Fruit-localized phytochromes regulate plastid biogenesis, starch synthesis, and carotenoid metabolism in tomato

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Abstract

Light signaling has long been reported to influence fruit biology, although the regulatory impact of fruit-localized photoreceptors on fruit development and metabolism remains unclear. Studies performed in phytochrome (PHY)-deficient tomato (Solanum lycopersicum) mutants suggest that SlPHYA, SlPHYB2, and to a lesser extent SlPHYB1 influence fruit development and ripening. By employing fruit-specific RNAi-mediated silencing of SlPHY genes, we demonstrated that fruit-localized SlPHYA and SlPHYB2 play contrasting roles in regulating plastid biogenesis and maturation in tomato. Our data revealed that fruit-localized SlPHYA, rather than SlPHYB1 or SlPHYB2, positively influences tomato plastid differentiation and division machinery via changes in both light and cytokinin signaling-related gene expression. Fruit-localized SlPHYA and SlPHYB2 were also shown to modulate sugar metabolism in early developing fruits via overlapping, yet distinct, mechanisms involving the co-ordinated transcriptional regulation of genes related to sink strength and starch biosynthesis. Fruit-specific SLPHY silencing also drastically altered the transcriptional profile of genes encoding light-repressor proteins and carotenoid-biosynthesis regulators, leading to reduced carotenoid biosynthesis during fruit ripening. Together, our data reveal the existence of an intricate PHY–hormonal interplay during fruit development and ripening, and provide conclusive evidence on the regulation of tomato quality by fruit-localized phytochromes.

Keywords: Auxin, carotenoid, cytokinin, fleshy fruit, phytochrome, plastid division, tomato, Solanum lycopersicum, starch.

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). Among environmental cues, light plays a significant role in determining fruit growth, pigmentation, and timing of ripening (Carvalho et al., 2011; Gupta et al., 2014; Llorente et al., 2016a). In tomato (Solanum lycopersicum), a major crop and important 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., 2010b; Bianchetti et al., 2017).

One of the earliest pieces of evidence of the influence of light on tomato fruit biology dates back to 1954, when...
fruit pigmentation was shown to be regulated by red/far red (R/FR) light in a reversible manner (Pringner 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 homodimers of two independently reversible subunits. Once activated by R light, PHYs are transported from the cytosol to the nucleus, where they counteract light, existing as homodimers of two independently reversibly subunits. Once activated by R light, PHYs are transported from the cytosol to the nucleus, where they counteract light, existing as homodimers of two independently reversible subunits.

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. Our data also reveal 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.

Materials and methods

Plant material and growth conditions

Tomato (Solanum lycopersicum L.) plants cv. Micro-Tom, which harbors the wild-type SIGLK2 allele (Carvalho et al., 2011), were grown under controlled conditions of 250 µmol m⁻² s⁻¹, a 12-h photoperiod, and air temperature of 27/22 °C day/ night. The fruit stages examined were immature green, mature green, breaker, and red ripe, which were harvested on average at 8, 25, 32, and 44 d post-anthesis. All fruits were harvested at the same time of the day with four biological replicates (each replicate was composed of a pool of at least five fruits from different plants). Columellae, 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 fragments specific to the coding sequences of SIPHYA, SIPHYB2, and both SIPHYB1 and SIPHYB2 were selected using BLAST queries against the Sol Genomics Network database (https://solgenomics.net/). The web-based computational tool psRNAit (Dai and Zhao, 2011) was employed to avoid off-target silencing. Each fragment was independently cloned into pENTR D-TOPO plasmids (Invitrogen) using the primers listed in Supplementary Table S1 at ITAG release 2.40 and both are.

Attempts to define the influence of fruit-localized PHYs on fruit development and ripening have been relatively limited. Brief R-light treatments of detached mature-green tomato fruits promote lycopene accumulation, a response reversed by subsequent treatment with FR light (SlPHYB2, 2000a), which is consistent with the hypothesis that fruit-localized PHYs play a regulatory role in controlling tomato fruit carotenogenesis. The marked accumulation of SIPHY4 transcripts during fruit ripening (SlPHYA, 2000a) associated with the reduced fruit lycopene levels observed in phyA tomato mutants (SlPHYB2, 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, SlPHYB2 is the most highly expressed PHY in tomato fruits (SlPHYB2, 2017). Moreover, the phyB2 mutant also displays considerable changes in the fruit carotenoid profile (SlPHYB2, 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, sink activity, and hormonal signaling (SlPHYB2, 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 PHYs or are merely consequences of the collateral negative effects of PHY deficiency on vegetative plant growth remains to be elucidated.

For carotenoid extraction, approximately 200 mg FW of pericarp samples were ground in liquid nitrogen and sequentially homogenized with a solution of 100 µl of saturated NaCl, then 200 µl of dichloromethane, and finally 1 ml of hexane:diethyl ether (1:1, v/v). The supernatant absorbance was recorded at 647 and 664 nm, and the total chlorophyll content was estimated using the equations given by Porra et al. (1989).
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 neurosporene levels were determined by high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA) as described by Lira et al. (2017).

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 distilled water. Soluble sugars (i.e. sucrose, fructose, and glucose) were measured using a 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 Sugiyama et al. (2014).

Antioxidant capacity and total phenolics
Hydrophilic and lipophilic Trolox equivalent antioxidant capacities (TEACs) were spectrophotometrically determined as described in Lira et al. (2016). Total phenolic content was determined in hydrophilic extracts by using the Folin–Ciocalteu method (Singleton and Rossi, 1965).

Plastid ultrastructure and abundance
Pericarp fragments taken from the pedicle region (green shoulder) of immature fruits were fixed at 4 °C in 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). Subsequently, the samples were post-fixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2), dehydrated in a graded acetone series, and embedded in Spurr’s resin. Ultrathin sections were stained with saturated uranyl acetate and lead citrate (Melo et al., 2016) and observed using a JEOL JEM1011 transmission electron microscope. Sections from three immature fruits picked from different plants were analysed per genotype.

Plastid abundance was determined as described in Bianchetti et al. (2017). Briefly, small pieces (1 × 1 mm) of pericarp were fixed in 3.5% (v/v) glutaraldehyde for 1 h. 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 softly disrupted and transferred to microscope slides. Isolated cells were visualized using a Leica microscope. Plastid densities in individual cells were estimated using the ImageJ program (https://imagej.nih.gov/ij/). At least 40 individual cells were analysed per sample.

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 S1. Quantitative real-time (qRT)-PCR reactions were performed in a StepOnePlus PCR Real-Time thermocycler (Applied Biosystems) in a final volume of 10 µl using 2× SYBR Green Master Mix reagent (Thermo Fisher Scientific). Melting curves were checked for unspecific amplifications and primer dimerization. Absolute fluorescence data were analysed using the LinRegPCR software package (Ruijter et al., 2009) to obtain quantification cycle (Cq) values and to calculate primer efficiency. Transcript abundances were normalized against the geometric mean of two reference genes, CAC and EXPRESSED (Expósito-Rodriguez et al., 2008).

Gene promoter analysis
Gene promoter analysis was performed using the promoter sequences available at the Sol Genomics Network. Typically, 3 kb upstream of the initial ATG codon of each sequence was analysed 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 (CAGCTG), CA-hybrid (GACGTA), CG-hybrid (CAGTG), canonical AuxRE (TGTGTC), and degenerate AuxRE (TGTGNC) motifs (Martínez-García et al., 2000; Song et al., 2008; Chabouni et al., 2009).

Statistical analysis
ANOVA and Student’s t-test were performed using the JMP statistical software package (14th edition; http://jmp.com). Comparisons with P<0.05 were considered statistically significant. Data from wild-type and all independent transgenic lines were also compared with principal component analysis (PCA) using the InfoStat software (http://infostat.com.ar).

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 transgenic plants obtained, hereafter designated as SIPHYA<sub>RNAi</sub>, SIPHYB2<sub>RNAi</sub>, and SIPHYB1/B2<sub>RNAi</sub> (Fig. 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 fruits (Powell et al., 2012).

Transcript abundance analysis revealed that SIPHYA, SIPHYB2, and both SIPHYB1 and SIPHYB2 were down-regulated in the SIPHYA<sub>RNAi</sub>, SIPHYB2<sub>RNAi</sub>, and SIPHYB1/B2<sub>RNAi</sub> lines, respectively (Fig. 1B). A search for potential tomato off-targets via BLAST queries against the Sol Genomics Network database or via the public web-based computational tool pss-RNAit (Dai and Zhao, 2011) failed to identify regions in the tomato coding that exhibited the 21-nucleotide perfect identity threshold reported to cause off-target silencing (Xu et al., 2006). The percentage of identity of the silencing fragments was below 60% with non-target tomato PHY genes (Supplementary Table S2). Moreover, the length of stretches with perfect identity between the RNAi fragments and non-target tomato PHY genes was ≤15 nucleotides (Supplementary Table S2). In line with this, no off-target SIPHY silencing was detected in the transgenic lines generated (Supplementary Fig. S1).

In all the transgenic lines, PHY knockdown was restricted to the fruit tissues as no significant PHY silencing was observed in leaf samples (Fig. 1B). Transgenic lines exhibited normal plant growth and visual phenotypic features similar to those found in wild-type (WT) plants (Supplementary Fig. S2). Overall, fruit-specific PHY knockdown caused no marked changes in fruit size and ripening progression (Supplementary Fig. S3).

Fruit-localized SIPHYA and SIPHYB2 differentially impact chloroplast biogenesis and differentiation 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 have also indicated 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 SlPHYA and SlPHYB2 knockdown reduced and increased the chlorophyll content in immature fruits, respectively (Fig. 2A). However, chlorophyll levels in immature fruits from SlPHYB1/B2 RNAi plants were similar to WT counterparts.

Microscopy analysis of pericarp cells revealed that the reduced chlorophyll content detected in SlPHYA RNAi immature fruits was associated with a reduction of up to 40% in the number of chloroplasts per pericarp cell compared to WT fruits (Fig. 2B). However, the higher chlorophyll content observed in SlPHYB2 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, SlGLK2 (Fig. 2C). SlPHYB1/B2 knockdown lines showed an intermediate impact on fruit chlorophyll content, plastid density, and SlGLK2 mRNA levels, exhibiting unaltered chlorophyll levels and chloroplast abundance in pericarp cells and slightly higher expression of SlGLK2 compared to the WT (Fig. 2).

Plastids of WT, SlPHYB2 RNAi, and SlPHYB1/B2 RNAi immature fruits exhibited remarkably similar internal membranous structures,
Fruit-localized phytochromes influence tomato quality traits |

displaying well-developed grana and stroma thylakoids as well as numerous plastoglobuli (Fig. 2D, Supplementary Fig. S4). In contrast, fruit-specific SlPHYA knockdown resulted in the formation of chloroplasts with highly reduced grana, suggesting a promotive role of PHYA-mediated light perception on fruit plastid granal development. Plastoglobuli and starch grains were observed equally in fruit chloroplasts of the WT and all transgenic lines.

As neither SlPHYB2 nor the SlPHYB1/B2 knockdown altered chloroplast density per cell or plastid ultrastructure (Fig. 2), fruit-localized SlPHYA seems to play a preponderant role in controlling chloroplast biogenesis and differentiation in early developing fruits. Transcript abundance analysis revealed that the reduced plastid abundance observed in SlPHYA-silenced fruits was 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 (Fig. 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 TRRs analysed were significantly down-regulated in SlPHYA RNAi lines, whereas SlCRF3 and SlCRF9 mRNA levels remained unchanged (Fig. 3C). As AtCRF2 is responsible for inducing AtPDV2, subsequently increasing plastid division rates in Arabidopsis (Okazaki et al., 2009), the drastic down-regulation of both SlCRF2 and SlPDV2 in SlPHYA-silenced fruits suggests that a similar regulatory mechanism also takes place early in the development of tomato fruits.

Alongside the down-regulation of cytokinin signaling genes, fruit-specific SlPHYA-silencing resulted in the up-regulation of tomato genes encoding light-signaling repressor proteins such as COP1, CUL4, DDB1, and DET1 (Fig. 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., 2010b).

Collectively, these data suggest that fruit-localized PHYA positively influences 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, SlGLK2.

Fruit-localized PHYs regulate starch metabolism during early fruit development

Fruit-specific SlPHYA and SlPHYB2 knockdown promoted starch accumulation during early fruit development (Fig. 4A). In both the WT and transgenic lines, the highest starch content

Fig. 2. Fruit-localized SlPHYA and SlPHYB2 differentially impact on chloroplast biogenesis and differentiation 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 (SlGLK2) normalized against the wild-type (WT) sample. Statistically significant differences compared with the WT sample were determined using Student's t-test: *P<0.05. Chlorophyll content and transcript abundance data are means (±SE) of at least three biological replicates. For plastid density, three fruits of each genotype were randomly picked, and two technical replicates were taken at the pedicel region of each fruit. Plastid density was determined in at least 40 individual cells per sample. (D) Representative TEM images of plastids in the pedicel region of immature fruits. Arrows indicate plastoglobuli. G, granal thylakoid.
was observed in immature green (IG) fruits, followed by slightly more reduced levels at the mature green (MG) stage, and undetectable levels from the breaker (Bk) stage onwards (Supplementary Fig. S5).

Compared to the WT genotype, marked differences in the transcript profiles of starch biosynthesis genes were observed in both SlPHYA- and SlPHYB2-silenced fruits (Fig. 4A, Supplementary Fig. S6). Catalysing 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 SlAGPaseL1 and SlAGPaseL3 were up-regulated whereas SlAGPaseL2 mRNA levels remained unchanged in immature fruits of SlPHYA<sup>RNAi</sup> plants. It is worth mentioning that SlAGPaseL1 was the large AGPase subunit most expressed in immature tomato fruits (Supplementary Table S3; 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 SlPHYA<sup>RNAi</sup> immature fruits compared to the WT counterparts (Fig. 4, Supplementary Figs S5, S6).

SlAGPaseS1, which encodes the small/catalytic AGPase subunit, was consistently down-regulated throughout fruit development and ripening in both the SlPHYA<sup>RNAi</sup> and SlPHYB2<sup>RNAi</sup> lines. However, despite the negative impact of either SlPHYA- or SlPHYB2-silencing on SlAGPaseS1 expression, this gene exhibited higher expression levels than those encoding AGPase large subunits (Supplementary Table S3), suggesting that the catalytic AGPase subunit was not limiting for starch biosynthesis in tomato fruits.

In both SlPHYA<sup>RNAi</sup> and SlPHYB2<sup>RNAi</sup> immature fruits, the starch synthase (STS)-encoding genes SlSTS1 and SlSTS2 were markedly up-regulated compared to WT fruits, whereas SlSTS3 was slightly down-regulated. For SlSTS6, higher transcript accumulation was observed in SlPHYA<sup>RNAi</sup> than in the WT throughout fruit development and ripening (i.e. IG to RR stage) (Supplementary Fig. S6). Finally, distinct expression patterns were observed for the starch branching enzyme (SBE)-encoding genes, as SlSBE1 was up-regulated in all the transgenic lines from MG to Bk stage whereas SlSBE2 was down-regulated in both SlPHYA<sup>RNAi</sup> and SlPHYB2<sup>RNAi</sup> from IG to RR stage (Supplementary Fig. S6).

The increased accumulation of starch in SlPHYA<sup>RNAi</sup> fruits correlated well with higher mRNA levels of SlLIN5 and SlLIN6 (Fig. 4D), which encode cell-wall invertases critically important for sink activity in tomato (Fridman and Zamir, 2003; Kocal et al., 2008). By applying an unsupervised method
Fruit-localized phytochromes influence tomato quality traits (i.e. principal component analysis, PCA) to search for patterns in the expression profiles of genes related to sink- and starch-biosynthesis, we demonstrated a clear separation of the WT, SlPHYARNAi, and SlPHYB2RNAi groups (Supplementary Fig. S7).

Previous findings have indicated that AUXIN RESPONSE FACTOR4 (SlARF4) is a major negative regulator of starch biosynthesis in early developing tomato fruits (Sagar et al., 2013; Bianchetti et al., 2017). Recent evidence also indicates that SlARF4 plays a repressor role in controlling the transcript abundance of sink-related genes, including SlLIN5 and SlLIN6 (Bianchetti et al., 2017). In accordance with this, fruit-specific SlPHYA and SlPHYB2 knockdown drastically reduced SlARF4 mRNA abundance in early developing tomato fruits (Fig. 4E). Although the direct transcriptional regulation of tomato AGPase, STS, and SBE genes by transcription factors associated with auxin- or light-signaling 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 sequences of these genes (Supplementary Fig. S8) is consistent with the hypothesis that light- and/or auxin-related transcription factors might directly control the expression of starch biosynthesis-related genes. Similarly, PIF, HY5, and/or ARF-binding motifs have also been identified within the promoter sequences of SILIN5 and SILIN6 genes (Bianchetti et al., 2017).

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

The very well-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., 2016b, 2017). Carotenoid profiling revealed a significant reduction in lycopene content in red ripe (RR) fruits of both the SlPHYARNAi and SlPHYB2RNAi lines compared to the WT (Fig. 5A, Supplementary Table S4). In contrast, the content of all other carotenoids analysed (i.e. phytoene, phytofluene, β-carotene, and lutein) remained virtually unchanged in ripe fruits of the transgenic lines compared to WT counterparts. As lycopene is the main carotenoid accumulated in ripe tomato, fruit-specific SlPHYA- or SlPHYB2-knockdown led to a slight, yet significant, reduction in total carotenoid content compared to the WT genotype (Fig. 5A, Supplementary Table S4). In accordance with this, significantly lower mRNA levels of genes encoding carotenoid biosynthesis-related enzymes such as GERANYLGERANYL...
DIPHOSPHATE SYNTHASE (GGPS), PHYTOENE SYNTHASE 1 (PSY1), and PHYTOENE DESATURASE (PDS) were observed in ripe fruits of SlPHYA and SlPHYB2-silenced lines than in WT counterparts (Fig. 5B, Supplementary Fig. S9). In line with the relatively limited reduction in total carotenoids, no significant differences in lipophilic antioxidant activity were observed between ripe WT and transgenic fruits (Supplementary Table S5). Interestingly, however, red ripe SlPHYB2-down-regulated fruits exhibited increased hydrophilic antioxidant activity compared to the WT, which may be associated with the higher content of total phenolics also detected in SlPHYB2RNAi ripe fruits (Supplementary Table S5).

Accumulating evidence indicates that light-signaling repressors such as SlPIF1a, SlCOP1, SlCUL4, SlDDB1, and SlDET1 negatively regulate carotenoid biosynthesis in tomato fruits (Azari et al., 2010b; Llorente et al., 2016) whereas auxin response factors such as SIARF2a and SIARF2b 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 SlPHYARA and SlPHYB2RNAi ripening fruits (Fig. 6, Supplementary Fig. S10). Among the four SlPIF genes most highly expressed in fruits (Rosado et al., 2016), SlPIF1a, SlPIF1b, and SlPIF4/5 mRNA levels were significantly higher in SlPHYB2RNAi ripening fruits (Fig. 6, Supplementary Fig. S10).
Fruit-localized phytochromes influence tomato quality traits | 3581

Fig. 6. PHY-dependent regulation of fruit carotenogenesis is associated with transcriptional changes in auxin- and light-signaling genes. (A) Transcript abundance of tomato genes encoding PHYTOCHROME INTERACTING FACTOR3s (SIPIFs). (B) Transcript abundance of tomato genes encoding the light-signaling repressors CONSTITUTIVE PHOTOMORPHOGENIC 1 (SICOP1), CULLIN 4 (SICULA), UV-DAMAGED DNA BINDING PROTEIN 1 (SLDDB1), and DE-ETIOLATED1 (SIDET1). (C) Transcript abundance of the tomato AUXIN RESPONSIVE FACTOR 2a and 2b (SIARF2a and SIARF2b) genes. For simplicity, the mean of the three values for the transgenic lines is shown. Values for each transgenic line are presented in Supplementary Fig. S10. Data are means (±SE) of at least three biological replicates. Statistically significant differences compared with the wild-type (WT) sample were determined using Student’s t-test: *P<0.05. MG, mature green; Bk, breaker; RR, red ripe.

profiles were similar to those observed in the SIPHYB2RNAi lines (Fig. 6, Supplementary Fig. S10).

Among the genes encoding light-signaling repressors, SICULA, SIDDDB1, and SIDET1 exhibited significantly higher mRNA levels in SIPHY4-silenced fruits in comparison to the WT at all fruit development stages analysed (Fig. 6B, Supplementary Fig. S10). Moreover, strikingly higher SIDET1 transcript abundance was also detected in SIPHYB2-knockdown compared to WT fruits at all ripening stages (i.e. MG, Bk, and RR) whereas SICOP1 and SIDDDB1 mRNA levels were also up-regulated in SIPHYB2RNAi fruits exclusively at the MG stage. Transcript levels of the positive regulators of tomato fruit carotenogenesis SIARF2a and SIARF2b were considerably lower in SIPHY4RNAi and SIPHYB2RNAi fruits, particularly at the Bk and RR stages (Fig. 6C, Supplementary Fig. S10). A PCA plot in which the expression profile of carotenoid biosynthesis-related genes as well as SIPIFs, SICOP1, SICULA, SIDDDB1, SIDET1, SIARF2a, and SIARF2b were represented revealed that the WT, SIPHY4RNAi, and SIPHYB2RNAi groups clearly separated from each other at the red ripe stage (Supplementary Fig. S11).

Altogether, these data suggest that both SIPHYA and SIPHYB2 play overlapping roles in promoting the paralogues SIARF2a and SIARF2b and repressing light-signaling repressors such as SIPIF1a, SIPIF1b, SIPIF4/5, SICOP1, SICULA, SIDDDB1, and SIDET1, which in turn mediate the PHY-dependent regulation of carotenoid biosynthesis in ripening tomato fruits.

Discussion

Studies performed on PHY-deficient mutants have suggested 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 a 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 a 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 early in the development of tomato fruits. In this current work, it is further demonstrated that fruit-localized SIPHYA and SIPHYB2 play distinct roles in controlling chloroplast biogenesis and activity during early stages of tomato fruit development.

The results indicate that SIPHYA-mediated light perception promotes fruit chloroplast biogenesis and differentiation, as inferred from the reduced chlorophyll content, lower chloroplast abundance, and poorly-developed grana stacking detected in SIPHY4RNAi immature fruits (Fig. 2). In line with this observation, an analysis of single and multiple phy mutants also suggested that SIPHYA is a major regulator of chlorophyll accumulation in tomato fruits (Gupta et al., 2014). In land plants, chloroplast division depends on nucleus-encoded proteins that form ring structures at the division site (Jarvis and López-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 SIARC5 (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; Cordleven and Schmülling, 2015). SICRF2, along with other SICRF and TRR genes, were drastically repressed in PHYA-down-regulated fruits, implying that changes in cytokinin signaling mediate the PHYA-dependent regulation of plastid division during early stages of tomato fruit development. In agreement with this, accumulating evidence indicates that there is an intensive crosstalk between the PHY and cytokinin signaling cascades, with particular involvement of CRF and type-A ARR proteins (Salomé et al., 2006; Oh et al., 2009).

Fruit-specific SIPHYA-silencing also promoted the mRNA accumulation of genes encoding all the major light-signaling repressor proteins already described to negatively regulate chloroplast biogenesis in tomato fruits, i.e. SICOP1, SICUL4, SIDDDB1, and SIDET1 (Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010a). Defective mutants or transgenic lines with reduced levels of each of these genes are known to develop more chloroplasts containing more grana/thylakoids in both leaves and immature fruits (Cookson et al., 2003; Liu et al., 2004; Kolotilin et al., 2007; Wang et al., 2008; Azari et al., 2010a), which in some cases, such as in the SIDET1-knockout mutant, is associated with the up-regulation of plastid biogenesis-related genes (Kolotilin et al., 2007). Therefore, the presence of fewer chloroplasts with poorly developed or almost no grana in immature fruits of the SIPHYA-suppressed lines agrees with the higher transcript abundance of SICOP1, SIDDDB1, and particularly SICUL4 and SIDET1 in these transgenic lines compared to the WT genotype.

In contrast, fruit-localized SIPHYA2 was shown to play a negative role in chlorophyll accumulation, as evidenced by the increment in chlorophyll content in immature fruits of SIPHYA2RNAi plants with no impact in chloroplast number in pericarp cells. As SIPHYA2 fruit-specific silencing led to higher SIGLK2 mRNA levels compared to the WT genotype, it seems plausible to suggest that the effect of SIPHYA2 on fruit chloroplasts is mediated by SIGLK2, the master regulator of chloroplast development in tomato fruits (Powell et al., 2012). Further suggesting that the SIPHYA2-mediated regulation of SIGLK2 expression is essential for the consequent changes in fruit chlorophyll accumulation, no obvious changes in chlorophyll content were observed in phyb2 mutants from tomato varieties that lacked functional SIGLK2 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, internal structure, and size during the early development of tomato fruits significantly impact the abundance of metabolites associated with organoleptic and nutritional quality at the ripe stage (Galpaz et al., 2008; Cocaládis et al., 2014). Intense starch synthesis and degradation take place in tomato fruit chloroplasts at the 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 SIPHYA2 suppression increased fruit starch levels and markedly altered the transcriptional profile of starch biosynthesis-related genes at the immature green stage (Fig. 4). AGPase, which catalyses the rate-limiting reaction in the starch synthesis pathway, is both transcriptionally and post-translationally regulated by light (Harn et al., 2000; Geigenberger, 2011), although the role played by PHYs in this regulatory process remains elusive. During early fruit development, SIPHYA-suppressed fruits exhibited increased mRNA levels of both SAGPaseL1 and SAGPaseL3, which encode AGPase large subunits, and SISTS1, SISTS2, and SISTS6, which encode starch synthase enzymes, along with an 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. Whether the up-regulation of starch biosynthesis-related genes is a compensatory mechanism to cope with the few and poorly developed chloroplasts observed in SIPHYA RNAi immature fruits remains to be investigated. In contrast, the increased starch accumulation detected in SIPHYA2-silenced immature fruits was not associated with increments in transcript abundance of AGPase-encoding genes nor with prominent reductions in soluble sugars, but instead were accompanied by increments in SISTS1 and SISTS2 mRNA levels. Furthermore, as no significant alterations in plastid abundance or internal structure were observed in SIPHYA2RNAi immature fruits, it seems likely that this genetic manipulation caused less prominent changes than SIPHYA-silencing on reactions taking place within fruit chloroplasts, including starch biosynthesis. Altogether, these findings suggest that SIPHYA and SIPHYA2 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 via the transcriptional and post-transcriptional down-regulation of AGPase (Sagar et al., 2013). Recent findings have also indicated 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 SIPHYA2-silenced fruits correlated well with the down-regulation of SLARF4 in these transgenic lines (Fig. 4). In fact, SIPHYA RNAi rather than SIPHYA2RNAi 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 SIPHYA2, positively modulates SLARF4, which in turn represses starch biosynthetic enzymes, such as AGPase and STS, consequently limiting starch synthesis in pre-ripening tomato fruits.

Previous findings indicated that a global deficiency in functional phytochromes transcriptionally represses both sink-related and starch biosynthesis-related enzymes in early developing tomato fruits, suggesting a promotive role of PHYs on the regulation of these processes (Bianchetti et al., 2017). However, it remained unclear whether these responses were dependent on fruit-localized PHYs or were the consequence of collateral negative effects of the global PHY deficiency on vegetative plant growth. Here, we shed light on this topic
by showing that fruit-localized SIPHYA, and to some extent SIPHYB2, repress both starch metabolism and key determinants of tomato fruit sink strength, including SLIN5 transcript accumulation (Fridman and Zamir, 2003; Kocal et al., 2008). Consequently, the down-regulation in starch synthesis and sink activity previously observed in fruits of the PHY-deficient mutant aurea (Bianchetti et al., 2017) may be due either to limitations in vegetative growth and metabolism or to the combinatorial effect of the deficiency in all phytochromes instead of only in SIPHYA or SIPHYB2. Moreover, it also seems tempting to suggest that the fewer and poorly-developed chloroplasts detected in SIPHYA-silenced immature fruits restrict photoassimilate production via fruit photosynthesis; therefore, the observed up-regulation of sink-related genes in transgenic fruits may represent a compensatory mechanism to maintain fruit growth and intense starch accumulation despite potential limitations in fruit-localized photoassimilation.

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., 2000a; Llorente et al., 2016b). Exposure of wild-type tomato fruits to red light (Alba et al., 2000a) or constitutively silencing of SlARF2 (Llorente et al., 2016b) promotes tomato fruit lycopene accumulation, thereby implying a positive role of PHY-dependent signaling cascades in the fruit carotenoid biosynthetic pathway. Consistent with this, our findings indicate that fruit-localized SIPHYA and SIPHYB2 positively influence the transcript accumulation of all the major carotenoid biosynthesis-related genes, including SIGGPS, SIPSY1, SIPDS, SICYCB, and SLYCβ, consequently modifying the lycopene and total carotenoid content 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., 2010a; Llorente et al., 2016b). Among these, the transcription factor SlPIF1a was shown to directly bind to the promoter of SIPSY1 to repress fruit carotenogenesis (Llorente et al., 2016b), thus resembling the action of its ortholog 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 together with the overall repression of carotenoid biosynthesis observed in both SIPHYA- and SIPHYB2-silenced fruits imply that light-signaling repressor proteins participate in SIPHYA- and SIPHYB2-mediated regulation of fruit carotenogenesis.

In addition, it is becoming increasingly well established that auxin represses tomato ripening and down-regulates lycopene accumulation, thereby implying a positive role of PHY-dependent signaling cascades in the fruit carotenoid biosynthetic pathway. Consistent with this, our findings indicate that fruit-localized SIPHYA and SIPHYB2 positively influence the transcript accumulation of all the major carotenoid biosynthesis-related genes, including SIGGPS, SIPSY1, SIPDS, SICYCB, and SLYCβ, consequently modifying the lycopene and total carotenoid content 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., 2010a; Llorente et al., 2016b). Among these, the transcription factor SlPIF1a was shown to directly bind to the promoter of SIPSY1 to repress fruit carotenogenesis (Llorente et al., 2016b), thus resembling the action of its ortholog 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 together with the overall repression of carotenoid biosynthesis observed in both SIPHYA- and SIPHYB2-silenced fruits imply that light-signaling repressor proteins participate in SIPHYA- and SIPHYB2-mediated regulation of fruit carotenogenesis.

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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 metabolism, 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 Fig. 7. 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. Compared to SIPHYB2, SIPHYA-dependent light perception seems to play a major role in promoting plastid division and differentiation as well as in controlling sink-related transcripts in tomato fruits. The data implicate cytokinin signaling-related proteins as mediators of the SIPHYA-dependent regulation of the plastid division machinery, and specific ARF genes as potential intermediates in the PHY-mediated regulation of fruit sugar and carotenoid metabolism. Altogether, these findings show that fruit-specific manipulation of PHY genes represents a promising approach to differentially regulate multiple biosynthetic pathways and, consequently, to modify the nutritional value of edible fleshy fruits.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Transcriptional profile of tomato PHY genes in PHY-silenced fruits.

Fig. S2. Vegetative phenotypes of the transgenic plants.

Fig. S3. Visual phenotypes and color changes of PHY-silenced fruits.

Fig. S4. Plastid structure in PHY-silenced fruits.

Fig. S5. Carbohydrate profile in PHY-silenced fruits.

Fig. S6. Transcript abundance of starch biosynthetic genes in PHY-silenced fruits.

Fig. S7. PCA of the expression profile of sink-related and starch biosynthesis-related genes.

Fig. S8. HY5-, PIF-, and ARF-binding motifs identified in the promoter regions of starch biosynthesis-related tomato genes.

Fig. S9. Carotenoid metabolism during ripening in PHY-silenced fruits.

Fig. S10. Transcript abundance of photomorphogenesis- and auxin-related genes in PHY-silenced fruits.

Fig. S11. PCA of the expression profiles of photomorphogenesis-related, auxin-related, and carotenoid biosynthesis-related genes.

Table S1. Primer sequences.

Table S2. Homology of the RNAi fragments.

Table S3. Relative transcript ratios of SLAGPase in immature fruits.

Table S4. Carotenoid profiles in red ripe fruits.

Table S5. Antioxidant activity and total phenolics in red ripe fruits.

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