Beyond the limits of photoperception: constitutively active PHYTOCHROME B2 overexpression as a means of improving fruit nutritional quality in tomato

Frederico Rocha Rodrigues Alves1,2, Bruno Silvestre Lira1, Filipe Christian Pikart1, Scarlet Santos Monteiro1, Cláudia Maria Furlan1, Eduardo Purgatto3, Grazieli Benedetti Pascoal3,4, Sônia Cristina da Silva Andrade5, Diego Demarco1, Magdalena Rossi1 and Luciano Freschi1.∗

1Departamento de Botânica, Universidade de São Paulo, São Paulo, SP, Brazil
2Departamento de Botânica, Universidade Federal de Goiás, Goiás, GO, Brazil
3Departamento de Alimentos e Nutrição Experimental, Universidade de São Paulo, São Paulo, SP, Brazil
4Curso de Graduação em Nutrição, Universidade Federal de Uberlândia, Minas Gerais, MG, Brazil
5Departamento de Genética e Biologia Evolutiva, Universidade de São Paulo, São Paulo, SP, Brazil

Received 28 September 2019; revised 28 January 2020; accepted 10 February 2020.
*Correspondence (Tel 551130918068; fax 551130917594; email freschi@usp.br)

Summary

Photoreceptor engineering has recently emerged as a means for improving agronomically beneficial traits in crop species. Despite the central role played by the red/far-red photoreceptor phytochromes (PHYS) in controlling fruit physiology, the applicability of PHY engineering for increasing fleshy fruit nutritional content remains poorly exploited. In this study, we demonstrated that the fruit-specific overexpression of a constitutively active GAF domain Tyr₅₂⁵-to-His PHYB2 mutant version (PHYB2Y₂₅₂H) significantly enhances the accumulation of multiple health-promoting antioxidants in tomato fruits, without negative collateral consequences on vegetative development. Compared with the native PHYB2 overexpression, PHYB2Y₂₅₂H-overexpressing lines exhibited more extensive increments in transcript abundance of genes associated with fruit plastid development, chlorophyll biosynthesis and metabolic pathways responsible for the accumulation of antioxidant compounds. Accordingly, PHYB2Y₂₅₂H-overexpressing fruits developed more chloroplasts containing voluminous grana at the green stage and overaccumulated carotenoids, tocopherols, flavonoids and ascorbate in ripe fruits compared with both wild-type and PHYB2-overexpressing lines. The impacts of PHYB2 or PHYB2Y₂₅₂H overexpression on fruit primary metabolism were limited to a slight promotion in lipid biosynthesis and reduction in sugar accumulation. Altogether, these findings indicate that mutation-based adjustments in PHY properties represent a valuable photobiotechnological tool for tomato biofortification, highlighting the potential of photoreceptor engineering for improving quality traits in fleshy fruits.

Keywords: photobiotechnology, Solanum lycopersicum, carotenoids, vitamin E, flavonoids, vitamin C, biofortification, antioxidants.

Introduction

Tomato (Solanum lycopersicum L.) is one of the most consumed vegetable crops in the world, both fresh or as processed juices and sauces (Bergougnoux, 2014). It is an important source of health-promoting substances (Gerszberg et al., 1997; Pratt et al., 2017). In tomato, five PHY-encoding genes, namely PHYA, PHYB1, PHYB2, PHYE and PHYF, have been identified (Alba et al., 2000a). PHYB2 is the most expressed in tomato fruits (Bianchetti et al., 2017; Hauser et al., 1997; Pratt et al., 2000a). Among these, PHYB2 has been predominantly used for overexpression, and phyB2 overexpression on fruit primary metabolism were limited to a slight promotion in lipids and a decrease in sugar accumulation. However, more extensive tomato fruit biofortification by simultaneously overexpressing PHYB2 and other genes with pleiotropic effects remains a challenging task.

Most attempts to improve fruit nutritional composition have focused on altering the expression of specific genes directly involved in the production of carotenoids, flavonoids and other health-promoting substances (Gerszberg et al., 2015). In contrast, the manipulation of key players of light perception and signalling transduction pathway has emerged as an alternative to promote more extensively tomato fruit biofortification by simultaneously altering the accumulation of multiple nutraceutical compounds (Azari et al., 2015; Davuluri et al., 2005; Ganesan et al., 2017; Gururani et al., 2015). Responsible for the red/far-red light wavelength perception, phytochromes (PHYS) regulate a wide range of photomorphogenic responses throughout plant life cycle (Demotes-Mainard et al., 2016). Once photoactivated by red light exposure, PHYS allosterically change their conformation, triggering their translocation from cytosol to nucleus (Smith, 2000). In the nucleus, active PHYs interact with light signal transduction components, initiating highly complex and extensively interconnected signalling cascades that ultimately lead to the differential expression of photomorphogenesis-related genes and proteins-depending protein degradation events (Chen and Chory, 2011; Shin et al., 2016). Upon far-red light or dark exposure, active PHYs are converted back to the biologically inactive form (Seluzicki et al., 2017). In tomato, five PHY-encoding genes, namely PHYA, PHYB1, PHYB2, PHYE and PHYF, have been identified (Alba et al., 2000a). PHYB2 is the most expressed in tomato fruits.
et al., 1995) and a major regulator of fruit chloroplast maturation and carotenoid accumulation (Bianchetti et al., 2018; Gupta et al., 2014).

Besides the mRNA levels, PHY protein activity can also be manipulated. In Arabidopsis thaliana and Avena sativa, specific amino acid changes in the photosensor module domains result in a light-independent constitutive biological activity (Jeong et al., 2016; Su and Lagarias, 2007). This is the case of the mutation Ty276R to-His in the conserved GAF domain of Arabidopsis PHYB that renders constitutive PHY-dependent photomorphogenic responses, even in the darkness (Hu et al., 2009; Oku et al., 2011; Su and Lagarias, 2007).

Here, we investigated whether the overexpression of the constitutively active GAF domain Tyr-to-His PHYB2 mutant version (PHYB2Y252H) is suitable to improve PHY-dependent developmental and metabolic responses associated with tomato fruit nutritional composition, such as plastid development (Bianchetti et al., 2017, 2018), carotenogenesis (Alba et al., 2000b; Bianchetti et al., 2018; Gupta et al., 2014) and tocopherol biosynthesis (Gramenga et al., 2018). To circumvent adverse collateral effects on vegetative growth (e.g. reduced plant height, apical dominance and leaf size) normally caused by whole-plant PHY overexpression (Garg et al., 2006; Husainied et al., 2007; Thiele et al., 1999), a fruit-specific overexpression strategy was employed. Our data indicated that the expression of the constitutively active PHYB2Y252H promotes the biosynthesis and accumulation of carotenoids, flavonoids and vitamins C and E in tomato fruits to significantly higher levels than those detected when the native PHYB2 was overexpressed. Fruit plastid biogenesis and differentiation were particularly promoted by PHYB2Y252H overexpression, accompanied by an overall up-regulation of genes encoding chloroplast components and photosynthesis-related proteins. Fruit primary metabolism was only marginally affected by the fruit-specific PHYB2 or PHYB2Y252H overexpression, which further highlights the potential application of this genetic manipulation to enhance tomato fruit nutritional quality.

Results

Fruit-specific PHYB2 and PHYB2Y252H overexpression in tomato plants

To identify the conserved tyrosine residue to generate hyperactive GAF domain Tyr-to-His PHYB2 mutants, the coding sequence of Solanum lycopersicum PHYB2 was aligned and compared with Arabidopsis thaliana PHYB. The conserved Y276 residue of the Arabidopsis PHYB GAF domain corresponds to Y252 in tomato PHYB2 (Figure S1). Next, transgenic lines of tomato (Micro-Tom cultivar) expressing the native PHYB2 gene or its mutant hyperactive form PHYB2Y252H under the control of fruit-specific PHOSPHOENOLPYRUVATE CARBOXYLASE 2 (PPC2) promoter (Fernandez et al., 2009) were generated. Overexpression of the transgenes was confirmed by RT-qPCR in two PPC2::PHYB2 (PPC::B2) lines (namely L1 and L3) and three PPC2::PHYB2Y252H (PPC::B2Y252H) lines (namely L5, L6 and L15) (Figure 1a). PHYB2 or PHYB2Y252H transcript levels were between twofold and sevenfold higher in the transgenic lines compared with PHYB2 mRNA levels detected in WT fruits throughout initial fruit development (immature green, IMG; mature green, MG) and ripening stages (breaker, BK; red ripe, RR) (Figure 1a). Transcript abundance of other PHY genes remained virtually indistinguishable between the WT and transgenic lines throughout fruit development and ripening (Table S1).

The fruit-specific overexpression of either PHYB2 or PHYB2Y252H resulted in no apparent phenotypical alterations on plant vegetative growth (Figure S2). In contrast, green fruits (IMG and MG) from the PPC::B2Y252H lines exhibited significantly darker green coloration at the fruit pedicellar (shoulder) region than WT counterparts (Figure 1b). At the BK stage, the green shoulder phenotype persisted in both PPC::B2 and PPC::B2Y252H fruits, whereas it was significantly attenuated in WT fruits. The green shoulder phenotype was subsequently lost in all genotypes at the RR stage (Figure 1b).

RNA-seq transcriptomic profiling performed in BK fruits of representative PPC::B2 (L1) and PPC::B2Y252H (L6) lines revealed that up to 5.4% of the 23 685 transcriptionally active genes at this developmental stage was affected either by the PHYB2 or PHYB2Y252H overexpression. Compared with the WT, 906 and 719 genes were differentially expressed exclusively in PPC::B2 and PPC::B2Y252H fruits, respectively. Only 379 differentially expressed genes (DEGs) were common in both transgenics when compared to the WT, with a predominance of up-regulated (331) over down-regulated (48) genes (Figure 1c, Table S2). RNA-seq results were further validated by RT-qPCR analysis, demonstrating consistency (r² correlation above 0.9) between both methods (Figure S3).

According to the Gene Set Enrichment Analysis (GSEA), the exclusive PPC::B2 DEGs were predominantly associated with global cell functioning and diverse metabolic processes, grouping around GO terms such as sequence-specific DNA binding, cell wall organization or biogenesis, oxoacid metabolic process and small molecule biosynthetic process (Figure 1c, Table S3). In contrast, the majority of DEGs exclusively detected in PPC::B2Y252H fruits were associated with chloroplast components and related processes, including GO terms as photosynthesis, thylakoid, photosynthetic membrane and generation of precursor metabolites and energy (Figure 1c, Table S3).

Either PHYB2 or PHYB2Y252H overexpression promotes fruit plastid biogenesis but only PHYB2Y252H enhances chlorophyll accumulation

The greener fruit phenotype and up-regulation of plastid-related genes in the transgenic lines prompted us to investigate the impacts of either PHYB2 or PHYB2Y252H overexpression on fruit chloroplast biogenesis and development. Microscopy analysis revealed increments of up to 50% in plastid abundance in pericarp cells of PPC::B2 and PPC::B2Y252H green fruits compared with WT counterparts (Figure 2a,b). The increased number of plastids detected in PPC::B2 and PPC::B2Y252H fruits was associated with up-regulation of PLASTID DIVISION 2 (PDV2) (Figure 2c), which encodes a vital component of the plastid division machinery (Okazaki et al., 2009).

Increased thylakoid stacking with more voluminous grana was observed in plastids of PPC::B2 green fruits, a phenotype further intensified in PPC::B2Y252H fruits (Figure 2a, Figure S4). In agreement, transcript abundances of several photosynthesis- and plastid-related genes, including those encoding Rubisco subunits (i.e. RBCS2a), were up-regulated in IMG fruits of PPC::B2Y252H lines, but not in PPC::B2 fruits (Figure 2c). Similarly, two tomato homologs of Arabidopsis mediator of thylakoid membrane bending at the grana margins CURVATURE THYLAKOID 1a (CURT1a, Armbruster et al., 2013), namely CURT1a1 (Soly-c01g095430) and CURT1a2 (Soly-10g011770) (Figure S5), were also exclusively up-regulated in PPC::B2Y252H IMG fruits (Figure 2c). In contrast, mRNA levels encoding transcription factors
previously associated with altered plastid maturation and maintenance in green tomato fruits, such as \textit{GOLDEN 2-LIKE 2} (\textit{GLK2}), \textit{AUXIN RESPONSE FACTOR 4} (\textit{ARF4}), \textit{ARABIDOPSIS PSEUDORESPONSE REGULATOR 2-LIKE} (\textit{APRR2Like}) and \textit{BEL-1 LIKE HOMEODOMAIN 11} (\textit{BEL11}) were virtually indistinguishable between the wild-type and transgenic lines (Table S4).

Total chlorophyll content was about threefold higher in \textit{PPC::B2Y252H} green fruits than in the WT (Figure 2d, Table S5), which was associated with increments of approximately twofold in transcript levels of \textit{PROTOCHLOROPHYLLIDE OXIDOREDUCTASE 1} (\textit{POR1}) (Figure 2c), which encodes an essential light-triggered chlorophyll biosynthesis-related enzyme (Heyes and Hunter, 2005). Moreover, transcriptome analysis carried out in MG fruits revealed enrichment of transcripts associated with photosynthesis, thylakoid and plastid membranes in \textit{PPC::B2Y252H} compared with WT fruits (Figure 2e). Most chlorophyll biosynthetic genes were significantly up-regulated in \textit{PPC::B2Y252H} MG fruits compared with the WT (Figure 2f, Table S6), which agrees with the higher chlorophyll content detected in the transgenic fruits. In contrast, \textit{PPC::B2} green fruits showed no significant changes in total chlorophyll levels nor in \textit{POR1} mRNA levels compared with the WT (Figure 2c,d, Tables S4 and S5).

\textit{PHYB2Y252H} overexpression promotes fruit isoprenoid metabolism

Besides photosynthesis, chloroplasts are essential organelles for the synthesis and storage of secondary metabolites (Armbruster et al., 2011). Therefore, increments in fruit plastid abundance and size have consistently been associated with increased tomato fruit nutritional composition (Bianchetti et al., 2017; Bino et al., 2005; Cocaliadis et al., 2014; Cruz et al., 2018; Enfissi et al., 2010; Galpaz et al., 2008; Kolotilin et al., 2007; Lupi et al., 2019; Nguyen et al., 2014). Besides, light signalling can also directly influence the expression of genes involved in the production of...
Figure 2  Fruit-specific PHYB2 and PHYB2<sup>Y252H</sup> overexpression promote chloroplast biogenesis and differentiation and chlorophyll biosynthesis. (a) Representative optical microscopy (upper panels) and transmission electron microscopy images (bottom panels) of plastids at the pedicel region of immature green (IMG) fruits of wild-type (WT), PPC::PHYB2 (PPC::B2) and PPC::PHYB2<sup>Y252H</sup> (PPC::B2<sup>Y252H</sup>) plants of Micro-Tom cultivar. Black asterisks indicate stacked thylakoids and white asterisks indicate plastoglobules. (b) Plastid number per pericarp cell of WT, PPC::B2 and PPC::B2<sup>Y252H</sup> fruits at mature green (MG) stage. (c) Heatmap representation of the statistically significant differences in mRNA abundance of plastid-related genes normalized against WT transcript levels at IMG stage (Dunnett’s test, α = 0.05) via RT-qPCR. Gene abbreviations and relative transcript values are detailed in Table S4. (d) Total chlorophyll content of WT, PPC::B2 and PPC::B2<sup>Y252H</sup> fruits. (e) Gene Set Enrichment Analysis (GSEA) of exclusive differentially expressed genes (DEGs) of PPC::B2<sup>Y252H</sup> MG fruits compared with WT counterparts. GO terms are detailed in Table S3. (f) Simplified chlorophyll biosynthetic pathway. Intermediate reactions are omitted. Up-regulated chlorophyll biosynthesis-related genes in PPC::B2<sup>Y252H</sup> MG fruits according to RNA-seq analysis are highlighted in red. Gene abbreviations, as well as logFC and FDR values, are detailed in Table S6. In (b) and (d), data are mean ± SE, dots represent individual values, and statistical differences within each stage are given by different letters (Tukey’s Test, α = 0.05). BK, breaker; ALA, 5-aminolevulinic acid.
isoprenoids (e.g., carotenoids, tocopherols) and other health-promoting substances accumulated in tomato fruits (Alba et al., 2006b; Gramenega et al., 2018; Kas et al., 2015; Llorente et al., 2016).

Carotenoid and tocopherol profiling revealed that PHYB2 overexpression promoted exclusively β-carotene and lutein accumulation in RR fruits (Figures 3a and S6, Table S5). In contrast, PPC::B2Y252H RR fruits exhibited significant increments in virtually all individual carotenoids analysed, with increments of about 50% in phytoene, phytofluene and lycopene levels and between 100% and 200% in β-carotene and lutein levels, respectively. Total tocopherol content in red fruits exhibited a similar trend, with increments of up to twofold in PPC::B2Y252H lines (Figures 3a and S6, Table S5). The levels of α-carotene, the dominant tocopherol form in tomato fruits, as well as β-carotene and lutein contents were significantly higher in PHYB2Y252H-overexpressing lines than in the WT at all sampled stages (Table S5). This indicates that the PHYB2Y252H-triggered intensification of fruit isoprenoid accumulation was not restricted to the ripening stages.

Transcript levels of many isoprenoid biosynthetic genes were up-regulated in response to PHYB2 and PHYB2Y252H overexpression (Figure 3b). Genes encoding rate-limiting enzymes responsible for the synthesis of isoprenoid precursor geranylgeranyl diposphate (GGDP), such as 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE 1 (DXS1) and GGDP SYNTHASE 2 (GGPS2), were up-regulated in PPC::B2Y252H and in all transgenic lines, respectively. In contrast, except LYCOPENE CYCLASE (LcyC), other core carotenoid biosynthesis genes were not consistently up-regulated in the transgenic fruits during ripening. Likewise, mRNA levels of transcription factors associated with the ripening-related regulation of carotenoid biosynthesis, such as RIPENING INHIBITOR (RIN), NON-RIPENING (NOR), COLORLESS NON-RIPENING (CNR), APFELA2a (AP2a) and FRUITFULL1 (FUL1) (Liu et al., 2015), were also unaltered in the transgenic lines during fruit ripening (Table S7).

The activities of the carotenoid desaturase enzymes PHYTOENE DESATURASE (PDS) and ζ-CAROTENE DESATURASE (ZDS) are especially sensitive to the cellular redox state, requiring plastoquinones (PQs) as co-factors to accept electrons produced during the desaturation steps of GGDP to lycopene (Fanciullino et al., 2014; Norris et al., 1995). The transcript levels of the PQ biosynthetic enzyme SOLANESYL DIPHOSPHATE SYNTHASE (SPS) were up-regulated in PPC::B2Y252H lines, likely boosting PQ biosynthesis in the transgenic fruits. Moreover, ORANGE RIPENING (ORR), a gene encoding a NADH dehydrogenase (Ndh) complex subunit responsible for PQ reduction (Endo et al., 2008), was also up-regulated in PPC::B2Y252H lines (Figure 3b).

GERANYLGERANYL DIPHOSPHATE REDUCTASE (GGDR), which encodes the enzyme responsible for the de novo synthesis of tocopherol precursor phytyl diphasphate (PDP), was markedly up-regulated exclusively in PPC::B2Y252H fruits at all sampling stages, suggesting increased conversion of GGDP to PDP in these transgenic lines. During green stages of fruit development, GGDR up-regulation presumably promoted PDP production, feeding the enhanced chlorophyll biosynthetic pathway mentioned above (Figure 3b). From the onset of ripening onwards, chlorophyll biosynthesis ceases and the precursor GGDP are channelled towards carotenoid production by the transcriptional inhibition of GGDR in WT (Table S7, Quadrana et al., 2013), whereas chlorophyll degradation releases phytoil, which is, in part, incorporated into tocopherol (Zhang et al., 2014), leading to its increment during ripening (Table S5). Interestingly, at the BK stage in PPC::B2Y252H lines, PHYTOL KINASE (VTES), which is responsible for the first step of phytol phosphorylation (Valentin et al., 2006), was up-regulated compared with WT counterparts (Figure 3b), probably enhancing the production of PDP and contributing to the increase in total tocopherol content observed all along fruit development (Figure 3b). Accordingly, tocopherol biosynthetic genes, particularly 2,3-DIMETHYL-5-PHTYLQUINOL METHYLTRANSFERASE B (VTE3), TOCOPHEROL CYCLASE (VTE1) and TOCOPHEROL C-METHYL TRANSFERASE (VTE4), were also predominantly up-regulated in PPC::B2Y252H lines from the IMG to the BK stage (Figure 3b).

Both flavonoid and ascorbate metabolisms are up-regulated in PHYB2Y252H ripe fruits

In our study, the fruit-specific PHYB2Y252H overexpression significantly promoted both flavonoid and ascorbate contents in RR fruits, whereas the levels of these antioxidants were slightly, but not significantly, increased in PPC::B2 fruits. Increments between twofold and threefold in rutin, kaempferol rutinoside, naringenin chalcone and naringenin glucoside contents were registered in PPC::B2Y252H RR fruits compared with WT counterparts (Figures 4a and S7, Table S8). Both ascorbate and dehydroascorbate levels were overaccumulated in PPC::B2Y252H fruits (Figure S7), resulting in increments of approximately 20% in the total pool of this antioxidant (Figure 4b).

On the other hand, the PPC::B2Y252H-induced increments in rutin, which is a major flavonoid accumulated in tomato fruits (Slimestad and Verheul, 2009), as well as kaempferol rutinoside, were associated with significantly higher transcript abundance of FLAVONOL SYNTHASE (FLS) in the transgenic fruits at the BK stage (Figure 4c). The higher ascorbate content was associated with increased transcript levels of GDP-MANNNOSE-3,5-EPIMERASE 1 (GME1) (Figure 4d), which encodes an enzyme responsible for an initial step in the ascorbate biosynthetic pathway (Wolucka and Van Montagu, 2007). RNA-seq data revealed that GME1 was the only core ascorbate biosynthetic gene modulated by PHYB2Y252H overexpression (Figure S8), with up to twofold increments in mRNA levels at the BK and RR stages as confirmed by qPCR analysis (Figure 4d).

Consistent with the differential effect of PHYB2 and PHYB2Y252H overexpression on isoprenoid flavonoids and ascorbate metabolism in RR fruits, only PPC::B2Y252H lines consistently exhibited higher total antioxidant activity, as estimated by the DPPH assay, reaching increments of up to 25% compared with WT counterparts (Figure 4e, Table S8).

Lipid and sugar metabolisms are the main primary metabolic pathways affected in the transgenic fruits

Mass spectrometry (MS)-based metabolite profiling and high-performance liquid chromatography (HPLC)-based soluble carbohydrate (sucrose, glucose, fructose) and organic acid (citric acid) analysis were used to investigate the impacts of PHYB2 or PHYB2Y252H overexpression on primary metabolism of ripe fruits. About 25% of the metabolites identified via GC–MS profiling was consistently altered in the transgenic lines compared with WT counterparts (Table S10). Hierarchical clustering analysis supported the differences between the clades composed by the PPC::B2 and PPC::B2Y252H lines based on polar and apolar metabolite profiles, evidencing that fruit metabolome was differentially affected by PHYB2 or PHYB2Y252H overexpression (Figure 5a-b).
Minor organic acids such as acetic, benzoic, lactic and succinic acids were under-accumulated in all transgenic lines, whereas oxalic acid was overaccumulated in PPC::B2 lines. Citric acid, which is the most abundant organic acid in tomato (Morgan et al., 2013), was not significantly altered in transgenic fruits throughout fruit development and ripening (Table S11). Except for aspartic acid, aminoacids were not consistently altered in the transgenic fruits compared with WT counterparts (Figure 5a).

Saturated fatty acids such as arachidic, lauric and lignoceric acids were significantly more abundant in all transgenic lines, whereas the levels of the polyunsaturated ω-6 linolenic acid were consistently increased only in PPC::B2Y252H fruits (Figure 5b). RNA-seq data analysis revealed that some genes encoding important lipid metabolism-related enzymes are up-regulated, particularly in PPC::B2Y252H lines (Table S2). For instance, GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (Soly-c08g076470), which encodes the enzyme catalysing an initial reaction in the biosynthesis of phospholipids (Chen et al., 2011), and some FATTY ACID DESATURASES (FADs, Solyc06g051400 and Solyc07g005510) encoding genes, associated with the accumulation of polyunsaturated fatty acids (Dar et al., 2017), were up-regulated in the PPC::B2Y252H (Table S2).
Glucose, fructose and sucrose contents were indistinguishable in WT and transgenic green fruits (Table S11). However, glucose and fructose levels were predominantly lower in the RR transgenic fruits than in the WT counterparts, whereas sucrose remained at similar levels in all genotypes (Figure 5c and S9, Table S11). Accordingly, total soluble solids content (°C/Brix) values in transgenic ripe fruits were approximately 5% lower than in the WT counterparts. Data are mean ± SE, and dots represent individual values.

Metabolites, gene abbreviations and relative transcript values are detailed in Tables S8 and S9. Statistical differences within each stage are given by asterisks (Dunnett’s test with WT as control group, α = 0.05) or different letters (Tukey’s test, α = 0.05). DHA, dehydroascorbate; AsA, ascorbic acid; BK, breaker.

Glucose, fructose and sucrose contents were indistinguishable in WT and transgenic green fruits (Table S11). However, glucose and fructose levels were predominantly lower in the RR transgenic fruits than in the WT counterparts, whereas sucrose remained at similar levels in all genotypes (Figure S5c and S9, Table S11). Accordingly, total soluble solids content (°C/Brix) values in transgenic ripe fruits were approximately 5% lower than in the WT counterparts. Data are mean ± SE, and dots represent individual values. Metabolites, gene abbreviations and relative transcript values are detailed in Tables S8 and S9. Statistical differences within each stage are given by asterisks (Dunnett’s test with WT as control group, α = 0.05) or different letters (Tukey’s test, α = 0.05). DHA, dehydroascorbate; AsA, ascorbic acid; BK, breaker.

## Figure 4

Flavonoid and ascorbate levels are increased in PHYB2Y2S2H-overexpressing fruits. (a) Flavonoid content in wild-type (WT), PPC2::PHYB2 (PPC::B2) and PPC2::PHYB2Y2S2H (PPC::B2Y2S2H) red ripe (RR) fruits of Micro-Tom cultivar. (b) Total ascorbate pool in RR fruits. (c,d) FLS and GME1 mRNA levels throughout fruit development. Transcript abundance was normalized against wild-type (WT) samples at the mature green (MG) stage. (e) Total antioxidant activity of polar extracts expressed as Trolox Equivalent Antioxidant Capacity (TEAC) in RR fruits. Data are mean ± SE, and dots represent individual values. Metabolites, gene abbreviations and relative transcript values are detailed in Tables S8 and S9. Statistical differences within each stage are given by asterisks (Dunnett’s test with WT as control group, α = 0.05) or different letters (Tukey’s test, α = 0.05). DHA, dehydroascorbate; AsA, ascorbic acid; BK, breaker.

© 2020 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 18, 2027–2041
PHYB2Y252H overexpression leads to phytonutrient overaccumulation in fruits of distinct tomato genetic backgrounds

To evaluate whether fruit-specific PHYB2 and PHYB2Y252H overexpression can promote fruit quality regardless of the tomato genetic background, this phenomenon was also investigated in the well-characterized tomato cultivar Ailsa Craig (AC). Wild-type AC plants were crossed with Micro-Tom (MT) wild-type or homozygous PHYB- or PHYB2Y252H-overexpressing plants generating F1 plants (AC-WT, AC-PPC::B2 and AC-PPC::B2Y252H, respectively) that were cultivated under greenhouse, semi-controlled conditions until fruit harvesting.

As observed for the MT background, MG fruits of AC plants overexpressing PHYB2Y252H exhibited darker green fruit coloration than AC-WT, a phenotype explained by the higher chlorophyll accumulation and increased POR1 transcript levels of the transgenic fruits (Figure 6a–c and S10). At the ripe stage, fruits from both AC-PPC::B2 and AC-PPC::B2Y252H genotypes displayed higher lycopene accumulation and more intense red coloration than the AC-WT counterparts (Figure 6a,d).

Compared with AC-WT, ripe AC-PPC::B2Y252H fruits overaccumulated β-carotene, lutein and all four (α, β, δ and γ) tocopherol forms (Figure 6d and S10). PHYB2Y252H overexpression in AC cultivar also resulted in the up-regulation of key tocopherol biosynthetic genes as GGDR and VTE3b (Figure 6e). The levels of flavonoids, such as rutin and quercetin derivatives, and ascorbate were also increased in response to PHYB2Y252H overexpression, and reductions of around 10% in °Brix were reported in red fruits of both AC-PPC::B2 and AC-PPC::B2Y252H genotypes compared with the AC-WT counterparts (Figure S10). Comparing data obtained from transgenic lines of MT and AC cultivars reveals that PHYB2Y252H overexpression can promote fruit nutritional quality in different tomato genetic backgrounds as well as distinct growth conditions (i.e. laboratory and greenhouse conditions).

Discussion

Manipulation of light signalling pathway components has been increasingly investigated as a means to improve desired quality traits in many crops, including adjustments in plant architecture,
shade avoidance responses, abiotic stresses tolerance and flowering time (Ganesan et al., 2017; Gururani et al., 2015). In tomato, accumulating genetic evidence indicates that alterations in downstream components of photoreceptor signal transduction pathways can impact fruit chemical composition by simultaneously affecting multiple metabolic pathways (Azari et al., 2019). PHYB2 or PHYB2Y252H mRNA levels in MG fruits. (c) Total chlorophyll content and POR1 mRNA levels MG fruits. (d) Carotenoid (lycopene, phytoene, phytofluene, lutein and β-carotene) and tocopherol (α, β, δ and γ forms) content in RR fruits. (d) GGDR and VTE3b mRNA levels in RR fruits. Transcript abundance was normalized against the WT samples. Data are mean ± SE, and dots represent individual values. Statistical differences are given by different letters (Tukey’s test, α = 0.05). POR1, protochlorophyllide oxidoreductase; GGDR, geranylgeranyl diphosphate reductase; VTE3b, 2,3-dimethyl-5-phytylquinol methyltransferase.

Making room for more: PHYB2-positive impacts on fruit plastid abundance and ultrastructure

The most striking visual phenotype of PCC::B2 and particularly PPC::B2Y252H transgenic lines was the production of greener immature fruits and the persistence of the green shoulder during initial ripening, which were attributed to a higher abundance of chloroplasts in the pedicular region compared with the WT (Figure 1). In agreement, PHY-mediated light perception has long been associated with chloroplast development and thylakoid formation from early seedling de-etiolation throughout adult plant life (Girnth et al., 1978; Mohr, 1977). In tomato fruits, PHY deficiency leads to reduced plaid size and density per cell (Bianchetti et al., 2017), whereas the opposite phenotype has been observed in the light-hyperresponsive tomato high-pigment (hp) mutants hp1 and hp2 (Davuluri et al., 2005; Kendrick et al., 1997; Wang et al., 2008). Fruit-localized PHY deficiency has also been demonstrated to regulate mRNA levels of multiple tomato genes encoding key components of the plastid division machinery (Bianchetti et al., 2018). Among these genes, PDV2 was particularly up-regulated in the PHYB2- or PHYB2Y252H-overexpressing lines (Figure 2), which corroborates the proposed central role of this gene in determining plastid division rates in immature tomato fruits (Bianchetti et al., 2018) and Arabidopsis leaves (Okazaki et al., 2009).

In line with the identification of CURT1a protein accumulation as requisite for grana stacking in Arabidopsis leaf chloroplasts (Armbuster et al., 2013; Pribil et al., 2018), the up-regulation of...
Curtiala1 and CURT1a2 in PPC::B2Y252H IMG fruits may be linked to the conspicuously incremented grana size registered in these transgenic lines (Figure 2). Also, genes associated with photosynthesis and chloroplast structure and functioning predominate among those up-regulated in response to PHYB2 or PHYB2Y252H overexpression (Figure 1), which agrees with the well-reported positive influence of PHY signalling pathway on these gene categories in de-etiolating seedlings and developing leaves (Dubreuil et al., 2011; Hu et al., 2009; Oh and Montgomery, 2014; Voo et al., 2019).

Increments in plastid ultrastructure positively affected isoprenoid accumulation once many active biosynthetic enzymes and their hydrophobic products are physically associated with plastid membranes (Llorente et al., 2017; Yuan et al., 2015). In tomato, such positive correlation is evident in several mutants and transgenic lines that produce plastid-rich, dark green immature fruits, such as hp1 and hp2 mutants (Koleti et al., 2007) and GLK2-overexpressing lines (Lupi et al., 2019; Powell et al., 2012). Therefore, the increments in plastid abundance and ultrastructure observed in PPC::B2 and PPC::B2Y252H fruits can be interpreted as a physical facilitator of isoprenoid biosynthesis and accumulation in the pericarp cells. However, many isoprenoid biosynthetic genes are also transcriptionally regulated by components of PHY signalling pathway (Gramegna et al., 2018; Inagaki et al., 2015; Llorente et al., 2016), which possibly explains the differential impacts of PHYB2 and PHYB2Y252H overexpression on the abundance of isoprenoids in green (chlorophylls) and ripening fruits (carotenoids and tocopherols). In fact, only PPC::B2Y252H fruits presented higher chlorophyll content than the WT, a differential response associated with the exclusive up-regulation of chlorophyll biosynthetic genes in the PPC::B2Y252H lines (Figures 2, 3 and 6).

**PHYB2Y252H**-induced overaccumulation of bioactive compounds mainly relies on the up-regulation of target biosynthetic genes

Tomato fruits are a major source of essential antioxidants in the human diet, such as carotenoids, tocopherols, flavonoids and ascorbate, which are associated with reducing the risks of cancer and cardiovascular diseases (Doria et al., 2008; Frusciante et al., 2007). Many of these antioxidant classes accumulate or display altered composition as tomato fruit ripens via complex metabolic pathways tightly coordinated at the transcriptional level (Bovy et al., 2007; Li et al., 2019; Quadrania et al., 2013; Verhoeven et al., 2002; Yuan et al., 2015).

Carotenoids and tocopherols have as a precursor the MEP pathway-derived GGDP (Pulido et al., 2012). Transcriptional analysis revealed that DXS1 and GGPS2 were particularly up-regulated in ripening PPC::B2 and PPC::B2Y252H fruits, possibly boosting the production of GGDP to sustain higher carotenoid and tocopherol biosynthesis fluxes (Figure 3). Although PHYB2Y252H overexpression resulted in significantly higher levels of lycopene, β-carotene and lutein, the most significant bioactive carotenoids in ripe tomatoes (Laiola et al., 2014), core carotenoid biosynthetic genes (e.g. PSY1, PDS) were not consistently modulated at the transcriptional level in these transgenic lines (Figure 3). This seemingly contradictory result suggests that the PHYB2Y252H-triggered up-regulation of GGDP-biosynthetic genes and/or the increased plastid abundance with more developed grana may suffice to sustain the higher carotenoid synthesis and accumulation detected in the ripe transgenic fruits. Alternatively, the presence of the constitutively active PHYB2Y252H molecules may also have affected fruit carotenogenesis via additional regulatory levels.

Carotenoid-related enzymes can also be post-translationally regulated, primarily relying on PQs as electron acceptors during the desaturation steps of phytoene and subsequent metabolites, driving lycopene biosynthesis (Norris et al., 1995). SPS, a PQ biosynthetic gene, was up-regulated in PPC::B2Y252H fruits, suggesting that PQ pool was increased in the transgenic fruits. Accordingly, silencing of tomato SPS reduces the levels of PQs, modifying carotenoid composition and leading to phytoene accumulation, possibly due to the impairment of desaturases activity (Jones et al., 2013). The positive relation between the levels of PQs and carotenoid biosynthesis has also been reported in Arabidopsis (Kim et al., 2015) and tomato hp2 leaves (Jones et al., 2013). The reduction of PQs is catalysed by the Ndh complex, essential for the control of carotenoid accumulation (Endo et al., 2008). In tomato fruits, mutations in the ORR gene, encoding a Ndh subunit, led to decreased carotenoid content (Nashilevitz et al., 2010), highlighting the importance of proper functioning of this redox chain to sustain tomato carotenogenesis. In line with this and in agreement with the enhanced carotenoids accumulation, ORR was up-regulated in PPC::B2Y252H fruits.

The marked increase in total tocopherol content in the PPC::B2Y252H lines can be attributed to the up-regulation of genes related to bottlenecks of PDP biosynthesis. VTE5, which catalyses the first and limiting step of channelling phytol chain into tocopherol biosynthetic pathway (Almeida et al., 2016), was transcriptionally up-regulated in PPC::B2Y252H BK fruits, providing PDP from chlorophyll turnover. Also, GGDR levels were up-regulated in PPC::B2Y252H BK and RR fruits, possibly increasing PDP supply via MEP pathway. In line with our findings, the up-regulation of GGDR in MG stages of hp1 tomato mutants was also associated with increased tocopherol content in this mutant (Enfissi et al., 2010). It has also been shown that GGDR transcription is negatively regulated by PIF3 in tomato fruits (Gramegna et al., 2018). Thus, the intensification of PHYB2Y252H, PIF3 interaction due to the constant presence of PHYB2Y252H in pericarp cell nuclei, and consequent reduction in PIF3 protein accumulation, explains the markedly higher GGDR expression in PPC::B2Y252H from early stages of fruit development until the completion of ripening. Besides GGDR, PHYB2Y252H overexpression promoted the expression of VTE1, VTE4 and VTE3b, the latter two already described to be positively correlated with fruit vitamin E content in tomato genotypes (Fritsche et al., 2017; Quadrania et al., 2013).

Light signals have been widely recognized as major environmental factors controlling flavonoid and vitamin C accumulation in tomato (Løvdal et al., 2010; Massot et al., 2012). In agreement, our findings indicate that the higher content of naringenin chalcone, rutin and kaempferol registered in the PPC::B2Y252H lines can be partially explained by the marked up-regulation of FLS mRNA levels detected in the transgenic fruits at BK stage, when this gene is at peak expression (Figure 4c). Similarly, among all core ascorbate biosynthetic genes, only GME1 was positively correlated with the vitamin C overaccumulation of PPC::B2Y252H fruits, corroborating the proposition of GME1 expression as a major determinant of ascorbate levels in tomato fruits (Gilbert et al., 2009; Li et al., 2019; Massot et al., 2012).
Fruit primary metabolism is marginally affected by PHYB2 manipulation

Besides playing vital roles in primary metabolism, soluble sugars, organic acids and some amino acids are also essential determinants of tomato fruit flavour (Carli et al., 2009). Different from the overall PHYB2-dependent light-induced changes in secondary metabolism, more limited differences in the abundance of primary metabolites were observed between the WT and transgenic ripe fruits, except for the over- and under-accumulation of some lipids and soluble sugars, respectively (Figure 5). Such reduction in soluble sugars agrees with the slightly lower °Brix values detected in ripe transgenic fruits, and the use of alternative promoters or further engineering in PHY sequence may be necessary to circumvent this negative collateral effect.

Accumulation of sugars in ripe tomato is directly influenced by the pool of starch synthesized at the early stages of fruit development, which is under the influence of multiple endogenous and environmental factors (Sagar et al., 2013; Schaffer et al., 2000; Yin et al., 2010). After confirming that the fruit-specific transgene overexpression had no impact on leaf photosynthetic capacity nor in the expression of predominant invertase genes, we identified a selective under-accumulation of transcripts encoding a specific subunit of AGPase, an enzyme responsible for catalysing the first and rate-limiting step of starch accumulation (Petreikov et al., 2006; Stark et al., 1992). In tomato, AGPaseL1 is the predominant large AGPase subunit in immature tomato fruits (Bianchetti et al., 2018; Petreikov et al., 2006), whose gene transcript accumulation is affected by fruit-localized PHYs (Bianchetti et al., 2018). Interestingly, PHYB2 and PHYB2Y252H overexpression significantly reduced AGPaseL1 mRNA levels specifically during early fruit development, when starch biosynthetic rates are maximum, suggesting a possible restriction in AGPase-dependent production of the starch precursor ADP-glucose.

As carbohydrate and fatty acids biosynthesis compete for the same carbon precursors (Rawsthorne, 2002; Yu et al., 2018), it seems plausible to suggest a possible link between the lower sugar and higher lipid abundance in PPC::B2Y252H ripe fruits compared with the WT (Figure 5). Additionally, it has been demonstrated that tocopherol deficiency affects fatty acid metabolism, with consequences in sugar metabolism in tomato. By two distinct genetic interventions, tocopherol-deficient plants showed higher amounts of starch and enhanced unsaturated fatty acid accumulation than WT. The changes in lipid profiles correlated with the reduction in FADs expression (Almeida et al., 2016; Bermúdez et al., 2018). Interestingly, an inverse scenario was described by our results. PPC::B2Y252H fruits showed higher tocopherol content, reduced amount of starch and up-regulation of FADs. How tocopherol influences sugar metabolism has not yet been precisely addressed but it might be the result of the interaction between lipid and sugar metabolism machineries as observed here.

Conclusion remarks

Our findings indicate that fruit-specific overexpression of native PHYB2 sequence is not sufficient to drive significant increments in health-promoting secondary metabolites, a limitation resolved with the use of a light-independent constitutively active version of this photoreceptor. The presence of continuously active PHYB2Y252H molecules all over the fruit cells, regardless of the surrounding light conditions, probably leads to saturated PHYB2-dependent light signalling. As a consequence, PHYB2 signalling-dependent gene transcription is intensified, including many plastid-related genes as well as biosynthetic enzymes responsible for the production of antioxidants. Experiments conducted using two genetic backgrounds (i.e. Micro-Tom and Ailsa Craig) and two cultivation conditions (i.e. laboratory and greenhouse) allowed us to infer that PHYB2Y252H overexpression is a valid tool for promoting fruit nutritional content across tomato cultivars and at distinct growth conditions. Moreover, as phytochrome GAF domain Tyr residues are well conserved across plant species, the gain-of-function engineering of PHYs employed in this work may also be applicable as a means to promote biofortification in other fleshy fruits.

Methods

Plasmid constructs and tomato transformation

Solanum lycopersicum PHYB2 full-length coding sequence was amplified with Platinum SuperFi Green PCR Master Mix (Thermo Fischer Scientific, Waltham, USA) using tomato fruit cDNA as a template and the oligonucleotide sequences detailed in Table S14. The fragment was inserted into entry vectors pDONR211 (Thermo Scientific) and recombined with pK7m24GW3 and pEN-L4-PPC-R1 plasmids (Fernandez et al., 2009) via LR clonase II Plus (Thermo Scientific) reaction to generate the final construct PPC2::PHYB2. The single T-to-C base change required to modify the amino acid translation from Tyr252 to His252 was inserted using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent), resulting in the PPC2::PHYB2Y252H construct. The Agrobacterium-mediated transformation of tomato (Solanum lycopersicum L.) plants cv. Micro-Tom, which harbours the wild-type GLK2 allele (Carvalho et al., 2011), was conducted as described by Bianchetti et al. (2018). Oligonucleotides used for plasmid construction and selection of transgenic plants are listed in Table S14. The homozygous Micro-Tom (MT) lines were obtained from the T2 or T3 progeny by seeking 100% kanamycin resistance in the T3 or T4 seed population, respectively. Experiments were performed in homozygous T4 (or later) generations.

Homozygous MT PHYB2 or PHYB2Y252H-overexpressing lines were used to generate F1 plants in Ailsa Craig (AC) background. Flowers from wild-type AC plants were emasculated at anthesis and hand-pollinated with MT-WT, MT-PPC2::PHYB2 or MT-PPC2::PHYB2Y252H pollen. F1 seedlings were selected via kanamycin resistance assay, selected using oligonucleotides listed in Table S14.

Growth conditions

Micro-Tom wild-type and transgenic lines were grown in a chamber under controlled conditions: 250 mmol m⁻² s⁻¹, 12-h photoperiod, air temperature of 27°C day/22°C night and 60% day/80% night relative air humidity. Fruits were collected at immature green (IMG, on average 13 days post-anthesis), mature green (MG, when green fruits are fully grown presenting locular gel), breaker (BK, when first signs of yellowing appear on the fruit bottom) and red ripe (RR, 12 days after BK) stages.

Ailsa Craig wild-type and transgenic lines were grown under greenhouse, semi-controlled conditions: average mean temperature of 25 ± 5°C, 11.5/13 light hours (winter/summer) and 250–350 mmol m⁻² s⁻¹ of incident photoirradiance. Fruits were harvested at MG and red ripe (RR, 12 days after BK) stages.

© 2020 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 18, 2027–2041
In all cases, fruits were harvested at the same time of the day (between the 4th and 6th hour of light period) in at least four biological replicates, with each replicate composed by at least four fruits from different plants. Seeds were removed, and the remaining tissues were immediately frozen in liquid nitrogen, powdered and stored at −80°C until use.

**RNA sequencing and data analysis**

RNA extraction was performed as described in Bianchetti et al. (2018), and RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Library preparation and sequencing using the Illumina HiSeq2500 system, as well as RNA-seq assembly, annotation and differential expression analysis are detailed in Methods S1. Gene Set Enrichment Analysis (GSEA) was performed on Blast2GO software (version 5.2.5) (http://www.blast2go.org/) with the following modifications on default parameters: Gene Set Minimal Size and FDR filter value were set to 10 and 0.25, respectively.

**RT-qPCR analysis**

RNA extraction, cDNA synthesis and RT-qPCR analyses were performed as described by Bianchetti et al. (2018). Oligonucleotide sequences used in the study are detailed in Table S14.

**Plastid ultrastructure and abundance**

Plastid ultrastructure was assessed following methods described by Bianchetti et al. (2018). Pericarp sections from three immature fruits harvested from different plants were analysed per genotype. Plastid abundance was determined as described in Bianchetti et al. (2017). At least 15 individual cells were analysed per sample.

**Chlorophyll, carotenoid and tocopherol profiling**

Chlorophyll a, chlorophyll b, phytone, phytoflouene, lycopene, β-carotene and lutein levels were determined by high-performance liquid chromatography with diode-array detection (HPLC-DAD) 1100 system (Agilent Technologies) as described in Cruz et al. (2018). Tocopherol extraction and quantification were performed as described in Lira et al. (2017). The endogenous metabolite concentration was obtained by comparing the peak areas of the chromatograms with commercial standards.

**Antioxidant activity, flavonoids and ascorbate quantification**

For the antioxidant activity of polar extracts and flavonoid content analysis, approximately 100 mg fresh weight (FW) of powdered fruit pericarp samples was extracted with 1 mL of 80% (v/v) methanol for 30 min in an ultrasonic bath at room temperature followed by the collection of the supernatants by centrifugation (12 000 g, 2 min, 25°C). DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity assay followed the protocol described by Furlan et al. (2015). Flavonoids were identified and quantified by HPLC systems according to the protocols and configurations described in Methods S1. Ascorbate extraction and detection were performed as described in Methods S1.

**Starch, soluble sugars and citrate quantification**

Starch and soluble sugar extractions were performed as described by Bianchetti et al. (2017). Starch levels were determined from the dried pellet as described in Suguiyama et al. (2014). Citrate quantification followed the protocol described in Amóros et al. (2003).

**Leaf gas exchange and fluorescence parameters**

Gas exchange parameters were measured between the 2nd and 4th hour of light period on the second fully expanded leaves from shoot to apex of approximately 2-month-old plants. Analyses were performed using a portable Li-6400XT infrared gas analyzer (LI-COR Biosciences, Lincoln, USA) adjusted to a constant chamber temperature of 25°C, reference CO2 concentration of 400 ppm and photosynthetic photon flux density of 1000 μmol photons m−2 s−1. Chlorophyll fluorescence parameters were measured on the third fully expanded leaves from shoot to apex using a portable fluorometer MINI-PAM (Heinz Walz GmbH, Effeltrich, Germany) following the protocol and derived calculations described in Alves et al. (2016).

**Metabolite profiling**

Mass spectrometry (MS)-based metabolite profiling followed the protocols described by Lisec et al. (2006) for polar compounds whereas that described in Bligh and Dyer (1959), Fiehn et al. (2000) and Ichihara and Fukubayashi (2010) was used for apolar compounds, with modifications described in Methods S1. Metabolite profile analyses were carried out in a gas chromatograph (Agilent Technologies 7890B) equipped with autoinjector (CombiPAL, CG sampler 80) and a mass-selective filter (Agilent Technologies 5977A). GC-MS configurations are described in Methods S1.

**Statistical analyses**

Experimental design was completely randomized. Statistical differences between groups were determined by ANOVA followed by Dunnnett’s test for transcript abundance analyses (with WT as a control group, α = 0.05) or Tukey’s HSD test (α = 0.05) for metabolites and remaining variables. Statistical analyses were performed using JMP statistical software package version 14 (https://www.jmp.com).

**Acknowledgments**

We also thank Aline Bertinatto Cruz, Diego dos Santos Brito, Ana Maria Rodrigues and Silvia Regina Blanco (University of São Paulo) for technical assistance.

**Funding**

This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, #2016/04924-0, #2016/01129-9, #2018/25774-2 and #2018/16389-8), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, #422287/2018-0 and #305012/2018-5) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (CAPES, Finance Code 001).

**Conflict of Interest**

The authors declare no conflict of interest.

**Author contributions**

FRRA, LF and MR designed the study; FRRA, BSL, FCP and SSM performed experiments; CMF, EP, GBP, SCXA and DD provided technical assistance and contributed to data analysis; FRRA and LF prepared the manuscript and collected contributions from all authors.
References

Alba, R., Kelmenson, P.M., Cordonnier-Pratt, M.-M. and Pratt, L.H. (2000a) The phytochrome gene family in tomato and the rapid differential evolution of this family in angiosperms. Mol. Biol. Evol. 17, 362–373.

Alba, R., Cordonnier-Pratt, M.-M. and Pratt, L.H. (2000b) Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato. Plant Physiol. 123, 363–370.

Almeida, J., Asil, R., Molinieri, V.N., Sestari, I., Lira, B.S., Carrari, F., Peres, L.E.P. et al. (2016) Fruits from ripening impaired, chlorophyll degraded and jasmonate insensitive tomato mutants have altered tocopherol content and composition. Phytochemistry 111, 72–83.

Alves, F.R.R., Melo, H.C., Crispim-Filho, A.J., Costa, A.C., Nascimento, K.J.T. and Carvalho, R.F. (2016) Physiological and biochemical responses of photomorphogenic tomato mutants (cv. Micro-Tom) under water withholding. Acta Physiol. Plant. 38, 155.

Amorós, A., Zapata, P., Pretel, M.T., Botella, M.A. and Serrano, M. (2003) Physico-chemical and physiological changes during fruit development and ripening of five loquat (Eriobotrya japonica Lindl.) cultivars. Food Sci. Technol. Int. 9, 43–51.

Armbruster, U., Pesaresi, P., Pribil, M., Hertle, A. and Leister, D. (2011) Update on chloroplast research: new tools, new topics, and new trends. Molecular Plant 4, 1–16.

Armbruster, U., Labs, M., Pribil, M., Viola, S., Xu, W., Scharfenberg, M., Hertle, A.P., Carvalho, R.F., Campos, M.L., Pino, L.E., Crestana, S.L., Zs et al. (2011) Convergence of developmental mutants into a single tomato model system: Micro-Tom as an effective toolkit for plant development research. Plant Methods 7, 18.

Chen, M. and Chory, J. (2011) Phytochrome signaling mechanisms and the control of plant development. Trends Cell Biol. 21, 646–671.

Chen, X., Snyder, C.I., Truksa, M., Shah, S. and Weelakere, R.J. (2011) sn-Glycerol-3-phosphate acyltransferases in plants. Plant Signal. Behav. 6, 1695–1699.

Cocaialidas, M.F., Fernández-Muñoz, R., Pons, C., Orzaez, D. and Granell, A. (2014) Increasing tomato fruit quality by enhancing fruit chloroplast function. A double-edged sword? J. Exp. Bot. 65, 4589–4598.

Cruz, A.B., Bianchetti, R.E., Alves, F.R.R., Purgatto, E., Peres, L.E.P., Rossi, M. and Freschi, L. (2018) Light, ethylene and auxin signaling interaction regulates carotenoid biosynthesis during tomato fruit ripening. Front. Plant Sci. 9, 1370.

Dar, A.A., Choudhury, A.R., Kancharla, P.K. and Anrumugam, N. (2017) The FAD2 gene in plants: occurrence, regulation, and role. Front. Plant Sci. 8, 1789.

Davies, J.W. and Cocking, E.C. (1965) Changes in carbohydrates, proteins and nucleic acids during cellular development in tomato fruit locule tissue. Planta, 67, 242–253.

Davuluri, G.R., Van Tuinen, A., Fraser, P.D., Manfredonia, A., Newman, R., Burgess, D., Brummel, D.A. et al. (2005) Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. Nat. Biotechnol. 23, 890–895.

Demotes-Mainard, S., Péron, T., Corat, A., Bertheloot, J., Gourrier, J.L., Pellechi-Travier, S., Crespel, L. et al. (2016) Plant responses to red and far-red lights, applications in horticulture. Environ. Exp. Bot. 121, 4–21.

Doras, M., Ehrat, D.L. and Papadopoulos, A.P. (2008) Tomato (Solanum lycopersicum) health components: from the seed to the consumer. Phytochem. Rev. 7, 231–250.

Dubreuil, C., J., Yi, Strand, A. and Grönlund, A. (2017) A quantitative model of the phytochrome-PF light signaling initiating chloroplast development. Sci. Rep. 7, 13884.

Endo, T., Ishida, S., Ishikawa, N. and Sato, F. (2008) Chloroplastic NAD(P)H dehydrogenase complex and cyclic electron transport around photosystem I. Mol. Cells 25, 158–162.

Enfissi, E.M.A., Barneche, F., Ahmed, I., Lichtlé, C., Gerrish, C., McQuinn, R.P., Giovannoni, J.J. et al. (2010) Integrative transcript and metabolite analysis of nutritionally enhanced DE-ETIOLATED1 downregulated tomato fruit. Plant Cell 22, 1190–1215.

Fauciullino, A.L., Bidél, L.P.R. and Urban, L. (2014) Carotenoid responses to environmental stimuli: integrating redox and carbon controls into a fruit model. Plant Cell Environ. 37, 273–289.

Fernandez, A.I., Viron, N., Alhagdow, M., Karimi, M., Jones, M., Amselem, Z. and Sicard, A. et al. (2009) Flexible tools for gene expression and silencing in tomato. Plant Physiol. 151, 1729–1740.

Fiehn, O., Kopka, J., Dörmann, P., Altman, T., Trethewey, R.N. and Willmitzer, L. (2000) Metabolite profiling for plant functional genomics. Nat. Biotechnol. 18, 1157–1161.

Fritsche, S., Wang, X. and Jung, C. (2017) Recent advances in our understanding of tocopherol biosynthesis in plants: an overview of key genes, functions, and breeding of vitamin E improved crops. Antioxidants 6, 99.

Fruciante, L., Carli, P., Ercolano, M.R., Pernice, R., Di Matteo, A., Fogliano, V., Frusciante, L., Carli, P., Ercolano, M.R., Pernice, R., Di Matteo, A., Fogliano, V. et al. (2014) Carotenoid responses to environmental stimuli: integrating redox and carbon controls into a fruit model. Plant Cell Environ. 37, 273–289.

Galpaz, N., Wang, Q., Menda, N., Zamir, D. and Hirschberg, J. (2008) Abscisic acid deficiency in the tomato mutant high-pigment 3 leading to increased plastid number and higher fruit lycopene content. Plant J. 53, 717–730.

Ganesan, M., Lee, H.-Y., Kim, J.-I. and Song, P.-S. (2017) Development of transgenic crops based on photo-biotechnology. Plant Cell Environ. 40, 2469–2486.

Garg, A.K., Sawers, R.J.H., Wang, H., Kim, J.-K., Walker, J.M., Brutnell, T.P., Parthasarathy, M.V. et al. (2006) Light-regulated overexpression of an Arabidopsis phytochrome A gene in rice alters plant architecture and increases grain yield. Plant Cell 223, 627–636.

Gerszberg, A., Hnatuszko-Konka, K., Kwak, K.Y. and Kononowicz, A.K. (2015) Tomato (Solanum lycopersicum L.) in the service of biotechnology. Plant Cell Tissue Organ Cult. 120, 881–902.

Gilbert, L., Alhagdow, M., Nunes-Nesi, A., Quemener, B., Guillou, F., Bouchet, B., Faurobert, M. et al. (2009) GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. Plant J. 60, 499–508.

© 2020 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 18, 2027–2041

Fruit biofortification via phytochrome engineering 2039
Giliberto, L., Perrotta, G., Pallara, P., Weller, J.L., Fraser, P.D., Bramley, P.M., Fiore, A. et al. (2005) Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. *Plant Physiol.* 137, 199–208.

Girnth, C., Bergfeld, R. and Kasemir, H. (1978) Phytochrome-mediated control of grana and stroma thylakoid formation in plastids of mustard cotyledons. *Planta* 141, 191–198.

Gramegna, G., Rosado, D., Carranza, A.P.S., Cruz, A.B., Simon-Moya, M., Llorente, B., Rodriguez-Concepcion, M. et al. (2018) PHOTOCROME INTERACTING FACTOR 3 mediates light-dependent induction of tocopherol biosynthesis during tomato fruit ripening. *Plant Cell Environ.* 42, 1328–1339.

Gupta, S.K., Sharma, S., Santisree, P., Kliambi, H.V., Appenroth, K., Sreekalashmi, Y. and Sharma, R. (2014) Complex and shifting interactions of phytochromes regulate fruit development in tomato. *Plant Cell Environ.* 37, 1688–1702.

Gururani, M.A., Ganesan, M. and Song, P.-S. (2015) Photo-biotechnology as a tool to improve agronomic traits in crops. *Biotechnol. Adv.* 33, 53–63.

Hauser, B.A., Pratt, L.H. and Cordonnier-Pratt, M.-M. (1997) Absolute quantification of five phytochrome transcripts in seedlings and mature plants of tomato (*Solanum lycopersicum* L.). *Planta* 201, 379–387.

Heyes, D.J. and Hunter, C.N. (2005) Making light work of enzyme catalysis: protoclorophyllide synthase. *Trends Biochem. Sci.* 30, 642–649.

Hu, W., Su, Y.-S. and Lagarias, J.C. (2009) A light-independent allele of cryptochrome 2 in tomato affects vegetative development and flowering time. *Plant Physiol.* 149, 2088–2102.

Kolotilin, I., Koltai, H., Tadmor, Y., Bar-Or, C., Reuveni, M., Meir, A., Nahon, S. et al. (2015) PHYTOCHROME INTERACTING FACTOR 3 mediates light-dependent induction of tocopherol biosynthesis during tomato fruit ripening. *Plant Cell Environ.* 42, 1328–1339.

Kolotilin, I., Koltai, H., Tadmor, Y., Bar-Or, C., Reuveni, M., Meir, A., Nahon, S. et al. (2015) PHYTOCHROME INTERACTING FACTOR 3 mediates the regulation of chlorophyll biosynthesis through transcriptional regulation of CHLH and GUN4 in rice seedlings. *PLoS ONE* 10, e0135408.

Kolotilin, I., Koltai, H., Tadmor, Y., Bar-Or, C., Reuveni, M., Meir, A., Nahon, S. et al. (2007) Transcriptional profiling of high pigment-2dg tomato mutant links early fruit plastid biogenesis with its overproduction of phytochromes. *Plant Physiol.* 145, 389–401.

Kolotilin, I., Koltai, H., Tadmor, Y., Bar-Or, C., Reuveni, M., Meir, A., Nahon, S. et al. (2015) Plant tolerance to excess light energy and photooxidative damage relies on plastoquinone-9 biosynthesis by binding to solaneryl diphasphate synthase in Arabidopsis. *Plant Cell* 27, 2956–2971.

Kolotilin, I., Koltai, H., Tadmor, Y., Bar-Or, C., Reuveni, M., Meir, A., Nahon, S. et al. (2007) Transcriptional profiling of high pigment-2dg tomato mutant links early fruit plastid biogenesis with its overproduction of phytochromes. *Plant Physiol.* 145, 389–401.

Kloetzel, B., Becuwe, N., Chevalier, A. and Havaux, M. (2015) Characterization of the AGPase large subunit isoforms from tomato indicates that the recombinant L3 subunit is active as a monomer. *Biochem. J.* 428, 201–212.

Liu, L., Shao, Z., Zhang, M. and Wang, Q. (2015) Regulation of carotenoid metabolism in tomato. *Molecular Plant* 8, 28–39.

Llorente, B., D’Andrea, L., Ruíz-Sola, M.A., Bottonew, E., Pulido, P., Andilla, J., Loza-Alvarez, P. et al. (2016) Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. *Plant J.* 85, 107–119.

Llorente, B., Martínez-Garcia, J.F., Stange, C. and Rodriguez-Concepcion, M. (2017) Illuminating colors: regulation of carotenoid biosynthesis and accumulation by light. *Curr. Opin. Plant Biol.* 37, 49–55.

Ludval, T., Olsen, K.M., Silimestad, R., Verheul, M. and Lillo, C. (2010) Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. *Phytochemistry* 71, 605–613.

Lupi, A.C.D., Lira, B.S., Gramegna, G., Trench, B., Alves, F.R.R., Demarco, P., Peres, L.E.P. et al. (2019) Solanum lycopersicum GOLDEN 2-LIKE 2 transcription factor affects fruit quality in a light- and auxin-dependent manner. *PLoS ONE* 14, e0212224.

Martin, C., Butelli, E., Petroni, K. and Tonelli, C. (2011) How can research on plants contribute to promoting human health? *Plant Cell* 23, 1685–1699.

Massot, C., Stevens, R., Génard, M., Longuena esse, J.-J. et al. (2012) Light affects ascorbate content and ascorbate-related gene expression in tomato leaves more than in fruits. *Planta* 235, 153–163.

Mohn, H. (1977) Phytochrome and chloroplast development. *Endeavour* 1, 107–114.

Morgan, M.I., Osorio, S., Baxter, C.I., Kruger, N.J., Ratcliffe, R.G., Fernie, A.R. and Sweetlove, L.J. (2013) Metabolic engineering of tomato fruit organic acid content guided by biochemical analysis of an introgression line. *Plant Physiol.* 161, 397–407.

Nashinevitz, S., Melamed-Bessudo, C., Izkovich, Y., Rogachev, I., Osorio, S., Itkin, M., Adato, A. et al. (2010) An Orange Ripening mutant links plastid NAD(P)H dehydrogenase complex activity to central and specialized metabolism during tomato fruit maturation. *Plant Cell* 22, 1977–1997.

Nguyen, C.V., Vrebalov, J.T., Gapper, N.E., Zheng, Y., Zhong, S., Fei, Z. and Giovannoni, J.J. (2014) Tomato GOLDEN2-LIKE transcription factors reveal molecular gradients that function during fruit development and ripening. *Plant Cell* 26, 585–601.

Norris, S.R., Barrette, R.T. and DellaPenna, D. (1995) Genetic dissection of carotenoid synthesis in Arabidopsis defines plastocyanine as an essential component of phytoene desaturase. *Plant Cell* 7, 2139–2149.

Oh, S. and Montgomery, B.L. (2014) Phytochrome-dependent coordinate control of distinct aspects of nuclear and plastid gene expression during anterograde signaling and photomorphogenesis. *Front. Plant Sci.* 5, 171.

Oka, Y., Kong, S.-G. and Matsuhashi, T. (2011) A non-covalently attached chromophore can mediate phytochrome B signaling in Arabidopsis. *Plant Cell Physiol.* 52, 2088–2102.

Okazaki, K., Kabeya, Y., Suzuki, K., Mori, T., Ichikawa, T., Matsui, M., Nakanishi, H. et al. (2009) The PLASTID DIVISION 1 and 2 components of the chloroplast division machinery determine the rate of plastid division in land plant cell differentiation. *Plant Cell* 21, 1769–1780.

Osorio, S., Ruan, Y.-L. and Fernie, A.R. (2014) An update on source-to-sink carbon partitioning in tomato. *Front. Plant Sci.* 5, 516.

Patrick, J.W., Botha, F.C. and Birch, R.G. (2013) Metabolic engineering of sugars and simple sugar derivatives in plants. *Plant Biotechnol. J.* 11, 142–156.

Peterekov, M., Shen, S., Yeselson, Y., Levin, I., Bar, M. and Schaffer, A.A. (2006) Temporarily extended gene expression of the ADP-Glc pyrophosphorylase large subunit (AgpL1) leads to increased enzyme activity in developing tomato fruit. *Plant Physiol.* 144, 1465–1479.

Peterekov, M., Eisenstein, M., Yeselson, Y., Preis, J. and Schaffer, A.A. (2010) Characterization of the AgpLase large subunit isoforms from tomato indicates that the recombinant L3 subunit is active as a monomer. *Biochem. J.* 428, 201–212.

Powell, A.L., Nguyen, C.V., Hill, T., Cheng, K.L., Figueroa-Balderas, R., Aktas, H., Ashrafi, H. et al. (2012) Uniform ripening encodes a Golden 2-like transcription factor regulating tomato fruit chloroplast development. *Science* 336, 1711–1715.

Pratt, L.H., Cordonnier-Pratt, M.-M., Hauser, B. and Caboche, M. (1995) Tomato contains two differentially expressed genes encoding B-type...
phytochromes, neither of which can be considered an ortholog of Arabidopsis phytochrome B. Planta, 197, 203–206.

Pribil, M., Sandoval-Ibáñez, O., Xu, W., Sharma, A., Labs, M., Liu, Q., Galgenmüller, C. et al. (2018) Fine-tuning of photosynthesis requires CURVATURE THYLAKOID1-mediated thylakoid plasticity. Plant Physiol. 176, 2351–2364.

Pulido, P., Perello, C. and Rodriguez-Concepción, M. (2012) New insights into plant isoprenoid metabolism. Molecular Plant 5, 964–967.

Quadraña, L., Almeida, J., Otaiza, S.N., Duffy, T., Silva, J.V.C., Godoy, F., Asís, R. et al. (2013) Transcriptional regulation of tocopherol biosynthesis in tomato. Plant Mol. Biol. 81, 309–325.

Raina, A., Rigano, M.M., Calafiore, R., Frusciante, L. and Barone, A. (2014) Enhancing the health-promoting effects of tomato fruit for biofortified food. Mediators Inflamm. 2014, 139873.

Rawsthorne, S. (2002) Carbon flux and fatty acid synthesis in plants. Prog. Lipid Res. 41, 182–196.

Sagar, M., Cherivin, C., Mila, I., Hao, Y., Roustan, J.P., Berichou, M., Gibon, Y. et al. (2013) SIAF4, an auxin response factor involved in the control of sugar metabolism during tomato fruit development. Plant Physiol. 161, 1362–1374.

Schaffer, A.A., Levin, I., Oguz, I., Petreikov, M., Cincarevsky, F., Yeselson, Y. et al. (2006) Arabidopsis phytochrome B in transgenic potato influences starch, soluble sugar and citric acid contents in wild-type and transgenic fruits. Plant Physiol. 142, 1270–1280.

Seluzicki, A., Burko, Y. and Chory, J. (2017) Dancing in the dark: darkness as a regulator of photosynthesis requires the tomato DDB1-interacting protein CUL4. Plant Cell 18, 2027–2041.

Shin, A.-Y., Han, Y.-J., Baek, A., Ahn, T., Kim, S.Y., Nguyen, T.S., Son, M. et al. (2014) Salinity induces carbohydrate accumulation and sugar-related genes in wild-type and transgenic fruits. Plant Cell Environ. 37, 563–574.

Shen, S. (2010) Salinity-induced carbohydrate accumulation during tomato fruit development. Plant Sci. Food Agric. 182, 574–585.