPHYE* and *SlPHYF* (Alba et al., 2000). Each phytochrome holoprotein is composed by a conserved chromophore – phytochromobilin (PΦB)

– responsible for perceiving the light stimuli and an apoprotein possessing regulatory proprieties (Wagner et al., 2005). Once activated by red light, PHYs are transported from the cytosol to the nucleus, where they interact with several regulatory proteins, initiating a highly regulated signaling cascade. Alongside with PHYs, light perception in plants also involves the conserved blue light photoreceptors cryptochromes (CRYs) and phototropins and the UV-B receptor proteins UVR8 (Cashmore, 1997; Christie, 2007; Wu et al., 2012).

Compared to the great strides made in recent years in the identification of several key components of the light signaling networks controlling plant vegetative photomorphogenesis (Jiao et al., 2007; Chen and Chory, 2011), relatively little is known about the light-dependent regulatory mechanisms controlling fleshy fruit biology. Experimental evidence indicates a role for PHYs in controlling carotenoid synthesis in tomato fruits (Alba et al., 2000; Schofield and Paliyath, 2005). Moreover, by comparing single and multiple *phy* tomato mutants, Gupta et al. (2014) revealed that SlPHYA, SlPHYB1 and SlPHYB2 differentially regulate the transcript accumulation of tomato *PHYTOENE SYNTHASE1* (*PSY1*), which encodes a key enzyme in the fruit carotenoid biosynthesis pathway. Moreover, studies have also identified R, but not FR, radiation as a promotive signal controlling tomato fruit carotenogenesis (Khudairi et al., 1971; Thomas and Jen, 1975; Alba et al., 2000).

Acting downstream of photoreceptors, a considerable number of transcription factors and regulatory proteins are responsible for transducing the light stimuli into changes in transcriptional profile (Figure 1.1). Activated by PHYs and stabilized by CRYs, LONG HYPOCOTYL5 (HY5) is a central transcript factor that promotes photomorphogenic-associated gene expression (Xu et al., 2015). HY5 and its homologous HYH display a leucine-zipper motif that binds to the promoter region of many light-responsive genes (Burman et al., 2017). Under continuous dark, HY5 is progressively marked for proteolysis by the photomorphogenic repressor protein CONSTITUTIVE PHOTOMORPHO-GENESIS1 (COP1) (Figure 1.1) (Chory, 2010; Boron and Vissenberg, 2014). Alongside with SUPRESSOR OF PHYA1 (SPA1), COP1 is part of a large protein complex, known as 'COP1-SPA complex', which closely interacts with other photomorphogenic repressor proteins such as DAMAGE DNA BINDING PROTEIN (DDB1); DETIOLATED1 (DET1) and CULLIN4 (CUL4) (Figure 1.1).

Also acting downstream of phytochromes, PHYTOCHROME-INTERACTING

FACTORs (PIFs) are members of a family of helix-looping-helix transcript factor known to down- and up-regulate photomorphogenic- and skotomorphogenic-related genes, respectively (Figure 1.1). Under inductive light conditions, PHYs interact with PIF proteins promoting their ubiquitination and subsequent degradation via proteasome 26S, thereby altering the transcriptional profile of PIF-target genes (Leivar et al., 2008; Chen and Chory, 2011). In *Arabidopsis thaliana*, the master transcription factor of chloroplast development and maintenance *GOLDEN-2 LIKE* (*GLK*) as well as key carotenoid biosynthetic genes, including *AtPSY1*, are important targets of PIF proteins (Toledo-Ortiz et al., 2010; Bou-Torrent et al., 2015). Among the six tomato PIF-encoding genes (Rosado et al., 2016), *SlPIF1a* has been shown to physically bind to *SlPSY1* promotor, inhibiting fruit carotenoid synthesis (Llorente et al., 2016). As highlighted by Llorente et al. (2016), the presence of chlorophylls may create a self-shading effect as sunlight passes through the flesh of green tomato fruits, resulting in a progressive reduction in R/FR ratios at increasing depths within the fruit tissues. As tomato fruit ripens, chlorophylls are degraded and inner fruit cells are irradiated with progressively more R-enriched light, facilitating the activation of PHYs and consequently promoting the PHY-mediated targeting of SlPIFs for degradation. According to the proposed model, by increasing R/FR ratio inside the fruit tissues and subsequently increasing SlPIF1a degradation, the reduction of fruit chlorophyll levels promotes *SlPSY1* expression and therefore intensifies carotenoid accumulation in this organ (Llorente et al., 2016).

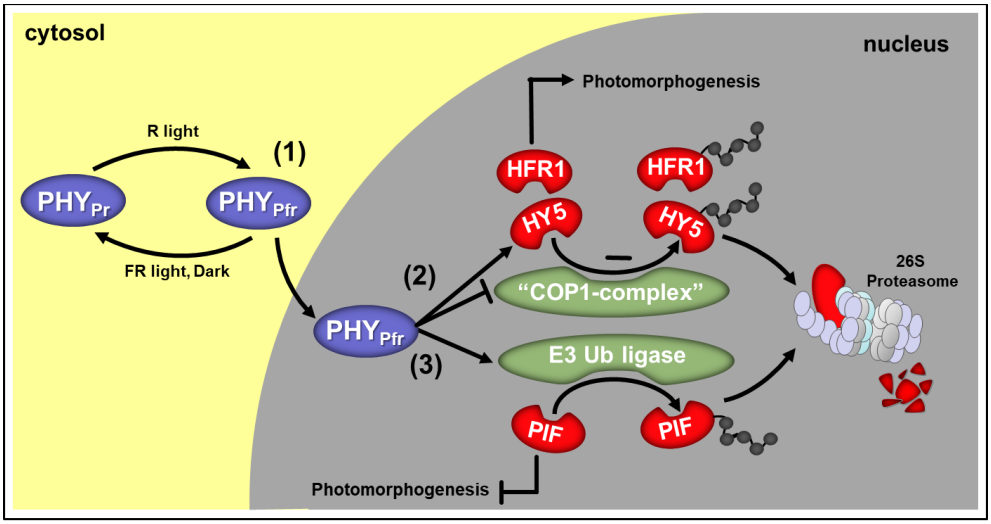

Figure 1.1 A simplified overview of light signal transduction via phytochromes (PHY) and cryptochromes (CRY) and their main interacting proteins. **(1)** Upon red (R) light irradiation, cytosolic PHY_{Pr} is converted

into its active Pfr form, which migrates to the nucleus. **(2)** In the cell nucleus, PHY_{Pfr} represses CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) E3-complex ("COP1complex"), which is responsible for targeting positive components of the light signal transduction pathway such as LONG HYPOCOTYL5 (HY5) for proteasomal degradation. PHY_{Pfr} also promotes *HY5* expression, whose encoding protein is responsible for stimulating the expression of numerous photomorphogenesis-associated genes. **(3)** In parallel, PHY_{Pfr} activates an unidentified E3 Ubiquitin (Ub) ligase responsible for targeting light signal transduction repressor proteins such as PHYTOCHROME-INTERACTING FACTORS (PIFs) for degradation via 26S proteasome. Adapted from Rodrigues et al. (2014).

Confirming the influence of light signaling on fruit biology, knockout/knockdown of tomato genes encoding HY5, COP1, DDB1, DET1, CUL4 and PIF1a has been shown to markedly impact fruit plastid biogenesis and nutritional quality (Liu et al., 2004; Wang et al., 2008; Kolotillin et al., 2007; Azari et al., 2010; Llorente et al., 2016).

3. Light and hormone interaction during plant development

Increasingly complex signaling cascades have been implicated in transducing the light stimuli in plants. Besides the vast array of signaling proteins and transcription factors directly associated with the regulatory cascades initiated by photoreceptors, intensive crosstalk between light and hormonal signaling pathways add even greater complexity to the regulatory cascades controlling plant photomorphogenic responses (Symons and Reid, 2003; Rodrigues et al., 2014).

Depending on the photomorphogenic response under consideration, light signaling proteins have been demonstrated to act either upstream or downstream of plant hormones (Vandenbussche et al., 2007; Franklin et al., 2011). When acting upstream of phytohormones, a given component of the light signaling cascade modulates either the plant hormone levels (*e.g.*, biosynthetic, degradation and conjugation enzymes), distribution (*e.g.*, transport proteins) or signaling (*e.g.*, receptors and signal transduction proteins) (Franklin et al., 2011; Zhong et al., 2009; Keuskamp et al., 2010). Conversely, plant hormones can also modulate the synthesis (Vandenbussche et al., 2007; Alabadi et al., 2008), stability (Sweere et al. 2001) or activity (Feng et al., 20008) of target components of the light perception and signaling cascades, thereby placing light signaling proteins as downstream elements of hormone-triggered signaling cascades during certain photomorphogenic responses. Light-hormonal interaction frequently occurs at the transcriptional (Khanna et al., 2007) or posttranslational levels (Colón-Carmona et al., 2000); however, some posttranscriptional or even translational regulation of hormone-

related proteins by light signaling and *vice-versa*, although not yet demonstrated, cannot be ruled out.

Significant advances have been made in recent years in understanding the mechanistic relationship between PHYs and plant hormones in certain physiological responses (Hersch et al., 2014; Warpeha and Montgomery, 2016). Among the huge diversity of light-hormonal signaling crosstalk already described in the literature, those involving auxins, cytokinins and ethylene will be the main focus of this Thesis.

During some photomorphogenic events, including seedling deetiolation and shade avoidance responses, PHYs have been shown to negatively regulate auxin synthesis. Indolyl-acetic acid (IAA), which is the predominant auxin in plant tissues (Ender & Strader, 2015), is mainly synthesized via the combined action of the flavin monooxygenases TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) using the aminoacid tryptophan as a precursor (Zhao et al., 2000; Tao et al., 2008). In *Arabidopsis*, AtPIF4 has been shown to bind to G-box motifs present on both *AtTAA* and *AtYUC* promotor regions, activating the expression of these key auxin biosynthetic genes (Stavang et al., 2009; Franklin et al., 2011; Sun et al., 2012). In agreement, by targeting AtPIF4 for proteolysis, AtPHYB acts as a repressor of both *AtTAA* or *AtYUC* gene expression, consequently inhibiting auxin biosynthesis (Tao et al., 2008). Besides repressing auxin synthesis, AtPHYB-mediated light perception also promotes the expression of *GRETCHEN HAGEN 3* (*GH3*) genes, whose encoding proteins are responsible for auxin conjugation (Tanaka et al., 2002; Park et al., 2007). Evidence also indicates that PHY-dependent light perception can influence auxin transport in tomato seedlings, though the mechanisms behind this interaction remain to be elucidated (Liu et al., 2011). With few exceptions (Colón-Carmono et al., 2000; Cluis et al., 2004; Melo et al., 2016), crosstalk events between light and auxin signaling proteins are still poorly described in the literature.

Whereas negative interactions have been predominantly observed between PHY and auxins (Cluis et al., 2004; Franklin et al., 2011; Sun et al., 2012), the opposite has been described for the PHY-cytokinin regulatory interplays (Buchanan-Wollaston et al., 2005, Van der Graff et al., 2006). Cytokinin can mimic the impact of the light stimuli in several photomorphogenic responses, including the etioplast-to-chloroplast conversion and the pigment accumulation in deetiolating seedlings (Chory et al., 1994; Halliday and Fankhauser, 2003;

Vandenbussche et al., 2007). Also, PHY-dependent light perception has been shown to modulate cytokinin biosynthesis and conjugation. For example, PHY-deficient tobacco (*Nicotiana tabacum*) mutants display lower cytokinin levels than wildtype counterparts (Krapiel et al., 1995), and active PHYs have been shown to promote cytokinin levels by down-regulating the expression of *CYTOKININ OXIDASE* (*CKX*) genes, which encode key enzymes responsible for the conjugation of this plant hormone (Buchanan-Wollaston et al., 2005; Van der Graff et al., 2006). Several lines of evidence also indicate extensive crosstalk between light and cytokinin signaling cascades. For instance, *Arabidopsis* PHYB has been shown to physically interact with type-A ARABIDOPSIS RESPONSE REGULATOR (ARR) proteins, which are major downstream targets of CKs receptors (Sweere et al., 2001; Halliday et al., 2003). Accordingly, a central role has been described for type-A ARRs in red light signaling and in the control of photoresponsive events in vegetative tissues of *Arabidopsis* (Heyl and Schmulling, 2003; Salome et al., 2006).

PHY-dependent light perception also strongly influences ethylene metabolism during plant vegetative growth (reviewed in Rodrigues et al., 2014). In plants, ethylene is synthetized from methionine via the action of the rate-limiting enzymes 1-AMINOCYCLO-PROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE (ACS) and ACC OXIDASE (ACO) (Van de Poel et al., 2012), which are codified by multigenic gene families, whose members are differentially regulated by multiple endogenous and environmental signals (Lin et al., 2009; Wolters and Jürgens, 2009). Among the environmental cues regulating ethylene biosynthesis, PHY-dependent red light perception has received particular attention (de Goeschl et al., 1967; Kang and Burg, 1972; Imaseki et al., 1971; Vandenbussche et al., 2003), indicating a negative influence on ACC levels (Jiao et al., 1987; Melo et al., 2016) as well as on the *ACO* and *ACS* transcript levels (Foo et al., 2006). Studies performed in *Arabidopsis* indicated that AtPIF5 promotes *AtACS* expression, and the PHYB-mediated targeting of AtPIF5 for proteasomal degradation proteolysis seems to play a major role in the light-dependent repression of *AtACS* expression (Khanna et al., 2007).

Light has also been shown to alter tissue sensitivity to ethylene (Solano et al., 1998; Laing et al., 2012). When ethylene is perceived by its dedicated receptors, a signaling cascade initiates by releasing the repression caused by CONSTITUTIVE TRIPLE RESPONSE (CTR1) on ETHYLENE INSENSITIVE 2 (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) (McMurchie et al., 1972; Nakatsuka et al., 1998; Zhong et al., 2009; Vandenbussche et al., 2012). Evidence suggests that light signaling can decrease tissue ethylene sensitivity throught the physical interaction between COP1 and EIN3 (Zhong et al., 2009). COP1 post-transcriptionally regulate the accumulation of EIN3, affecting the downstream genes expression, such as several *ERFs*. On the other hand, PHY promotes the degradation of COP1 and consequently the destabilization of EIN3, which in turn, act as an inhibitor of the ethylene signaling pathway (Zhong et al., 2009).

Despite the massive recent advances in our knowledge on the mechanisms behind light-hormonal crosstalk during plant vegetative development, whether and how such signaling interplay influence fruit development and ripening remains elusive.

4. Hormonal control of fruit development and ripening

Fruit development can be divided into several stages, including fruit set, expansion, maturation, ripening and senescence. Unsurprisingly, each one of these stages is strictly regulated by highly interconnected hormonal signaling networks (Kumar et al., 2014). Data indicate that the successful fruit set after pollination depends on the combined action of auxins, gibberellins and cytokinins (Kumar et al., 2014). In both dry and fleshy fruits, gibberellins play a central role in controlling organogenesis in the ovary tissues after pollination (Serrani et al. 2008; Arnaud et al., 2010). However, a growing number of evidence place auxins as the master regulators of gibberellin biosynthesis during fruit set (Serrani et al., 2008; Jong et al., 2009). Accordingly, the suppression of either *SlARF7* or *SlIAA9*, two negative regulators of auxin signaling response, leads to up-regulation of gibberellin levels and the formation of parthenocarpic fruits (Wang et al., 2005; Jong 2009; 2011). Though the interaction of cytokinins with auxins and gibberellins during fruit set remains elusive, it has been shown that cytokinin biosynthesis genes, as well as type-A TRRs, are up-regulated soon after pollination, and treating unpollinated ovaries with cytokinins leads to parthenocarpy (Matsuo et al., 2012).

After pollination and fruit set, intense mitotic activity is observed in fleshy fruits, followed by a gradual reduction in cell division rate and maximal rates of cell enlargement (Srivastava and Handa, 2005). As indicated by both genetic and pharmacological evidence (McAtee et al., 2013; Kumar et al., 2014), the combined action of auxins and cytokinins is

crucial for maintaining the intense cell division rates observed after pollination whereas auxins and gibberellins assume a central role in controlling cell expansion soon after the decline in cell division rates. Also, cytokinins have been identified as positive regulators of fruit sink strength (Roitsch and Ehneβ, 2000), invertase activity (Godt and Roitsch, 1997; Albacete et al., 2014) and columella formation (Matsuo et al., 2012) during initial fruit development.

After fruit growth is completed, the maturation phase is marked by a progressive reduction in both cytokinin and auxin levels and a transient rise in abscisic acid (ABA) content (Srivastava and Handa, 2005). In recent years, ABA has emerged as a promoter of ethylene synthesis during fruit ripening (Zhang et al., 2009; Wang et al., 2012; Sun et al., 2012) and also important regulator of cell-wall degradation, sugar accumulation and color change in ripening fruits (Galpaz et al., 2008; Ogawa et al., 2009; Bastias et al., 2011).

During ripening, fruits undergo dramatic physiological and biochemical changes, resulting in marked alterations in fruit color, composition and texture (Seymour et al., 2013). Although increasingly more complex multi-hormonal signaling cascades have been described during fruit ripening (McAtee et al., 2013), the central role of ethylene as a positive regulator of climacteric ripening remains indisputable (Cara and Giovanonni, 2008). Accordingly, the ripening-associated changes in fruit color, flavor, biochemical composition and texture is severely disturbed in mutants defective in either ethylene production or perception (Klee and Giovanonni, 2011). Similarly, these same ripening-related traits are drastically impaired in mutants deficient in transcription factors acting upstream of ethylene, such as *ripening inhibitor (rin;* Robinson and Tomes, 1968), *nonripening* (*nor*; Giovannoni, 2004) and *Colorless nonripening* (*Cnr*; Thompson et al., 1999).

5. Fruit plastids: a central place for reactions and storage

Critical steps of the biochemical routes responsible for the production of many health-promoting compounds accumulated in fleshy fruits (*e.g.*, phenylpropanoids such as carotenoids and tocopherols) are carried out inside plastids (Egea et al., 2011). Therefore, the biogenesis and maturation of these organelles in fruit tissues are known to impact both the nutritional and organoleptic features of fully ripe fruits (Barsan et al., 2012).

In land plants, plastid division depends on nucleus-encoded proteins that will form ring structures at the division site (TerBush et al., 2013). Some of these genes are derived from the ancestral prokaryotic cell division machinery, including the tubulin GTPase FILAMENTOUS

TEMPERATURE SENSITIVE-Z (FtsZ) (Basak and Moller, 2013) and some other components that interact with FtsZ proteins, such as ACCUMULATION AND REPLICATION OF CHLOROPLASTS (ARC) and CRUMPLED LEAF (CRL). Moreover, plastid division-related proteins specific to land plants, such as PLASTID DIVISION (PDV), facilitate the formation of the division ring at the final steps of the division process (Okazaki et al., 2009). Interestingly, *Arabidopsis PDV2* has been shown to be transcriptionally regulated by cytokinin signaling, thereby linking the plant hormonal status with the chloroplast division rates in plant cells (Okazaki et al., 2009). Data recently obtained by our research group also indicate that tomato genes encoding key components of the plastid division machinery are strictly regulated by PHY-dependent light perception in deetiolating tomato seedlings (Melo et al., 2016).

As in other plant species, the light-induced GLK transcription factors are positive regulators of chloroplast development in tomato leaf and fruit tissues. Whereas *SlGLK1* regulates chloroplast biogenesis and maturation in vegetative tissues, *SlGLK2* plays an equivalent role specifically in fruits (Powell et al., 2012). Accordingly, overexpression of *SlGLK2* in a *glk2* mutant background resulted in fruits with increased chlorophyll levels, well-developed chloroplasts and higher content of starch, soluble sugars and carotenoids (Powell et al. 2012; Nguyen et al. 2014).

In pre-ripening fruits, chloroplasts are not only responsible for fruit-localized photosynthesis but are also the site of starch accumulation. Starch can contribute with up to 30% of the total dry mass of certain fleshy fruits (Schaffer and Petreikov, 1997), directly impacting the final sugar concentration in climacteric fruits, such as banana, melon, apple and tomato (Keeling and Myres, 2009). Plastids are the site of action of ADP-glucose pyrophosphorylase (AGPase), responsible for catalyzing the conversion of glucose-6-phosphate and ATP to ADP-glucose, which is the first committed step in starch biosynthesis (Fernier et al., 2001; Neuhaus and Stitt, 1990; Denyer et al., 1995; Georgelis et al., 2007). AGPase is a heterotetramer comprising a pair of small/catalytic (AGPase-S) and a pair of large/regulatory (AGPase-L) subunits (Kim et al., 2007; Georgelis et al., 2007). Whereas AGPase-S is highly conserved among land plants, AGPase-L subunits are considerably more diverse, displaying organ-specific action and particular regulatory properties (Georgelis et al., 2007; Batra et al., 2017). The ADP-glucose molecules produced by AGPase activity are subsequently arranged by a set of enzymes – *e.g.*, starch synthesis enzymes (STS) and starch

branching synthesis enzymes (SBS) – to determine the final structure of starch molecules (Zeeman et al., 2010).

During ripening, fruit chloroplasts undergo a series of genetic, biochemical and morphological changes leading to their conversion into chromoplasts (Barsan et al., 2010). This is one of the most dramatic events during climacteric ripening, involving intense chlorophyll and starch degradation, disruption of thylakoids and progressive accumulation of carotenoids (Egea et al., 2011). In tomato, the accumulation of carotenoids such as β-carotene and lutein at the start of the ripening process (*i.e.*, Breaker stage) renders the yellowish coloration characteristic of this transitory ripening stage. Subsequently, massive lycopene amounts are accumulated in the chromoplasts giving rise to the distinctive red coloration of fully ripe tomato fruits (Fraser et al., 1994, 2001). 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.

Carotenoids are derived from the methylerythritol (MEP) pathway, which initiates with the combination of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to generate geranylgeranyl diphosphate (GGPP), the precursor of phytoene, the first carotenoid in the pathway (Bramley, 2013). The conversion of two molecules of GGPP into phytoene is catalyzed by PSY, the rate-limiting enzyme in the carotenoid biosynthetic pathway. Phytoene can be converted into lycopene, which can be cyclized forming β-carotene, zeaxanthin, violaxanthin, neoxanthin, δ-carotene, α-carotene or lutein (Saini et al., 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, PHYTOENE DESATURASE (PDS) and LYCOPENE β-CYCLASES (β-LCY and CYC-β), whose transcriptional regulation is tightly controlled by ripening-associated transcription factors, hormonal signaling and several environmental stimuli (Su et al., 2015).

The impact of plastid biogenesis and differentiation on the biochemical composition of fleshy fruits is far more extensive than its influence on carbohydrate and carotenoid metabolisms. Although the interconnection between plastid abundance and activity with other fruit metabolic routes are not covered in this Thesis, excellent reviews on this topic can be found in the literature (Neuhaus and Emes, 2000; Cocaliadis, 2014).

As I hope it becomes clear in the next Chapters of this Thesis, understanding the relevance of PHY-mediated light perception and its downstream signaling cascades controlling fruit development and metabolism holds enormous promise for the improvement of the nutritional quality of edible fleshy fruits.

In this Thesis, we present original experimental data (Chapter I) revealing that phytochrome chromophore phytochromobilin (PΦB) biosynthesis inhibits sugar accumulation in tomato fruits by transcriptionally downregulating sink- and starch biosynthesis-related enzymes. Moreover, we demonstrate that PΦB deficiency represses fruit chloroplast biogenesis, implicating more limited production of photoassimilates via fruit photosynthesis. As presented in Chapter I, the proposed mechanism relies on the action of both auxins and cytokinins as the mediators of the negative impact of PΦB deficiency on fruit sink strength and chloroplast formation.

By employing fruit-specific RNAi-mediated silencing of *SlPHY* genes, we demonstrate in Chapter II that fruit-localized phytochromes and their downstream signaling cascades not only modulate chloroplast biogenesis in immature tomato fruits but also regulate sugar and carotenoid accumulation, which are two essential features of tomato fruit quality. Evidence indicated that an intricate light-hormonal signaling network involving key components of both auxin and cytokinin signal transduction pathways is implicated in the PHY-dependent regulation of fruit plastid biogenesis, sugar metabolism and carotenoid accumulation.

Finally, we raise important considerations concerning the potential of the combined manipulation of photoreceptors, light signaling proteins and key components of auxin and cytokinin signaling cascades, which I personally consider to be a particularly promising strategy to judiciously alter multiple quality traits in tomato, and possibly other edible fleshy fruits.

FINAL CONSIDERATIONS

Whether and how light-hormonal signaling crosstalk influence fleshy fruit development and metabolism have remained important open questions in plant photobiology. Answering these questions may facilitate the judicious manipulation of key components of both the light and hormonal signaling cascades aiming fruit improvement.

By using two distinct strategies, this study provides a new, comprehensive set of clues to better understand the light-hormonal mechanisms regulating plastid biogenesis as well as carotenoid and sugar metabolism in tomato fruits. By using a tomato mutant characterized by a global deficiency in functional PHYs, we identified the major phenotypical impacts of PHY deficiency on tomato fruit development and metabolism (**Chapter I**). Subsequently, transgenic tomato lines with fruit-specific silencing of *PHY* genes were generated and characterized to provide information on the role played by specific fruit-localized PHYs on key tomato quality traits (**Chapter II**). In both these approaches, special attention was devoted to the impact of PHY manipulation on the fruit hormonal signaling.

Some interesting conclusions can be drawn based on data obtained via these two distinct approaches. When combined, results from these two strategies allowed the identification of several signaling components (*e.g.*, TRRs, CRFs, ARFs, PIFs, DET1, DDBD1 and COP1) as potential targets of PHY signaling in tomato fruit tissues. Moreover, both approaches implicated cytokinins as intermediates of the PHY-dependent regulation of plastid division machinery. Also, either the fruit-specific downregulation of specific *PHY*s or the global deficiency in functional PHYs drastically altered multiple components of the auxin signaling cascade, revealing a novel, intimate relationship between PHYs and auxins in fruit tissues. In contrast, whereas the global deficiency in functional PHYs negatively impacted fruit starch metabolism, the opposite was observed when either *SlPHYA* or *SlPHYB2* are downregulated specifically in the fruit tissues. This last observation highlights the potential limitations in investigating the influence of PHYs on fruit biology by exclusively using PHY-deficient mutants, as some of the fruit phenotypical changes observed can be a consequence of the collateral negative effects of PHY deficiency on plant vegetative growth rather than the action of fruit-localized PHYs.

Our findings also indicate that the fruit-specific manipulation of PHYs can be a particularly convenient system for future investigations focused on mechanistically understanding the light-hormonal signaling crosstalk controlling other important physiological and agronomic attributes in fleshy fruits, including fruit set and growth, accumulation of other health-promoting compounds (*e.g.*, vitamins C and E, flavonoids), production of volatile compounds, among others. Further studies focused on identifying which tomato *PHY* genes are more directly associated with the PHY-dependent changes in tomato fruit metabolism and hormonal signaling may provide important targets for the future manipulation of light-dependent responses in this fruit crop species. The fruit-specific silencing of *SlPHYE* and *SlPHYF*, as well as the fruit-specific overexpression of each one of the five tomato *PHY* genes, may be particularly promising strategies to gain additional, relevant information in this research field.

We hope the findings presented in this Thesis substantiate further advances in fruit improvement via intragenesis, synthetic biology and other biotechnological tools. Based on the data presented in this Thesis, we believe that the combined manipulation of phytochrome, auxin, cytokinin and ethylene signaling-related genes in tomato and other fruit crops represents a promising venue for adjusting fruit ripening and quality traits.

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