Universidade de São Paulo
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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

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Phytochrome and phytohormone interplay in tomato:
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Comissão Julgadora

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Prof. Dr. Luciano Freschi
Orientador

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

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ObriGALO!

*“O correr da vida embrulha tudo.
A vida é assim: esquentada e esfria,
aperta e daí afrouxa,
sossega e depois desinquieta.
O que ela quer da gente é coragem”*

João Guimarães Rosa

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RESUMO

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 açúcares, 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 *PHYs* 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 SIPHYB2 presentes no próprio fruto regulam negativamente o acúmulo de clorofilas nos frutos imaturos, já as de SIPHYA influenciam positivamente a maquinaria de divisão plastidial, e tanto SIPHYA quanto SIPHYB2 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 SIPHYA 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 SIPHYA e SIPHYB2 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 SIPHYB2 negatively regulates chlorophyll accumulation in immature fruits whereas SIPHYA positively influences the plastid division machinery. Both SIPHYA and SIPHYB2 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 SIPHYA-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 SIPHYA- and SIPHYB2-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 *SIPHYA*, *SIPHYB1*, *SIPHYB2*, *SIPHYE* and *SIPHYF* (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 SIPHYA, SIPHYB1 and SIPHYB2 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 PHOTOMORPHOGENESIS1 (COP1) (Figure 1.1) (Chory, 2010; Boron and Vissenberg, 2014). Alongside with SUPPRESSOR 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 SIPIFs for degradation. According to the proposed model, by increasing R/FR ratio inside the fruit tissues and subsequently increasing SIPIF1a degradation, the reduction of fruit chlorophyll levels promotes *SIPSYI* 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., 2008) 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 synthesized 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 through 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 *SIIAA9*, 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 Ehneß, 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 Giovannoni, 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 Giovannoni, 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 *SIPHY* 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.

6. References

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OBJECTIVES

General objective

The overall aim of this Thesis was to investigate the role of phytochromes and their interplay with auxins, cytokinins and ethylene during the regulation of tomato fruit development, maturation and ripening, with a focus on the control of the plastid biogenesis, sugar metabolism and carotenoid accumulation.

Specific objectives

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

1. By using sing a tomato mutant characterized by a global deficiency in functional PHYs, assess the overall impact of functional PHYs on the temporal and spatial distribution of auxin, cytokinin and ethylene signaling within fruit tissues and its influence on fruit plastid biogenesis, sugar metabolism and ripening. This specific goal was addressed in **Chapter I** of this Thesis.

2. By employing fruit-specific RNAi-mediated silencing of *SIPHY* genes, investigate the specific role played by fruit-localized SIPHYA, SIPHYB1 and SIPHYB2 and their downstream signaling cascades on the control of the plastid biogenesis, sugar metabolism and carotenoid accumulation in tomato fruits. This specific goal was addressed in **Chapter II** of this Thesis.

CHAPTER I: PHYTOCHROMOBILIN DEFICIENCY IMPAIRS SUGAR METABOLISM THROUGH THE REGULATION OF CYTOKININ AND AUXIN SIGNALING IN TOMATO FRUITS

This Chapter is organized as published in the scientific journal Scientific Reports.

Bianchetti, R.E., Cruz, A.B., Oliveira, B.S., Demarco, D., Purgatto, E., Peres, L.E.P., Rossi, M. and Freschi, L. (2017) Phytochromobilin deficiency impairs sugar metabolism through the regulation of cytokinin and auxin signaling in tomato fruits. *Scientific Reports*, **7**, 7822.

Abstract

Phytochromes and plant hormones have been emerging as important regulators of fleshy fruit biology and quality traits; however, the relevance of phytochrome-hormonal signaling crosstalk in controlling fruit development and metabolism remains elusive. Here, we show that the deficiency in phytochrome chromophore phytochromobilin (PΦB) biosynthesis inhibits sugar accumulation in tomato (*Solanum lycopersicum*) fruits by transcriptionally downregulating sink- and starch biosynthesis-related enzymes, such as cell-wall invertases, sucrose transporters and ADP-glucose pyrophosphorylases. PΦB deficiency was also shown to repress fruit chloroplast biogenesis, which implicates more limited production of photoassimilates via fruit photosynthesis. Genetic and physiological data revealed the involvement of auxins and cytokinins in mediating the negative impact of PΦB deficiency on fruit sink strength and chloroplast formation. PΦB deficiency was shown to transcriptionally repress type-A TOMATO RESPONSE REGULATORS and AUXIN RESPONSE FACTORS both in pericarp and columella, suggesting active phytochrome-hormonal signaling crosstalk in these tissues. Data also revealed that PΦB deficiency influences fruit ripening by delaying the climacteric rise in ethylene production and signaling. Altogether, the data uncover the involvement of phytochromes in fine-tuning sugar metabolism, chloroplast formation and the timing of fruit ripening and also reveal a link between auxins, cytokinins and phytochromes in regulating sugar import and accumulation in fruits.

Keywords: Fleshy fruits, Phytochrome, Sugar, Auxin, Cytokinin.

1. Introduction

Photoreceptor-mediated light perception and signaling have long been described to regulate the development and metabolism of fleshy fruits, ultimately affecting their final composition (McCollum, 1954; Thomas and Jen, 1975). In tomato (*Solanum lycopersicum*), the model species for fleshy fruit physiology (Carvalho et al., 2011), red/far-red light perception via phytochromes has emerged as a relevant source of environmental information controlling fruit ripening and carotenogenesis (Alba et al., 1997; Alba et al., 2000; Gupta et al., 2014). Phytochromes (PHYs) are part of a multigene family, which in tomato encompasses five members: *SIPHYA*, *SIPHYB1*, *SIPHYB2*, *SIPHYE* and *SIPHYF* (Alba et al., 2000). Functional PHYs are homodimers associated with a conserved chromophore known as phytochromobilin (PΦB), which upon red light exposure undergoes a conformational alteration that triggers light signaling (Rockwell et al., 2006). Hence, tomato chromophore-deficient mutants, such as *yellow-green-2* (*yg-2*) and *aurea* (*au*), fail to produce functional phytochromes, resulting in phenotypic changes such as increased stem and petiole elongation as well as pale-green leaves and fruits (Carvalho et al., 2011).

Once activated by light, phytochromes are transported to the nucleus (Rockwell et al., 2006), where they inhibit the PHYTOCHROME INTERACTION FACTORs (PIFs), negative regulators of light response, and promote photomorphogenesis-promoting factors such as LONG HYPOCOTYL 5 (HY5) by downregulating the protein complexes formed by CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), DETIOLATED1 (DET1), DAMAGE DNA BINDING1 (DDB1) and CULLIN4 (CUL4) (Rockwell et al., 2006; Leivar and Quail, 2011; Dong et al., 2015; Wang and Wang, 2015). In accordance with their key functions in light signal transduction, overexpression or knockout/knockdown of tomato genes encoding PIF, HY5, COP1, DET1, DDB1 or CUL4 have been shown to greatly impact the organoleptic and nutritional composition of tomato fruits (Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010; Llorente et al., 2016). Hence, manipulating light signaling has been suggested as a viable strategy for improving the nutritional composition of tomato fruits (Liu et al., 2004; Azari et al., 2010).

Tomato plants with altered light perception or signal transduction frequently show changes in fruit chloroplast biogenesis and development (Liu et al., 2004; Kolotilin et al., 2007; Azari et al., 2010). As developing fruits contain photosynthetically active chloroplasts (Piechulla et al., 1987; Cocaliadis et al., 2014), increments in the abundance or in the photosynthetic performance of these organelles in immature stages may contribute to some extent in supplying carbon and energy for the highly demanding processes of fruit

growth and maturation (Powell et al., 2012). Nevertheless, large fleshy fruits, including the modern tomato varieties, are regarded as essentially photosynthate sinks relying on carbon importing from leaves to complete their developmental program (Lytovchenko et al., 2011; Cocaliadis et al., 2014; Osorio et al., 2014). Therefore, carbohydrate metabolism-related enzymes that significantly contribute to the sink activity in developing tomato fruits, such as cell-wall invertases (LINs), sucrose transport proteins (SUTs) and ADP-glucose pyrophosphorylase (AGPases), play central roles in determining fruit growth rates (Baxter et al., 2005; Zanon et al., 2009; Albacete et al., 2014; Tieman et al., 2017). In line with their key role in fruit growth, many of these enzymes are subjected to transcriptional and posttranscriptional regulation by numerous environmental (*e.g.* light, drought, salinity) and endogenous stimuli (*e.g.* plant hormones, carbon availability) (Albacete et al., 2014; Osorio et al., 2014).

In tomato, as in many other fleshy fruits, the accumulation of soluble sugars and organic acids is one of the major determinants of the final organoleptic and nutritional quality of the fruits (Osorio et al., 2014). Remarkable changes in carbohydrate metabolism are observed during tomato fruit development and ripening, all of them directly influencing the final sugar content of the fruit (Salles et al., 2003). During early fruit development, starch synthesis and accumulation predominates in the pericarp, which is the most abundant tissue in a tomato fruit; however, the involvement of other tissues in fruit carbon metabolism is also widely accepted. The columella, for instance, plays a central role in importing sugars as this tissue connects the fruit to the rest of the plant (Baxter et al., 2005). Therefore, the signaling events controlling sugar import and accumulation are presumably coordinated within the different fruit tissues.

Compelling biochemical, genetic and molecular evidence is now revealing that both sources of carbohydrates in fruits (*i.e.* fruit photosynthesis and carbon importing from the leaves) are modulated by both light and hormonal signals (Sagar et al., 2013; Albacete et al., 2014; Cocaliadis et al., 2014). Among plant hormones, auxins and cytokinins are known to control chloroplast biogenesis and differentiation in vegetative and reproductive tissues (Chory et al., 1994; Ullanat and Jayabaskaran, 2002; Sagar et al., 2013; Cortleven and Schmölling, 2015) and also transcriptionally and post-transcriptionally regulate key proteins related to carbohydrate metabolism and sink activity (Roitsch and Ehneß, 2000; Sagar et al., 2013; Albacete et al., 2014). Despite the massive advances in clarifying the intricate phytochrome-hormonal signaling network controlling various developmental responses in vegetative tissues (Halliday and Fankhauser, 2003; Lau and Deng, 2010), the phytochrome and hormonal signaling crosstalk responsible for controlling sugar

metabolism in developing fruits remain elusive. Also, limited information is currently available on the role of phytochrome-hormonal interactions in controlling the initiation and progression of fruit ripening. Here, we provide evidence that deficiency in phytochrome chromophore biosynthesis impacts plastid biogenesis and sugar metabolism via coordinated alterations in auxin and cytokinin signaling in both pericarp and columella tissues. The upregulation of genes encoding phytochrome apoproteins in the columella was also identified as a compensatory mechanism to overcome the presumably less R-enriched light reaching fruit inner tissues. Moreover, our data also suggest that PΦB deficiency influences the timing of ripening by delaying the climacteric rise in ethylene production and signaling.

2. Material and Methods

Plant material and growth conditions

Seeds of wild type (WT) *Solanum lycopersicum* L. (cv. Micro-Tom), *aurea* (*au*) and *entire* mutants and transgenic lines carrying the synthetic auxin-responsive (*DR5*) and ethylene-responsive (*EBS*) promoters fused to the reporter gene *uid* (encoding a β -glucuronidase, GUS) were obtained from the Laboratory of Hormonal Control of Plant Development (www.esalq.usp.br/tomato)(Carvalho et al., 2011). The *EBS::GUS* construct was donated by Dr. Anna N. Stepanova and Dr. Jose M. Alonso (North Carolina State University, USA). Seeds of Micro-Tom *SIARF4*-silenced line (*SIARF4*-ASL) were obtained from Dr. Mohamed Zouine and Dr. Mondher Bouzayen (University of Toulouse)(Sagar et al., 2013). Crosses and phenotypical screening were carried out to generate *au-DR5::GUS* and *au-EBS::GUS*. All the analyzed plants were in the Micro-Tom background and homozygous for the mutations or transgenes.

Growth conditions and treatments

Plants were grown in 6-L rectangular pots containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, São Paulo, Brazil) and expanded vermiculite, supplemented with 1 g L⁻¹ of NPK 10:10:10, 4 g L⁻¹ of dolomite limestone (MgCO₃ + CaCO₃) and 2 g L⁻¹ thermophosphate (Yoorin Master®, Yoorin Fertilizantes, Brazil) in growth chamber at 250 μ mol m⁻² s⁻¹, 12-h photoperiod, air temperature of 27°C day / 22°C night and air humidity of approximately 60% day / 80% night. Immature green (IG) fruits were harvested about 5, 8, 10, 15 and 22 days after anthesis (dpa) corresponding to the stages IG1, IG2, IG3, IG4 and IG5, respectively. Fruits were also harvested after reaching the following ripening stages: MG (ca. 30 dpa, displaying jelly placental tissues), Bk (breaker, ca. 34 dpa, displaying the first external yellow color signals) and Bk1, Bk2, Bk3,

Bk6, Bk9, Bk12, Bk15, Bk18 and Bk21, corresponding to 1, 2, 3, 6, 9, 12, 15, 18 and 21 days after breaker, respectively. Fruits were divided into pericarp, columella and placental tissues + seeds. All biological samples were harvested at the same time of the day to avoid possible fluctuations in the parameters due to circadian rhythm. Four biological samples formed by at least five fruits each were harvested at each sampling time. Ethylene emission, iodine-staining and quantitative GUS analyses were performed immediately after harvesting. For other assays, fruits tissues were frozen in liquid nitrogen, ground and stored at -80°C.

Pigment quantification and fruit color

Chlorophyll extraction and quantification were carried out as described in Lira et al. (2016). Carotenoids were extracted and quantified as described in Sestari et al. (2014). Change in Hue angle and fruit color intensity (chroma) during tomato fruit development and ripening were measured using a Konica Minolta CR-400 colorimeter (Su et al., 2015).

Starch and soluble sugar quantification

Soluble sugars and starch were extracted as described in Lira et al. (Lira et al., 2016). The supernatant residue was measured in a HPLC system equipped with a pulsed amperometric detector (Dionex, Sunnyvale, USA) and a CarboPac PA1 (4 × 250 mm) column as described in Purgatto et al. (Purgatto et al., 2002). The endogenous metabolite concentration was obtained by comparing the peak areas of the chromatograms with commercial standards. Starch content was determined from dried pellets as described in Suguiyama et al. (Suguiyama et al., 2014).

Hormonal analysis

Endogenous indoleacetic acid (IAA) levels were determined by gas chromatography tandem mass spectrometry-selected ion monitoring (GC-MS-SIM). Frozen samples (100 mg FW) were extracted and methylated as described in Rigui et al. (2015). Approximately 0.5 µg of labeled [¹³C₆]IAA (Cambridge Isotopes, Inc.) was added to each sample as an internal standard. Extracts were analyzed on a gas chromatograph (GC) coupled to a mass spectrometer (MS) (model GCMS-QP2010 SE, Shimadzu) in selected ion monitoring mode as described in Melo et al. (2016). Ions with a mass ratio/charge (m/z) of 130 and 189 (corresponding to endogenous IAA) and 136 and 195 (corresponding to [¹³C₆]-IAA) were monitored. Endogenous concentrations were calculated based on extracted chromatograms at m/z 130 and 136.

Ethylene emission was determined in intact tomato fruits detached and enclosed in

a sealed transparent vial. The vial was flushed with ethylene-free air (1 L min^{-1}) for 5 min and incubated for 60 minutes under specific experimental conditions, as appropriate. After incubation, 1-mL gas samples were analyzed as described in Melo et al. (2016).

Endogenous ACC was extracted and analyzed as described by Bulens et al. (2011). Activity of ACO was determined according to Bulens et al. (2011), with the modifications described in Melo et al. (2016).

Hormone-responsive promoter analysis

Quantitative GUS activity assay was assayed according to Jefferson et al. (1987), with some modifications. Briefly, samples were ground in liquid nitrogen and subsequently homogenized in MUG extraction buffer composed of 50 mM Hepes-KOH (pH 7.0), 5 mM DTT and 0.5% (w/v) PVP. After centrifugation, 200 μL aliquots of the supernatant was mixed with 200 μL GUS assay buffer composed of 50 mM HEPES-KOH (pH 7.0), 5 mM DTT, 10 mM EDTA and 2 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) and incubated at 37°C for 30 minutes. Subsequently, aliquots of 100 μL were taken from each tube and the reactions were stopped and fluorescence was analyzed using a spectrofluorometer (LS55, Perkin Elmer) with 365 nm excitation and 460 nm emission wavelength (5 nm bandwidth).

Plastid abundance and structure

Pericarp samples were cut into small pieces (1 x 1 mm) and plastid ultrastructure was analyzed as described in Melo et al., (2016)

Plastid abundance was determined as in Li et al. (2016) with some modifications. Briefly, small pericarp pieces (1 x 1 mm) were incubated in glutaraldehyde 3.5% (v/v) for 60 min and cells were separated by incubation in Na-EDTA 0.1M pH 9.5 at 60°C for 180 min under continuous agitation. Isolated cells were visualized using a Leica microscope, and plastid densities were estimated on individual cells using the ImageJ program.

Transcriptional profile

Total RNA extraction, cDNA synthesis, primer design and qPCR assays were performed as described by Quadrana et al. (2013). Primer sequences used are detailed in Supplementary Table S2.1. qRT-PCR reactions were performed in a StepOnePlus PCR Real-Time thermocycler (Applied Biosystems) in a final volume of 10 μl using 2X SYBR Green Master Mix reagent (Thermo Fisher Scientific). Melting curves were checked for unspecific amplifications and primer dimerization. Absolute fluorescence data were analyzed using the LinRegPCR software package to obtain quantitation cycle (C_q) values and calculate primer efficiency. Transcript abundances were normalized against the

geometric mean of two reference genes, *CAC* and *EXPRESSED* (Exposito-Rodriguez et al., 2008).

Gene promoter analyses

Promoter sequences were retrieved from Sol Genomics Network (<https://solgenomics.net/>) and analyzed using PlantPAN 2.0 platform (<http://plantpan2.itps.ncku.edu.tw/>) (Chow et al., 2016) to identify the regulatory motifs. Typical 3 kb upstream from the initial codon ATG was analyzed for the presence of PBE-box (CACATG), G-box (CACGTG), CA-hybrid (GACGTA) and CG-hybrid (GACGTG) motifs, which are recognized by HY5 and/or PIFs (Martínez-García et al., 2000; Song et al., 2008), as well as the presence of canonical AuxRE (TGTGTC) and degenerate AuxRE (TGTGNC), which are recognized by ARFs (Chaabouni et al., 2009).

3. Results

PΦB deficiency impacts tomato fruit ripening

Previous studies have shown that mutations in specific phytochromes can significantly alter tomato fruit ripening (Gupta et al., 2014). Here, the impact of the deficiency in PΦB biosynthesis, which impairs the formation of functional phytochromes, on tomato fruit ripening was determined by monitoring fruit color, carotenoid content as well as ethylene biosynthesis and metabolism during the on-the-vine ripening of fruits from wild type (WT) and PΦB-deficient mutant *aurea* (*au*).

Time course analysis revealed that *au* and WT fruits attained MG stage at virtually the same number of days post anthesis (dpa) and showed no significant differences in fruit diameter throughout the growth and ripening phases (Supplementary Figure S2.1A-B). In contrast, the change in fruit coloration from green to yellow, which marks the transition from the MG to the Bk stage, was delayed by approximately 8 days in *au* compared to the WT (Figure 2.1A and Supplementary Figure S2.1C). After the Bk stage, the progression in the acquisition of distinctive red fruit coloration and the accumulation of carotenoids was indistinguishable in both genotypes (Figure 2.1A-B). The delayed-ripening phenotype of *au* fruits ripening on the vine under photoperiodic conditions (Figure 2.1) was also observed when fruits of this mutant were harvested at MG and left to ripen off the vine under constant white light or dark conditions (Supplementary Figure S2.2). During the off-the-vine ripening, the time required for the transition from the MG to the Bk stage was exactly the same in *au* fruits incubated under either light or dark conditions (Supplementary Figure S2.2). Conversely, ripening was initiated earlier in the WT fruits exposed to light than in those maintained under absolute darkness (Supplementary Figure S2.2).

In agreement with the changes in fruit coloration, the accumulation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the climacteric rise in ethylene emission in *au* fruits were also delayed by approximately 8 days compared to the WT (Figure 2.1C-D). Despite such temporal differences, the maximal levels of both ACC content and ethylene emission were only slightly lower in *au* fruits than in the WT (Figure 2.1C-D and Supplementary Figure S2.3A-B). In contrast, the activity of ACC oxidase (ACO), a key enzyme in ethylene production (Bulens et al., 2011), was both delayed and significantly reduced in *au* compared to WT (Figure 2.1E). This reduction in ACO activity in *au* was particularly evident in pericarp and columella tissues (Supplementary Figure S2.3C). In both WT and *au* fruits, the climacteric rise in ethylene production coincided with increases in ACO activity and preceded ACC accumulation (Figure 2.1C-E). The rise in ACC levels after the climacteric peak of ethylene production suggests that most ACC formed during the climacteric phase is converted into ethylene and the accumulation of the ethylene precursor at the post-climacteric phase may be a consequence of the progressive reduction in ACO activity (Van de Poel et al., 2014).

Figure 2.1. Deficiency in phytochromobilin biosynthesis delays tomato fruit ripening. Ripening-associated traits and ethylene metabolism and signaling were monitored in developing and ripening fruits of wild-type (WT) and *aurea* (*au*) mutant plants. (a) Fruit color estimated by Hue angle. (b) Total carotenoids content. (c) 1-aminocyclopropane-1-carboxylic acid (ACC) content. (d) Ethylene emission. (e) ACC oxidase (ACO) activity. (f) *In vitro* GUS activity assayed in fruits carrying the synthetic ethylene-responsive promoter *EBS* fused to the GUS reporter protein (*EBS::GUS* and *au-EBS::GUS*). Values shown are mean $\pm$ SE.

The impact of PΦB deficiency on ethylene signaling was evaluated by measuring the activity of the reporter protein GUS under the control of the ethylene-responsive

promoter *EBS* (Stepanova et al., 2007) and also by analyzing the transcript abundance of tomato genes encoding three ethylene responsive factors (ERFs) – *Sl-ERF.E1*, *Sl-ERF.E2* and *Sl-ERF.E4* – whose expression patterns are closely associated with the ripening process (Liu et al., 2016). The peak in ethylene signaling output took place approximately 8 days later in *au* fruits compared to WT, temporally coinciding with the climacteric rise in the production of this hormone in each of these genotypes (Figure 2.1F). In both genotypes, the maximum activation of *EBS* promoter was observed earlier in pericarp and columella (Bk1) than in the placental tissues and seeds (Bk12) (Supplementary Figure S2.4). In both genotypes, transcript abundance of *Sl-ERF.E2* and *Sl-ERF.E4* progressively increased during ripening (Supplementary Figure S2.4). Overall, no marked differences in *Sl-ERF.E1*, *Sl-ERF.E2* and *Sl-ERF.E4* transcript abundance were observed between *au* and WT at each fruit development stage (Supplementary Figure S2.4).

Therefore, our data suggest that the *PΦB* deficiency significantly delays the initiation of the ripening in tomato but has a very limited impact on the progression of ripening once started. Consequently, from this point forward, stage-based comparisons between WT and *au* genotypes will be presented rather than time-course comparisons.

PΦB deficiency reduces plastid abundance in pericarp cells

Among many other processes, phytochromes are known to regulate the accumulation of photosynthetic pigments (Waters and Langdale, 2009). Consistently, immature fruits of the *au* mutant exhibited a distinctive pale-green coloration as confirmed both by the reduced total chlorophyll content and significantly lower color saturation (chroma, which is indicative of color intensity) compared to WT (Figure 2.2A-B).

As phytochrome signaling can influence both plastid biogenesis and differentiation (Shin et al., 2009; Waters and Langdale, 2009), it became relevant to assess whether the pale-green phenotype and the associated reduced chlorophyll content in *au* fruits resulted from changes in plastid abundance or alterations in plastid ultrastructural features. Microscopy analysis of pericarp cells revealed a reduction of approximately 70% in the number of chloroplasts per cell in *au* compared to WT (Figure 2.2C). Chloroplast were not only less abundant but also smaller in diameter in *au* than in WT (Figure 2.2D).

The internal membranous structure of the plastids was remarkably similar in pericarp cells of *au* and WT immature fruits (Supplementary Figure S2.5). Well-developed grana and stroma thylakoids were equally observed in both genotypes. In WT fruits, the dismantling of grana thylakoids, indicative of the conversion of chloroplasts into chromoplasts, became evident from the Bk stage onwards. In contrast, dismantlement of

grana thylakoids in plastids of *au* pericarp cells was initiated earlier, at the MG stage (Supplementary Figure S2.5). During pre-climacteric (IG to MG) and climacteric phases (Bk to Bk3), plastids from *au* pericarp cells exhibited a tendency to present more numerous plastoglobuli than the WT (Supplementary Figure S2.5), presumably associated with the accumulation of phytol molecules not diverted to chlorophyll production in this pale-green mutant (Gauze et al., 2007).

Figure 2.2. Deficiency in phytochromobilin leads to impaired plastid biogenesis and reduced chlorophyll in pericarp cells. Chlorophyll content, fruit color intensity and plastid abundance per cell were monitored in developing and ripening fruits of wild-type (WT) and *aurea* (*au*) mutant plants. **(a)** Pericarp chlorophyll content. **(b)** Chroma, which indicates color intensity. **(c)** Plastid density per pericarp cell. Values shown are mean $\pm$ SE. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit developmental stage. **(d)** Light microscopy of isolated pericarp cells of immature fruits. Increased chloroplast size and abundance can be observed in WT compared to *au* cells. IG1 to IG5, immature green stages (corresponding to fruit development); MG, mature green; Bk to Bk21, 0 to 21 days after breaker stage (corresponding to the ripening phase).

Despite the lower abundance and size of the chloroplasts in unripe fruits of *au* compared to the WT (Figure 2.2D), the total carotenoid content was virtually indistinguishable in fully ripe fruits of both genotypes (Figure 2.1 and Supplementary Figure S2.2). In agreement, chromoplast number per pericarp cell in fully ripe *au* fruits (317.5 ± 71.1) was statistically similar to that observed in WT counterparts (351.8 ± 75.7). These data suggest that intensified plastid replication may occur during the ripening of *au* fruits allowing chromoplasts to accumulate to levels as high as those observed in the WT.

This assumption is supported by previous observations in other tomato mutants that also possess extremely reduced chloroplast abundance in unripe fruits but exhibits chloroplast densities similar to the WT at fully ripe stage (Forth and Pyke, 2006).

Collectively, these data suggest that the distinctive pale-green phenotype and associated reduced chlorophyll content observed in *au* mutant primarily result from a significant reduction in chloroplast abundance and size at pre-climacteric fruit development rather than changes in chloroplast ultrastructure. Data also suggest that intensified plastid replication during the climacteric phase compensate for the limited plastid biogenesis detected in unripe fruits of the PΦB-deficient mutant.

PΦB deficiency leads to reduced sugar import and accumulation in tomato fruits

It has been estimated that fruit photosynthesis provides up to 20% of the photoassimilates accumulated in this organ (Tanaka et al., 1974; Hetherington et al., 1998; Cocaliadis et al., 2014). Thus, we further addressed whether the reduced chlorophyll content and plastid abundance are associated with alterations in sugar content in *au* fruits. Indeed, at the onset of fruit expansion, which corresponds to immature green 3 and 4 (IG3 and IG4) stages, reductions of up to 50% in starch content were observed in the pericarp of *au* fruits compared to WT (Figure 2.3A). Iodine-staining analysis revealed conspicuously more intense blue-purple color, indicative of the presence of starch, in WT than *au* immature green fruits (Figure 2.3B). As expected, starch levels progressively decreased towards undetectable levels at the ripening phase (Figure 2.3A). As ADP-glucose pyrophosphorylase (AGPase) represents a limiting enzyme in starch production in tomato fruit (Petreikov et al., 2009), the mRNA levels of genes encoding AGPase large (*Sl-AGPaseL1*, *Sl-AGPaseL2* and *Sl-AGPaseL3*) and small (*Sl-AGPaseS1*) subunits were profiled in immature green fruits (IG2 to IG4). Overall, the mRNA levels of AGPase-encoding genes were significantly reduced in pericarp and columella tissues of *au* compared to the WT (Figure 2.3C).

Sucrose and glucose were also significantly less abundant in *au* than in WT during early stages of fruit development (Figure 2.3D and Supplementary Figure S2.6). At the end of the ripening phase (Bk12), columella and pericarp sucrose levels were approximately 40% lower in *au* than in WT fruits (Figure 2.3D). When sucrose, glucose and fructose levels were combined at each fruit development stage, an overall tendency of reduced soluble sugar content in *au* through development was revealed (Supplementary Figure S2.6).

Figure 2.3. Deficiency in phytochromobilin negatively impacts fruit sugar accumulation and transcript abundance of sink- and starch biosynthesis-related genes. Carbohydrate content and transcript levels of sink-related genes were monitored in developing and ripening fruits of wild-type (WT) and *aurea* (*au*) mutant plants. **(a)** Starch content in pericarp cells. **(b)** Lugol staining in immature fruits (IG3). The blue-purple color indicates starch reaction with iodine. **(c)** Transcript abundance of tomato genes encoding ADP-glucose pyrophosphorylase (*AGPase*) in immature green (IG2 to IG4) fruits. **(d)** Sucrose content in pericarp and columella tissues. **(e)** Transcript abundance of tomato genes encoding invertases (*Sl-LIN*) and sucrose transporters (*Sl-SUT*). Values shown are mean $\pm$ SE. Transcript abundance was normalized against WT at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit developmental stage.

Although fruit photosynthesis may provide part of the carbohydrates accumulated in tomato fruits (Tanaka et al., 1974; Hetherington et al., 1998; Cocaliadis et al., 2014), this organ relies heavily on carbon and energy import from leaves to complete its growth and development (Lytovchenko et al., 2011; Cocaliadis et al., 2014; Osorio et al., 2014). Therefore, to investigate whether the PΦB deficiency also modulates sink-related genes in tomato fruits, a transcriptional profile of genes encoding cell-wall invertases (*Sl-LIN5*, *Sl-*

LIN6 and *Sl-LIN7*) and sucrose transport proteins (*Sl-SUT1* and *Sl-SUT2*) essential for fruit sink activity (Fridman and Zamir, 2003; Kocal et al., 2008) was performed in pericarp and columella samples from both *au* and WT genotypes. In line with the prevailing idea that columella plays a vital role in importing photoassimilates, mRNA levels of *Sl-LIN* and *Sl-SUT* genes were more abundant in this region than in the pericarp (Supplementary Figure 2.7). Moreover, consistent with the high demand of carbon and energy required for initial fruit growth, the highest transcript abundances of *Sl-LIN5*, *Sl-LIN6*, *Sl-LIN7* and *Sl-SUT2* in both genotypes were observed during early fruit development and were found to decrease during the ripening phase (Supplementary Figure S2.7). Overall, a trend of reduced mRNA levels of *LIN* and *SUT* genes in *au* compared to WT was observed in both columella and pericarp tissues (Figure 2.3E). At early fruit development (IG2 stage), all genes were downregulated in the columella of *au* compared to WT fruits (Figure 2.3E). Similarly, all *LIN* and *SUT* genes analyzed were also significantly downregulated in pericarp tissues of *au* during ripening (Bk3). Interestingly, *Sl-LIN5*, which encodes the most relevant cell-wall invertase to determine sink activity and brix content in developing tomato fruits (Fridman et al., 2004; Zanor et al., 2009), was significantly downregulated in *au* at all fruit development stages and tissues analyzed (Figure 2.3E).

The analysis of sugar metabolism showed that *PΦB* deficiency reduces starch biosynthesis and sink strength, which ultimately leads to decreased carbohydrate levels in fully ripe tomato fruits.

PΦB deficiency negatively impacts cytokinin and auxin signaling during early fruit development

Among plant hormones, cytokinins and auxins play significant roles in plastid biogenesis and development (Chory et al., 1994; Okazaki et al., 2009; Sagar et al., 2013) as well as sugar import, metabolism and accumulation in tomato fruits (Sagar et al., 2013; Albacete et al., 2014). To gain insight into the potential role played by these phytohormones during the phytochrome-dependent regulation of chloroplast development and sugar metabolism in tomato fruits, a spatiotemporal analysis of important cytokinin- and auxin-related signaling components was performed in developing fruits of *au* and WT tomato plants.

Among the downstream targets of CKs receptors, type-A ARABIDOPSIS RESPONSE REGULATORS (ARRs) represent an important convergence point between phytochrome and cytokinin signaling in vegetative tissues of Arabidopsis (Sweere et al., 2001; Salome et al., 2006). All five type-A *AtARR* homologous in tomato, namely *TOMATO RESPONSE REGULATOR (TRR) 3/4*, *TRR7/15*, *TRR8/9a*, *TRR8/9b* and

TRR16/17 (Shani et al., 2010), were significantly downregulated in the columella of early developing *au* fruits (IG2 stage) compared to the WT (Figure 2.4). As observed for *LIN* and *SUT* genes, mRNA levels of most type-A *TRR* genes were higher in columella of early developing fruits, decreasing during the ripening phase (Supplementary Figure S2.8).

Figure 2.4. Deficiency in phytochromobilin downregulates primary cytokinin response genes during early tomato fruit development. Transcriptional profile of *TOMATO RESPONSE REGULATOR* (*TRR*) genes was performed in pericarp and columella tissues of immature (IG), mature green (MG) and ripening (Bk3, 3 days after breaker) fruits of wild-type (WT) and *aurea* (*au*) mutant plants. Transcript abundance was normalized against WT at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, P<0.05) compared with the WT at each fruit development stage.

The impact of PΦB deficiency on auxins was first investigated by determining the endogenous content of indole-3-acetic acid (IAA), the main auxin in plants, and the activity of the reporter protein GUS expressed under the control of the *DR5* auxin-responsive promoter in fruits of *DR5::GUS* and *au-DR5::GUS* plants (Melo et al., 2016). Whereas *au* and WT fruits exhibited limited differences in IAA content (Figure 2.5A), *DR5* promoter activation was significantly lower in *au-DR5::GUS* than in *DR5::GUS* in both columella and pericarp (Figure 2.5B). Consistent with the idea that auxin signaling promotes tomato fruit set, cell division and enlargement at pre-climacteric phase while subsequently repressing fruit ripening (de Jong et al., 2011; Pattison and Catala, 2012; McAtee et al., 2013; Mazzucato et al., 2015; Su et al., 2015), analysis of the *DR5* promoter activation revealed that, regardless of the genotype or fruit tissue, the maximum auxin signaling

output occurs at immature and mature green stages, progressively decreasing during the ripening phase (Supplementary Figure S2.9). Histochemical GUS staining revealed that the auxin-responsive *DR5* promoter is more intensively active in the inner pericarp and columella tissues (Supplementary Figure S2.10), which is consistent with the higher *in vitro* GUS activity detected in the columella of *DR5::GUS* and *au-DR5::GUS* compared to other fruit tissues (Supplementary Figure S2.9).

Given the marked differences in *DR5* promoter activation between *au* and WT developing fruits, a transcriptional profile of genes encoding auxin-associated signaling proteins was performed. In the auxin signaling cascade, hormone perception leads to targeting Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE) proteins for degradation via 26S proteasome, thus reducing the cellular abundance of these key repressors of auxin response (Audran-Delalande et al., 2012; Sagar et al., 2013). Among the 26 members of the tomato *Aux/IAA* family, *Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9*, *Sl-IAA15*, *Sl-IAA27* and *Sl-IAA36* have been identified as the most prominently expressed in fruit tissues (Audran-Delalande et al., 2012); consequently, their transcript abundance was compared in developing *au* and WT fruits. At the MG stage, mRNA levels of most *Sl-IAA* genes in both columella and pericarp were significantly higher in *au* than WT (Figure 2.5C). Compared to WT, *Sl-IAA3* was upregulated in both columella and pericarp tissues of *au* in all fruit development stages analyzed. Except for *Sl-IAA3*, the expression of all *IAA* genes analyzed was significantly downregulated at ripening (Bk3) in columella and pericarp tissues of both genotypes (Supplementary Figure S2.11).

Aux/IAA proteins are known to inhibit the expression of auxin-associated genes by a constant physical inhibition of ARF transcript factors (Sagar et al., 2013; Zouine et al., 2014), which in tomato are encoded by a gene family comprising 22 members (Zouine et al., 2014). Therefore, the transcript abundance of eight *Sl-ARF* genes highly expressed in fruits (*i.e.* *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4*, *Sl-ARF5*, *Sl-ARF8a*, *Sl-ARF8b* and *Sl-ARF10a*) was also evaluated (Sagar et al., 2013; Hao et al., 2015). Compared to WT, most of the *Sl-ARF* genes analyzed were significantly downregulated in the columella and pericarp tissues of *au* early developing fruits (IG2 stage) (Figure 2.5D). With the exception of *Sl-ARF2a* and *Sl-ARF5*, mRNA levels of all other *Sl-ARF* genes were especially high during early fruit development, decreasing thereafter (Supplementary Figure S2.12).

Figure 2.5. Phytochromobilin deficiency negatively impacts auxin levels and signaling during early tomato fruit development. Auxin content, signaling output and transcript abundance of genes associated with auxin signal transduction were monitored in pericarp and columella tissues of immature (IG), mature green (MG), breaker (Bk) and ripening (Bk3, 3 days after breaker) fruits of wild-type (WT) and *aurea* (*au*) mutant plants. **(a)** Indole-3-acetic acid (IAA) content. **(b)** *In vitro* GUS activity assayed in fruits carrying the synthetic auxin-responsive promoter *DR5* fused to

the GUS reporter protein (*DR5::GUS* and *au-DR5::GUS*). (c) Relative transcript abundance of *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes. (d) Relative transcript abundance of *AUXIN RESPONSE FACTOR* (*ARF*) genes. The mean relative expression was calculated from means of two technical replicates of at least three biological replicates and normalized against WT at each fruit developmental stage. Values shown are mean $\pm$ SE. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage.

The analysis of the transcriptional profile of *TRR*, *Sl-IAA* and *Sl-ARF* genes revealed that the deficiency in P Φ B biosynthesis negatively impacts cytokinin and auxin signaling. Moreover, the presence of PBE-box, G-box, CA-hybrid and/or CG-hybrid motifs (Martínez-García et al., 2000; Song et al., 2008) within the 3-kb promoter sequence of most of these genes (Supplementary Figures S2.13 and S2.14) supports the hypothesis that HY5 and PIF transcription factors may directly control the expression of these cytokinin- and auxin-signalling genes.

Auxin signaling influences sugar metabolism in tomato fruit

The widely accepted positive role played by cytokinins in tomato fruit sink activity and sugar accumulation (Albacete et al., 2014) contrasts with the considerably more limited information regarding auxin influence on sugar import and accumulation during fruit development (Sagar et al., 2013). To further characterize the impacts of auxin signaling on tomato fruit sugar metabolism, the starch content, as well as the transcript levels of sink- and starch biosynthesis-related genes were determined in a *Sl-ARF4*-silenced line (*SlARF4-ASL*) (Sagar et al., 2013) and in the *Sl-IAA9* loss-of-function mutant *entire* (Zhang et al., 2007).

Overall, *Sl-ARF4-ASL* and *entire* exhibited significantly higher levels of starch than the WT during most of the initial fruit development (Figure 2.6A). These differences were particularly more pronounced at IG3 and IG4 stages, thus coinciding with the fruit development stages in which the starch levels were conspicuously lower in *au* than in the WT (Figure 2.6A). Accordingly, *SlAGPase* genes were upregulated in both pericarp and columella samples of immature fruits of the *Sl-ARF4-ASL* line (Figure 2.6B). Compared to WT, columella mRNA levels of *Sl-AGPaseL2*, *Sl-AGPaseL3* and *Sl-AGPaseS1* also increased in the *entire* mutant (Figure 2.6B). Among the *AGPase* genes, *Sl-AGPaseL2* expression was particularly disturbed in both the *au* mutant and the *Sl-ARF4*-silenced line (Figure 2.6B). Interestingly, *Sl-ARF4-ASL* and *entire* immature fruits exhibited increased transcript abundance of most sink-related genes analyzed, and the opposite was observed for the *au* mutant (Figure 2.6C).

Figure 2.6. Auxin promotes starch accumulation by increasing transcript abundance of sink- and starch biosynthesis-related genes. Starch content and transcript levels of sink- and starch biosynthesis-related genes were monitored during the early development of *aurea* (*au*), *entire* and *Sl-ARF4*-silenced line (*SIARF4-ASL*) fruits. **(a)** Starch content in pericarp and columella tissues of immature green (IG2 to IG4) fruits. **(b)** Transcript abundance of tomato genes encoding ADP-glucose pyrophosphorylase (*AGPase*) in pericarp and columella tissues of immature green 2 (IG2) fruits. **(c)** Transcript abundance of tomato genes encoding invertases (*Sl-LIN*) and sucrose transporters (*Sl-SUT*) in columella tissues of IG2 fruits. Values shown are mean $\pm$ SE. Transcript abundance was normalized against WT at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT. IG2 to IG4, immature green stages (corresponding to early fruit development).

Although the direct transcriptional regulation of tomato *AGPase*, *LIN* and *SUT* genes by auxin or light signaling-associated transcription factors remains to be determined, the presence of canonical and/or degenerated ARF-binding Auxin Response Element (AuxRE) motifs as well as *cis*-acting elements recognized by HY5 and/or PIFs within the 3 Kb fragment promoter of all these genes is consistent with this hypothesis (Supplementary Figure S2.15).

Phytochrome genes are highly expressed in tomato fruit columella

The most striking differences in transcript abundances of auxin-, cytokinin- and sink-related genes between *au* and WT were observed in the columella. As pericarp and columella are exposed to distinct light intensity and quality conditions (Llorente et al., 2016), the mRNA levels of phytochromes and light signaling-related genes were profiled.

Interestingly, the mRNA levels of all five genes encoding phytochromes in tomato were markedly higher in columella than in pericarp tissues (Figure 2.7). In early fruit

development (IG2), mRNA levels of *Sl-PHYA*, *Sl-PHYB1* and *Sl-PHYB2* were up to four-fold higher in columella than in pericarp, whereas for *Sl-PHYE* and *Sl-PHYF* the differences reached up to 20-30-fold. *Sl-PHYB1* and *Sl-PHYE* mRNA levels progressively reduced during ripening in columella, whereas less noticeable patterns in transcript abundances were observed for the other PHY-encoding genes (Figure 2.7). It is worth mentioning that *Sl-PHYB2* mRNA was the PHY-encoding gene most abundantly expressed in all the tissues and stages evaluated (Supplementary Table S2.1).

Transcript levels of all tomato PHY-encoding genes were drastically reduced in *au* fruits compared to the WT, which may reflect a negative feedback mechanism triggered by the deficiency in PΦB of this mutant (Figure 2.7). Regarding the light signaling-associated genes analyzed, *Sl-COP1*, *Sl-DDB1* and *Sl-DET1* showed similar mRNA levels in *au* and WT; however, *Sl-CUL4* transcript abundance was remarkably lower in *au* fruits in both, pericarp and columella (Supplementary Figure S2.16). Negligible differences in mRNA levels were observed between columella and pericarp regardless of genotype or fruit development stage analyzed.

Figure 2.7. Deficiency in phytochromobilin downregulates PHY-encoding genes. Relative mRNA levels of the five tomato genes encoding phytochromes (*Sl-PHY*) in the pericarp and columella tissues of wild type (WT) and *aurea* (*au*) fruits harvested at immature (IG2), mature green (MG) and ripening (Bk3) stages. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Collectively, these data suggest that light perception and signal transduction may be active even in the most internal tissues, and the more intensive expression of *PHYs* might compensate for the reduced amount of incident radiation in columella. Moreover, the data also revealed that all PHY-encoding genes are markedly downregulated in the *au*

mutant, which further suggests that this PΦB-deficient mutant may represent a suitable model for investigating the impact of global deficiency of functional phytochromes on tomato fruit development and metabolism.

4. Discussion

The role of light and hormones in plastid biogenesis and development as well as in sugar import, metabolism and accumulation in tomato fruits has been assessed in several studies (Kolotilin et al., 2007; Azari et al., 2010; Azari et al., 2010; Sagar et al., 2013); however, whether and how the signaling cascades triggered by these stimuli crosstalk remains elusive. Here, the impact of the potential interconnection between light and hormone signaling in tomato fruit metabolism was investigated by applying a mutant-based approach and time course analysis of two functionally and spatially contrasting fruit tissues: the pericarp and the columella.

It is widely accepted that climacteric tomato fruit ripening is triggered only after the cell expansion phase is complete and seeds are mature (Giovannoni, 2004). This dramatic change in the developmental program is marked by an intermittent rise in ethylene production and signaling (Giovannoni, 2004). Whereas distinct effects on fruit growth and ripening progression were observed in *phyA*, *phyB1*, *phyB2*, *phyAB1*, *phyB1B2* and *phyAB1B2* tomato mutants (Gupta et al., 2014), here we demonstrate that deficiency in phytochrome chromophore synthesis leads to a significant delay in the initiation of the fruit ripening process. However, no impact was observed on the time course of fruit growth, in the magnitude of climacteric ethylene production and signaling, nor in the progression of ripening once started (Figure 2.1). During normal vegetative growth, when auto-inhibitory ethylene production predominates, phytochrome-dependent light perception has been shown to downregulate ethylene biosynthesis (Vangronsveld et al., 1988; Melo et al., 2016). In contrast, here we provide genetic evidence that the deficiency in PΦB biosynthesis postpones the autostimulatory ethylene production in tomato fruit, consequently delaying the initiation of ripening.

Another distinctive phenotypical trait observed in early developing *au* fruits was the lower chloroplast abundance and the reduced levels of chlorophyll conferring their characteristic pale-green phenotype (Figure 2.2). These findings have several implications. First, they agree with the increased plastid biogenesis widely reported in loss-of-function or silencing of tomato genes encoding light signaling repressors such as DET1, DDB1, CUL4 and COP1 (Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010). Second, fruit photosynthesis and consequently the local production of photoassimilates might be limited, thus explaining, at least in part, the lower carbohydrate

levels observed in this mutant and reinforcing the fact that fruit photosynthesis is a source of carbon for this organ. Third, besides their role in fruit photosynthesis, plastids are also critical for the accumulation of starch granules in immature tomato fruits and are the site of action of AGPases, which are chloroplast-localized enzymes (Geigenberger, 2011). Therefore, the reduced starch accumulation and lower transcript abundance of *AGPase* genes observed in *au* fruits may simply reflect the limited chloroplast biogenesis observed in this PΦB-deficient mutant. Additionally, the impact of the impaired phytochrome chromophore biosynthesis on sugar metabolism was not only the result of the reduced number of chloroplasts but also the weaker sink strength in *au* fruits evidenced by the downregulation of cell wall invertases and sucrose transporters (Figure 2.3).

The simultaneous reduction in transcript abundance of *Sl-ARF* and type-A *TRR* genes, associated with the upregulation of some particular *Sl-IAA* genes (*e.g.*, *Sl-IAA3*) and the reduction in *DR5* promoter activity observed in *au* immature fruits (Figures 2.4 and 2.5) strongly suggest that phytochrome deficiency negatively influences cytokinin and auxin signaling in tomato fruits. The downregulation of type-A *TRR* genes observed in immature *au* fruits is in line with the key role 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), suggesting that similar phytochrome-cytokinin interaction mechanisms might also take place during the early development of tomato fruits. Whether the negative impact of the PΦB deficiency on type-A *TRR* mRNA levels correlates with changes in the endogenous levels of cytokinins, as demonstrated in other plant models (Roman et al., 2016), remains to be investigated.

Interestingly, as the reduction in the auxin-responsive *DR5* promoter activity observed in *au* fruits was not accompanied by significant changes in endogenous auxin content, auxin responsiveness rather than biosynthesis or transport seems to be the primary phytochrome-auxin link during the early stages of tomato fruit development. Considering the fact that *Aux/IAA* genes encode short-lived proteins that bind to and repress the ARF transcript factors (Zouine et al., 2014), one possible explanation for the altered auxin responsiveness observed in the PΦB-deficient mutant is the direct transcriptional regulation of *Aux/IAA* and *ARF* genes by light signaling-associated transcription factors. This is supported by the presence of PIF and/or HY5 binding motifs within the promoters of most of these genes. Moreover, biochemical evidence indicates that at least some Arabidopsis *Aux/IAA* proteins are post-translationally regulated by phytochrome-mediated phosphorylation (Colón-Carmona et al., 2000); consequently, the direct influence of phytochrome on tomato *Aux/IAA* protein stability and action is also a promising venue

deserving further investigation.

Genetic and pharmacological evidence clearly indicates that cytokinins are key promoters of invertase activity, sink activity and sugar accumulation in developing tomato fruits (Albacete et al., 2014). It is also known that exogenous auxins can promote both sugar metabolism (Tang et al., 2015) and activity of sucrose-cleaving enzymes (Albacete et al., 2014) in immature tomato fruits. In agreement, our data show that the downregulation of *Sl-ARF4*, which is known to act as a repressor rather than a promoter of the transcription of auxin-responsive genes (Sagar et al., 2013), results in increased starch content along with higher mRNA levels of most AGPase-encoding genes in early developing tomato fruits (Figure 2.6), which agrees with previous reports using this genotype (Sagar et al., 2013). Importantly, sink-related genes were also upregulated in the *SlARF4*-suppressed line, thus suggesting that not only starch accumulation (Sagar et al., 2013) but also sugar import may be negatively regulated by this auxin transcriptional regulator. Moreover, the detection of increased starch content and mRNA levels of genes involved in starch synthesis and sink activity in immature fruits of the auxin-constitutive response mutant *entire* (Figure 2.6) further supports the positive role of auxin on sugar metabolism in tomato fruits. In agreement with our findings, fruits of an *Aux/IAA9* frame-shift tomato mutant identified via TILLING-based screening exhibited an increased brix value compared to the WT in the fully ripe stage under either greenhouse or open-field conditions (Mazzucato et al., 2015). Therefore, although the direct regulation of tomato *AGPase*, *LIN* and *SUT* genes by light signaling-associated transcription factors also cannot be excluded due to the presence of HY5 and/or PIF bindings motifs in their promoter regions, it seems plausible to hypothesize that the reduced sugar content and transcript abundance of sink- and starch biosynthesis-related genes observed in early developing *au* fruits may be associated with the lower cytokinin and auxin signaling output detected in this PΦB-deficient mutant compared to the WT.

Finally, despite their contrasting localization within tomato fruits, pericarp and columella tissues exhibited surprisingly similar phytochrome-dependent hormonal changes during early fruit development (Figure 2.8), which suggests that phytochrome signaling cascade may be active in both of these fruit regions. Recent reports indicate a self-shading effect as sunlight passes through the flesh of green tomato fruits, resulting in a progressive reduction in both light transmittance and R/FR ratios at increasing depths within the fruit tissues (Llorente et al., 2016). Therefore, internal fruit regions, such as the columella, presumably have a significantly higher proportion of phytochromes in their inactive form compared to more externally positioned fruit tissue layers such as the pericarp. As

markedly higher transcript abundance of all tomato phytochrome-encoding genes was observed in tomato columella than in pericarp tissues, we are left to speculate whether potential increments in phytochrome apoprotein abundance in the columella represent a compensatory mechanism to overcome the presumably less R-enriched light reaching the inner tissues of tomato fruit. The limited difference in transcript abundance of genes encoding light signaling-associated proteins, such as COP1, DDB1, DET1 and CUL4, in pericarp and columella tissues is consistent with such a hypothesis.

Figure 2.8. Impact of phytochrome chromophore deficiency on auxin and cytokinin signaling and its potential influence on sugar metabolism and plastid development during early tomato fruit development. At immature green stage, phytochrome chromophore phytochromobilin (PΦB) deficiency upregulates *AUXIN/INDOLE-3-ACETIC ACID3* (*Aux/IAA3*) and downregulates *AUXIN RESPONSE FACTOR* (*ARF*) and type-A *TOMATO RESPONSE REGULATOR* (*TRR*) genes in both columella and pericarp tissues, consequently leading to reduced auxin (AUX) and cytokinin (CK) signaling output in both of these fruit regions. In columella cells, the PΦB-deficiency-triggered reduction in AUX and CK signaling downregulates genes encoding key enzymes involved in starch synthesis, such as ADP-glucose pyrophosphorylase (AGPases), and sink activity, such as cell-wall invertases (LINs) and sucrose transport proteins (SUTs). In pericarp cells, similar PΦB-deficiency-triggered changes in hormonal signaling inhibit both starch biosynthesis and chloroplast formation. The downregulation of sink-related genes in columella is believed to reduce sugar import to the fruit, whereas the impaired chloroplast formation in pericarp cells may restrict local photoassimilate production via fruit-localized photosynthesis. Therefore, the reduced fruit starch accumulation in PΦB-deficient plants may be associated with the more limited sugar import and fruit photosynthesis in this genotype. Arrows at the ends of lines indicate stimulatory influence. Dotted lines indicate hypothesized mechanisms. Transcript abundance data were expressed as the ratio between qPCR values obtained in PΦB-deficient (*aurea*) and wild-type samples at the immature green 2 (IG2) stage, corresponding to early fruit development.

As the deficiency in PΦB inexorably limits the formation of functional phytochromes, the downregulation of all tomato PHY-encoding genes in *au* suggests that the production of the PHY apoproteins in the plant cells may be inhibited via a negative feedback loop mediated by the accumulation of nonfunctional molecules of this photoreceptor. Therefore, besides the limited abundance of PΦB molecules, the *au* mutation also leads to a global deficiency in PHY apoproteins, thus reinforcing the relevance of this genotype for investigating the impacts of global phytochrome deficiency on fruit biology and quality traits. However, it is plausible to assume that not all phenotypical alterations detected in *au* may be directly associated with its deficiency in functional phytochromes as the impaired production of PΦB molecules may also affect other physiological processes (Terry and Kendrick, 1999). Hence, additional studies including single and multiple *phy* tomato mutants might be instrumental to further evaluate the phytochrome-dependent regulation of tomato fruit sugar metabolism and plastid development.

Altogether, the data showed that PΦB deficiency impacts chloroplast formation and sugar metabolism during early tomato fruit development via changes in both cytokinin and auxin signaling, revealing a link between hormones and phytochromes that regulates sugar import, metabolism and accumulation in tomato fruits. This study also opens up a window of opportunity for improving fruit ripening, sink strength and sugar content through the combined manipulation of phytochrome- and hormone-related genes.

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6. Supplementary Information

Supplementary Figure S2.1 Comparative fruit development and ripening in wild type and phytochromobilin-deficient mutant.

Supplementary Figure S2.2 Impact of light treatment on tomato fruit ripening.

Supplementary Figure S2.3 Ethylene metabolism in developing tomato fruits.

Supplementary Figure S2.4 Ethylene signaling in developing tomato fruits.

Supplementary Figure S2.5 Transmission electron microscopy of plastids from pericarp cells.

Supplementary Figure S2.6 Soluble carbohydrate profile in developing tomato fruits.

Supplementary Figure S2.7 Transcript abundance of sink-related genes in tomato fruits.

Supplementary Figure S2.8 Transcript abundance of *TRR* genes in tomato fruits.

Supplementary Figure S2.9 Auxin signaling output in developing tomato fruits.

Supplementary Figure S2.10 Spatial pattern of *DR5::GUS* expression during fruit development.

Supplementary Figure S2.11 Transcript abundance of *Aux/IAA* genes in tomato fruits.

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Supplementary Figure S2.13 HY5- and PIF-binding motifs identified in the promoter region of *TRR* genes.

Supplementary Figure S2.14 HY5- and PIF-binding motifs identified in the promoter region of *Aux/IAA* and *ARF* tomato genes.

Supplementary Figure S2.15 HY5-, PIF- and ARF-binding motifs identified in the promoter region of sink- and starch biosynthesis-related tomato genes.

Supplementary Figure S2.16 Transcript abundance of light signaling-related genes in developing tomato fruits.

Supplementary Table S2.1 Relative transcript ratio of *Sl-PHY* genes in pericarp and columella tissues.

Supplementary Table S2.2 Primer sequences used for qPCR.

Supplementary Figure S2.1. Comparative fruit development and ripening in wild type and phytochromobilin-deficient mutant. Chronological development and time course analysis of increase in diameter of wild-type (WT) and *aurea* (*au*) fruits harvested at five immature green stages (IG1 to IG5), mature green (MG), Bk (breaker), Bk1 (1 day after Bk), Bk2, Bk3, Bk6, Bk9, Bk12, Bk15, Bk18 and Bk21. **(a)** Time interval in days post anthesis (dpa) to attain each of these fruit sampling stages. **(b)** Time course of increase in fruit diameter from anthesis until fully ripe stage. Diameter was recorded using a digital caliper and the time interval to attain specific development stages was monitored in on-the-vine ripening fruits ($n \geq 20$). **(c)** Time interval (dpa) from anthesis to MG and from MG to Bk stage. **(d)** Representative image of WT (above) and *au* (below) fruits at distinct development stages. Asterisks indicate statistically significant differences compared with the WT at each fruit development stage (Student's t-test, $P < 0.05$).

Supplementary Figure S2.2. Impact of light treatment on tomato fruit ripening. Fruits of wild-type (WT) and *aurea* (*au*) mutant were left to ripen off-the-vine under constant white light (approximately $50 \mu\text{mol m}^{-2} \text{s}^{-1}$) or absolute dark conditions. Samples were harvested at mature green (MG, two days after the beginning of the treatment), breaker (Bk), Bk1 (1 day after Bk), Bk3, Bk6 and Bk12 stages. **(a)** Time required for the transition from MG to Bk stage. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within genotypes and light treatments. **(b)** Ripening-related changes in fruit color (Hue angle), fruit color intensity (Chroma) and total carotenoid content. Values shown are mean $\pm$ SE.

Supplementary Figure S2.3. Ethylene metabolism in developing tomato fruits. Comparison of ethylene metabolism in wild-type (WT) and *aurea* (*au*) fruits harvested at five immature green stages (IG1 to IG5), mature green (MG), Bk (breaker), Bk1 (1 day after Bk), Bk2, Bk3, Bk6, Bk9, Bk12, Bk15, Bk18 and Bk21. **(a)** Ethylene emission in whole fruits. **(b)** 1-aminocyclopropane-1-carboxylic acid (ACC) content in pericarp tissues. **(c)** ACC oxidase (ACO) activity in the pericarp, columella and placental tissues + seeds. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.4. Ethylene signaling in developing tomato fruits. Comparison of ethylene signaling output in developing wild-type (WT) and *aurea* (*au*) fruits. **(a)** *In vitro* GUS activity assayed in the pericarp, columella and placental tissues + seeds of ripening fruits carrying the ethylene-responsive promoter *EBS* fused to the GUS reporter protein (*EBS::GUS* and *au-EBS::GUS*). **(b)** Transcript abundance of genes encoding the ethylene-responsive factors (ERFs) *SI-ERF.E1*, *SI-ERF.E2* and *SI-ERF.E4* in the pericarp and columella tissues of immature (IG2), mature green (MG) and ripening (Bk3) fruits. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.5. Transmission electron microscopy of plastids from pericarp cells. (a) Immature green fruits (IG1 to IG3). (b) Mature green (MG) and ripening fruits (Bk1 and Bk3, 1 and 3 days after breaker stage, respectively). Typical thylakoids including grana stacks can be observed in both wild-type (WT) and *aurea* (*au*) immature green fruits. Precocious chloroplast-to-chromoplast conversion and accumulation of plastoglobuli can also be observed in *au* mutant at MG stage.

Supplementary Figure S2.6. Soluble carbohydrate profile in developing tomato fruits. Comparison of carbohydrate profile in wild-type (WT) and *aurea* (*au*) fruits harvested at two immature green stages (IG2 and IG3), mature green (MG), Bk3 (3 day after Breaker), Bk6 and BK12. **(a)** Glucose, fructose and sucrose content in pericarp and columella tissues. **(b)** Summed values of these three soluble carbohydrates in pericarp and columella tissues. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.7. Transcript abundance of sink-related genes in developing tomato fruits. Relative mRNA levels of tomato genes encoding invertases (*SI-LIN*) and sucrose transporters (*SI-SUT*) in the pericarp and columella tissues of wild-type (WT) and *aurea* (*au*) fruits harvested at immature (IG2), mature green (MG) and ripening (Bk3) stages. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.8. Transcript abundance of *TRR* genes in developing tomato fruits. Relative mRNA levels of *TOMATO RESPONSE REGULATOR* (*TRR*) in the pericarp and columella tissues of wild-type (WT) and *aurea* (*au*) fruits harvested at immature (IG2), mature green (MG) and ripening (Bk3) stages. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.9. Auxin signaling output in developing tomato fruits. *In vitro* GUS activity assayed in the pericarp, columella and placental tissues + seeds of developing wild-type (WT) and *aurea* (*au*) fruits carrying the auxin-responsive promoter *DR5* fused to the GUS reporter protein (*DR5::GUS* and *au-DR5::GUS*). Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.10. Spatial pattern of *DR5::GUS* expression during fruit development. Histochemical GUS staining assayed in immature green fruits (IG2 to IG4) and mature green (MG) fruits of wild-type (WT) and *aurea* (*au*) carrying the auxin-responsive promoter *DR5* fused to the GUS reporter protein (*DR5::GUS* and *au-DR5::GUS*). Bar: 1 cm.

Supplementary Figure S2.11. Transcript abundance of *Aux/IAA* genes in developing tomato fruits. Relative mRNA levels of the *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) tomato genes *SI-IAA3*, *SI-IAA4*, *SI-IAA9*, *SI-IAA15*, *SI-IAA27* and *SI-IAA36* in pericarp and columella tissues of wild-type (WT) and *aurea* (*au*) fruits harvested at immature (IG2), mature green (MG) and ripening (Bk3) stages. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.12. Transcript abundance of *ARF* genes in developing tomato fruits. Relative mRNA levels of *AUXIN RESPONSE FACTOR* (*ARF*) tomato genes *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4*, *Sl-ARF5*, *Sl-ARF8a*, *Sl-ARF8b* and *Sl-ARF10a* in pericarp and columella tissues of wild-type (WT) and *aurea* (*au*) fruits harvested at immature (IG2), mature green (MG) and ripening (Bk3) stages. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between *au* and WT at each fruit development stage.

Supplementary Figure S2.13. HY5- and PIF-binding motifs identified in the promoter region of *TRR* genes. Fragments of 3 kb upstream ATG initiation site of genes encoding type-A TOMATO RESPONSE REGULATORS (TRRs) are represented by a blue line. Motif positions are indicated by triangles: PBE-box (green), recognized by PHYTOCHROME-INTERACTING FACTORS (PIFs). CA hybrid (black) and CG hybrid (yellow), recognized by LONG HYPOCOTYL 5 (HY5). G-box (red), recognized by both PIFs and HY5.

Supplementary Figure S2.14. HY5- and PIF-binding motifs identified in the promoter region of *Aux/IAA* and *ARF* tomato genes. Fragments of 3 kb upstream ATG initiation site of *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) and *AUXIN RESPONSE FACTOR* (*ARF*) tomato genes are represented by a blue line. Motif positions are indicated by triangles: PBE-box (green), recognized by PHYTOCHROME-INTERACTING FACTORS (PIFs). CA-hybrid (black) and CG-hybrid (yellow), recognized by LONG HYPOCOTYL 5 (HY5). G-box (red), recognized by both PIFs and HY5.

Supplementary Figure S2.15. HY5-, PIF- and ARF-binding motifs identified in the promoter region of sink- and starch biosynthesis-related tomato genes. Fragments of 3 kb upstream ATG initiation site of the tomato genes encoding cell-wall invertases (*SI-LIN*), sucrose transporters (*SI-SUT*) and the small and large subunits of AGPases (*SI-AGPase*) are represented by a blue line. Motif positions are indicated by triangles: PBE-box (green), recognized by PHYTOCHROME-INTERACTING FACTORS (PIFs). CA-hybrid (black) and CG-hybrid (yellow), recognized by LONG HYPOCOTYL 5 (HY5). G-box (red), recognized by both PIFs and HY5. Canonical AuxRE (purple) and degenerated AuxRE (gray), recognized by AUXIN RESPONSE FACTORS (ARFs).

Supplementary Figure S2. 16. Transcript abundance of light signaling-related genes in developing tomato fruits. Relative mRNA levels of tomato genes encoding light signaling associated genes in pericarp and columella tissues of wild-type (WT) and *aurea* (*au*) fruits harvested at immature (IG2), mature green (MG) and ripening (Bk3) stages. Mean relative expression was normalized against pericarp WT samples at IG2 stage. Values shown are mean $\pm$ SE. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among fruit development stages within each genotype. Asterisks indicate statistically significant differences (Student's t-test, $p < 0.05$) between *au* and WT at each fruit development stage. The abbreviations indicate the following: *SI-COP1*, *CONSTITUTIVE PHOTOMORPHOGENIC 1*; *SI-CUL4*, *CULLIN 4*; *SI-DET1*, *DE-ETIOLATED1*; *SI-DDB1*, *UV-DAMAGED DNA BINDING PROTEIN 1*.

Supplementary Table S2.1. Relative transcript ratio of *SI-PHY* genes in pericarp and columella tissues.

		<i>SI-PHYA</i>	<i>SI-PHYB1</i>	<i>SI-PHYB2</i>	<i>SI-PHYE</i>	<i>SI-PHYF</i>
IG2 Pericarp	<i>SI-PHYA</i>	1.0 ± 0.2	3.0 ± 0.6	0.2 ± 0.03	2.0 ± 0.4	0.8 ± 0.1
	<i>SI-PHYB1</i>	0.3 ± 0.1	1.0 ± 0.4	0.05 ± 0.02	1.2 ± 0.5	0.3 ± 0.1
	<i>SI-PHYB2</i>	6.8 ± 2.2	20.2 ± 6.4	1.0 ± 0.3	13.9 ± 4.4	5.3 ± 1.6
	<i>SI-PHYE</i>	0.5 ± 0.2	1.4 ± 0.6	0.1 ± 0.0	1.0 ± 0.4	0.4 ± 0.2
	<i>SI-PHYF</i>	1.3 ± 0.46	3.8 ± 1.3	0.2 ± 0.1	2.6 ± 0.9	1.0 ± 0.4
MG Pericarp	<i>SI-PHYA</i>	1.0 ± 0.2	0.6 ± 0.13	0.1 ± 0.02	1.6 ± 0.4	0.9 ± 0.1
	<i>SI-PHYB1</i>	1.4 ± 0.4	1.0 ± 0.28	0.1 ± 0.04	1.1 ± 0.3	0.9 ± 0.2
	<i>SI-PHYB2</i>	9.8 ± 1.9	7.0 ± 1.3	1.0 ± 0.2	15.7 ± 3	6.4 ± 1.2
	<i>SI-PHYE</i>	0.6 ± 0.3	0.5 ± 0.2	0.06 ± 0.03	1.0 ± 0.4	0.4 ± 0.2
	<i>SI-PHYF</i>	1.5 ± 0.4	1.1 ± 0.3	0.2 ± 0.04	3.8 ± 0.6	1.0 ± 0.2
Bk3 Pericarp	<i>SI-PHYA</i>	1.0 ± 0.5	0.5 ± 0.2	0.1 ± 0.06	0.7 ± 0.3	1.6 ± 0.7
	<i>SI-PHYB1</i>	2.2 ± 0.2	1.0 ± 0.10	0.3 ± 0.03	1.0 ± 0.1	1.0 ± 0.2
	<i>SI-PHYB2</i>	7.6 ± 0.8	3.6 ± 0.4	1.0 ± 0.1	5.3 ± 0.6	6.9 ± 0.8
	<i>SI-PHYE</i>	1.5 ± 0.2	0.7 ± 0.07	0.2 ± 0.02	1.0 ± 0.1	1.3 ± 0.1
	<i>SI-PHYF</i>	1.1 ± 0.2	0.5 ± 0.08	0.2 ± 0.02	1.0 ± 0.1	1.0 ± 0.2
IG2 Columella	<i>SI-PHYA</i>	1.0 ± 0.3	1.2 ± 0.2	0.1 ± 0.04	8.1 ± 2.2	1.0 ± 0.1
	<i>SI-PHYB1</i>	0.8 ± 0.2	1.0 ± 0.3	0.1 ± 0.03	1.1 ± 0.3	0.9 ± 0.2
	<i>SI-PHYB2</i>	7.8 ± 1.5	9.6 ± 1.6	1.0 ± 0.2	63 ± 12.4	9.1 ± 1.5
	<i>SI-PHYE</i>	0.1 ± 0.04	0.2 ± 0.04	0.02 ± 0.01	1.0 ± 0.32	0.1 ± 0.04
	<i>SI-PHYF</i>	0.8 ± 0.2	1.0 ± 0.2	0.1 ± 0.02	6.3 ± 1.3	1.0 ± 0.2
MG Columella	<i>SI-PHYA</i>	1.0 ± 0.4	0.4 ± 0.2	0.1 ± 0.03	2.5 ± 1.1	0.5 ± 0.1
	<i>SI-PHYB1</i>	2.6 ± 0.5	1.0 ± 0.2	0.2 ± 0.03	1.1 ± 0.2	1.7 ± 0.3
	<i>SI-PHYB2</i>	16.5 ± 4.1	6.4 ± 1.6	1.0 ± 0.3	42 ± 10.7	11.0 ± 2.7
	<i>SI-PHYE</i>	0.4 ± 0.1	0.2 ± 0.05	0.02 ± 0.01	1.0 ± 0.3	0.3 ± 0.1
	<i>SI-PHYF</i>	1.5 ± 1.1	0.6 ± 0.4	0.1 ± 0.07	2.4 ± 1.8	1.0 ± 0.3
Bk3 Columella	<i>SI-PHYA</i>	1.0 ± 0.5	0.7 ± 0.2	0.4 ± 0.1	2.7 ± 0.8	6.5 ± 0.9
	<i>SI-PHYB1</i>	3.0 ± 1.0	1.0 ± 0.6	0.9 ± 0.3	1.6 ± 0.8	8.1 ± 2.8
	<i>SI-PHYB2</i>	4.8 ± 0.9	2.3 ± 0.4	1.0 ± 0.5	9.1 ± 1.7	13.0 ± 2.4
	<i>SI-PHYE</i>	0.7 ± 0.3	0.3 ± 0.1	0.2 ± 0.08	1.0 ± 0.5	1.9 ± 0.7
	<i>SI-PHYF</i>	0.5 ± 0.1	0.3 ± 0.07	0.2 ± 0.05	0.8 ± 0.3	1.0 ± 0.5

SI-PHYB2 relative expression compared to other *SI-PHYs* is highlighted in bold terms. Values represent means ± SD from at least three biological replicates and are expressed as relative transcript amount comparing each *SI-PHY* gene. The abbreviations *SI-PHYA*, *SI-PHYB1*, *SI-PHYB2*, *SI-PHYE*, *SI-PHYF* indicate genes encoding tomato PHYTOCHROME A, B1, B2, E and F, respectively. Stages: immature (IG2), mature green (MG) and ripening (Bk3) stages.

Supplementary Table S2.2. Primer sequences used for qPCR.

Gene	Primers for qRT-PCR ¹	Locus ²
<i>SI-PHYA</i>	F: 5' - CACTCTCGTGGAGGATTCAT - 3' / R: 5' - GAGCCATAAAACACACACCC - 3'	Solyc10g044670
<i>SI-PHYB1</i>	F: 5' - ACTTCTGTTCCGGTCCATTCC - 3' / R: 5' - TCTCAGACAACCTGTGATGCC - 3'	Solyc01g059870
<i>SI-PHYB2</i>	F: 5' - GTGAGGGTTATTCCAGGATGA - 3' / R: 5' - TGACCATATACTGAGGGTGAC - 3'	Solyc05g053410
<i>SI-PHYE</i>	F: 5' - CGCTATTGAGGAACCCACTT - 3' / R: 5' - GCATCAACACCAATCAGACC - 3'	Solyc02g071260
<i>SI-PHYF</i>	F: 5' - ACTAGCCAAGATCATTGACG - 3' / R: 5' - CTCCAAGATTGAACTCACAAG - 3'	Solyc07g04548
<i>SI-COP1</i>	F: 5' - TTGCCCCTGTTGTTCTCA-3' / R: 5' - CCAATGAATGACGAACTGTTT - 3'	Solyc12g005950
<i>SI-CUL4</i>	F: 5' - GGAGGAATTGGAGGGGACATTA - 3' / R: 5' - GAAATCATGGACTTTTCAGCATCA - 3'	Solyc02g021470
<i>SI-DDB1</i>	F: 5' - ATCTCGTGCCTGGACATAAA - 3' / R: 5' - CGAAGGAACACATCAGAACA - 3'	Solyc02g021650
<i>SI-DET1</i>	F: 5' - GCTATTGACCGCCATAGACA - 3' / R: 5' - TCTTAGTTCGCCCATTCTGTG - 3'	Solyc01g056340
<i>SI-LIN5</i>	F: 5' - TTGGAAGGGATTGAGAATCG - 3' / R: 5' - AATTCCAGCCCATTCTTTCT - 3'	Solyc09g010080
<i>SI-LIN6</i>	F: 5' - AACCCGCTATCTACCCGTCT - 3' / R: 5' - GGGCTTGATCCACTTACGAA - 3'	Solyc10g083290
<i>SI-LIN7</i>	F: 5' - TCTTGACTTTGGCTGGGTTT - 3' / R: 5' - TTCACGACGCACTGAGTTTC - 3'	Solyc09g010090
<i>SI-SUT1</i>	F: 5' - AGGAGGAACTTGCCTGGAT - 3' / R: 5' - CGACTGCTGGTTTAGCATCA - 3'	Solyc11g017010
<i>SI-SUT2</i>	F: 5' - GGAGAGAAGTCTATCAG - 3' / R: 5' - GACAAATACAATGAAGTTGC - 3'	Solyc05g007190
<i>SI-AGPaseL1</i>	F: 5' - AGCAGACTACTACCAAACAG - 3' / R: 5' - ATTCCAATCGGTACTTTCC - 3'	Solyc01g109790
<i>SI-AGPaseL2</i>	F: 5' - CTGAAATTATCCCTTCTGCTG - 3' / R: 5' - ACTTCACTGTTCCAATATCCTC - 3'	Solyc07g019440
<i>SI-AGPaseL3</i>	F: 5' - CGCGCTACTTCGTAATAACC - 3' / R: 5' - CCATCAATTCTCCATTGCA - 3'	Solyc01g079790
<i>SI-AGPaseS1</i>	F: 5' - TGTAAGATTACCATTCCTG - 3' / R: 5' - TCTTCTATAATTGCTCCCTCTG - 3'	Solyc07g056140
<i>TRR 3/4</i>	F: 5' - CCAACATCACCAACAAGACG - 3' / R: 5' - ATGGGAGAGGAGGGCTTAA - 3'	Solyc05g006420
<i>TRR 7/15</i>	F: 5' - ATGTTTGAGGAAGGTGCTG - 3' / R: 5' - TGATTGTGTCGTTGGATCGT - 3'	Solyc03g113720
<i>TRR 8/9a</i>	F: 5' - TGCTTAGAAGAAGGGGCAGA - 3' / R: 5' - GGGGGCTTTTACATTGTT - 3'	Solyc10g079600
<i>TRR 8/9b</i>	F: 5' - CAGCAGCAACAACAACAG - 3' / R: 5' - TCTGCCAGCCCTTCTTAGATC - 3'	Solyc02g071220
<i>TRR 16/17</i>	F: 5' - GGTCTAAGGGCGTTGGAGTA - 3' / R: 5' - ATCTGAGCCCCTTCTTCCAT - 3'	Solyc06g048930
<i>SI-IAA3</i>	F: 5' - GCCACCAGTTCGATCATACA - 3' / R: 5' - ATAAGGTGCTCCATCCATGC - 3'	Solyc09g065850
<i>SI-IAA4</i>	F: 5' - ACTCCACCTGTTGCCAAGAC - 3' / R: 5' - AGATAAGGGGCTCCATCCAT - 3'	Solyc06g053840
<i>SI-IAA9</i>	F: 5' - CAGAGGGGAAGTTTCTGTCG - 3' / R: 5' - CAACCTGTGCCTTTGTAGCA - 3'	Solyc04g076850
<i>SI-IAA15</i>	F: 5' - ATCGGAGACAGCCAAATCAG - 3' / R: 5' - TTTGCTGGAGGTTTGTTC - 3'	Solyc03g120390
<i>SI-IAA27</i>	F: 5' - GCAAGAGAAGCTCAGTGA - 3' / R: 5' - ACATCTCCCAAGGAACATCG - 3'	Solyc03g120500
<i>SI-IAA36</i>	F: 5' - TGGAAGGAGTAGCCATTGGA - 3' / R: 5' - TGGTACATCTCCAGCAAGCA - 3'	Solyc06g066020
<i>SI-ARF2a</i>	F: 5' - GCAAGGTCAAGAGTTATCGA - 3' / R: 5' - CATTGTTTCTCAGACAAGTC - 3'	Solyc03g118290
<i>SI-ARF2b</i>	F: 5' - CTGGGTTAAGCGACAAGCTC - 3' / R: 5' - CCCCGCATTTGATACAGAGT - 3'	Solyc12g042070
<i>SI-ARF3</i>	F: 5' - GATTGTTTGTCTCCCTTGA - 3' / R: 5' - GTGGCTGACCCCGATAGATA - 3'	Solyc02g077560
<i>SI-ARF4</i>	F: 5' - TGAAAGCCATCAACTCTCGG - 3' / R: 5' - ATCCCATCTGACCATCAAGCATC - 3'	Solyc11g069190
<i>SIARF5</i>	F: 5' - TTCCGAGCCAAGAAAAGAAA - 3' / R: 5' - CACTCGCATCAGTTGGAAGA - 3'	Solyc04g081240
<i>SIARF8a</i>	F: 5' - AGCCCGTCCAATATGTTTCA - 3' / R: 5' - TTTGATGTTGCTTCTGCTG - 3'	Solyc03g031970
<i>SIARF8b</i>	F: 5' - CATCTCCTTCCGACCACAGT - 3' / R: 5' - TGGTGGATCAATTTGCTCTGC - 3'	Solyc02g037530
<i>SI-ARF10a</i>	F: 5' - ATTCTCTGTGCCTAGATACTG - 3' / R: 5' - CTATAAATGTGCCTAACTTCCA - 3'	Solyc11g069500
<i>SI-ERF.E1</i>	F: 5' - GTTCCTCTCAACCCCAAACG - 3' / R: 5' - TTCATCTGCTCACCACCTGTAGA - 3'	Solyc09g075420
<i>SI-ERF.E2</i>	F: 5' - ACTTCGTGAGGAAACCTGAAC - 3' / R: 5' - GTTACTAATATAAGTCATGTTGGG - 3'	Solyc06g063070
<i>SI-ERF.E4</i>	F: 5' - AGGCCAAGGAAGAACAAGTACAGA - 3' / R: 5' - CCAAGCCAAACGCGTACAC - 3'	Solyc01g065980
<i>CAC (constitutive)</i>	F: 5' - CCTCCGTTGTGATGTAAGTGG - 3' / R: 5' - ATTGGTGGAAAGTAACATCATCG - 3'	Solyc08g006960
<i>Expressed (constitutive)</i>	F: 5' - GCTAAGAACGCTGGACCTAATG - 3' / R: 5' - TGGGTGTGCCTTTCTGAATG - 3'	Solyc07g025390

¹F: forward, R: reverse²Locus according to the Sol Genomics Network database (<http://solgenomics.net/>).

CHAPTER II: FRUIT-LOCALIZED PHYTOCHROMES REGULATE PLASTID DIVISION, STARCH SYNTHESIS AND CAROTENOID METABOLISM IN TOMATO FRUITS

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Abstract

Light signaling has long been reported to influence fruit biology, though the regulatory impact of fruit-localized photoreceptors on fruit development, metabolism and quality traits remains elusive. Studies performed in phytochrome(PHY)-deficient tomato (*Solanum lycopersicum*) mutants suggest that SIPHYA, SIPHYB2 and to a lesser extent SIPHYB1 influence fruit development and ripening. By employing fruit-specific RNAi-mediated silencing of *SIPHY* genes, we demonstrated that fruit-localized SIPHYA and SIPHYB2 play contrasting roles in regulating plastid biogenesis and maturation in tomato. Data revealed that fruit-localized SIPHYA, rather than SIPHYB1 or SIPHYB2, positively influence tomato plastid division machinery via changes in both light and cytokinin signaling-related gene expression. In contrast, SIPHYB2 negatively regulates chlorophyll accumulation by controlling the expression of the master transcription factor of chloroplast development GOLDEN2-LIKE-2. Fruit-localized SIPHYA and SIPHYB2 were also shown to modulate starch metabolism in early developing fruits via overlapping, yet distinct, mechanisms involving the coordinated transcriptional regulation of the negative regulator of starch biosynthesis AUXIN RESPONSE FACTOR4 (SlARF4) and most starch biosynthetic genes. At the ripening stage, fruit-specific silencing of either *SIPHYA* or *SIPHYB2* resulted in an overall reduction in carotenoid biosynthesis along with the up- and down-regulation of genes encoding light repressor proteins and the carotenoid biosynthesis positive regulators SlARF2a/SlARF2b, respectively. Altogether, the data shed light on the specific role played by fruit-localized phytochromes and their downstream signaling cascades, showing that both SIPHYA- and SIPHYB2-mediated light perception regulate fruit plastid biogenesis and essential features of tomato fruit quality such as sugar and carotenoid accumulation.

Keywords: Carotenoid, Sugar, Auxin, Cytokinin, Fleshy fruits, Plastid division..

1. Introduction

Fleshy fruit growth, maturation and ripening are under strict developmental, hormonal and epigenetic regulation, which in turn are fine-tuned by a plethora of environmental stimuli (Kumar et al., 2014; Giovannoni et al., 2017). Besides providing energy for photosynthesis, light also influences many aspects of fruit biology, including size, pigmentation and timing of ripening (Carvalho et al., 2011; Gupta et al., 2014). In tomato (*Solanum lycopersicum*), a leading vegetable crop and major model species for fleshy fruits, several lines of evidence indicate that changes in light perception and signaling can lead to significant alterations in fruit development and quality traits (Giliberto et al., 2005; Schofield and Paliyath, 2005; Azari et al., 2010; Bianchetti et al., 2017).

One of the earliest pieces of evidence of light influence on tomato fruit biology dates back to 1954, when fruit pigmentation was shown to be regulated by red (R)/far red (FR) light in a reversible manner (Piringer and Heinze, 1954). First isolated only a few years later, phytochromes (PHYs) act as molecular switches in response to R and FR light, existing as a homodimer of two independently reversible subunits. Once activated by R light, PHYs are transported from the cytosol to the nucleus, where they counteract light signaling repressor proteins, such as CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1), CULLIN4 (CUL4), DNA DAMAGE-BINDING PROTEIN 1 (DDB1), DETIOLATED1 (DET1) and PHYTOCHROME INTERACTION FACTOR (PIF) (Rockwell et al., 2006; Dong et al., 2015; Seluzicki et al., 2017). In line with their role as repressors of photomorphogenic responses, either the down-regulation or loss-of-function of tomato genes encoding COP1, CUL4, DDB1, DET1 and PIF1a profoundly alter tomato fruit physiology and nutritional composition (Cookson et al., 2003; Liu et al., 2004; Davuluri et al., 2005; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010; Enfissi et al., 2010; Llorente et al., 2016).

In tomato, five PHY-encoding genes, namely *SIPHYA*, *SIPHYB1*, *SIPHYB2*, *SIPHYE* and *SIPHYF*, have been identified (Alba et al., 2000). The paralogous *SIPHYB1* and *SIPHYB2*, which originated during the *Solanum* whole genome triplication event (The Tomato Genome Consortium, 2012), display distinct expression profiles within tomato organs pointing to functional diversification (Hauser et al., 1997; Weller et al., 2000). *SIPHYB1* is more prominently expressed in vegetative tissues, whereas the highest *SIPHYB2* expression levels are detected in fruits (Hauser et al., 1997; Bianchetti et al., 2017). Moreover, evidence also suggests a more direct involvement of *SIPHYB1*, rather than *SIPHYB2*, during early seedling photomorphogenic responses (van Tuinen et al., 1995, 1995; Weller et al., 2000). Very little is known about the influence of *SIPHYE* and *SIPHYF* on tomato vegetative and reproductive development (Schrager-Lavelle et al., 2016).

Attempts to define the influence of fruit-localized PHY on fruit development and ripening have been relatively limited. Brief R-light treatments of detached mature-green tomato fruits have been shown to promote lycopene accumulation, a response reversed by subsequent treatment with FR light (Alba et al., 2000), which is consistent with the hypothesis that fruit-localized PHYs play a regulatory role in controlling tomato fruit carotenogenesis. The marked accumulation of *SIPHYA* transcripts during fruit ripening (Alba et al., 2000) associated with the reduced fruit lycopene levels observed in *phyA* tomato mutants (Gupta et al., 2014) raise the possibility that this PHY may be an important regulator of tomato fruit carotenoid biosynthesis. However, regardless of the development stage or tissue considered, *SIPHYB2* is the most highly expressed *PHY* in tomato fruits (Bianchetti et al., 2017). Moreover, *phyB2* mutant also display considerable changes in fruit carotenoid profile (Gupta et al., 2014), suggesting that multiple PHYs are involved in regulating this metabolic process.

Besides carotenogenesis, PHYs have also been found to control other aspects of tomato fruit development and metabolism, including chloroplast biogenesis, chlorophyll accumulation, sugar metabolism and hormonal signaling (Gupta et al., 2014; Bianchetti et al., 2017). However, as the existing evidence supporting these findings is exclusively based on studies performed in *phy* mutants, whether these responses are dependent on fruit-localized PHY or are merely consequences of the collateral negative effects of PHY deficiency on vegetative plant growth remains to be elucidated.

By employing fruit-specific RNAi-mediated silencing of *SIPHY* genes, we shed light on the functional specificity of fruit-localized SIPHYs in controlling developmental and metabolic processes associated with sugar and carotenoid accumulation, two essential nutritional quality traits of this edible fruit. Data also revealed 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.

2. Material and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L.) plants cv. Micro-Tom, which harbors the wild-type *SLGLK2* allele (Carvalho et al., 2011), were grown under controlled conditions: 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 12h photoperiod and air temperature of 27°C day/22°C night. The fruit stages used were immature green, mature green, breaker and red ripe, which were harvested on average 8, 25, 32 and 44 days post-anthesis. All fruits were harvested at the same time of the day in four biological replicates (each replicate was composed of a pool of at least five fruits from

different plants). Columella, placenta and seeds were immediately removed, and the remaining tissues were frozen in liquid nitrogen and stored at -80°C until use.

Generation of transgenic tomato plants

Three cDNA fragments specific to *SIPHYA*, *SIPHYB2* and both *SIPHYB1* and *SIPHYB2* were independently cloned into pENTR D-TOPO plasmid (Invitrogen) using the primers listed in the Supplementary Table S3.2. Subsequently, each fragment was recombined into the plant transformation vector pK8GWIWG (Fernandez et al., 2009). Transgenic Micro-Tom plants were generated by *Agrobacterium*-mediated transformation according to Pino et al. (2010), with minor changes: cotyledons from 5-day-old seedlings were used for the transformation; the zeatin and kanamycin concentration were 5 µM and 70 mg L⁻¹, respectively.

Fruit color, pigment quantification and plastid abundance analysis

Change in fruit color (Hue angle) was determined using a Konic Minolta CR-200 colorimeter as described in Su et al. (2015). Chlorophyll extraction and quantification were carried out as described in Lira et al. (2016) with some modifications. Pericarp sample were weighed (typically 100 mg fresh weight – FW), ground in liquid nitrogen, immersed in a 10-times excess volume of N,N-dimethylformamide and incubated at room temperature for 24 h in absolute darkness and constant agitation (200 rpm). After centrifugation (9,000 g 5 min, 4°C) the supernatant absorbance was recorded at 647 and 664 nm, and the total chlorophyll content was estimated using the equation published by Porra et al., (1989).

For carotenoid extraction, approximately 200 mg FW of pericarp sample were ground in liquid nitrogen and sequentially homogenized with a solution of saturated 100 µL of NaCl, 200 µL of dichloromethane and 1 mL of hexane:diethyl ether (1:1, v/v). The supernatant was collected after centrifugation (5,000 g, 10 min, 4°C). The remaining carotenoids in the pellet were extracted three more times with 500 µL of hexane:diethyl ether (1:1, v/v). All supernatant fractions were combined, completely vacuum dried and suspended with 200 µL of acetonitrile. Lycopene, β-carotene, lutein and neoxanthin levels were determined by high-pressure liquid chromatography (HPLC) with photodiode array detector (PDA) as described by Lira et al. (2017).

Plastid abundance was determined as in Bianchetti et al. (2017). Briefly, small pieces of (1 x 1 mm) os pericarp were fixed in 3,5% (v/v) glutaraldehyde for 1 hour. Samples were washed twice and transferred to 0.1 M NaEDTA pH 9.5 solution for 4 h at 60°C in complete darkness. Pieces were macerated and transferred to microscope slides. Isolated cells were visualized using a Leica microscope. Plastid densities in individual cell were estimates using

the ImageJ program. At least 40 individual cells were analyzed per sample.

Starch and soluble sugar quantification

Starch and soluble sugar extractions were performed as described in Bianchetti et al. (2017). Briefly, approximately 200 mg FW of pericarp samples was extracted with 1 mL of 80% (v/v) methanol for 10 min at 80°C followed by the collection of the supernatants by centrifugation (13,000 g, 10 min, 4°C). The remaining pellets were re-extracted five times, and all supernatants were combined, completely vacuum dried and suspended in 200 µL water. Soluble sugars (*i.e.*, sucrose, fructose and glucose) were measured using an HPLC system equipped with an amperometric detector (Dionex, Sunnyvale, USA) and a CarboPac PA1 (4 × 250 mm) column (Purgatto et al., 2002). Starch levels were determined from dried pellet as described in Suguiyama et al. (2014).

Transcriptional profile

Total RNA extraction, cDNA synthesis, primer design and qPCR assays were performed as described by Quadrana et al. (2013). Primer sequences used are detailed in Supplementary Table S3.2. qRT-PCR reactions were performed in a StepOnePlus PCR Real-Time thermocycler (Applied Biosystems) in a final volume of 10 µl using 2X SYBR Green Master Mix reagent (Thermo Fisher Scientific). Melting curves were checked for unspecific amplifications and primer dimerization. Absolute fluorescence data were analyzed using the LinRegPCR software package to obtain quantitation cycle (Cq) values and calculate primer efficiency. Transcript abundances were normalized against the geometric mean of two reference genes, *CAC* and *EXPRESSED* (Exposito-Rodriguez et al., 2008).

Gene promoter analysis

Gene promoter analysis was performed using the promoter sequences available at Sol Genomics Network (<https://solgenomics.net/>). Typically, 3kb upstream to initial codon ATG of each sequence was analyzed using the PlantPAN 2.0 platform (<http://plantpan2.itps.ncku.edu.tw/>) (Chow et al., 2016) for the presence of PBE-box (CACATG), G-box (CACGTG), CA-hybrid (GACGTA), CG-hybrid (GACGTG), canonical AuxRE (TGTGTC) and degenerate AuxRE (TGTGNC) motifs (Martínez-García et al., 2000; Song et al., 2008; Chaabouni et al., 2009).

Statistical analysis

ANOVA and Student's T test were calculated using the Jump statistical software package (14th edition). Comparisons with $P \leq 0.05$ were considered statistically significant.

3. Results

Fruit-specific PHY knockdown in transgenic tomato plants

To investigate the role played by distinct PHYs in tomato fruit development and ripening, we generated fruit-specific silenced tomato plants with reduced mRNA levels of *SIPHYA*, *SIPHYB2* or both *SIPHYB1* and *SIPHYB2*. This was achieved using a hairpin-mediated RNAi approach based on the expression of specific fragment sequences of these genes under the control of the fruit-specific *PPC2* promoter (Fernandez et al., 2009). The obtained transgenic plants, hereafter designated as *SIPHYA*^{RNAi}, *SIPHYB2*^{RNAi} and *SIPHYB1/B2*^{RNAi} (Figure 3.1A), were generated in a Micro-Tom background homozygous for the wild-type *GOLDEN2-LIKE-2* (*SIGLK2*) allele (Carvalho et al., 2011), which encodes a transcription factor critically important for chloroplast development in tomato fruit tissues (Powell et al., 2012).

Figure 3.1. Fruit-specific PHY knockdown in transgenic tomato plants. (a) Constructs designed for *SIPHYA*^{RNAi}, *SIPHYB1/B2*^{RNAi} and *SIPHYB2*^{RNAi} transgenic line generation. A: *SIPHYA*-specific

fragment of the mRNA 5' UTR. B1/B2: *SIPHY1/B2*-specific fragment of the mRNA 5' UTR. B2: *SIPHYB2*-specific fragment of the mRNA 5' UTR. **(b)** Relative mRNA levels of *SIPHYA*, *SIPHYB1* and *SIPHYB2* in mature green fruits of *SIPHYA^{RNAi}*, *SIPHYB2^{RNAi}* and *SIPHYB1/B2^{RNAi}* lines. **(c)** Relative *SIPHY* mRNA levels in leaves and immature green (IG), mature green (MG) and breaker (Bk) fruits of *SIPHYA^{RNAi}*, *SIPHYB2^{RNAi}* and *SIPHYB1/B2^{RNAi}* lines. Values shown are mean $\pm$ SE. Transcript abundance was normalized against the wild type (WT). Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT.

Transcript abundance analysis revealed that *SIPHYA*, *SIPHYB2* and both *SIPHYB1* and *SIPHYB2* were down-regulated in *SIPHYA^{RNAi}*, *SIPHYB2^{RNAi}* and *SIPHYB1/B2^{RNAi}* lines, respectively (Figure 3.1B). In all transgenic lines, *PHY* knockdown was restricted to the fruit tissues as no significant *PHY* silencing was observed in leaf samples (Figure 3.1C). Transgenic lines exhibited normal plant growth and visual phenotypic features similar to those found in wild-type (WT) plants. Overall, fruit-specific *PHY* knockdown caused no marked changes in fruit size and ripening progression (Supplementary Figure S3.1).

Fruit-localized SIPHYA and SIPHYB2 differentially impact chloroplast biogenesis and chlorophyll accumulation during early fruit development

The *PHY*-dependent regulation of chloroplast development has been extensively reported in leaf tissues of several species (Stephenson et al., 2009; Inagaki et al., 2015). Moreover, some recent reports also indicate altered chlorophyll accumulation and chloroplast biogenesis in immature fruits of *PHY*-deficient tomato mutants (Gupta et al., 2014; Bianchetti et al., 2017). Compared to the WT, fruit-specific *SIPHYA* and *SIPHYB2* knockdown reduced and increased the chlorophyll content in immature fruits by approximately 30%, respectively (Figure 3.2A). In contrast, chlorophyll levels in immature fruits from *SIPHYB1/B2^{RNAi}* plants were similar to WT counterparts.

Microscopy analysis of pericarp cells revealed that the reduced chlorophyll content detected in *SIPHYA^{RNAi}* immature fruits (Figure 3.2A) was associated with a reduction of up to 40% in the number of chloroplasts per pericarp cell compared to WT fruits (Figure 3.2B). In contrast, the higher chlorophyll content observed in *SIPHYB2^{RNAi}* immature fruits was not accompanied by changes in plastid abundance but instead was linked to the up-regulation of the master regulator of chloroplast development and maintenance *SIGLK2* (Figure 3.2C). *SIPHYB1/B2* knockdown lines showed an intermediate impact on fruit chlorophyll content, plastid density and *SIGLK2* mRNA levels, exhibiting unaltered chlorophyll level and chloroplast abundance in pericarp cells and slightly higher expression of *SIGLK2* compared to the WT (Figure 3.2).

Figure 3.2. Fruit-localized SIPHYA and SIPHYB2 differentially impact chloroplast biogenesis and chlorophyll accumulation during early fruit development. (a) Total chlorophyll content in immature fruits. (b) Plastid abundance per pericarp cell of immature fruits. (c) Relative mRNA levels of *GOLDEN2-LIKE-2* (*SIGLK2*) in immature fruits. Values shown are mean $\pm$ SE. Transcript abundance was normalized against the wild type (WT). Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT.

As neither the *SIPHYB2* nor the *SIPHYB1/B2* knockdown altered chloroplast density per cell (Figure 3.2B), fruit-localized SIPHYA seems to play a preponderant role in controlling chloroplast biogenesis in early developing fruits. Transcript abundance analysis revealed that the reduced plastid abundance observed in *SIPHYA*-silenced fruits is most probably explained by a drastic reduction in mRNA levels of genes encoding key components of the plastid division machinery, such as FILAMENTOUS TEMPERATURE SENSITIVE-Z (FtsZs), ACCUMULATION AND REPLICATION OF CHLOROPLASTS (ARCs) and PLASTID DIVISION 2 (PDV2), compared to the WT genotype (Figure 3.3A).

Given the key role played by cytokinins in regulating plastid division and maturation in plants and the widely reported crosstalk between this hormonal class and PHY signaling (Okazaki et al., 2009; Cortleven and Schmülling, 2015), a transcriptional profiling of type-A *TOMATO RESPONSE REGULATOR* (*TRR*) was performed. Four out of the five type-A *TRR* analyzed were significantly down-regulated in immature fruits of *SIPHYA*^{RNAi} compared to the WT genotype (Figure 3.3B). Moreover, among the five *CYTOKININ RESPONSE*

FACTOR genes most highly expressed in tomato fruit tissues (Shi et al., 2012), *SICRF1*, *SICRF2* and *SICRF5* were markedly down-regulated in *SIPHYA*^{RNAi} plants, whereas *SICRF3* or *SICRF9* mRNA levels remained unchanged (Figure 3.3C). As *AtCRF2* is responsible for inducing *AtPDV2*, subsequently promoting plastid division rates in *Arabidopsis* (Okazaki et al., 2009), the drastic down-regulation of both *SICRF2* and *SIPDV2* in *SIPHYA*-silenced fruits suggests that a similar regulatory mechanism also takes place in early-developing tomato fruits.

Figure 3.3. *SIPHYA*-mediated regulation of chloroplast division machinery is associated with changes in the transcript abundance of light and cytokinin signaling genes. (a) Relative mRNA levels of genes encoding components of the plastid division machinery in immature fruits. (b) Relative mRNA levels of type-A *TOMATO RESPONSE REGULATOR* (*TRR*) genes in immature fruits. (c) Relative mRNA levels of *CYTOKININ RESPONSE FACTOR* (*SICRF*) genes in immature fruits. (d) Relative mRNA levels of genes encoding light signaling repressor proteins. Values shown are mean $\pm$ SE. Transcript abundance was normalized against the wild type (WT). Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT. The abbreviations indicate the following: FtsZ, Filamentous temperature sensitive-Z; ARC, Accumulation and replication of chloroplasts; PDV2, Plastid Division 2; COP1, Constitutive photomorphogenic 1; CUL4, Cullin 4; DDB1, UV-damaged DNA binding protein 1; DET1, De-etiolated1.

Alongside the down-regulation of cytokinin signaling genes, fruit-specific *SIPHYA* silencing resulted in the up-regulation of tomato genes encoding light signaling repressor proteins such as COP1, CUL4, DDB1 and DET1 (Figure 3.3D), which are negative regulators of plastid division and maturation in tomato and other species (Chory and Peto, 1990;

Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010).

Collectively, these data suggest that fruit-localized PHYA positively influence tomato plastid division machinery via changes in the transcript abundance of both light and cytokinin signaling genes, whereas PHYB2 negatively regulates chlorophyll accumulation by controlling the expression of the master transcription factor of chloroplast development and maintenance SIGLK2.

Fruit-localized PHYs regulate starch metabolism during early fruit development

Fruit-specific *SIPHYA*, *SIPHYB2* and *SIPHYB1/B2* knockdown increased starch accumulation by up to 30% during early fruit development (Figure 3.4A). Overall, the higher increments in starch content were observed in *SIPHYA*- and *SIPHYB2*-suppressed fruits than in *SIPHYB1/B2*-suppressed ones. In both WT and transgenic lines, maximum starch content was observed in immature green (IG) fruits, followed by slightly more reduced levels at the mature green (MG) stage, and undetectable levels from breaker (Bk) stage onwards (Figure S2).

Compared to the WT genotype, marked differences in the transcript profile of starch biosynthesis genes were observed in both *SIPHYA*- and *SIPHYB2*-silenced fruits (Figure 3.4B and Supplementary Figure S3.3). Catalyzing the first committed step in starch biosynthesis, ADP-glucose pyrophosphorylase (AGPase) is a heterotetramer comprising a pair of small/catalytic and a pair of large/regulatory subunits (Kim et al., 2007; Figueroa et al., 2013). Among the tomato genes encoding the large AGPase subunits, both *SIAGPaseL1* and *SIAGPaseL3* were up-regulated whereas *SIAGPaseL2* mRNA levels remained unchanged in immature fruits of *SIPHYA^{RNAi}* plants. It is worth mentioning that *SIAGPaseL1* is the large AGPase subunit most highly expressed in immature tomato fruits (Supplementary Table S3.1; Petreikov et al. 2006); therefore, the 3-fold increment in its mRNA levels correlates well with the higher starch levels and reduced soluble sugar levels detected in *SIPHYA^{RNAi}* immature fruits compared to the WT counterparts (Figure 3.4C-D and Supplementary Figure S3.2).

SIAGPaseS1, which encodes the small/catalytic AGPase subunit, was consistently down-regulated throughout the fruit development and ripening in both *SIPHYA^{RNAi}* and *SIPHYB2^{RNAi}* lines (Figure 3.4B and Supplementary Figure S3.3). However, despite the negative impact of either *SIPHYA* or *SIPHYB2* silencing on *SIAGPaseS1* expression, this gene exhibited higher expression levels than those encoding AGPase large subunits (Supplementary Table S3.1), suggesting that the catalytic AGPase subunit is not limiting for starch biosynthesis in tomato fruits.

In both *SIPHYA^{RNAi}* and *SIPHYB2^{RNAi}* immature fruits, the starch synthase (STS)-

encoding genes *SISTS1* and *SISTS2* were markedly up-regulated compared to WT fruits, whereas *SISTS3* was down-regulated (Figure 3.4B). For *SISTS6*, higher transcript accumulation was observed in *SIPHYA*^{RNAi} than in the WT throughout fruit development and ripening. Finally, opposite expression patterns for the starch branching enzyme (SBE)-encoding genes were observed, as *SISBE1* and *SISBE2* were respectively up- and down-regulated in all transgenic lines compared to the WT genotype (Figure 3.4B).

Figure 3.4. Fruit-localized phytochromes regulate starch metabolism during early fruit development. (a) Starch content in pericarp cells of immature fruits. (c) Schematic representation of the major steps of starch biosynthesis. Heatmap represents statistically significant differences in mRNA levels of starch biosynthesis genes in *SIPHYA*^{RNAi} and *SIPHYB2*^{RNAi} lines compared with the wild type (WT) (Student's t-test, $P < 0.05$). For simplicity, the mean of the three transgenic line values is represented when at least two lines exhibited mean values statistically different from those detected in the WT. Relative transcript values for each transgenic line is presented in Supplementary Figure S3.3. (d) Glucose content in immature fruits. (e) Fructose content in immature fruits. (f) Relative mRNA levels of *AUXIN RESPONSE FACTOR 4* (*SIARF4*) in immature fruits. Values shown are mean $\pm$ SE. Transcript abundance was normalized against the WT. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT. The abbreviations indicate the following:

IG, immature green; MG, mature green; Bk, breaker; RR, red ripe; Glc-1-P, Glucose 1-phosphate; ADPG, Adenosine diphosphate glucose; AGPase, ADP-glucose pyrophosphorylase; STS, Starch synthase; SBE, Starch branching enzyme.

Previous findings indicate that AUXIN RESPONSE FACTOR4 (ARF4) is a major negative regulator of starch biosynthesis in early developing tomato fruits (Sagar et al., 2013; Bianchetti et al., 2017). Accordingly, fruit-specific *SIPHYA* and *SIPHYB2* knockdown drastically reduced *SIARF4* mRNA in early developing tomato fruits (Figure 3.4E). Although the direct transcriptional regulation of tomato *AGPase*, *STS* and *SBE* genes by auxin or light signaling-associated transcription factors remains to be determined, the presence of PBE-box, G-box, CA-hybrid and/or CG-hybrid motifs (Martínez-García et al., 2000; Song et al., 2008) as well as canonical and/or degenerated ARF-binding Auxin Response Element (AuxRE) motifs within the 3-kb promoter sequence of these genes (Supplementary Figure S3.4) is consistent with the hypothesis that light- and/or auxin-related transcription factors might directly control the expression of starch biosynthetic genes.

PHY-dependent regulation of fruit carotenoid biosynthesis is associated with transcriptional changes in light and auxin signaling genes

The vastly characterized PHY-mediated signaling networks controlling carotenogenesis in vegetative tissues (Toledo-Ortiz et al., 2010) contrasts with the considerably more limited information regarding the fruit-localized PHY-dependent signaling cascades regulating carotenoid biosynthesis in fleshy fruits (Llorente et al., 2016; Llorente et al., 2017). Analysis of carotenoid levels and transcript abundance of key carotenoid metabolism-associated genes revealed an overall reduction in fruit carotenogenesis in both *SIPHYA*^{RNAi} and *SIPHYB2*^{RNAi} lines compared to the WT (Figure 3.5 and Supplementary Figure S3.5). During the ripening phase, corresponding to the Bk and red ripe (RR) stages, transcripts of genes encoding carotenoid biosynthesis enzymes such as GERANYLGERANYL DIPHOSPHATE SYNTHASE (GGPS), PHYTOENE SYNTHASE 1 (PSY1) and PHYTOENE DESATURASE (PDS) were up to 10-fold less abundant in *SIPHYA*- and *SIPHYB2*-downregulated fruits than in WT counterparts. As a consequence, total carotenoid levels in ripe *SIPHYA*^{RNAi} and *SIPHYB2*^{RNAi} fruits were approximately 30% lower than in the WT (Supplementary Figure S3.5).

Figure 3.5. Fruit-specific *SIPHYA* or *SIPHYB2* knockdown represses carotenoid biosynthesis during tomato fruit ripening. Schematic representation of carotenoid biosynthetic pathway in tomato. Gray lines indicate that intermediate reactions were omitted. Heatmap represents statistically significant differences in metabolites and in mRNA levels of carotenoid biosynthesis genes in *SIPHYA^{RNAi}* and *SIPHYB2^{RNAi}* lines compared to the wildtype (WT) (Student's t-test, P<0.05). For simplicity, the mean of the three transgenic line values is represented when at least two lines exhibited mean values statistically different from those detected in the WT. The absolute metabolite and relative transcript values for each transgenic line are presented in Supplementary Figure S3.5. The abbreviations indicate the following: MG, mature green; Bk, breaker; RR, red ripe; MEP, Methylerythritol 4-phosphate; GGDP, Geranylgeranyl diphosphate; GGPS, GGDP synthase; PSY, Phytoene synthase; PDS, Phytoene desaturase; LCYβ, Chloroplast-specific β-lycopene cyclase; CYCβ, Chromoplast-specific β-lycopene cyclase.

Accumulating evidence indicates that light signaling repressors such as *SIPIF1a*, *SICOP1*, *SICUL4*, *SIDDB1* and *SIDET1* negatively regulate carotenoid biosynthesis in tomato fruits (Azari et al., 2010; Llorente et al., 2016) whereas auxin response factors such as *SlARF2a* and *SlARF2b*, play the opposite role (Hao et al., 2015). To gain insight into the potential role played by these signaling components during the PHY-dependent regulation of carotenoid biosynthesis in tomato fruits, the transcript abundance of their encoding genes was profiled in both *SIPHYA^{RNAi}* and *SIPHYB2^{RNAi}* ripening fruits (Figure 3.6 and Supplementary Figures S3.6 and S3.7). Among the four *SIPIF* genes most highly expressed in fruits (Rosado et al., 2016), *SIPIF1a*, *SIPIF1b* and *SIPIF4/5* mRNA levels were up to 20-fold higher in *SIPHYA*- and *SIPHYB2*-downregulated fruits compared to the WT counterparts. In contrast, *SIPIF3* was drastically reduced in both *SIPHYA^{RNAi}* and *SIPHYB2^{RNAi}* ripening fruits.

Overall, *SICOP1*-, *SICUL4*-, *SIDDB1*- and *SIDET1*-encoding genes were also up-regulated in both *SIPHYA*- and *SIPHYB2*-knockdown lines. Compared to the WT, *SIPHYA*- and *SIPHYB2*-silenced fruits exhibited strikingly higher *SIDET1* transcript levels throughout the ripening. In contrast, the transcript levels of positive regulators of tomato fruit carotenogenesis *SlARF2a* and *SlARF2b*, were considerably lower in *SIPHYA^{RNAi}* and

SIPHYB2^{RNAi} ripening fruits (Figure 3.6 and Supplementary Figure S3.7)

Figure 3.6. PHY-dependent regulation of fruit carotenogenesis is associated with transcriptional changes in auxin and light signaling genes. Heatmap represents statistically significant differences in mRNA levels of auxin and light signaling genes in *SIPHYAas* and *SIPHYB2as* lines compared to the wildtype (WT) (Student's t-test, $P < 0.05$). For simplicity, the mean of the three transgenic line values is represented when at least two lines presented mean values statistically different from those detected in the WT. The absolute metabolite and relative transcript values for each transgenic line are presented in Supplementary Figures S3.6 and S3.7. The abbreviations indicate the following: MG, mature green; Bk, breaker; RR, red ripe; PIF, Phytochrome interacting factor; COP1, Constitutive photomorphogenic 1; CUL4, Cullin 4; DDB1, UV-damaged DNA binding protein 1; DET1, De-etiolated1, ARF, Auxin response factor.

4. Discussion

Studies performed on PHY-deficient mutants suggest that PHY-dependent light perception participates in the regulation of several aspects of tomato fruit biology (Gupta et al., 2014; Bianchetti et al., 2017). Here, we applied an RNAi-mediated organ-specific silencing approach to investigate the impact of fruit-localized SIPHYs on tomato fruit physiology and quality traits. Differently from the pleiotropic phenotypical alterations observed in *phy* mutants (Gupta et al., 2014; Bianchetti et al., 2017), the fruit-specific silencing of the target *SIPHY* genes resulted in no obvious impacts on plant vegetative growth and overall yield. This suggests that the perturbation in fruit metabolism caused by the fruit-specific SIPHY manipulation does not propagate from fruits to the rest of the plant, which agrees with the limited transference of substances out of this predominantly sink organ.

In a previous work, we demonstrated that the global deficiency in functional PHYs drastically reduces chlorophyll content and chloroplast abundance in tomato fruits (Bianchetti et al., 2017). Therefore, the PHY-mediated regulation of plastid biogenesis and maturation

widely reported for leaf tissues (Stephenson et al., 2009; Oh and Montgomery, 2014; Melo et al., 2016) seems to be conserved in early developing tomato fruits. In this work, we further demonstrate that fruit-localized *SIPHYA* and *SIPHYB2* play distinct roles in controlling chloroplast biogenesis and activity during early stages of tomato fruit development.

The results demonstrated that *SIPHYA*-mediated light perception promotes fruit chloroplast biogenesis, as inferred from the reduced chlorophyll content and chloroplast abundance detected in *SIPHYA*^{RNAi} immature fruits (Figure 3.2). In line with this observation, the analysis of single and multiple *phy* mutants also suggested *SIPHYA* as a major regulator of chlorophyll accumulation in tomato fruits (Gupta et al., 2014). In land plants, chloroplast division depends on nucleus-encoded proteins that will form ring structures at the division site (Jarvis and Lopez-Juez, 2013). Our findings clearly demonstrate that fruit-localized *SIPHYA* influences the transcript levels of genes derived from the ancestral prokaryotic cell division machinery, such as *SIFtsZ* (*i.e.* *SIFtsZ1*, *SIFtsZ2*) and *SIARCs* (*i.e.* *SIARC3* and *SIARC6*), as well as those encoding chloroplast division-related proteins specific to land plants, such as *SIPDV2*. In *Arabidopsis*, PDV2 determines the rate of chloroplast division and is positively regulated by cytokinins, being strongly promoted in transgenic plants overexpressing the cytokinin signaling-related transcription factor CRF2 (Okazaki et al., 2009; Cortleven and Schmölling, 2015). *SI-CRF2*, along with other *SI-CRF* and *TRR* genes, were drastically repressed in *PHYA*-downregulated fruits, implying that changes in cytokinin signaling mediate the *PHYA*-dependent regulation of plastid division during early stages of tomato development. In agreement, accumulating evidence indicates an intensive crosstalk between *PHY* and cytokinin signaling cascades, with particular involvement of CRF and type-A ARR proteins (Salome et al., 2006; Oh et al., 2009).

Fruit-specific *SIPHYA* silencing also promoted the mRNA accumulation of genes encoding all major light signaling repressor proteins already described to negatively regulate chloroplast biogenesis in tomato fruits, *i.e.*, *SICOP1*, *SICULA*, *SIDDB1* and *SIDET1* (Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010). Defective mutants or transgenic lines with reduced levels of each of these genes are known to display increased fruit plastid abundance and development (Cookson et al., 2003; Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010), which in some cases, such as in the *SIDET1*-knockout mutant, is associated with the up-regulation of plastid biogenesis genes (Kolotilin et al., 2007).

In contrast, fruit-localized *SIPHYB2* was shown to play a negative role in fruit chloroplast development, as evidenced by the increment in chlorophyll content in immature fruits of *SIPHYB2*^{RNAi} plants with no impact in chloroplast number in pericarp cells. As

SIPHYB2 fruit-specific silencing led to higher *SlGLK2* mRNA levels compared to the WT genotype, it seems plausible to suggest that the effect of *SIPHYB2* on fruit chloroplasts is mediated by *SlGLK2*, the master regulator of plastid development in tomato fruits (Powell et al., 2012). Further suggesting that the *SIPHYB2*-mediated regulation of *SlGLK2* expression is essential for the consequent changes in fruit chlorophyll levels, no obvious changes in this parameter were observed in *phyb2* mutants from tomato varieties that lack functional *SlGLK2* proteins (Gupta et al., 2014). In agreement with these findings, PHY-dependent transcriptional regulation of *GLK* genes has been increasingly reported in vegetative tissues of other plant species (Oh and Montgomery, 2014; Song et al., 2014).

Alterations in chloroplast number and size in early developing tomatoes significantly impact the abundance of metabolites associated with organoleptic and nutritional quality at the ripe stage (Galpaz et al., 2008; Cocaliadis et al., 2014). Intense starch synthesis and degradation take place in tomato fruit chloroplasts at unripe and breaker stages, respectively (Schaffer and Petreikov, 1997). Whereas the global deficiency in PHYs significantly reduces the starch content in immature tomato fruits (Bianchetti et al., 2017), fruit-localized *SIPHYA* or *SIPHYB2* suppression increased fruit starch levels by up to 30% and markedly altered the transcriptional profile of starch biosynthesis genes at the immature green stage (Figure 3.4). AGPase, which catalyzes the rate-limiting reaction in the starch synthesis pathway, is both transcriptionally and posttranslational regulated by light (Harn et al., 2000; Geigenberger, 2011), though the role played by PHYs in this regulatory process remains elusive. During early fruit development, *SIPHYA*-suppressed fruits exhibited increased mRNA levels of both *SlAGPaseL1* and *SlAGPaseL3*, which encode AGPase large subunits, and *SlSTS1*, *SlSTS2* and *SlSTS6*, which encode starch synthase enzymes, along with the increase in starch accumulation and reduced soluble sugar content, thus indicating a repressor role for fruit-localized *SIPHYA* on the first steps of starch synthesis in tomato fruits. In contrast, the increased starch accumulation detected in *SIPHYB2*-silenced immature fruits was not associated with increments in transcript abundance of AGPase-encoding genes nor prominent reductions in soluble sugars but instead were accompanied by an increment in *SlSTS1* and *SlSTS2* mRNA levels. This suggests that *SIPHYA* and *SIPHYB2* negatively regulate starch synthesis via overlapping, yet distinct, mechanisms.

The influence of auxin on fruit sugar metabolism has been increasingly reported (Purgatto et al., 2002; Yuan and Carbaugh, 2007; Bianchetti et al., 2017). In tomato, *SlARF4* has been described as a key negative regulator of starch synthesis during early fruit development by the transcriptional and post-transcriptional down-regulation of AGPase (Sagar et al., 2013). Recent findings also indicate that PHYs strictly regulate the transcript

abundance of this particular auxin response factor in both vegetative (Melo et al., 2016) and fruit tissues (Bianchetti et al., 2017). In line with this, the increased starch accumulation in pre-ripening *SIPHYA*- and *SIPHYB2*-silenced fruits correlates well with the down-regulation of *SlARF4* in these transgenic lines (Figure 3.4). In fact, *SIPHYA*^{RNAi} rather than *SIPHYB2*^{RNAi} exhibited the most expressive decrease in *SlARF4*, and only the former displayed increased mRNA levels of AGPase-encoding genes in immature fruits. Together, these data strongly suggest that fruit-localized PHYA, and to some extent SIPHYB2, positively modulates *SlARF4*, which in turn represses starch biosynthetic enzymes, such as AGPase and STS, consequently limiting starch synthesis in pre-ripening tomato fruits.

The link between PHY-dependent light perception and carotenoid metabolism in both vegetative and fruit tissues has been highlighted by a number of studies (Alba et al., 2000; Llorente et al., 2016). Exposure of wild-type tomato fruits to red light (Alba et al., 2000) or constitutively silencing of *SlPIF1a* (Llorente et al., 2016) promotes tomato fruit lycopene accumulation, thereby implying a positive role of PHY-dependent signaling cascades on fruit carotenoid biosynthetic pathway. Consistent with this, our findings indicate that fruit-localized SIPHYA and SIPHYB2 positively influence the transcript accumulation of all major carotenoid biosynthesis-related genes, including *SlGGPS*, *SlPSY1*, *SlPDS*, *SlCYCβ* and *SlLYCβ*, consequently modifying carotenoid levels in this fleshy fruit. Light signaling repressor proteins such as *SIDET1*, *SIDDB1*, *SICOP1*, *SICUL4*, and more recently *SlPIF1a*, have been identified as key negative regulators of tomato fruit carotenoid synthesis (Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010; Llorente et al., 2016). Among these, the transcription factor *SlPIF1a* was shown to directly bind to the promoter of the *SlPSY1* to repress fruit carotenogenesis (Llorente et al., 2016), thus resembling the action of its homolog in Arabidopsis (*AtPIF1*) in controlling carotenoid biosynthesis in leaf tissues (Toledo-Ortiz et al., 2010). Therefore, the marked up-regulation of *SIDET1*, *SIDDB1*, *SICOP1*, *SICUL4*, *SlPIF1a* and *SlPIF1b* along with the overall repression of carotenoid biosynthesis observed in both *SIPHYA*- and *SIPHYB2*-silenced fruits implicate that light signaling repressor proteins participate in *SIPHYA*- and *SIPHYB2*-mediated regulation of fruit carotenogenesis.

Additionally, it is becoming increasingly well established that auxin represses tomato ripening and down-regulates lycopene biosynthetic genes (Su et al., 2015). Among tomato *ARF* genes, two paralogs, *SlARF2a* and *SlARF2b*, have emerged as key positive regulators of tomato fruit ripening and lycopene accumulation (Hao et al., 2015). Either *SIPHYA* or *SIPHYB2* fruit-specific silencing profoundly reduced both *SlARF2a* and *SlARF2b*, suggesting the involvement of these auxin signaling elements in the PHY-dependent regulation of

carotenoid biosynthesis in tomato fruits.

Overall, our results shed light on the specific role played by fruit-localized phytochromes and their downstream signaling cascades, showing that plastid division, as well as sugar and carotenoid metabolisms, are profoundly regulated by SIPHYA- and SIPHYB2-mediated light perception. A model summarizing the influence of fruit-localized SIPHYs on tomato fruit physiology is presented in Figure 3.7.

Figure 3.7. Proposed model for phytochrome-mediated signaling events controlling chloroplast biogenesis and sugar and carotenoid metabolisms in tomato fruits. (a) SIPHYA- and SIPHYB2-dependent light perception regulate fruit plastid division and maturation, respectively. By promoting key members of the cytokinin signaling-related *CRF* and *TRR* gene family, SIPHYA up-regulates SIPDV2, a rate-limiting component of plastid division machinery. Moreover, the SIPHYA-mediated down-regulation of light signaling repressors, such as SICOP1, SIDET1, SIDDB1 and SICUL4, induces other major components of the chloroplast division machinery, such as SIFTSZs and SIARCs. In contrast, SI-PHYB2 represses the chloroplast differentiation transcription factor SIGLK2, consequently limiting chloroplast differentiation in early developing fruits. (b) Fruit-localized SIPHYA and SIPHYB2 play overlapping roles in repressing and promoting starch and carotenoid biosynthesis, respectively. Both phytochromes induce SIARF4, a negative regulator of AGPase and starch accumulation in tomato fruits. In contrast, these same photoreceptors promote both *SIARF2* paralogues and inhibit all major genes encoding light signaling repressor proteins, consequently up-regulating most components of the tomato carotenoid biosynthetic route. Arrows at the ends of lines indicate stimulatory effects, whereas bars indicate inhibitory effects. The abbreviations indicate the following: AGPase, ADP-glucose pyrophosphorylase; ARC, Accumulation and replication of chloroplasts; ARF, Auxin response factor; COP1, Constitutive photomorphogenic 1; CRF, Cytokinin response factor; CUL4, Cullin 4; DDB1, UV-damaged DNA binding protein 1; DET1, De-etiolated1; FtsZ, Filamentous temperature sensitive-Z; GGPS, Geranylgeranyl pyrophosphate synthase; GLK2, Golden2-like-2; PDS, Phytoene desaturase; PDV2, Plastid Division 2; PIF, Phytochrome interacting factor; PSY, Phytoene synthase; TRR, Tomato response regulator.

According to this model, SIPHYA and SIPHYB2 play overlapping roles in regulating starch and carotenoid biosynthesis whereas they differentially regulate distinct aspects of fruit plastid biogenesis and maturation. Our data implicate cytokinin signaling-

related proteins as mediators of the SIPHYA-dependent regulation of plastid division machinery, and specific *ARF* genes as potential intermediates in the PHY-mediated regulation of fruit sugar and carotenoid metabolism. Altogether, these findings reveal that fruit-specific manipulation of *PHY* genes represents a promising approach to differentially regulate multiple biosynthetic pathways and, consequently, modify the nutritional value of edible fleshy fruits.

5. References

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6. Supplementary information

Supplementary Figure S3.1. Visual phenotype and color change of *PHY*-silenced fruits.

Supplementary Figure S3.2. Carbohydrate profile in *PHY*-silenced fruits.

Supplementary Figure S3.3. Transcript abundance of starch biosynthetic genes in *PHY*-silenced fruits.

Supplementary Figure S3.4. HY5-, PIF- and ARF-binding motifs identified in the promoter region of starch biosynthesis-related tomato genes.

Supplementary Figure S3.5. Carotenoid metabolism during the ripening in *PHY*-silenced fruits.

Supplementary Figure S3.6. Transcript abundance of photomorphogenesis-related genes in *PHY*-silenced fruits.

Supplementary Figure S3.7. Transcript abundance of the tomato *AUXIN RESPONSIVE FACTOR 2a* and *2b* (*SlARF2a* and *SlARF2b*) genes in *PHY*-silenced fruits.

Supplementary Table S3.1. Relative transcript ratio of *SlAGPase* in immature green fruits.

Supplementary Table S3.2. Primer sequences.

Supplementary Figure S3.1. Visual phenotype and color change of *PHY*-silenced fruits. (a) Representative fruits at each sampling stage. (b) Fruit color estimated by Hue angle. Values shown are mean $\pm$ SE. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage. The abbreviations indicate the following: IG, immature green; MG, mature green; Bk, breaker; RR, red ripe.

Supplementary Figure S3.2. Carbohydrate profile in *PHY*-silenced fruits. (a) Starch, sucrose, glucose, and fructose content. (b) Summed values of the three soluble sugars analyzed (*i.e.* sucrose + glucose + fructose). Values shown are mean $\pm$ SE. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage. The abbreviations indicate the following: IG, immature green; MG, mature green; Bk, breaker; RR, red ripe.

Supplementary Figure S3.3. Transcript abundance of starch biosynthetic genes in *PHY*-silenced fruits. (a) Large (L1, L2 and L3) and small (S1) subunits of ADP-glucose pyrophosphorylase (AGPase). (b) Starch synthase (STS). (c) Starch branching enzyme (SBE). Values shown are mean $\pm$ SE. Transcript abundance was normalized against WT at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage. The abbreviations indicate the following: IG, immature green; MG, mature green; Bk, breaker; RR, red ripe.

Supplementary Figure S3.4. HY5-, PIF- and ARF-binding motifs identified in the promoter region of starch biosynthesis-related tomato genes. Fragments of 3 kb upstream ATG initiation site of the tomato genes encoding ADP-glucose pyrophosphorylase small and large subunits (*SIAGPase*), Starch synthase (*SISTS*) and Starch branching enzyme (*SISBE*) are represented by a blue line. Motif positions are indicated by triangles: PBE-box (green), recognized by PHYTOCHROME-INTERACTING FACTORS (PIFs). CA-hybrid (black) and CG-hybrid (yellow), recognized by LONG HYPOCOTYL 5 (HY5). G-box (red), recognized by both PIFs and HY5. Canonical AuxRE (purple) and degenerated AuxRE (gray), recognized by AUXIN RESPONSE FACTORS (ARFs).

Supplementary Figure S3.5. Carotenoid metabolism during the ripening in *PHY*-silenced fruits.

(a) Lycopene, β -carotene, lutein, neurosporene and total carotenoid levels. **(b)** Relative mRNA levels of tomato genes encoding carotenoid biosynthetic enzymes. Values shown are mean $\pm$ SE. Transcript abundance was normalized against the wild type (WT) at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage. The abbreviations indicate the following: MG, mature green; Bk, breaker; RR, red ripe; SPSY, phytoene synthase; SIPDS, phytoene desaturase; SILCY β , chloroplast-specific β -lycopene cyclase; SICYC β , chromoplast-specific β -lycopene cyclase.

Supplementary Figure S3.6. Transcript abundance of photomorphogenesis-related genes in *PHY*-silenced fruits. (a) Tomato genes encoding PHYTOCHROME INTERACTING FACTORS (SIPIFs). (b) Tomato genes encoding the light signaling repressors CONSTITUTIVE PHOTOMORPHOGENIC 1 (SICOP1), CULLIN 4 (SICUL4), UV-DAMAGED DNA BINDING PROTEIN 1 (SIDDB1) and DE-ETIOLATED1 (SIDET1). Values shown are mean $\pm$ SE. Transcript abundance was normalized against WT at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage. The abbreviations indicate the following: MG, mature green; Bk, breaker; RR, red ripe.

Supplementary Figure S3.7. Transcript abundance of the tomato *AUXIN RESPONSIVE FACTOR 2a* and *2b* (*SIARF2a* and *SIARF2b*) genes in *PHY*-silenced fruits. Values shown are mean $\pm$ SE. Transcript abundance was normalized against the wild type (WT) at each fruit developmental stage. Asterisks indicate statistically significant differences (Student's t-test, $P < 0.05$) compared with the WT at each fruit development stage. The abbreviations indicate the following: MG, mature green; Bk, breaker; RR, red ripe, ARF, auxin response factor.

Supplementary Table S3.1. Relative transcript ratio of tomato *AGPase* genes in immature green fruits.

		<i>Sl-AGPaseS1</i>	<i>Sl-AGPaseL1</i>	<i>Sl-AGpaseL2</i>	<i>Sl-AGPaseL3</i>
WT		1.00 ± 0.15	0.33 ± 0.08	0.09 ± 0.01	0.06 ± 0.01
<i>PHYAas</i>	L1	1.00 ± 0.07	0.65 ± 0.05	0.21 ± 0.02	0.15 ± 0.01
	L3	1.00 ± 0.21	1.01 ± 0.21	0.24 ± 0.05	0.20 ± 0.04
	L5	1.00 ± 0.08	0.64 ± 0.06	0.13 ± 0.01	0.17 ± 0.01
<i>PHYB2as</i>	L2	1.00 ± 0.36	1.40 ± 0.35	0.02 ± 0.00	0.18 ± 0.04
	L3	1.00 ± 0.21	1.53 ± 0.33	0.11 ± 0.02	0.29 ± 0.06
	L4	1.00 ± 0.09	1.48 ± 0.13	0.07 ± 0.00	0.22 ± 0.02

Statistically significant differences in the transcript ratio between *Sl-AGPase* genes are indicated in bold terms (Student's t-test, $p < 0.05$). Values represent means ± SD from at least three biological replicates and are expressed as relative transcript amount compared to *Sl-AGPaseS1* for each genotype.

Supplementary Table S3.2. Primer sequences.

Gene	Primer sequences ¹	Locus ²
<i>SIPHYA-RNAi</i>	F: 5'- CACCATGTCGTCTTCAAGACCTAG - 3' / R: 5'- CGGTGGTAACCTTTGTCTGACTTTGG - 3'	Solyc10g044670
<i>SIPHYB2-RNAi</i>	F: 5'- CCACCCTTCAACTACCAATGCTGATTC - 3' / R: 5'- GTCCTGTACAGTAGATGCTTC - 3'	Solyc05g053410
<i>SIPHYB1/B2-RNAi</i>	F: 5'- CACCATGGCTTCTGGAAGTAG - 3' / R: 5'- GTAGTTTAAACAGACTGTGAATAATC - 3'	Solyc01g059870
<i>SIPHYA</i>	F: 5'- CACTCTCGTGGAGGATTCAT - 3' / R: 5'- GAGCCATAAAACACACACCC - 3'	Solyc10g044670
<i>SIPHYB1</i>	F: 5'- ACTTCTGTTCGGTCCATTCC - 3' / R: 5'- TCTCAGACAACTGTGATGCC - 3'	Solyc01g059870
<i>SIPHYB2</i>	F: 5'- GTGAGGGTTATTCAAGATGA - 3' / R: 5'- TGACCATATACTGAGGGTGAC - 3'	Solyc05g053410
<i>SICOP1</i>	F: 5'- TTGCCCTGTGTCTCTCA - 3' / R: 5'- CCAATGAATGACGAAACTGTTC - 3'	Solyc12g005950
<i>SICUL4</i>	F: 5'- GGAGGAATTGGAGGGGACATTA - 3' / R: 5'- GAAATCATGGACTTTTCAGCATCA - 3'	Solyc02g021470
<i>SIDDB1</i>	F: 5'- ATCTCGTGCCTGGACATAAA - 3' / R: 5'- CGAAGGAACACATCAGAAACA - 3'	Solyc02g021650
<i>SIDET1</i>	F: 5'- GCTATTGACCGCCATAGACA - 3' / R: 5'- TCTTAGTTTCGCCCATCTGTG - 3'	Solyc01g056340
<i>SIPIF1a</i>	F: 5'- AACTTCTTGCTTTGCTCTCTG - 3' / R: 5'- GCTCCGCCCATAAATCA - 3'	Solyc09g063010
<i>SIPIF1b</i>	F: 5'- TAGTATGGCAAAATGGTGGAG - 3' / R: 5'- CGGCGTCACAACTCGGTG - 3'	Solyc06g008030
<i>SIPIF3</i>	F: 5'- AAGGCTTCCCAATAATGC - 3' / R: 5'- CCATCAGACCAAACCTCCC - 3'	Solyc01g102300
<i>SIPIF4/5</i>	F: 5'- GGCTTAGGTTCACATACAG - 3' / R: 5'- TGATGGTGTCTGTTGTCTC - 3'	Solyc07g043580
<i>SIGLK2</i>	F: 5'- ATGTTTGGGGGCATCCACATG - 3' / R: 5'- GCAAATCAGAGGCAACTGTGTC - 3'	Solyc10g008160
<i>SIARC3</i>	F: 5'- GGAAGTGATGATAGAGTCGT - 3' / R: 5'- GCTGAATCTGGAATCTTGCTGTC - 3'	Solyc04g017620
<i>SIARC6</i>	F: 5'- GCTTACGGCAGTTCTGTG - 3' / R: 5'- CGTTGTCGTCCTTTGAGTG - 3'	Solyc04g081070
<i>SIFTsZ1</i>	F: 5'- AGCAGCCGAACAAGCAACTC - 3' / R: 5'- CTGAAACGACTGGGTGAAAC - 3'	Solyc07g065050
<i>SIFTsZ2</i>	F: 5'- GAGCAGTTCAAGCCCAAGAAG - 3' / R: 5'- CACGCACATCAGCAAAATCC - 3'	Solyc09g009430
<i>SIPDV2</i>	F: 5'- TCTTGACTTTGGCTGGGTTC - 3' / R: 5'- TTCACGACGCACTGAGTTTC - 3'	Solyc03g007980
<i>TRR ¾</i>	F: 5'- CCAACATCACCAACAAGACG - 3' / R: 5'- ATGGGAGAGGAGGGCTTAAA - 3'	Solyc05g006420
<i>TRR 7/15</i>	F: 5'- ATGTTTGGAGGAAGGTGCTG - 3' / R: 5'- TGATTGTGTCGTTGGATCGT - 3'	Solyc03g113720
<i>TRR 8/9a</i>	F: 5'- TGCTTAGAAGAAGGGGCAGA - 3' / R: 5'- GGGGGCTTTTACATTTGGTT - 3'	Solyc10g079600
<i>TRR 8/9b</i>	F: 5'- CAGCAGCAACAACAACAACAG - 3' / R: 5'- TCTGCCAGCCCTTCTTAGATC - 3'	Solyc02g071220
<i>TRR 16/17</i>	F: 5'- GGTCTAAGGGCGTTGGAGTA - 3' / R: 5'- ATCTGAGCCCTTCTTCCAT - 3'	Solyc06g048930
<i>SICRF1</i>	F: 5'- CTGACGCCGTTACCAATTTT - 3' / R: 5'- ACGTCGAAACCAAAAGCATC - 3'	Solyc06g082590
<i>SICRF2</i>	F: 5'- GAAGGGGAACAGGGGTTTAA - 3' / R: 5'- GCGGTCTTCTTCTCCTCTCTT - 3'	Solyc08g081960
<i>SICRF3</i>	F: 5'- CGGACCATGACACAACAGAC - 3' / R: 5'- CTTACCCCATTTTCGTCGTC - 3'	Solyc01g008890
<i>SICRF5</i>	F: 5'- CCTCTGTCTCCACCTCA - 3' / R: 5'- AAGGGGATCGAAAATCGAAG - 3'	Solyc01g095500
<i>SICRF9</i>	F: 5'- AGGAAATGGGGGAAATATGC - 3' / R: 5'- AGGACCAGGGCTCAAATTCT - 3'	Solyc03g119580
<i>SIAGPaseL1</i>	F: 5'- AGCAGACTACTACCAAACAG - 3' / R: 5'- ATTCCAATCGGTAATTTCC - 3'	Solyc01g109790
<i>SIAGPaseL2</i>	F: 5'- CTGAAATTATCCCTTCTGCTG - 3' / R: 5'- ACTTCACTGTTCCAATATCCTC - 3'	Solyc07g019440
<i>SIAGPaseL3</i>	F: 5'- CGCGCTACTTCGTAATAACC - 3' / R: 5'- CCATCAATTCTCCATTGCA - 3'	Solyc01g079790
<i>SIAGPaseS1</i>	F: 5'- TGTAAGATTACCAATTCCTG - 3' / R: 5'- TCTTCTATAATTGCTCCCTCTG - 3'	Solyc07g056140
<i>SISTS1</i>	F: 5'- GCTCAAATGTCCAAACTTCAC - 3' / R: 5'- TATGGTTCCTGAGTCTATCTG - 3'	Solyc08g083320
<i>SISTS2</i>	F: 5'- CGCTGATTGGATTATTGGG - 3' / R: 5'- GGATTATGTCAACACCTTTCTG - 3'	Solyc03g083090
<i>SISTS3</i>	F: 5'- AGGATATTGCGAACCATTTC - 3' / R: 5'- AGCCTCATCATACTTCAATAC - 3'	Solyc02g071040
<i>SISTS6</i>	F: 5'- AGTTGGGAGGGGATTCAGAC - 3' / R: 5'- ACTTGTGTCATCCTCTCGTG - 3'	Solyc02g088000
<i>SISBE1</i>	F: 5'- GTGATCGGAGGAATGCTAATGT - 3' / R: 5'- GAAGTTCGGGATTCGGAATC - 3'	Solyc09g009190
<i>SISBE2</i>	F: 5'- TCAACTTCCACCCAATAACAC - 3' / R: 5'- ACCAAATTCCTCAAGCATCAC - 3'	Solyc04g082400
<i>SIGGPS</i>	F: 5'- GCTGTTGGTGTCTTATATCGTG - 3' / R: 5'- CTTCTCAATGCCATAAACGCTG - 3'	Solyc09g008920
<i>SIPSY1</i>	F: 5'- CGATGGTGCTTTGTCCGATAC - 3' / R: 5'- CTCATCAACCCAACCGTACC - 3'	Solyc03g031860
<i>SIPDS</i>	F: 5'- CGTTCCGTGCTTCTCCGC - 3' / R: 5'- CTAGAATCCCTTGCCTCCAG - 3'	Solyc03g123760
<i>SILYCβ</i>	F: 5'- TTGACTTAGAACCTCGTTATTGG - 3' / R: 5'- AACAGTTCCTTTGTCAATTATCT - 3'	Solyc04g040190
<i>SICYCβ</i>	F: 5'- GCACCCACATCAAAGCCAGAG - 3' / R: 5'- GCCACATGGAGAGTGGTGAAG - 3'	Solyc06g074240
<i>SIARF2a</i>	F: 5'- GCAAGGTCAAGAGTTATCGA - 3' / R: 5'- CATTGGTTTCTCAGACAAGTC - 3'	Solyc03g118290
<i>SIARF2b</i>	F: 5'- CTGGGTAAAGCGACAAGCTC - 3' / R: 5'- CCCCGCATTGATACAGAGT - 3'	Solyc12g042070
<i>SIARF4</i>	F: 5'- TGAAAGCCATCAACTCTCGG - 3' / R: 5'- ATCCCATCTGACCATCAAGCATC - 3'	Solyc11g069190
<i>CAC (constitutive)</i>	F: 5'- CCTCCGTTGTGATGTAAGTGG - 3' / R: 5'- ATTGGTGGAAAGTAACATCATCG - 3'	Solyc08g006960
<i>Expressed (constitutive)</i>	F: 5'- GCTAAGAACGCTGGACCTAATG - 3' / R: 5'- TGGGTGTGCCTTTCTGAATG - 3'	Solyc07g025390

¹F: forward, R: reverse;²Locus according to the Sol Genomics Network database (<http://solgenomics.net/>).

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 *PHYs* 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 *SIPHYA* or *SIPHYB2* 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 *SIPHYE* and *SIPHYF*, 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.

ARTICLES PUBLISHED

Directly related to this thesis (publications as first author)

Bianchetti, R.E., Cruz, A.B., Oliveira, B.S., Demarco, D., Purgatto, E., Peres, L.E.P., Rossi, M. and Freschi, L. (2017) Phytochromobilin deficiency impairs sugar metabolism through the regulation of cytokinin and auxin signaling in tomato fruits. *Scientific Reports*, **7**, 7822.

Bianchetti, R.E., Purgatto, E., Rothan, C., Rossi, M. and Freschi, L. Fruit-localized phytochromes regulate plastid division, starch synthesis and carotenoid metabolism in tomato fruits. Submitted to *Plant Journal*.

Related to this thesis, but not part of it (publications as co-author)

Melo N.K.G., **Bianchetti R.E.**, Lira B.S., Oliveira P.M.R., Zuccarelli R., Dias D.L.O., Demarco D., Peres L.E.P., Rossi M. and Freschi L. (2016) Nitric oxide, ethylene, and auxin crosstalk mediates greening and plastid development in deetioloating tomato seedlings. *Plant Physiology* **170**: 2278-2294

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