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FREDERICO ROCHA RODRIGUES ALVES

MANIPULAÇÃO DE FITOCROMOS COMO ESTRATÉGIA DE BIOFORTIFICAÇÃO DE FRUTOS DE TOMATEIRO (*Solanum lycopersicum* L.)

PHYTOCHROME MANIPULATION AS A BIOFORTIFICATION STRATEGY OF TOMATO FRUITS (*Solanum lycopersicum* L.)

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo para a obtenção do título de Doutor em Ciências (Área Botânica)

Orientador: Prof. Dr. Luciano Freschi

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At.te,
F

“ *Feliz aquele que transfere o que sabe e aprende o que ensina.*”

Cora Coralina (1889-1985)

Art. 205. *A educação, direito de todos e dever do Estado e da família, será promovida e incentivada com a colaboração da sociedade, visando ao pleno desenvolvimento da pessoa, seu preparo para o exercício da cidadania e sua qualificação para o trabalho.*

- Constituição da República Federativa do Brasil, 1988

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RESUMO

ALVES, Frederico Rocha Rodrigues. Manipulação de fitocromos como estratégia de biofortificação de frutos de tomateiro (*Solanum lycopersicum* L.). 2019. 117f. Tese (Doutorado em Ciências Biológicas – Área Botânica) – Instituto de Biociências, Universidade de São Paulo, São Paulo, Brasil.

A manipulação de elementos da cadeia de sinalização luminosa tem sido amplamente empregada como estratégia de melhoramento de plantas de interesse agronômico. Os fitocromos (PHYs), fotorreceptores de luz vermelha/vermelha-distante, possuem papéis centrais na transdução do sinal luminoso controlando diversos processos fisiológicos em frutos, inclusive aqueles relacionados com a síntese de compostos de valor nutricional. Entretanto, informações a respeito da possibilidade de aplicação da manipulação de PHYs como ferramenta fotobiotecnológica visando a biofortificação de frutos ainda são limitadas. Nesta tese, os impactos da sobre-expressão fruto-específica de *PHYTOCHROME B2* (*PHYB2*) e de uma forma mutante constitutivamente ativa desse fotorreceptor ($PHYB2^{Y252H}$) sobre o desenvolvimento de frutos de tomateiro foram avaliados. Em frutos verdes, tanto a sobre-expressão de *PHYB2* quanto de $PHYB2^{Y252H}$ aumentou o número de plastídios e o desenvolvimento de tilacoides, incrementando a capacidade de síntese e estocagem de importantes compostos de valor nutricional. Além disso, os frutos transgênicos maduros sobre-expressando $PHYB2^{Y252H}$ acumularam maior conteúdo de carotenoides, vitamina E, flavonoides e vitamina C, estando esse fenótipo associado a modulações específicas na transcrição de genes biossintéticos das respectivas rotas metabólicas ao longo do desenvolvimento do fruto. Comparados ao tipo selvagem, os frutos transgênicos apresentaram limitadas alterações em seu metabolismo primário, com destaque para o aumento e diminuição nos teores de compostos lipídicos e açúcares solúveis, respectivamente. O acúmulo de compostos antioxidantes foi substancialmente intensificado pela sobre-expressão de $PHYB2^{Y252H}$ em comparação à forma nativa *PHYB2*. Em conjunto, os dados obtidos indicam que a manipulação da atividade de PHYs constitui uma relevante ferramenta de fotobiotecnologia para a biofortificação de frutos carnosos.

Palavras-chave: fotobiotecnologia, metabolismo secundário, antioxidantes, carotenoides, tocoferois, flavonoides, ascorbato.

ABSTRACT

ALVES, Frederico Rocha Rodrigues. Phytochrome manipulation as a biofortification strategy of tomato fruits (*Solanum lycopersicum* L.). 2019. 117p. Thesis (Ph.D. in Biological Sciences – Botany) – Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil.

Manipulation of light signaling pathway components has been widely applied as a molecular plant breeding strategy in crop species. Phytochromes (PHYs), red/far-red photoreceptors, play central roles concerning light signal transduction, controlling a plethora of physiological processes in fruits, including the biosynthesis of compounds with nutritional value. However, information regarding the applicability of PHY manipulation as a photobiotechnological tool aiming fruit biofortification are limited. In the present thesis, the impacts of fruit-specific overexpression of *PHYTOCHROME B2* (*PHYB2*) and a mutated constitutively active form of this photoreceptor (*PHYB2*^{Y252H}) on tomato fruit development were evaluated. In green fruits, either *PHYB2* or *PHYB2*^{Y252H} overexpression increased plastid number and thylakoid development, boosting up the synthesis and storage capacities of important compounds with nutritional value. Accordingly, red ripe *PHYB2*^{Y252H}-overexpressing fruits accumulated more carotenoids, vitamin E, flavonoids and vitamin C as a consequence of specific modulation of biosynthetic genes associated with the respective metabolic pathways throughout fruit development. Compared to the wildtype, transgenic fruits exhibited limited changes in primary metabolism, including increments and reductions in lipid and soluble sugar levels, respectively. Accumulation of antioxidant compounds was substantially intensified by PHYB2^{Y252H}-overexpression compared to the native form PHYB2. Altogether, our findings indicate that PHY activity manipulation represents a relevant photobiotechnological tool for fleshy fruit biofortification.

Keywords: photobiotechnology, secondary metabolism, antioxidants, carotenoids, tocopherols, flavonoids, ascorbate.

O tomate como alvo de manipulação fotobiotecnológica

O fruto do tomateiro está entre os alimentos mais consumidos no mundo, seja *in natura* ou processado na forma de molhos e sucos, e tanto a sua produção quanto a área mundial dedicada ao seu cultivo mais que dobrou nos últimos 20 anos (Bergougnoux, 2014). O tomate possui grande importância na alimentação humana devido ao seu alto valor nutricional, associado especialmente aos seus elevados teores de antioxidantes.

O licopeno é um dos principais antioxidantes com ação sobre o corpo humano, desempenhando papel crítico no combate aos radicais livres e na prevenção de diversas doenças e, em termos mundiais, o tomate constitui a principal fonte desse composto nutracêutico para o homem (Khachik *et al.*, 2002). Aumentos no consumo de licopeno estão associados à prevenção de doenças cardiovasculares, hepáticas e do sistema nervoso central, além de alguns tipos de câncer como o de próstata e do trato digestivo (Heber & Lu, 2002; Stice *et al.*, 2018). O licopeno é responsável pela coloração avermelhada dos frutos e é o carotenoide presente em maior concentração no tomate.

O segundo carotenoide mais abundantemente encontrado no tomate é o β-caroteno, precursor da vitamina A, relacionado com a saúde dos olhos e proteção da pele (Grune *et al.*, 2010), cuja deficiência é um dos principais problemas nutricionais que afetam crianças e mulheres grávidas especialmente em países em desenvolvimento (West *et al.*, 2002).

Além dos carotenoides, teores significativos de tocoferóis (vitamina E), flavonoides e ácido ascórbico (vitamina C) também podem ser encontrados em frutos maduros de tomate, os quais são igualmente benéficos para a saúde humana, possuindo propriedades antioxidantes e anti-carcinogênicas (Dorais *et al.*, 2008). Os flavonoides e o ácido ascórbico, por exemplo, são antioxidantes hidrofílicos que complementam a natureza hidrofóbica dos carotenoides e tocoferois. Dietas ricas

em diversas classes de antioxidantes têm sido associadas à redução nos riscos de doenças cardíacas, certos tipos de câncer e outras doenças degenerativas relacionadas ao envelhecimento (Wilson *et al.*, 2017).

O valor econômico e as propriedades nutricionais atribuídas aos frutos de tomateiro fazem desta espécie um importante alvo para biofortificação, ou seja, o emprego de estratégias para aumentar o conteúdo nutricional tanto por métodos tradicionais de melhoramento genético quanto por manipulações via técnicas de transgenia (Garg *et al.*, 2018; Martin *et al.*, 2011).

O aumento na concentração de compostos nutracêuticos tem sido buscado através da modulação da expressão de genes que codificam enzimas envolvidas nas rotas biossintéticas específicas desses compostos ou fatores de transcrição envolvidos na regulação de tais enzimas (Bovy *et al.*, 2007; Butelli *et al.*, 2008). Por exemplo, várias tentativas têm sido realizadas com o intuito de aumentar o conteúdo de carotenoides em frutos de tomate utilizando genes bacterianos que codificam enzimas envolvidas na biossíntese destas substâncias (Fraser *et al.*, 2001; Römer *et al.*, 2000). Estas abordagens normalmente resultam no incremento dos teores de um ou poucos metabólitos, sem desencadear elevações no fluxo através de toda a rota biossintética desses compostos.

De forma semelhante, os níveis de flavonoides e vitamina C em tomate têm sido aumentados tanto pela modulação direta de genes envolvidos em etapas biossintéticas (Li *et al.*, 2019; Muir *et al.*, 2001) quanto por meio de fatores de transcrição relacionados ao controle das rotas bioquímicas (Bovy *et al.*, 2002; Xu *et al.*, 2015a). Embora ambas estratégias tenham sido efetivas na elevação do conteúdo de flavonoides, raramente os teores de carotenoides em frutos dessas linhagens foram alterados. Assim sendo, a sobre-expressão de genes que codificam enzimas biossintéticas geralmente resulta em tomates com conteúdo aumentado específico para apenas uma classe de metabólitos.

Uma das alternativas para a obtenção de aumentos simultâneos em diversos metabólitos nutracêuticos é a modulação de genes regulatórios cujos produtos influenciam simultaneamente as rotas de biossíntese das diferentes classes de

compostos de interesse, mesmo que essas rotas sejam relativamente independentes. Estes genes, frequentemente são oriundos das próprias plantas e consequentemente, este método seria mais facilmente aceito pelo público consumidor dos produtos. Uma abordagem capaz de produzir aumentos concomitantes em diversas classes de compostos nutracêuticos consiste na modulação de fatores associados à percepção ou transdução do sinal luminoso (Azari *et al.*, 2010a; Wang *et al.*, 2008).

As plantas são capazes de perceber a luz por meio dos fotorreceptores, que atuam integrando os sinais luminosos do ambiente externo ao ambiente celular interno. Os diversos fotorreceptores desempenham papel central no ajuste e na integração da sinalização da luz para o crescimento, desenvolvimento, sobrevivência e reprodução, controlando processos diversos tais como a fotossíntese, germinação, floração, fototropismos e estabelecimento e manutenção de ritmos circadianos (Jiao *et al.*, 2007; Quail, 2002).

Os fotorreceptores possuem um módulo fotossensor, responsável pela absorção da luz por moléculas específicas denominadas cromóforos, e um módulo efetor que exerce a atividade biológica, por exemplo, catálise e/ou interação com outra proteína (Ziegler & Möglich, 2015). De uma maneira geral, as foto-isomerizações que ocorrem nos cromóforos após a absorção de luz no comprimento de onda adequado fazem com que os fotorreceptores sejam capazes de interagir com proteínas sinalizadoras e/ou fatores transcrição que levarão à alteração no padrão de expressão de genes relacionados à resposta luminosa. Consequentemente, não é motivo de surpresa que mutações em genes relacionados à percepção ou transdução do sinal luminoso resultem em alterações profundas nos perfis de expressão gênica com efeitos marcantes no crescimento, desenvolvimento e metabolismo vegetal (Jiao *et al.*, 2007; Kolotilin *et al.*, 2007). Tais fatores podem, portanto, representar alvos genéticos prioritários para o incremento da qualidade dos frutos tanto por abordagens clássicas de melhoramento genético quanto por técnicas de transgenia.

Os estudos realizados até o momento dentro desta concepção vêm apresentando resultados promissores (Azari *et al.*, 2010a; Azari *et al.*, 2010b;

Davuluri *et al.*, 2005; Kolotilin *et al.*, 2007; Liu *et al.*, 2004; Wang *et al.*, 2008) e, portanto, um crescente interesse da comunidade científica tem sido despertado no que concerne à identificação, análise e modulação de elementos envolvidos na transdução do sinal luminoso a fim de se utilizar ao máximo o impacto de tal estratégia fotobiotecnológica (Ganesan *et al.*, 2017; Gururani *et al.*, 2015). Adicionalmente, a maioria dos genes que codificam fatores relacionados à transdução dos sinais luminosos são altamente conservados dentro do reino vegetal e desta forma, parece provável que seus efeitos sejam reprodutíveis em diversas espécies vegetais de interesse agronômico (Azari *et al.*, 2010a).

Apesar desse considerável potencial biotecnológico e do fato que as primeiras constatações do papel estimulatório da luz sobre o conteúdo de fitonutrientes em frutos de tomate terem sido obtidas há mais de 70 anos (Piringer & Heinze, 1954), apenas recentemente alguns avanços foram obtidos no que tange ao conhecimento da relação entre a percepção e transdução do sinal luminoso e a concentração de compostos nutracêuticos em tomate e outros frutos carnosos.

A transdução do sinal luminoso pelos fitocromos

O fitocromo foi o primeiro dos fotorreceptores a ser identificado através de pesquisas envolvendo o efeito da luz vermelha e vermelho-distante sobre a germinação de sementes de alface (Borthwick *et al.*, 1954). Nestes estudos iniciais, o fitocromo foi identificado e caracterizado como sendo uma cromoproteína fotorreversível: sua forma biologicamente inativa Fv absorve a luz em comprimentos de onda na região do vermelho (λ entre 650 e 670 nm) e se converte na forma ativa Fvd, que, por sua vez, é capaz de absorver a luz na região do vermelho-distante (λ entre 705 e 730 nm) e retornar à sua forma inicial Fv. Em condições de escuro, Fvd também é convertida em Fv (Borthwick, 1957; Borthwick & Hendricks, 1960; Borthwick *et al.*, 1952; Butler *et al.*, 1959).

O fitocromo é formado pela dimerização de duas subunidades proteicas. Cada um dos monômeros é composto por uma molécula de pigmento denominada cromóforo, responsável pela absorção de luz, e uma cadeia polipeptídica chamada apoproteína. Nas plantas superiores, o cromóforo do fitocromo é um tetrapirrol linear denominado fitocromobilina, sintetizado nos plastídios. Ao ser exportada do plastídio para o citosol, a fitocromobilina se liga à apoproteína de forma autocatalítica por meio de uma ligação tioéster a um resíduo de cisteína, formando a holoproteína (Kevei *et al.*, 2007; Li & Lagarias, 1992; Rockwell *et al.*, 2006).

As apoproteínas PHY são codificadas por uma pequena família de genes nas angiospermas. Por exemplo, *Arabidopsis* contem cinco genes PHY (PHYA a PHYE), a mesma quantidade em tomateiro (PHYA, PHYB1, PHYB2, PHYE e PHYF), enquanto o arroz possui apenas três (PHYA a PHYC) (Bae & Choi, 2008; Clack *et al.*, 1994; Hauser et al., 1995; Sharrock & Quail, 1989). Devido a isso, os diferentes fitocromos apresentam propriedades moleculares distintas e podem agir isoladamente ou em conjunto na determinação das diversas respostas dependentes de luz, apresentando funções semelhantes ou diferentes nas plantas (Bae & Choi, 2008).

Estruturalmente, os fitocromos são organizados em dois módulos: a porção N-terminal fotossensora e a porção C-terminal efetora, conectadas por uma região de dobradiça flexível. Cada módulo é constituído por determinados domínios. O módulo fotossensor consiste dos domínios P1/NTE (extensão N-terminal), P2/PAS (Per/Arnt/Sim), P3/GAF (cGMP fosfodiesterase/Adenilciclase/FhlA) e P4/PHY (fitocromo-específico) (Figura R1). O resíduo conservado de cisteína ao qual o cromóforo fitocromobilina se encontra covalentemente ligado se encontra no domínio GAF (Wagner *et al.*, 2005). Recentemente, a estrutura do módulo fotossensor de PHYB foi melhor elucidada (Burgie *et al.*, 2014; Von Horsten *et al.*, 2016), demonstrando duas conformações interessantes: o motivo do laço com nó sensor de luz entre os domínios PAS e GAF, responsável pela estabilidade entre os domínios durante a fotoconversão Fv-Fvd e interação com determinados fatores de transcrição (Kikis *et al.*, 2009); e o motivo da língua no dominío PHY, que medeia a associação com

o módulo efetor durante a fotoconversão (Burgie *et al.*, 2017). O módulo efetor C-terminal consiste em um domínio PRD (domínio relacionado ao PAS) composto por um par de repetições (PAS-A e PAS-B) e um domínio HKRD (semelhante à histidina cinase). As funções do módulo efetor estão relacionadas à dimerização dos fitocromos, localização nuclear e interação proteína-proteína com alguns componentes da cadeia de sinalização (Bae & Choi, 2008; Klose *et al.*, 2015; Pham *et al.*, 2018; Qiu *et al.*, 2017).

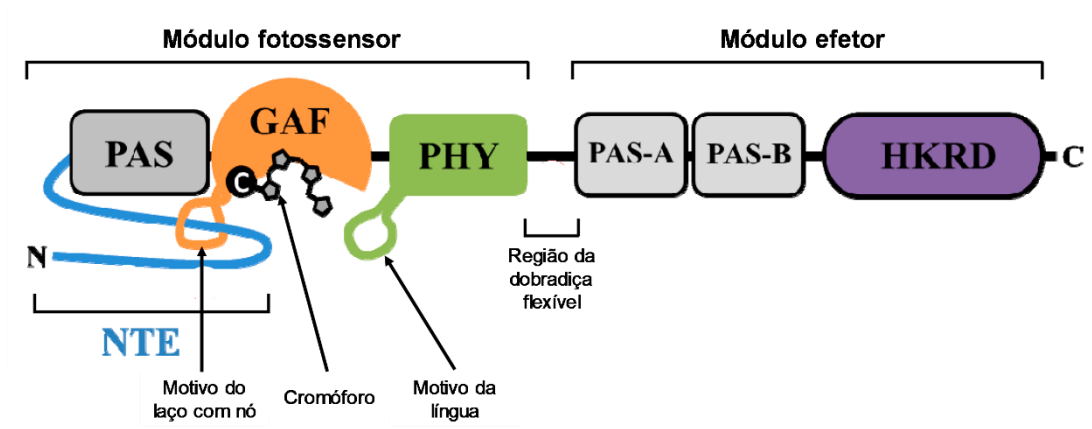

Figura R1. Esquema da estrutura do fitocromo e identificação dos seus domínios constituintes. Abreviações dos domínios se encontram descritas no texto. Adaptado de Hoang *et al.* (2019).

Em síntese, a sinalização luminosa dependente de fitocromos segue os seguintes passos: fotoativação, translocação para o núcleo, interação com outras proteínas, transdução do sinal (mudanças nos níveis dos fatores de transcrição, por exemplo) e controle transcricional dos genes fotorresponsivos (Xu *et al.*, 2015b).

No citosol, o fitocromo se encontra dimerizado em seu estado inativo Fv. A absorção de luz vermelha pela fitocromobilina em sua forma inativa Fv resulta na fotoisomerização *cis* para *trans* da dupla ligação dos carbonos entre os anéis C e D do cromóforo acompanhada de uma rotação do anel D (Hughes, 2010; Yang *et al.*, 2014). Essa importante alteração estrutural desencadeia mudanças nas interações intermoleculares da proteína que levam à conformação ativa Fvd, translocada para o núcleo (Chen *et al.*, 2005; Fankhauser & Chen, 2008). Os

fitocromos, porém, não possuem sinais de localização nuclear (SLNs) endógenos ativos e, portanto, alguns facilitadores de transporte que possuem SLNs precisam ser recrutados para a translocação. Para PHYA, as proteínas FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) e FHY1-LIKE (FHL) interagem com as proteínas fotoativadas e o complexo formado é importado para o núcleo (Hiltbrunner *et al.*, 2006). Para os demais fitocromos (PHYB-PHYE), proteínas facilitadoras semelhantes ainda não foram identificadas e se hipotetiza de que fatores de transcrição envolvidos em sua rota de sinalização sejam mediadores de sua translocação para o núcleo (Pfeiffer *et al.*, 2012).

No núcleo, os fitocromos interagem e inibem duas classes principais de repressores de respostas fotomorfogênicas: o complexo formado pelas proteínas CONSTITUTIVE PHOTOMORPHOGENESIS e SUPPRESSOR OF PHYA (COP1/SPA), atuando na degradação mediada por ubiquitina de fatores relacionados à sinalização luminosa (Podolec & Ulm, 2018) e as proteínas PHYTOCHROME INTERACTING FACTORS (PIFs), atuando sobre a transcrição de genes responsivos à luz (Leivar & Quail, 2011).

O mecanismo de proteólise envolve a ligação covalente de uma proteína ubiquitina ao seu substrato, com as atividades sequenciais de uma enzima ativadora da ubiquitina (E1), uma enzima conjugadora da ubiquitina (E2) e uma ubiquitina ligase (E3), levando à degradação do substrato pelo proteassomo 26S (Pickart 2001). A enzima ubiquitina ligase E3 determina a especificidade do substrato a ser degradado. COP1 é uma ligase E3, interagindo com SPA na repressão da fotomorfogênese. O complexo COP1-SPA interage com múltiplos fatores de transcrição chaves para respostas fotomorfogênicas, marcando-os por ubiquitinação para degradação no proteossomo (Podolec & Ulm, 2018). Alvo da ação desse complexo repressor, LONG HYPOCOTYL 5 (HY5) é um dos fatores de transcrição centrais da sinalização luminosa, se ligando a vários promotores de genes responsivos à luz, promovendo a sua expressão e o desenvolvimento fotomorfogênico (Gangappa & Botto, 2016). PHYs interagem com o complexo COP1-SPA, dissociando-os e fazendo com que COP1 seja transportado para fora do núcleo, permitindo o acúmulo da proteína HY5 (Lu *et al.*, 2015).

Em contrapartida, as PIFs são fatores de transcrição principalmente associados com a regulação dos genes responsáveis pelas respostas de desenvolvimento em condições de ausência de luz (escotomorfogênese), caracterizado por indução do alongamento celular e inibição da síntese de pigmentos fotossintéticos (Leivar & Monte, 2014). Com a translocação dos fitocromos para o núcleo após estímulo luminoso, PHYs interagem com PIFs, sendo fosforiladas, ubiquitinadas e degradadas pelo proteassomo 26S (Leivar & Quail, 2011; Shen *et al.*, 2008). A representação das interações dos fitocromos com as proteínas PIFs e o complexo COP1/SPA regulando a transcrição de genes responsivos à luz está indicada na Figura R2.

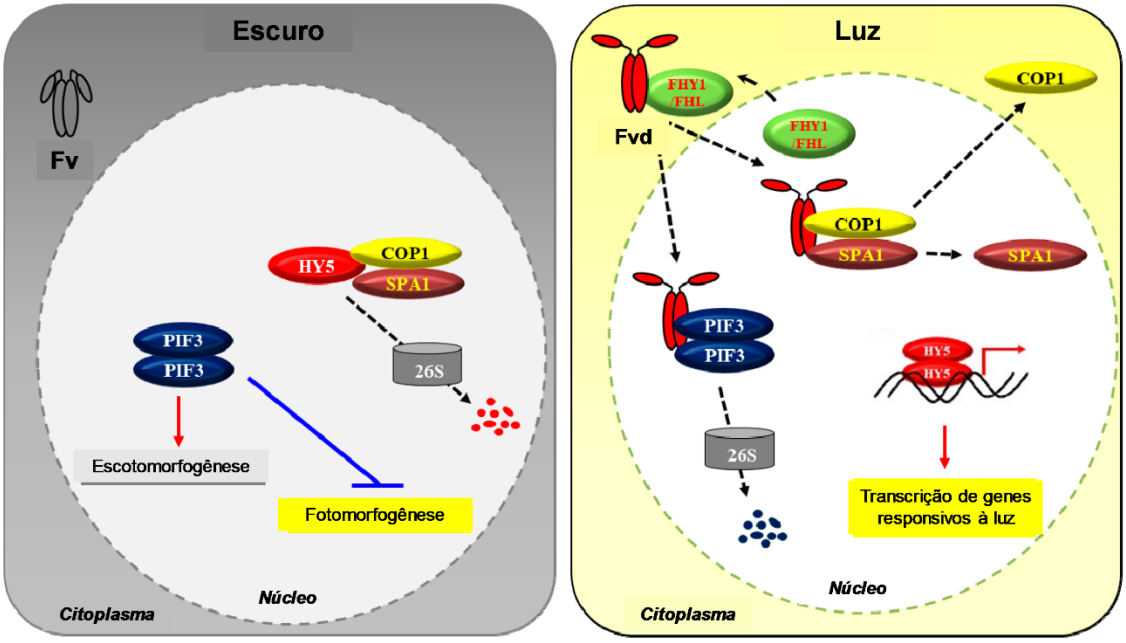

Figura R2. Modelo simplificado de sinalização do sinal luminoso dependente de fitocromos. Na ausência de luz, as proteínas PIFs induzem a expressão de genes responsáveis pelo desenvolvimento escotomorfogênico e as proteínas HY5, fatores de transcrição de genes responsivos à luz, são ubiquitinadas e degradadas por ação do complexo COP/SPA. Sob iluminação e fotoativação do fitocromo, a forma ativa Fvd é translocada para o núcleo, marcando as proteínas PIFs para degradação pelo proteassomo 26S e dissociando o complexo COP/SPA. HY5, dessa forma, se encontra livre para induzir a transcrição de genes fotomorfogênicos. Abreviações das proteínas se encontram descritas no texto. Adaptado de Hoang *et al.* (2019).

Os mecanismos moleculares pelos quais os PHYs dissociam o complexo COP1-SPA e também fosforilam PIFs ainda não estão bem elucidados (Hoang *et al.*, 2019; Pham *et al.*, 2018).

Assim sendo, o fitocromo atua como um interruptor ativado por luz vermelha, a depender do balanço entre suas formas ativa e inativa, para realizar alterações na transcrição de genes por meio da sua interação com componentes da rota de sinalização luminosa. Porém, certas mutações que resultam em mudanças conformacionais dos fitocromos podem alterar a sua atividade e sua sensibilidade ao sinal luminoso para a fotoativação.

Em *Arabidopsis*, mutações na porção N-terminal dos fitocromos foram mapeadas e envolvem deficiência na incorporação do cromóforo, na estabilização da forma ativa Fvd ou na transdução do sinal (Nagatani 2010; Rockwell & Lagarias, 2006). Algumas mutações de ganho de função também foram encontradas no domínio GAF, promovendo tanto maior estabilidade da proteína (PHYA^{E229K}) (Dieterle *et al.*, 2005) ou até mesmo respostas constitutivas independentes do sinal luminoso (PHYB^{Y276H}) (Hu *et al.*, 2009; Su & Lagarias, 2007). Um resumo das mutações mapeadas no domínio fotossensor dos fitocromos está detalhada na Figura R3.

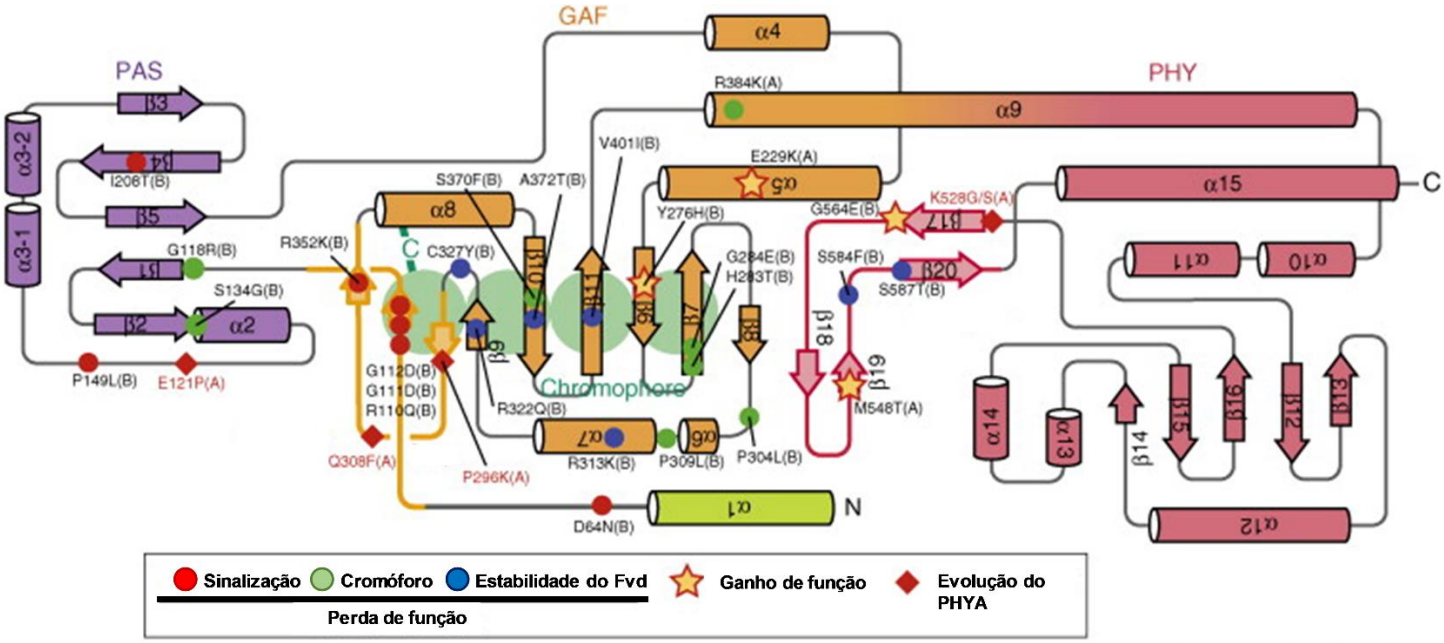

Figura R3. Mutações mapeadas no domínio fotossensor dos fitocromos de *Arabidopsis*. Mutações de perda de função são classificadas na deficiência da transdução do sinal luminoso (em vermelho), incorporação do cromóforo (em verde) e estabilização do Fvd (em azul). Mutações de ganho de função estão representadas por estrelas. Substituições de aminoácidos importantes para a diversificação das isoformas dos fitocromos estão indicadas por losangos vermelhos. As posições dos aminoácidos estão enumeradas de acordo com PHYA (A) ou PHYB (B). Traduzido de Nagatani (2010).

O resíduo Y^{276} do PHYB de *Arabidopsis* provavelmente interage diretamente com o anel D tetrapirrólico do cromóforo, responsável pela alteração conformacional desencadeadora do sinal e, dessa forma, a proteína mutante $PHYB^{Y276H}$ é translocada para o núcleo mesmo na ausência de luz (Oka *et al.*, 2011; Su & Lagarias, 2007). O perfil transcricional de plântulas de *Arabidopsis* portadoras de $PHYB^{Y276H}$ crescidas no escuro é bastante semelhante ao daquelas crescidas sob luz vermelha contínua, apresentando também um fenótipo de desestiolamento parcial (Hu *et al.*, 2009). Atualmente, alelos de $PHYB^{Y276H}$ têm sido empregados no desenvolvimento de novos marcadores moleculares para seleção de linhagens transgênicas devido à intensa autofluorescência na faixa do vermelho da proteína mutante, possibilitando monitoramento não-invasivo de sua expressão, substituindo métodos dependentes de antibióticos ou agrotóxicos (Hu & Lagarias, 2017). Mutações semelhantes de ganho de função em resíduos de tirosina dos fitocromos também foram verificados em aveia (Jeong *et al.*, 2016), indicando possivelmente respostas conservadas entre os vegetais.

A influência da luz e do fitocromo na síntese de metabólitos de valor nutricional em frutos de tomateiro

Através da modulação da expressão gênica, o fitocromo está diretamente relacionado com importantes respostas fisiológicas durante o ciclo vegetal, como a fotossíntese, a carotenogênese e desenvolvimento de frutos. A manipulação da expressão de genes que codificam as proteínas do fitocromo, bem como a modulação de sua atividade por meio de introdução de mutações, constituem estratégias promissoras para o desenvolvimento de culturas com características

agronômicas de interesse melhoradas (Boccalandro *et al.*, 2003; Ganesan *et al.*, 2017; Garg *et al.*, 2006; Gupta *et al.*, 2014; Gururani *et al.*, 2015; Thiele *et al.*, 1999; Zhang *et al.*, 2013).

Para o tomateiro, estudos pioneiros identificaram que fitocromos estariam envolvidos no controle da síntese de pigmentos, modulando tanto o acúmulo de carotenoides quanto de flavonoides (Khudairi & Arboleda, 1971; Piringer & Heinze, 1954). Plantas de tomateiro sobre-expressando o gene *PHYA* de aveia apresentaram folhas e frutos verde-escuros, entretanto, nenhuma caracterização metabólica foi realizada nessas linhagens transgênicas (Boylan & Quail, 1989). Alba *et al.* (2000) constataram que os níveis de transcritos de *PHYA* apresentam aumentos consideráveis durante a maturação de frutos de tomateiro na presença de luz e que, de forma interessante, um breve pulso de luz vermelha era capaz de dobrar os teores de licopeno em frutos de tomate mantidos no escuro. A sobre-expressão constitutiva de *PHYB1* e *PHYB2* em tomateiro levou à inibição do alongamento caulinar e a um acúmulo quase três vezes maior de antocianinas nos tecidos vegetativos (Husaineid *et al.*, 2007), mas nenhuma análise dos impactos dessas manipulações foi realizada no processo de amadurecimento ou sobre a qualidade nutricional dos frutos. Através de estudos realizados com mutantes deficientes em fitocromos específicos, Gupta *et al.* (2014) indicaram que PHYA possui papel no acúmulo de clorofilas nos frutos e, juntamente com PHYB1, atuam inibindo o acúmulo de licopeno durante o estágio de crescimento do fruto. Os autores afirmam ainda que os diferentes fitocromos atuam de forma a estabelecer um equilíbrio dinâmico entre as fases de maturação do fruto e o controle da síntese e acúmulo de carotenoides nesse órgão. Ademais, o silenciamento fruto-específico de *PHYA* e *PHYB2* em tomateiro levou à redução no conteúdo de carotenoides totais em frutos maduros em virtude de alterações diretas nos níveis de transcritos de enzimas relacionadas à carotenogênese (Bianchetti *et al.*, 2018).

As proteínas PIFs também influenciam no acúmulo de fitonutrientes. Em tomateiro, Llorente *et al.* (2016) demonstrou que a biossíntese de carotenoides é reprimida por PIF1a devido à sua ligação ao promotor da *PHYTOENE SYNTHASE 1* (*PSY1*), enzima que atua na etapa inicial da rota carotenogênica, inibindo a sua

expressão. Os autores ainda verificaram que o silenciamento de *PIF1a* aumenta significativamente o conteúdo de carotenoides totais em frutos maduros. Gramegna *et al.* (2018) verificou que PIF3 inibe a expressão de *GERANYLGERANYL DIPHOSPHATE REDUCTASE* (*GGDR*), responsável pela síntese do precursor dos tocoferóis. Dessa forma, impactos relacionados ao acúmulo de compostos nutracêuticos pelos PHYs podem ser resultado da sua interação com as proteínas PIFs.

Uma das evidências mais marcantes da influência da luz sobre a qualidade nutricional do tomate originou-se do isolamento dos mutantes carregando as mutações monogênicas recessivas *high pigment 1* e *2* (*hp1* e *hp2*), as quais conferem respostas exageradas à luz. Entre outras peculiaridades, tais mutantes são classicamente caracterizados por apresentarem hipocótilos encurtados e altos níveis de antocianinas em suas plântulas, bem como uma pigmentação mais escura em folhas e frutos (Bino *et al.*, 2005; Lieberman *et al.*, 2004). De modo interessante, a pigmentação aumentada nos frutos desses mutantes origina-se de um aumento significativo no número de cloroplastos e nos teores de clorofilas em frutos imaturos, bem como numa maior abundância de cromoplastos e elevação no conteúdo de carotenoides, especialmente licopeno, em frutos maduros. Devido ao aumento em seu conteúdo de licopeno, as mutações *hp* têm sido introgredidas em várias cultivares de tomateiro, recebendo a denominação geral "Lycopen Rich Tomatoes (LRT)", as quais tem sido utilizadas na extração industrial de licopeno para uso como complemento nutricional de alimentos processados, corantes alimentícios, bem como na indústria de cosméticos e farmacêutica (Levin *et al.*, 2006).

Proposições iniciais sugeriam que as mutações *hp1* e *hp2* seriam lesões nas estruturas de genes da via biossintética dos carotenoides (Kerckhoffs *et al.*, 1997), todavia, posteriormente demonstrou-se que algumas delas representam diferentes mutações em genes que codificam os ortólogos em tomate dos genes *DEETIOLATED1* (*DET1*) e *UV DAMAGE DNA BINDING PROTEIN 1* (*DDB1*) de *A. thaliana*, respectivamente, os quais interagem genética e bioquimicamente e atuam como reguladores negativos da fotomorfogênese (Liu *et al.*, 2004; Mustilli

et al., 1999). Para validar esses resultados genéticos e estudar a importância funcional de DET1 e DDB1 em tomate, plantas transgênicas com alterações na expressão desses genes foram produzidas, permitindo confirmar que a redução na expressão desses elementos promove aumentos significativos nos teores de clorofilas em frutos imaturos e de carotenoides, flavonoides, tocoferóis (vitamina E), ascorbato (vitamina C) e outros fitonutrientes em frutos maduros (Bino *et al.*, 2005; Cruz *et al.*, 2018; Davuluri *et al.*, 2004; Davuluri *et al.*, 2005; Wang *et al.*, 2008).

A repressão dos transcritos de reguladores negativos da transdução do sinal luminoso em tomate, como CULLIN 4 (CUL4) e COP1, igualmente resultaram no acúmulo de carotenoides e flavonoides (Liu *et al.*, 2004; Wang *et al.*, 2008). Em contrapartida, a supressão constitutiva por RNAi de LONG HYPOCOTYL 5 (HY5), um regulador positivo da transdução do sinal luminoso, reduziu os teores de clorofilas e a formação de tilacóides nos cloroplastos de frutos imaturos, bem como diminuiu o conteúdo de carotenoides totais em frutos maduros (Liu *et al.*, 2004). Portanto, baseado nesses resultados pode-se concluir que ao contrário de COP1, DET1 e DDB1, a proteína HY5 estimula a biogênese de plastídios e a pigmentação de frutos de tomate, o que corrobora sua atuação como um regulador positivo da transmissão do sinal luminoso em plantas (Gangappa & Botto, 2016). A atuação das proteínas COP1 e DET1 como repressoras de respostas fotomorfogênicas em plantas se baseia na promoção da degradação, via ubiquitinação, de proteínas que atuam como reguladoras positivas na transdução do sinal luminoso, tais como HY5, sendo que esse mecanismo de degradação pós-traducional de fatores promotores da fotomorfogênese tem sido interpretado como um aspecto regulatório chave na cascata de sinalização desencadeada pelos fitocromos (Gyula *et al.*, 2003; Wang & Wang, 2015).

Como mencionado anteriormente, incrementos no conteúdo de carotenoides em frutos de tomate desencadeados por mutações ou modificações por transgenia em genes relacionados à percepção/transdução do sinal luminoso são quase sempre acompanhados de mudanças conspícuas na abundância, tamanho e/ou ultraestrutura dos plastídios. Grande parte dos estudos sobre a formação de

plastídios e a diferenciação de cloroplastos em cromoplastos tem sido realizada em tomateiro, uma vez que durante a maturação do fruto carnoso desta espécie desenvolvem-se cromoplastos programados para sintetizar e acumular grandes quantidades de carotenoides, tocoferois e outros fitonutrientes lipofílicos (Egea *et al.*, 2010; Li & Yuan, 2013).

Ao contrário do observado em linhagens transgênicas com alterações em genes de biossíntese de carotenoides, mutantes e transgênicas com mudanças em genes relacionados à transdução do sinal luminoso (como as mutações *hp*) são caracterizadas não apenas por aumentos nos fluxos das rotas biossintéticas de compostos nutracêuticos, mas também apresentam incrementos significativos na capacidade de síntese e estocagem dessas substâncias em seus frutos (Azari *et al.*, 2010b; Cookson *et al.*, 2003; Liu *et al.*, 2004). Essa constatação corrobora o atual entendimento de que um dos fatores limitantes ao aumento no conteúdo de fitonutrientes em frutos carnosos consiste em restrições na capacidade de produção e armazenamento dos mesmos e, portanto, esforços devem ser canalizados para a geração de frutos com plastídios maiores, mais abundantes e com estrutura interna mais favorável ao acúmulo desses compostos (Azari *et al.*, 2010b; Cocaliadis *et al.*, 2014; Kolotilin *et al.*, 2007; Wang *et al.*, 2008).

Diante desse cenário, faz-se necessário aquilatar o conhecimento atual acerca do modo pelo qual o sinal luminoso controla a biogênese plastidial, especialmente no que tange ao papel de fotorreceptores como fitocromos durante a formação dessa organela.

Dada a importância central dos fitocromos na cadeia de transdução dos sinais luminosos e seus consequentes impactos na regulação da biossíntese de vários compostos de valor nutricional, parece-nos plausível hipotetizar que alterações quantitativas advindas da sobre-expressão dos genes de fitocromos bem como da modulação de sua ativação por luz especificamente nos tecidos do fruto alterariam os processos de produção e acúmulo de fitonutrientes, gerando tomates com maior conteúdo de compostos nutracêuticos de importância para a saúde humana, como carotenoides, tocoferóis, vitamina C e flavonoides, sem afetar o desenvolvimento vegetativo das plantas.

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OBJETIVOS

A presente tese tem como objetivo principal investigar os impactos da manipulação dos níveis de fitocromos bem como de sua atividade como estratégia para a biofortificação de frutos do tomateiro.

De forma específica, empregamos uma abordagem fruto-específica para a sobre-expressão da forma nativa do FITOCROMO B2 (PHYB2) e da forma mutada $PHYB2^{Y252H}$ (constitutivamente ativa independente do sinal luminoso) e avaliamos os impactos de ambas manipulações especialmente sobre o perfil global de transcritos, biogênese dos plastídios e acúmulo de compostos de valor nutricional durante o desenvolvimento e amadurecimento dos frutos de tomateiro.

CAPÍTULO ÚNICO: Beyond the limits of photoperception: constitutively active PHYTOCHROME B2 overexpression as a strategy of tomato biofortification

O presente capítulo está apresentado em inglês e organizado conforme as normas de publicação do periódico *Plant Biotechnology Journal*.

SUMMARY

Photoreceptor engineering has recently emerged as 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 elusive. In this study, we demonstrated that the fruit-specific overexpression of a constitutively active GAF domain Tyr^{252}-to-His PHYB2 mutant version ($PHYB2^{Y252H}$) significantly promoted the accumulation of multiple health-promoting antioxidants in tomato fruits, without negative collateral consequences on vegetative development. Compared to the native *PHYB2* overexpression, $PHYB2^{Y252H}$-overexpressing lines exhibited more extensive increments in transcript abundance of genes associated with fruit plastid development, chlorophyll synthesis and metabolic pathways responsible for fruit antioxidant accumulation. Accordingly, $PHYB2^{Y252H}$ overexpression caused increments in plastid ultrastructure in green fruits and overaccumulation of carotenoids, tocopherols, flavonoids and ascorbate in ripe fruits compared to both wildtype and *PHYB2*-overexpressing lines. Limited impacts over the fruit primary metabolism were registered due to either *PHYB2* or $PHYB2^{Y252H}$ overexpression, slightly reducing and promoting sugar and lipid biosynthesis, respectively. Taken together, these findings indicate that mutation-based adjustments in PHY activity represents a valuable photobiotechnological tool for tomato biofortification, highlighting the potential of photoreceptor engineering in improving quality traits in fleshy fruits.

Keywords: photobiotechnology, *Solanum lycopersicum*, carotenoids, vitamin E, flavonoids, vitamin C, secondary metabolism, antioxidants.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetable crops in the world, both *in natura* or as processed juices and sauces (Bergougnoux, 2014). It is an important source of health-promoting substances in the human diet, including carotenoids (e.g., lycopene and β-carotene), flavonoids, ascorbic acid (i.e., vitamin C) and tocopherol (i.e., vitamin E) (Frusciante *et al.*, 2007; Dorais *et al.*, 2008). Accordingly, increasing attention has been devoted worldwide to improve tomato productivity and fruit quality traits both via traditional breeding or transgenic-based approaches (Levin *et al.*, 2006; Liu *et al.*, 2004; Martin *et al.*, 2011).

Most attempts to improve fruit nutritional composition have focused on manipulating 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 signaling transduction pathway has been emerged as an alternative to promote more extensively tomato fruit biofortification by simultaneously altering the production of multiple nutraceutical compounds (Azari *et al.*, 2010a; 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 (Demotes-Mainard *et al.*, 2016). Once photoactivated by red light exposure, PHYs allosterically changes its conformation, triggering its translocation from cytosol to nucleus (Smith 2000). In the nucleus, active PHYs interact with light signaling components, initiating highly complex and extensively interconnected signaling cascades that ultimately lead to the differential expression of photomorphogenesis-related genes and proteasome-dependent 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.*, 2000). *PHYB2* is the most expressed *PHY* in tomato fruits (Bianchetti *et al.*, 2017; Hauser *et al.*, 1997;

Pratt *et al.*, 1995) and a major regulator of fruit chloroplast maturation and carotenoid accumulation (Gupta *et al.*, 2014, Bianchetti *et al.*, 2018).

Besides PHY levels, their activity can also be manipulated. In *Arabidopsis thaliana* (L.) Heynh. and *Avena sativa* L., specific aminoacid changes in the photosensor module domains result in a light-independent constitutive biological activity (Jeong *et al.*, 2016; Su and Lagarias, 2007). A well-characterized modulation in PHY activity is the mutation of the *Arabidopsis* PHYB conserved GAF domain Tyr^{276}-to-His that confers constitutive PHY-dependent photomorphogenic responses, even in dark conditions (Hu *et al.*, 2009; Oka *et al.*, 2011; Su and Lagarias, 2007).

As PHY photoperception domains are well conserved across plant species, here we investigated whether the overexpression of the constitutively active GAF domain Tyr-to-His PHYB2 mutant version ($PHYB2^{Y252H}$) can be applied as a means to promote PHY-dependent developmental and metabolic responses intrinsically associated with the final fruit nutritional composition, such as plastid development (Bianchetti *et al.*, 2017, 2018), carotenogenesis (Bianchetti *et al.*, 2018; Gupta *et al.*, 2014), tocopherol biosynthesis (Gramegna *et al.*, 2018), among others. To circumvent the 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; Husaineid *et al.*, 2007; Thiele *et al.*, 1999), a fruit-specific overexpression strategy was employed. Our data indicated that overexpression of the light-independent constitutively active mutant $PHYB2^{Y252H}$ promotes carotenoid, flavonoid and vitamins C and E in tomato fruits to significantly higher levels than those detected when the native sequence of this photoreceptor was overexpressed. Fruit plastid biogenesis and differentiation were particularly promoted by $PHYB2^{Y252H}$ overexpression, accompanied by an overall upregulation of genes encoding chloroplast components and photosynthesis-related proteins. Fruit primary metabolism was only marginally affected by the fruit-specific *PHYB2* or $PHYB2^{Y252H}$ overexpression, which further highlights the potential application of this genetic manipulation as a means to promote tomato fruit nutritional quality.

RESULTS

Fruit-specific *PHYB2* and *PHYB2*^{Y252H} overexpression in tomato plants

To identify the conserved tyrosine residue to generate hyperactive GAF domain Tyr-to-His mutants of PHY, the coding sequence of *Solanum lycopersicum* PHYB2 was aligned and compared to *Arabidopsis thaliana* PHYB. The conserved tyrosine residue of the phytochrome GAF domain Y^{276} in *Arabidopsis PHYB* corresponds to Y^{252} in tomato *PHYB2* (Figure S1). Next, transgenic tomato plants expressing the native *PHYB2* gene or its mutant hyperactive form *PHYB2*^{Y252H} 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::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) lines (namely L5, L6 and L15) (Figure 1a). *PHYB2* or *PHYB2*^{Y252H} transcript levels were between 2- and 7-fold higher in the transgenic lines compared to *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 *PHYB2*^{Y252H} resulted in no apparent phenotypical alterations on plant vegetative growth and overall yield (Figure S2). In contrast, green fruits (IMG and MG) from the *PPC::B2*^{Y252H} lines exhibited significantly darker green coloration at fruit pedicellar (shoulder) region than WT counterparts (Figure 1b). At the BK stage, the green shoulder phenotype persisted in both *PPC::B2* and *PPC::B2*^{Y252H} fruits, whereas it was significantly attenuated in WT fruits. The green shoulder phenotype was subsequently lost in all genotypes (RR, Figure 1b).

RNA-Seq transcriptomic profiling performed in BK fruits of representative *PPC::B2* (L1) and *PPC::B2*^{Y252H} (L6) lines revealed that up to 3.6% of the total tomato transcriptome was affected either by the *PHYB2* or *PHYB2*^{Y252H} overexpression. Compared to the WT, 906 and 719 genes were differentially expressed exclusively in *PPC::B2* and *PPC::B2*^{Y252H} 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 (r^2 correlation above 0.9) between both methods (Figure S3).

According to the Gene Set Enrichment Analysis (GSEA), the exclusive *PPC::B2* DEGs are 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::B2*^{Y252H} 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).

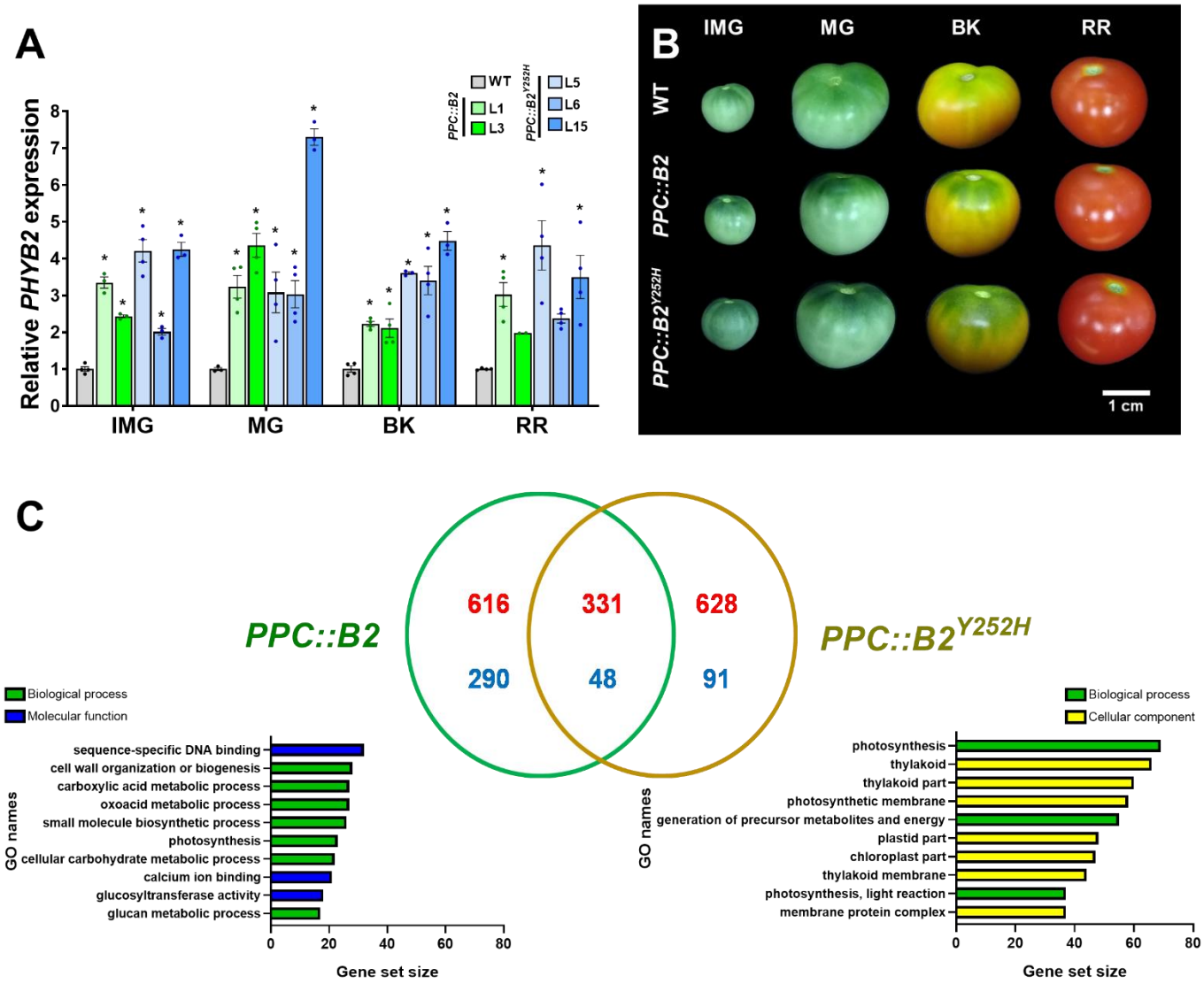

Figure 1. *PHYB2* and *PHYB2*^{Y252H} overexpression modifies fruit pigmentation and global transcriptomic profile. A. *PHYB2* or *PHYB2*^{Y252H} mRNA levels throughout fruit development of the transgenic lines. Transcript abundance was normalized against *PHYB2* mRNA levels detected in wild-type (WT) at each stage. Data are mean ± SE and dots represent individual values. Statistical differences within each stage are given by asterisks (Dunnett's Test with WT as control group, $\alpha = 0.05$). **B.** Visual phenotype of transgenic fruits in different development stages. **C.** Venn's diagram analysis and Gene Set Enrichment Analysis (GSEA) in *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) BK fruits compared to the WT counterparts. Up- and down-regulated genes are indicated in red and blue colors, respectively. Numbers represent overlapping changes among differentially expressed genes (DEGs) (adjusted p-value < 0.05). TMM values and GO terms are detailed in Tables S2 and S3, respectively. IMG, immature green; MG, mature green; BK, breaker; RR, red ripe.

Either *PHYB2* or *PHYB2^{Y252H}* overexpression promotes plastid biogenesis but only *PHYB2^{Y252H}* enhances chlorophyll accumulation

The greener fruit phenotype and up-regulation of plastid-related genes in *PPC::B2* and *PPC::B2^{Y252H}* lines prompted us to investigate the impacts of both transgenic manipulations 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::B2^{Y252H}* green fruits compared with WT counterparts (Figure 2a-b). The increased number of plastids detected in *PPC::B2* and *PPC::B2^{Y252H}* fruits was associated with up-regulation of *PLASTID DIVISION 2* (*PDV2*), which encodes a vital component of the plastid division machinery (Okazaki *et al.*, 2009) (Figure 2c).

Increased thylakoid stacking with more voluminous grana was observed in plastids of *PPC::B2* MG fruits, a phenotype further intensified in *PPC::B2^{Y252H}* fruits (Figure 2a, Figure S4). In agreement, transcript abundances of several photosynthesis- and plastid-related genes, including those encoding RuBisCO subunits (e.g., *RBCS2a*), were up-regulated in IMG fruits of *PPC::B2^{Y252H}* 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* (Solyc01g095430) and *CURT1a2* (Solyc10g011770) (Figure S5), were also exclusively up-regulated in *PPC::B2^{Y252H}* IMG fruits. In contrast, mRNA levels encoding transcription factors previously associated with altered plastid maturation and maintenance in green tomato fruits, such as *GOLDEN 2-LIKE 2* (*GLK2*), *AUXIN RESPONSE FACTOR 4* (*ARF4*), *ARABIDOPSIS PSEUDORESPONSE REGULATOR 2-LIKE* (*APRR2Like*) and *BEL-1 LIKE HOMEODOMAIN 11* (*BEL11*) were virtually indistinguishable between the wildtype and transgenic lines (Table S4).

Total chlorophyll content was about 3-fold higher in *PPC::B2^{Y252H}* green fruits than in the WT (Figure 2d, Table S5), which was associated with increments of approximately 2-fold in transcript levels of *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE 1* (*POR1*), which encodes an essential light-triggered

chlorophyll biosynthesis-related enzyme (Heyes and Hunter, 2005). In contrast, *PPC::B2* green fruits showed no significant changes in chlorophyll levels nor in *POR1* mRNA levels compared to the WT (Figure 2c, Table S4).

Transcriptome analysis carried out in MG fruits revealed enriched abundance of transcripts associated with photosynthesis, thylakoid and plastid membranes in *PPC::B2*^{Y252H} compared to WT fruits (Figure S6). Most chlorophyll biosynthetic genes were significantly up-regulated in *PPC::B2*^{Y252H} MG fruits compared to the WT (Figure 2e, Table S6), which agrees with the higher chlorophyll content detected in the transgenic fruits.

Altogether, these results indicate that fruit-specific overexpression of either *PHYB2* or *PHYB2*^{Y252H} promotes fruit chloroplast biogenesis and differentiation compared to the WT. *PHYB2*^{Y252H} overexpression caused more extensive grana formation compared to native *PHYB2*, which was explained by the upregulation of *CURT1a* homologs and other plastid-related genes. Moreover, only the *PHYB2*^{Y252H}-overexpressing lines presented transcriptional up-regulation of all major chlorophyll biosynthetic steps, leading to significant increments in fruit chlorophyll accumulation compared to the WT.

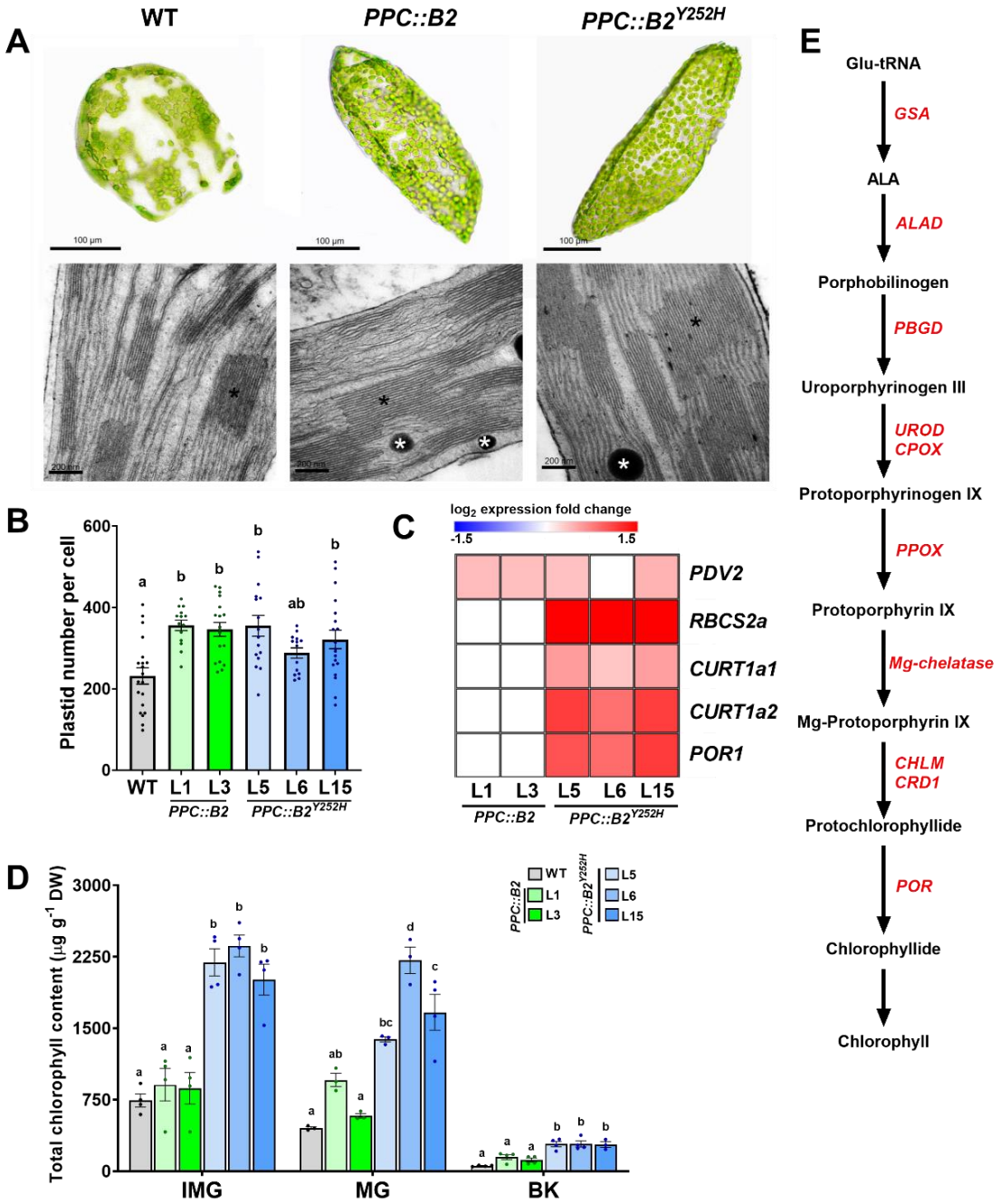

Figure 2. Fruit-specific *PHYB2* and *PHYB2*^{Y252H} overexpression promotes chloroplast biogenesis and differentiation and chlorophyll biosynthesis. A. Representative optical microscopy (upper panels) and transmission electronic microscopy images (bottom panels) of plastids of the pedicel region of immature green (IMG) fruits of wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) plants. Black asterisks indicate the increased grana volume in the transgenic lines and white asterisks indicate plastoglobules. **B.** Plastid number per cell on MG stage of WT, *PPC::B2* and *PPC::B2*^{Y252H} fruits. **C.** Heatmap representation of the statistically significative differences in mRNA levels of plastid-related genes between the transgenic and WT fruits at IMG stage

(Dunnett's test, α = 0.05). Gene abbreviations and relative transcript values are detailed in Table S4. **D.** Total chlorophyll content of WT, *PPC::B2* and *PPC::B2*^{Y252H} fruits. **E.** Simplified chlorophyll biosynthetic pathway. Intermediate reactions are omitted. Differentially expressed chlorophyll biosynthesis-related genes in *PPC::B2*^{Y252H} MG fruits according to RNASeq analysis are highlighted in red. Gene abbreviations, logFC and FDR values for genes 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). MG, mature green; BK, breaker; RR, red ripe; ALA, 5-aminolevulinic acid.

PHYB2^{Y252H} overexpression promotes fruit isoprenoid metabolism

Besides photosynthesis, chloroplasts are essential sites 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). Moreover, light signaling can also directly influence the expression of genes involved in the production of isoprenoids (e.g., carotenoids, tocopherols) and other health-promoting substances accumulated in tomato fruits (Gramegna et al., 2018; Ksas 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 S7). In contrast, *PPC::B2*^{Y252H} RR fruits exhibited significant increments in virtually all individual carotenoids analyzed, 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 about 40% and 100% in *PPC::B2* and *PPC::B2*^{Y252H} lines, respectively (Figures 3a and S7). Levels of α-tocopherol, the dominant tocopherol form of tomato fruits, as well as the β-carotene, lutein content were significantly higher in *PHYB2*^{Y252H}-overexpressing lines than in the WT at all sampled stages (Table S5). This indicates that the PHYB2^{Y252H}-triggered intensification in fruit isoprenoid metabolism was not restricted to the ripening stages.

Transcript levels of many isoprenoid biosynthetic genes were up-regulated in response to *PHYB2* and *PHYB2*^{Y252H} overexpression (Figure 3b). Genes encoding rate-limiting enzymes responsible for the synthesis of isoprenoid precursor geranylgeranyl diphosphate (GGDP), such as *1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE 1* (*DXS1*) and *GGDP SYNTHASE 2* (*GGPS2*) were up-regulated in *PPC::B2*^{Y252H} and in all transgenic lines, respectively. In contrast, except *LYCOPENE ε-CYCLASE* (*εLCY*), other 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*), *APETALA2a* (*AP2a*) and *FRUITFULL1* (*FUL1*) (Liu *et al.*, 2015), were also not significantly altered 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::B2*^{Y252H} lines, likely improving PQ content 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::B2*^{Y252H} lines (Figure 3b).

GERANYLGERANYL DIPHOSPHATE REDUCTASE (*GGDR*), which encodes the enzyme responsible for the synthesis of tocopherol precursor phytyl-diphosphate (PDP), was markedly up-regulated exclusively in *PPC::B2*^{Y252H} fruits at all sampling stages, suggesting increased conversion of GGDP to PDP in these transgenic lines. Tocopherol biosynthetic genes, particularly *2,3-DIMETHYL-5-PHYTYLQUINOL METHYLTRANSFERASE b* (*VTE3b*), *TOCOPHEROL CYCLASE* (*VTE1*) and *TOCOPHEROL C-METHYL TRANSFERASE* (*VTE4*), were also predominantly up-regulated in *PPC::B2*^{Y252H} lines from the IMG to the BK stage. The tocopherol precursor PDP can also be supplied by the chlorophyll degradation pathway and sequential phosphorylation of the phytol chain (Zhang *et al.*, 2014). Genes involved in ripening-related fruit chlorophyll breakdown, notably *PHEOPHYTINASE PHEOPHORBIDE HYDROLASE-LIKE 1* (*PPHL1*), as well as *PHYTOL KINASE* (*VTE5*), which is responsible for the first step of phytol phosphorylation (Valentin *et al.*, 2006), were also up-regulated at BK stage in *PPC::B2*^{Y252H} lines compared to WT counterparts (Figure 3b).

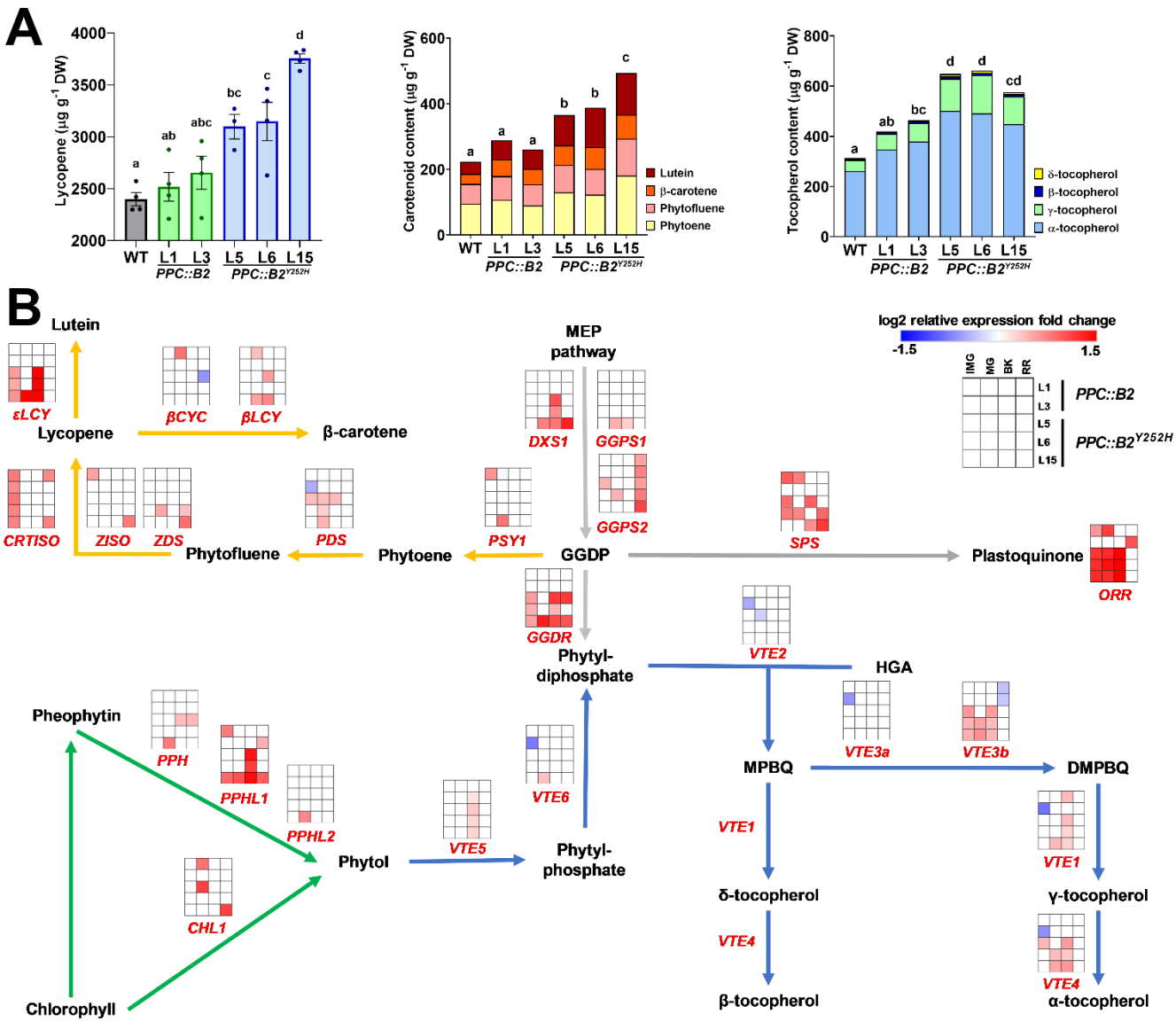

Figure 3. Fruit-specific *PHYB2* and *PHYB2*^{Y252H} overexpression promotes isoprenoid metabolism. A. Carotenoid (lycopene, phytoene, phytofluene, lutein and β-carotene) and total tocopherol content in wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) RR fruits. Data are mean ± SE and dots represent individual values. Statistical differences are given by different letters (Tukey's Test, α = 0.05). **B.** Schematic representation of isoprenoid metabolism interconnecting carotenoid (orange), tocopherol (blue) and chlorophyll (green) pathways. Intermediate reactions are omitted. Heatmap representation of the statistically significative differences mRNA levels of isoprenoid-related genes between the transgenic and WT fruits at each stage (Dunnett's test, α = 0.05). Gene abbreviations and relative transcript values are detailed in Table S7. IMG, immature green; MG, mature green; BK, breaker; RR, red ripe; MEP, methylerythritol phosphate; GGDP, geranylgeranyl diphosphate;

HGA, homogentisic acid; MBPQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMBPQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone.

Collectively, these findings indicate that either *PHYB2* or *PHYB2*^{Y252H} overexpression can transcriptionally promote the production of the isoprenoid precursor GGDP. A comparatively larger number of genes involved in PDP production (e.g., *GGDR*, *VTE5*), tocopherol production (e.g., *VTE3b*, *VTE1*, *VTE4*) and plastoquinone biosynthesis (e.g., *SPS*, *VTE3b*), was up-regulated in response to *PHYB2*^{Y252H} overexpression, which explains the significantly higher tocopherol accumulation in *PPC::B2*^{Y252H} than in *PPC::B2* fruits. Most carotenoid biosynthesis genes were not transcriptionally altered in the transgenic lines, but *PHYB2*^{Y252H}-overexpressing lines consistently accumulated PQ metabolism-related transcripts (e.g., *SPS* and *ORR*). Therefore, besides the transcriptional induction of GGDP-generating enzymes, the PQ-dependent regulation of carotenoid biosynthetic enzyme activity may also be implicated with the overaccumulation of lycopene and other carotenoids in *PPC::B2*^{Y252H} fruits.

Both flavonoid and ascorbate metabolisms are upregulated in *PHYB2* and *PHYB2*^{Y252H} ripe fruits

In addition to carotenoids and tocopherols, flavonoids and ascorbate also contribute significantly in determining the antioxidant capacity of red tomato fruits (Frusciante *et al.*, 2007). Although PHY-dependent regulation of flavonoid and ascorbate metabolism have been demonstrated in plant vegetative tissues (Brödenfeldt and Mohr, 1988; Nimmagadda and Narayanaswamy, 2009), the influence of fruit-localized PHYs on the biosynthetic pathways of these antioxidant compounds have rarely been investigated (González *et al.*, 2015). In our study, the fruit-specific *PHYB2*^{Y252H} overexpression significantly promoted both total flavonoid and ascorbate content in RR fruits, whereas the levels of these antioxidants were slightly, but not significantly, increased in *PPC::B2* fruits. Increments between 2 and 3-fold in rutin, kaempferol rutinoside, naringenin chalcone and naringenin glucoside contents were registered in *PPC::B2*^{Y252H} RR fruits compared to WT counterparts (Figures 4a and S8, Table S8). Both ascorbate and dehydroascorbate levels were over-accumulated in *PPC::B2*^{Y252H} fruits (Figure S8), resulting in increments of approximately 20% in the total pool of this antioxidant (Figure 4b).

Transcript levels of *CHALCONE SYNTHASE 1* (*CHS1*) and *CHS2*, which encode isoforms responsible for the conversion of the flavonoid precursor 4-coumaryol-CoA to naringenin chalcone (Figure S9), were occasionally upregulated in some transgenic lines throughout ripening (Table S9). On the other hand, the *PPC::B2*^{Y252H}-induced increments in rutin, which is a major flavonoid accumulated in tomato fruits (Slimestad and Verheul, 2009), 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-D-MANNOSE-3,5-EPIMERASE 1* (*GME1*), which encodes an enzyme responsible for an initial step in the ascorbate biosynthetic pathway (Wolucka and Van Montagu, 2007). RNASeq data revealed that *GME1* was the only core ascorbate biosynthetic gene modulated by

PHYB2^{Y252H} overexpression (Figure S10), with up to 2-fold 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 *PHYB2*^{Y252H} overexpression on isoprenoid metabolism, flavonoids and ascorbate accumulation in RR fruits, only *PPC::B2*^{Y252H} lines consistently exhibited higher total antioxidant activity, as estimated by the DPPH assay, reaching increments of up to 25% compared to WT counterparts (Figure 4e, Table S8).

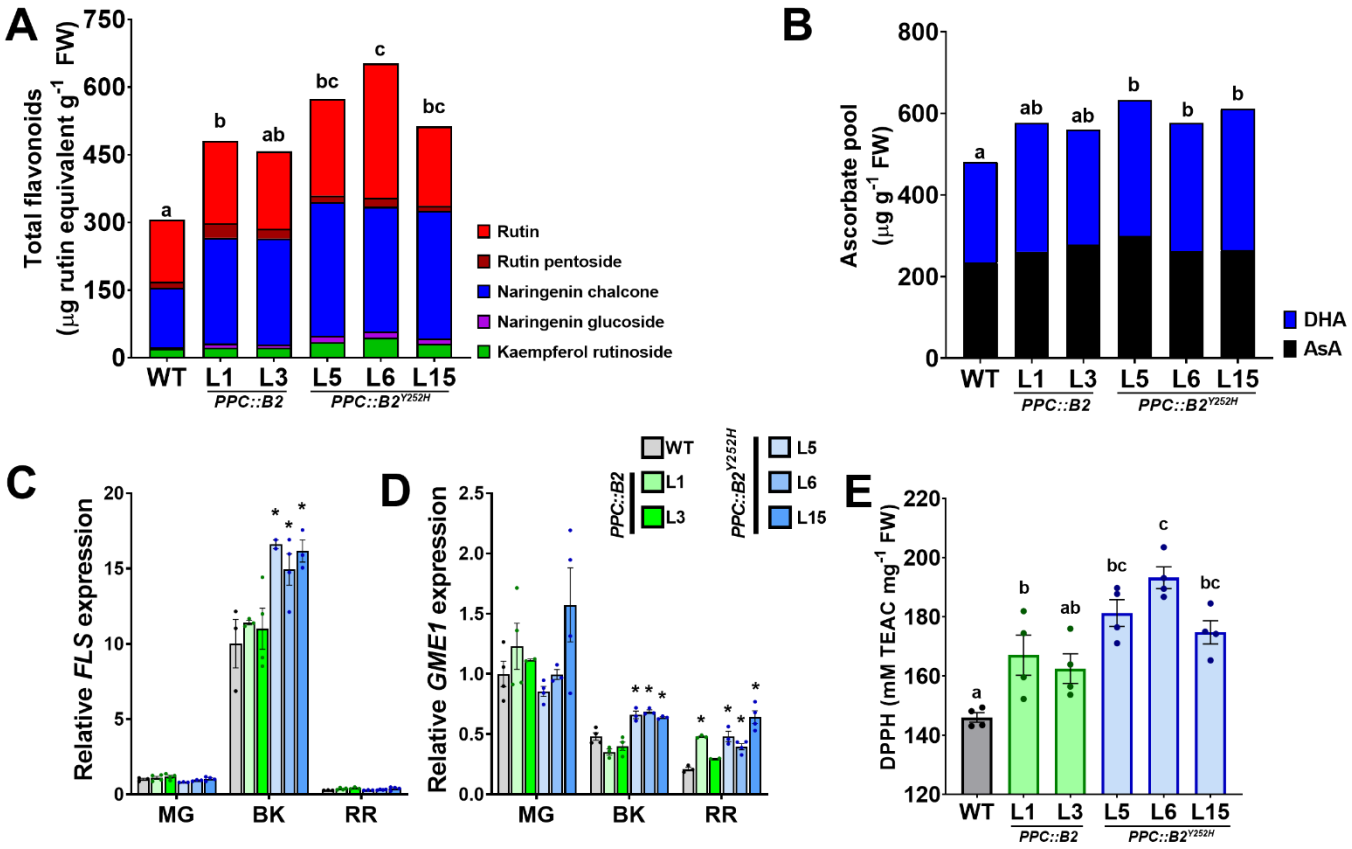

Figure 4. Flavonoid and ascorbate accumulation are increased in *PHYB2* and *PHYB2*^{Y252H} overexpressing fruits. A. Total flavonoids content in RR fruits of wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) lines. **B.** Total ascorbate pool in RR fruits. **C-D.** *FLS* and *GME1* mRNA levels throughout fruit development. Transcript abundance was normalized against *PHYB2* mRNA levels detected in wild-type (WT) at MG stage. **D.** Total antioxidant activity 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; MG, mature green; BK, breaker; RR, red ripe.

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

Nontargeted mass spectrometry (MS)-based metabolite profiling and HPLC-based soluble carbohydrate and organic acid analysis were used to investigate the impacts of the *PHYB2* or *PHYB2*^{Y252H} overexpression on primary metabolism of ripe fruits. About 25% of the metabolites identified via nontargeted GC–MS profiling was consistently altered in the transgenic lines compared to WT counterparts (Table S10). Hierarchical clustering analysis supported the differences between the clades composed by the *PPC::B2* and *PPC::B2*^{Y252H} lines based on polar and apolar metabolite profiles, evidencing that fruit metabolome was differentially affected by *PHYB2* or *PHYB2*^{Y252H} overexpression (Figure 5a-b).

Minor organic acids such as acetic, benzoic, lactic and succinic acids were consistently under-accumulated in all transgenic lines, whereas oxalic acid was over-accumulated 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 to WT counterparts (Figure 5a).

Saturated fatty acids such as arachidic, lauric, 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::B2*^{Y252H} fruits (Figure 5b). RNASeq data analysis revealed that some genes encoding important lipid metabolism-related enzymes are up-regulated, particularly in *PPC::B2*^{Y252H} lines (Table S2). For instance, *GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE* (Solyc08g076470), which encodes the enzyme catalyzing an initial reaction in the biosynthesis of phospholipids (Chen *et al.*, 2011), and some *FATTY ACID DESATURASES* (Solyc06g051400 and Solyc07g005510), associated with the accumulation of polyunsaturated fatty acids (Dar *et al.*, 2017) were upregulated in the *PPC::B2*^{Y252H}, particularly at BK stage (Table S2).

Glucose, fructose and sucrose content 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 (Figures 5c and S11). The levels of soluble sugars accumulated in ripe tomato fruits can be influenced by several factors, including the plant photosynthetic capacity, dynamics of starch biosynthesis and breakdown, and sucrose import from vegetative tissues and conversion into hexoses (Patrick *et al.*, 2013). To investigate the possible causes of the reduced levels of soluble sugars in the transgenic lines, we first estimated the plant photosynthetic capacity by measuring gas exchange, fluorescence parameters, chlorophyll content and starch levels in source leaves of all genotypes. Overall, net photosynthesis rate, stomatal conductance, photosystem II efficiency as well as chlorophyll and starch content in leaves of the transgenic lines were indistinguishable from the WT counterparts (Table S12).

As the transient starch accumulation during early tomato fruit development also directly influence total soluble sugar content at ripe stage (Davies and Cocking, 1965; Yin *et al.*, 2010), we next characterized the dynamics of starch accumulation in green WT and transgenic fruits. Both *PHYB2* and *PHYB2*^{Y252H} overexpression negatively impacted starch accumulation, reducing the levels of this carbohydrate at IMG stage in 27% and 44%, respectively (Figure 5d, Table S11). The lower starch content in the transgenic lines at IMG stage was associated with a significant reduction in mRNA levels of the gene responsible for encoding the subunit L1 of ADP-GLUCOSE PYROPHOSPHORYLASE (*AGPaseL1*) (Figure 5e), which is the AGPase large subunit-encoding gene most expressed in tomato sink tissues (Bianchetti *et al.*, 2018; Petreikov *et al.*, 2010).

Although reduced starch synthesis in immature tomato fruits can also be caused by reduced sink capacity by the fruit tissues (Osorio *et al.*, 2014), the transcript abundance of key genes associated with tomato fruit sugar import and breakdown, such as *LYCOPERSICUM INVERTASE 5* and *6* (*LIN5* and *LIN6*) and *ACID INVERTASE 1* (*TIV1*), were not consistently altered in the transgenic lines compared to the WT during early fruit development (Table S13).

Taken together, our data suggest that sugar and lipid metabolisms are the major fruit primary metabolic pathways affected by fruit-specific *PHYB2* or *PHYB2*^{Y252H}

overexpression. Rather than associated with changes in plant photosynthetic or fruit sink capacities, the reduced levels of soluble sugars in red ripe transgenic fruits seem to be associated with lower starch synthesis and accumulation during early fruit development.

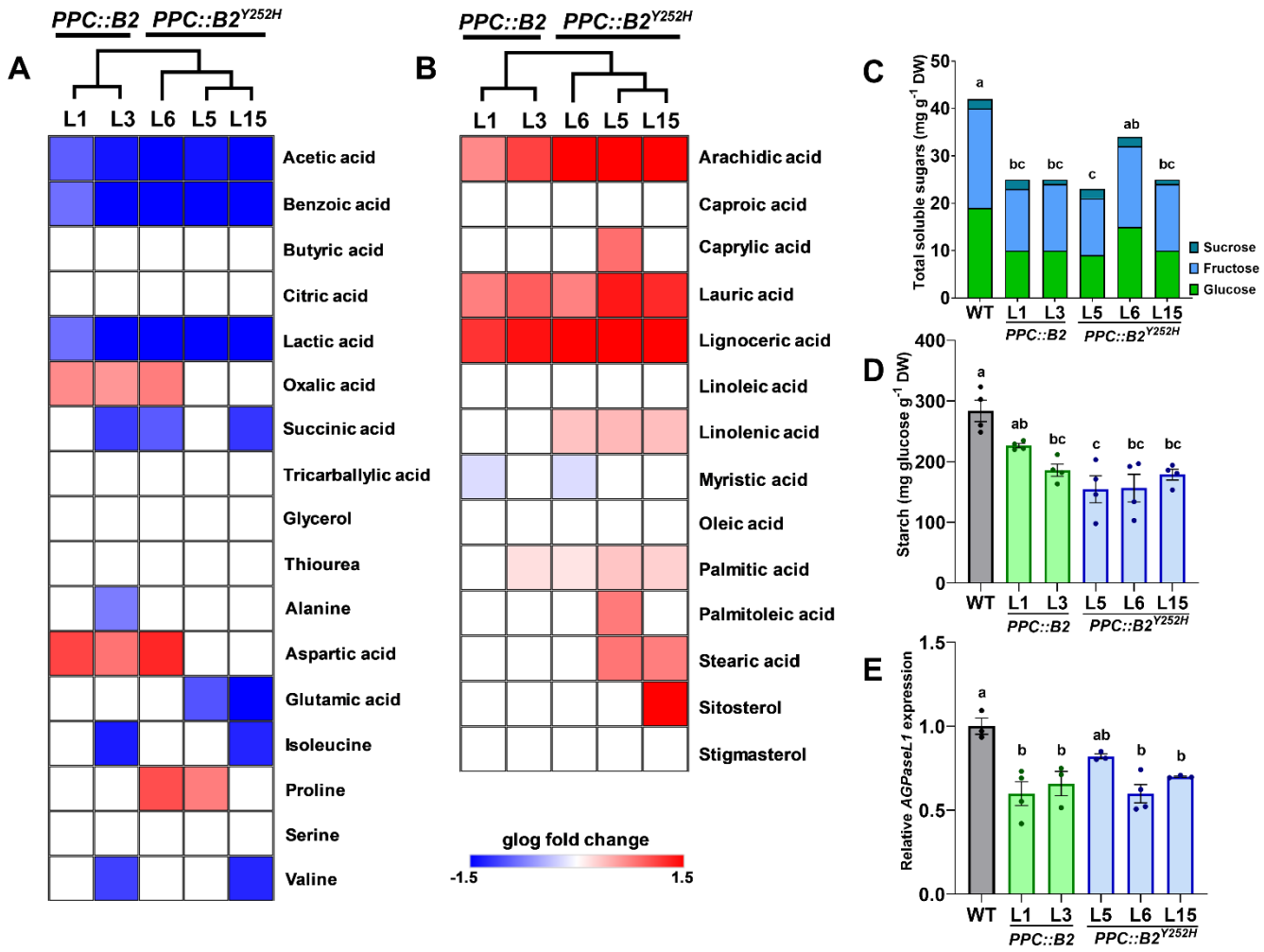

Figure 5. Fruit-specific PHYB2 and PHYB2^{Y252H} overexpression impacts on primary metabolism are limited to changes in lipid and sugar contents. A-B. Heatmap representation of the relative abundance of polar (A) and apolar (B) compounds in *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2^{Y252H}* (*PPC::B2^{Y252H}*) RR transgenic fruits. Only statistically different results are displayed in generalized logarithm (glog) normalized against the wild-type (WT) (Dunnett's test, α = 0.05). Relative metabolite abundances are detailed in Table S10. Dendrograms indicate hierarchical clustering relationships between lines. **C.** Total soluble sugars content in RR fruits. **D.** Starch content in IMG fruits. **E.** *AGPaseL1* mRNA levels in IMG fruits. In D-E, data are mean ± SE and dots represent individual values. Metabolites, gene abbreviations and relative transcript values are detailed in Tables S11 and S13. Statistical differences are given by different letters (Tukey's Test, α = 0.05). IMG, immature green; RR, red ripe.

DISCUSSION

Manipulation of light signaling pathway components has been increasingly investigated as a mean to improve desired quality traits in many crops, including adjustments in plant architecture, shade avoidance responses, abiotic stresses tolerance, flowering time, among others (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.*, 2010b; Davuluri *et al.*, 2005; Liu *et al.*, 2004; Wang *et al.*, 2008). In contrast, attempts to promote tomato biofortification via increments in photoreceptor protein abundance (Giliberto *et al.*, 2005) or by genetic engineering photoreceptor properties (e.g., light sensitivity, protein stability) are currently scarce or missing, respectively. Here, we demonstrated that the fruit-specific overexpression of the constitutively active Tyr^{252}-to-His PHYB2 mutant version leads to the overaccumulation of multiple health-promoting antioxidants in tomato fruits, without causing adverse collateral effects on vegetative development. In contrast, overexpression of native *PHYB2* under the control of the same fruit-specific promoter (*pPPC2*) resulted in less prominent, and sometimes non-significative, increases in bioactive compounds (e.g., carotenoids, flavonoids, vitamin C and E) in ripe tomato fruits. These findings confirm that engineered PHY forms, particularly light-independent constitutively active versions (Jeong *et al.*, 2016; Oka *et al.*, 2011; Su and Lagarias, 2007), provide opportunities to explore beyond the limits of photoperception conferred by the overexpression of native PHY sequences, thereby representing a powerful photobiotechnological tool to increment the nutritional quality of fruits.

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

The most striking visual phenotype of *PPC::B2* and particularly *PPC::B2*^{Y252H} 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 pedicelar region compared to 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 through adult plant life (Girnth *et al.*, 1978; Mohr 1977). In tomato fruits, PHY deficiency leads to reduced plastid 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 *PHYB2*^{Y252H}-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).

Although transcription factors such as GLK2, ARF4, APRR2Like and BEL11 have been closely associated with increased abundance and development of internal membranous structures in tomato fruit chloroplasts (Lupi *et al.*, 2019; Meng *et al.*, 2018; Pan *et al.*, 2013; Powell *et al.*, 2012; Sagar *et al.*, 2013), the transcript abundance of their encoding genes did not differ between WT and transgenic fruits (Table S4). However, in line with the identification of CURT1a protein accumulation as requisite for grana stacking in *Arabidopsis* leaf chloroplasts (Armbruster *et al.*, 2013; Pribil *et al.*, 2018), the upregulation of *CURT1a1* and *CURT1a2* in *PPC::B2*^{Y252H} 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 upregulated in response to *PHYB2* or *PHYB2*^{Y252H} overexpression (Figure 1), which agrees with the well-reported positive influence of PHY-signaling pathway on these gene categories in leaves (Dubreuil *et al.*, 2017; Hu et al., 2009; Oh and Montgomery, 2014; Yoo *et al.*, 2019).

Increments in plastid ultrastructure positively affected isoprenoid accumulation once many active biosynthetic enzymes and their hydrophobic products are physically associated to 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 (Kolotilin *et al.*, 2007) and *GLK2*-overexpressing lines (Powell *et al.*, 2012; Lupi *et al.*, 2019). Therefore, the increments in plastid abundance and ultrastructure observed in *PPC::B2* and *PPC::B2*^{Y252H} 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 signaling pathway (Llorente *et al.*, 2016; Gramegna *et al.*, 2018; Inagaki *et al.*, 2015), which possibly explains the differential impacts of *PHYB2* and *PHYB2*^{Y252H} overexpression on the abundance of isoprenoids in green (chlorophylls) and ripening fruits (carotenoids and tocopherols). In fact, only *PPC::B2*^{Y252H} fruits presented higher chlorophyll content than the WT, a differential response associated with the exclusive up-regulation of chlorophyll biosynthetic genes in the *PPC::B2*^{Y252H} lines (Figure 2). Among PHY signaling transduction components, PHYTOCHROME INTERACTING FACTORS (PIFs) transcription factors are known to directly repress the expression of a wide variety of photosynthesis-related and chlorophyll biosynthetic genes (Huq *et al.*, 2004; Shin *et al.*, 2009; Tang *et al.*, 2012). As PIF protein abundance is negatively regulated by active PHYs, the higher expression levels of chlorophyll biosynthetic genes in *PPC::B2*^{Y252H} fruits may be explained by the presumably constant PHYB2-PIF interaction provided by the presence of the light-independent constitutively active PHYB2^{Y252H} molecules inside the nuclei of fruit cells.

PHYB2^{Y252H}-induced overaccumulation of bioactive compounds mainly rely on the upregulation 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 (Dorais *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; Quadrana *et al.*, 2013; Verhoeyen *et al.*, 2002; Yuan *et al.*, 2015).

Carotenoids and tocopherols are derived from the MEP pathway-derived isoprenoid precursor GGDP (Pulido *et al.*, 2012). Overexpression of genes encoding rate-limiting enzymes responsible for GGDP production has been shown to improve overall isoprenoid content both in *Arabidopsis* seedlings (Estévez *et al.*, 2001) and potato tubers (Morris *et al.*, 2006), but has failed to alter isoprenoid levels under other circumstances (García-Alcázar *et al.*, 2017). In this context, transcriptional analysis revealed that *DXS1* and *GGPS2* were particularly up-regulated in ripening *PPC::B2* and *PPC::B2*^{Y252H} fruits, possibly boosting the production of GGDP to sustain higher carotenoid and tocopherol biosynthesis fluxes (Figure 3).

Although *PHYB2*^{Y252H} overexpression resulted in significantly higher levels of lycopene, β-carotene and lutein, the most significant bioactive carotenoids in ripe tomatoes (Raiola *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 apparently contradictory result suggests that the *PHYB2*^{Y252H}-triggered up-regulation of GGDP-biosynthetic genes and/or the increased in plastid size and abundance may suffice to sustain higher carotenoid synthesis and accumulation in the ripe transgenic fruits. Alternatively, the presence of the constitutively active PHYB2^{Y252H} molecules may also have affected fruit carotenogenesis via additional regulatory levels.

Carotenoid-related enzymes can also be post-transcriptionally regulated, primarily relying on PQs as electron acceptors during the desaturation steps of phytoene and subsequent metabolites, driving lycopene biosynthesis (Norris *et al.*, 1995). As *SPS*, a PQ-biosynthetic gene, was up-regulated in *PPC::B2*^{Y252H} fruits, it seems tempting to suggest 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 also reported

in *Arabidopsis* (Kim *et al.*, 2015) and tomato *hp2* leaves (Jones *et al.*, 2013). The reduction of PQs is catalyzed 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.

Differential transcriptional profile of tocopherol biosynthesis in *PPC::B2*^{Y252H} transgenic fruits revealed an overall upregulation of major enzymes, explaining the 2-fold increase in tocopherol content at ripe stage (Figure 3). Tocopherol accumulation is mainly limited by the supply of the prenyl precursor PDP (Zhang *et al.*, 2013). PDP can be derived from successive phosphorylations of phytol chain obtained via the action of dephytylating enzymes in chlorophyll breakdown, or from the activity of GGDR, channeling GGDP from the MEP pathway into tocopherol biosynthesis (Pellaud and Mène-Saffrané, 2017). As tomato fruits ripe, transcript levels of *GGDR* and *VTE5* decreases, restricting PDP availability and consequently, tocopherol accumulation (Quadrana *et al.*, 2013). The marked increase in total tocopherol content in the *PPC::B2*^{Y252H} lines can be attributed to the up-regulation of genes related to both bottlenecks of PDP biosynthesis. Moreover, the chlorophyll dephytylating enzyme PPHL1 (Lira *et al.*, 2016), and VTE5, which catalyzes the first and limiting step of channeling phytol chain into tocopherol biosynthetic pathway (Almeida *et al.*, 2016), were both transcriptionally up-regulated in *PPC::B2*^{Y252H} BK fruits, providing PDP from chlorophyll breakdown. Also, *GGDR* levels were up-regulated in *PPC::B2*^{Y252H} 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). Moreover, *GGDR* transcription has been recently shown to be negatively regulated by PIF3 in tomato (Gramegna *et al.*, 2018). Theoretically, the intensification of PHYB2^{Y252H}-PIF3 interaction due to the constant presence of PHYB2^{Y252H} in pericarp cells nuclei, and consequent reduction in PIF3 protein accumulation, can be at least one of the reasons behind the marked higher *GGDR* expression in *PPC::B2*^{Y252H} fruits from early stages of development until complete

ripening. Besides *GGDR*, *PHYB2*^{Y252H} overexpression promoted the expression of *VTE1*, *VTE4* and *VTE3b*, the latter two already described to positively correlate with fruit vitamin E content in tomato genotypes (Fritsche *et al.*, 2017; Quadrana *et al.*, 2013).

Alongside lycopene, flavonoids and ascorbate are the most abundant health-promoting antioxidants found in ripe tomato fruits (Frusciante *et al.*, 2007). 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::B2*^{Y252H} lines can be at least 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 4d). Similarly, among all core ascorbate biosynthetic genes, only *GME1* was positively correlated with the vitamin C overaccumulation of *PPC::B2*^{Y252H} 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).

PHYB2 manipulation impact over primary metabolism is mainly restricted to reduced soluble sugar levels

Besides playing vital roles in primary metabolism, soluble sugars, organic acids and some aminoacids are also essential determinants of tomato fruit flavor (Carli *et al.*, 2009). Different from the overall PHYB2^{Y252H}-mediated 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).

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 major invertase genes, we identified a selective under-accumulation of transcripts encoding a specific subunit of AGPase, an enzyme responsible for the catalyzing the first and rate-limiting step of starch accumulation (Petreikov *et al.*, 2006; Stark *et al.*, 1992). In tomato, AGPase acts as a heterotetramer composed by two large/regulatory subunits encoded by three *AGPaseL* genes, and two small/catalytic subunits encoded by a single *AGPaseS* gene (Chen *et al.*, 1998; Petreikov *et al.*, 2006). Among them, *AGPaseL1* is the large AGPase subunit most expressed in immature tomato fruits (Bianchetti *et al.*, 2018; Petreikov *et al.*, 2006), also influenced by fruit-localized PHYs (Bianchetti *et al.*, 2018). Interestingly, *PHYB2* and *PHYB2*^{Y252H} overexpression significantly reduced transcript levels of *AGPaseL1* 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::B2*^{Y252H} ripe fruits compared to the WT (Figure 5). Moreover, plastids are significant sites of lipid biosynthesis and storage of lipophilic compounds (Ohlrogge and Browse, 1995); therefore, the significative improvement in chloroplast number and structure registered in the transgenic lines can also be implicated in this phenotypical difference. In fact, a positive relation between plastid abundance and lipid content has also been described in *hp1* fruits (Lenucci *et al.*, 2012), but data are lacking for other tomato genotypes characterized by the production of plastid-rich dark green fruits.

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 clearly surpassed by the use of a light-independent constitutively active version of this photoreceptor. By constantly being active in

the fruit cell nuclei regardless of the surrounding light conditions, PHYB2^{Y252H} probably leads to saturated PHYB2-dependent light signaling. As a consequence, PHYB2 signaling-dependent gene transcription is intensified, including many plastid biogenesis and maturation genes as well as biosynthetic enzymes responsible for the production of antioxidants. Being conserved across species, specific GAF domain Tyr residues offer the opportunity of similar gain-of-function engineering of PHYs as a means to promote biofortification in other fleshy fruits, widening up the range of possible photobiotechnological applications of mutated PHY versions.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L.) plants cv. Micro-Tom, which harbors the wild-type *GLK2* allele (Carvalho *et al.*, 2011) were grown in a chamber under controlled conditions: 250 µmol m^{-2} s^{-1}, 12h photoperiod air temperature of 27ºC day/22ºC night and 60% day/80% night relative air humidity. The fruit stages used were 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 appears on the fruit bottom) and red ripe (RR, characterized as 12 days after the BK stage). All fruits were harvested at the same time of the day (between the 4th and 6th hour of light period) in four biological replicates (each replicate was composed of a pool of 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.

Plasmid constructs and tomato transformation

Solanum lycopersicum PHYB2 full-length coding sequence was amplified via Platinum SuperFi Green PCR Master Mix (Thermo Scientific) using tomato fruit cDNA as template and the oligonucleotide sequences detailed in Supplementary Table S1. Each fragment was inserted into entry vectors pDON221 (Thermo

Scientific) and recombined with pK7m24GW,3 and pEN-L4-PPC-R1 plasmids 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 aminoacid translation from Tyr^{252} to His^{252} was inserted using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent), resulting in the *PPC2::PHYB2*^{Y252H} construct.

All the plasmids were introduced in *Agrobacterium tumefasciens* EHA105 following the protocol described by Bianchetti *et al.* (2018). Oligonucleotides used for plasmid construction and selection of transgenic plants are listed in Table S14.

RNA sequencing, reads mapping and differential expression analysis

RNA extraction was performed as described in Bianchetti *et al.* (2018) and RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA sequencing was performed using Illumina HiSeq 2500 System. The filtering of low-quality reads, primers sequences and vectors were performed by the program Seqyclean v.1.9.10 (https://bitbucket.org/izhbannikov/seqyclean), using cutoff bases with average quality lower than 24QScore. The database used was Univec (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html). After filtering, reads with length less than 65pb were removed.

Filtered reads were mapped and counted using the package STAR 2.6.1 to the genome sequences of *Solanum lycopersicum* downloaded from SOL Genomics Network database (ITAG3.2 version, SGN, http://solgenomics.net/organism/Solanum_lycopersicum/genome).

Only the uniquely mapped reads were used for differential expression analysis. The edgeR program (Robinson *et al.*, 2010) from R/Bioconductor package was used to obtain the differentially expressed genes. Within the program, the data from each group were normalized with TMM (Robinson and Oshlack, 2010) using the calcNormFactors function. Contigs with expression profiles of zero in at least three samples were removed in order to avoid artifacts caused by low expression contigs. Differential expression analysis was performed using the negative binomial function, applying the Benjamini-Hochberg correction (Benjamini and

Hochberg, 1995) for multiple tests to avoid false positives. Only genes with FDR < 0.05 were considered differentially expressed.

RT-qPCR analysis

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

Plastid ultrastructure and abundance

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

Chlorophyll, carotenoid and tocopherol profiling

For chlorophyll and carotenoid extraction, approximately 20 mg dry weight (DW) of powdered pericarp samples were homogenized with a solution of 300 µL of saturated NaCl, then 200 µL of dichloromethane, and finally 1 mL of 1:1 (v/v) hexane: diethyl ether. The supernatant was collected after centrifugation (5000 *g*, 10 min, 4ºC). The remaining pigments in the pellet were extracted three more times with 500 µL of the 1:1 (v/v) hexane: diethyl ether solution. All supernatant fractions were combined, completely vacuum-dried, and suspended with 300 µL of acetonitrile. Chlorophyll *a*, chlorophyll *b*, phytoene, phytofluene, lycopene, β-carotene and lutein levels were determined by high-performance liquid chromatography carried out as described by Cruz *et al.* (2018). Tocopherol extraction and quantification were performed as described in Lira *et al.* (2017).

Antioxidant activity, flavonoids and ascorbate quantification

Approximately 100 mg 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). Rutin, kaempferol rutinoside, naringenin glucoside and naringenin chalcone were quantified by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) (LCI 1260 – Agilent Technologies) using a Zorbax C18 column (150 x 4.6 mm, 3.5 µm particle diameter) at 45ºC with a flow rate of 1 ml min^{-1}. The mobile phase was a gradient of 0.1% acetic acid (A) and acetonitrile (B): 0 to 6 min with 85% A:15% B, 6 to 20 min with 70% A: 30% B, 20 to 30 min with 100% B. Eluted compounds were detected and quantified at 280 and 352 nm wavelengths. Ascorbate extraction and quantification were performed as described by Davey *et al.* (2003).

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 (during the blooming period). Analyses were performed using a portable LI-6400XTR infrared gas analyzer (LI-COR Biosciences, Lincoln, NE, USA) adjusted to a constant chamber temperature of 25°C, reference CO_2 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 (Walz, Effeltrich, Germany) following the protocol and derived calculations described in Alves *et al.* (2016).

Nontargeted metabolite profiling

Nontargeted mass spectrometry (MS)-based metabolite profiling of red ripe fruits followed the protocols described by Lisec *et al.* (2006) for polar compounds and Bligh and Dyer (1959), Fiehn *et al.* (2000) and Ichihara and Fukubayashi (2010) for apolar compounds, with modifications.

For apolar metabolite extraction, 1g FW of powdered fruit pericarp was mixed with 2.5 mL of chloroform: methanol solution at a proportion of 1:2 (v:v) added with 65 µg of tridecanoic acid as the internal standard. The mixture was extracted for 30 min at 4°C under agitation, mixed with 1.25 mL chloroform and 1.25 mL sodium sulfate 1.5% (m:v) and shaken for further 5 min at 4°C. After centrifugation at 1,000 *g* for 5 min at 4°C, the upper phase was discarded, 0.25 g of sodium sulfate was added, vortexed and centrifuged at 2,000 *g* for 5 min. The supernatant was transferred to appropriated vials and reduced to dryness under N_2 flow. For the first derivatization, samples were resuspended in 1.2 mL of hexane:toluene 5:1 (v:v), 1.5 mL methanol and 300 µL HCl 8% (v:v methanol), vortexed for 30 s and incubated for 90 min at 100°C. Sequentially, 1 mL of hexane and 1 mL of $_dH_2O$ were added to the extract, vortexed for 30 s and dried under N_2 flow. For the second derivatization, samples were resuspended in 240 µL hexane, 20 µL pyridine and 20 µL MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide), vortexed until complete solubilization and transferred to vials for GC-MS injection.

Metabolite profile analyses were carried out in a gas chromatograph (Agilent Technologies 7890B, USA) equipped with auto-injector (CombiPAL, CG sampler 80, USA) and a mass-selective filter (Agilent Technologies 5977A, USA). Metabolites were separated in a HP 5MS UI column (30m x 0.25 mm x 0.25 µm/ Agilent Technologies, USA). Samples were injected at 250°C in splitless mode using helium as carrier gas under 1 mL min^{-1} flow rate. Column temperature

programming set was adjusted at 60°C for 1 min, ramp temperature of 5°C per minute until 325°C, maintained for 10 min. MS parameters were: ion source set at 230°C, interface set at 290°C, mass range 50-600 m/z scanned at 2.7 scans per second.

Statistical analyses

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

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

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Table S12. Photosynthetic parameters, chlorophyll and starch contents in leaves of wild-type and transgenic plants.
Table S13. Transcript abundance of sugar-related genes in wild-type and transgenic fruits.
Table S14. Oligonucleotides used in this study.

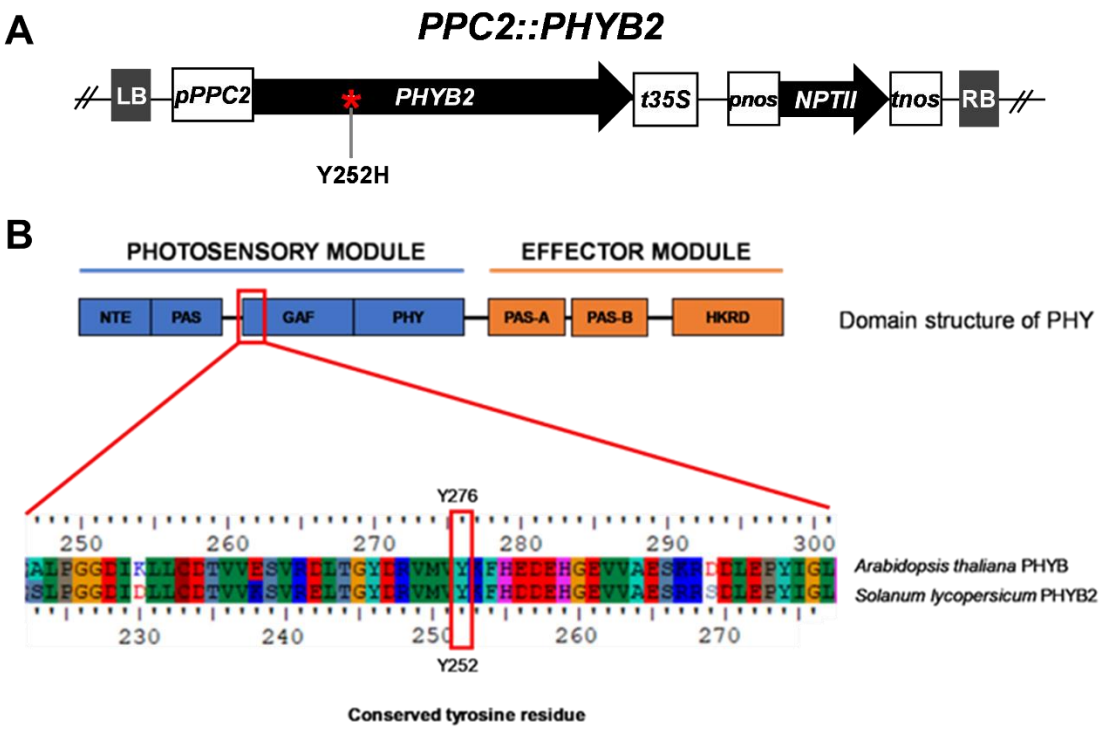

Figure S1. Construct designed for the generation of transgenic lines. A. Linearized scheme of the vector used for the generation of transgenic *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) lines. The tyrosine-to-histidine mutation is highlighted by the red asterisk. **B.** Partial alignment of *Arabidopsis* PHYB and tomato PHYB2. The conserved GAF domain Tyr residue is highlighted by the red box, which corresponds to Y^{276} and Y^{252} in *Arabidopsis* PHYB and tomato PHYB2, respectively. LB, left-border; pPPC2, *PHOSPHOENOLPYRUVATE CARBOXYLASE 2* promoter; PHYB2, *PHYTOCHROME B2* gene; t35S, 35S terminator; pnos, nos promoter; responsible for the control of *NPTII* gene expression; *NPTII*, *NEOMYCIN PHOSPHOTRANSFERASE II* gene; tnos, nos terminator; RB, right-border; NTE, N-terminal extension domain; PAS, Per/Arnt/Sim domain; GAF, cGMP fosfodiesterase/Adenylcyclase/FhlA domain; PHY, phytochrome-specific domain; HKRD, histidine kinase-relate domain.

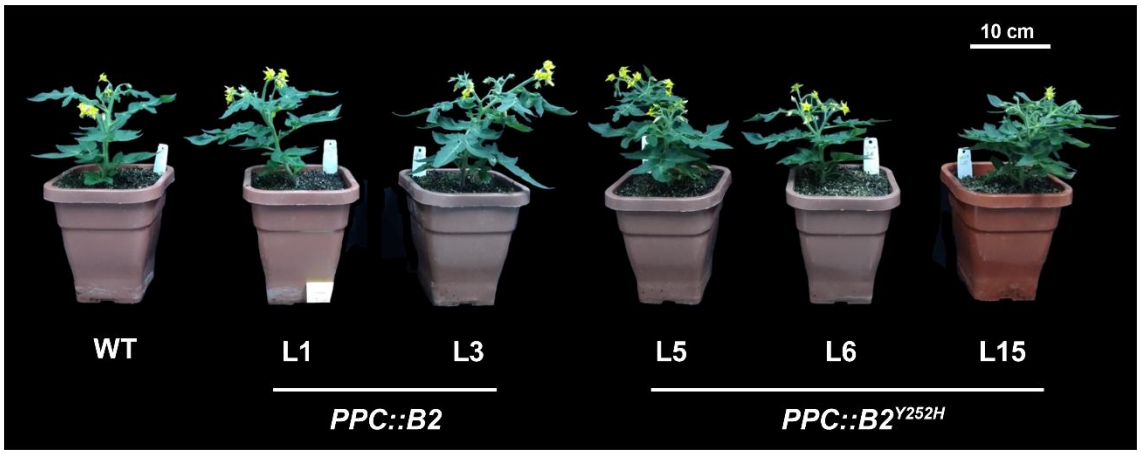

Figure S2. Representative individual tomato plants from wild-type and transgenic lines. WT, wildtype; *PPC::B2*, *PPC2::PHYB2*; *PPC::B2*^{Y252H}, *PPC2::PHYB2*^{Y252H}.

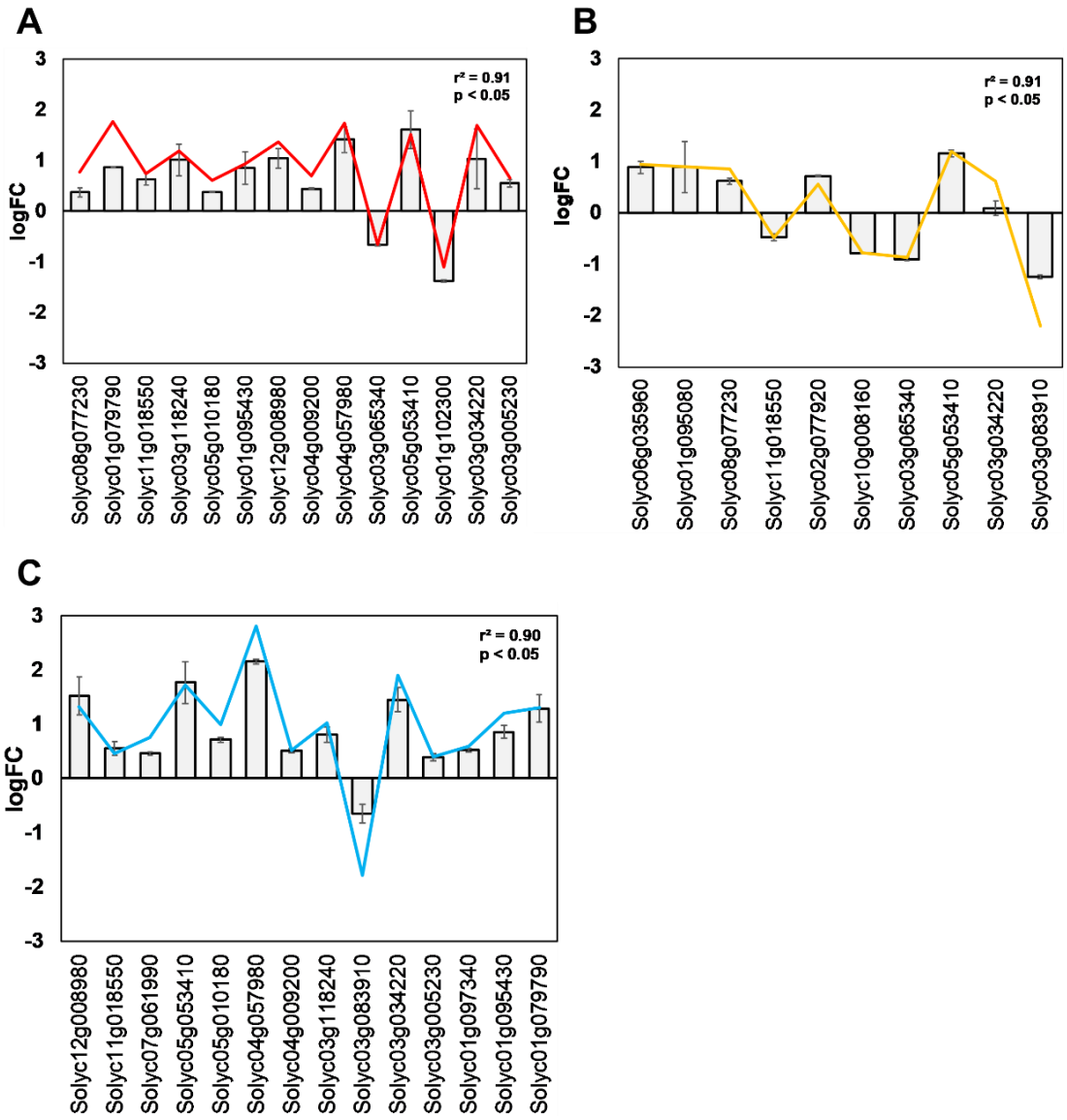

Figure S3. Validation of RNASeq analysis via RT-qPCR. Validation was performed for L6MGxWTMG (A), L1BKxWTBK (B) and L6BKxWTBK (C) comparisons as described in the Table S2. Bars represent mean $\log_2$ relative transcript abundances calculated in the RT-qPCR analyses. Lines represent mean $\log_2$ expression ratios calculated by pairwise DE analysis using edgeR. Pearson correlation was calculated for each comparison and r^2 and p-values are given in each figure.

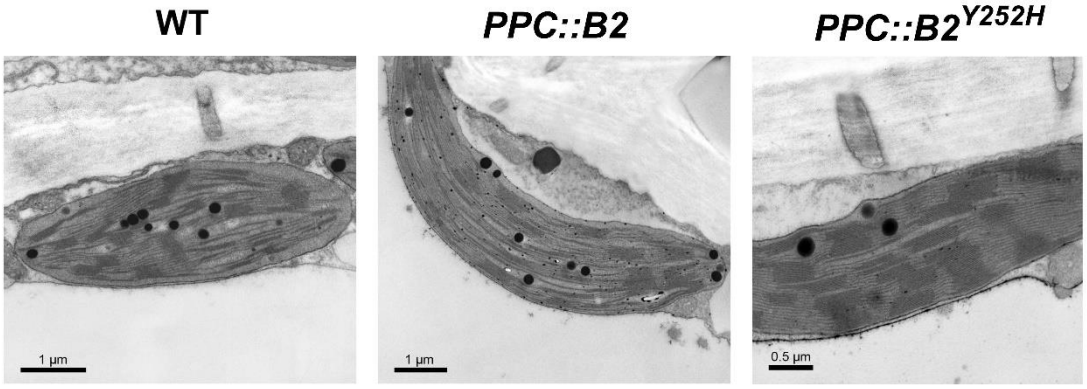

Figure S4. TEM images of plastids of wild-type and transgenic fruits. Transmission electronic microscopy images of plastids of the pedicel region of immature fruits of wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) tomato plants.

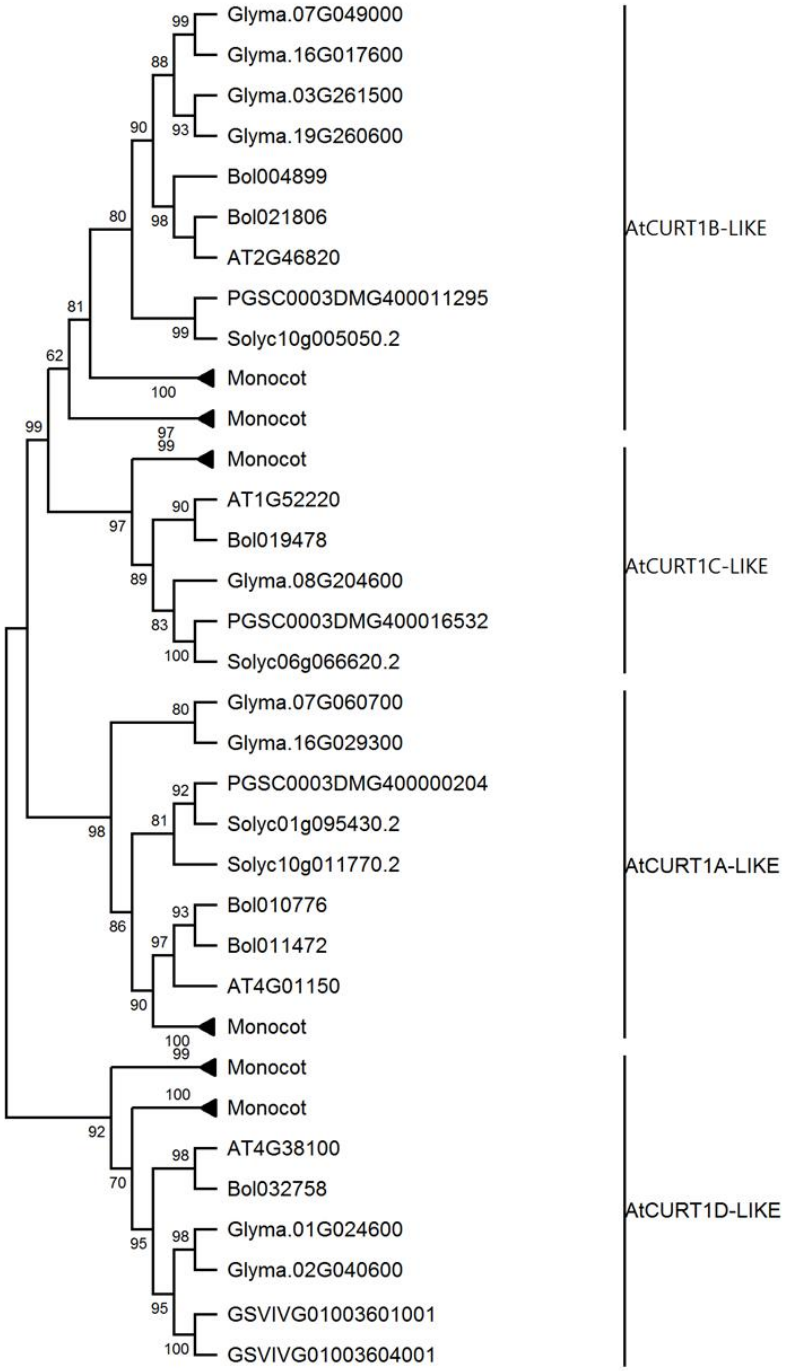

Figure S5. Phylogenetic reconstruction of the CURT protein family. Sequences from the families containing AtCURT proteins were retrieved from Phytozome 12.1 database (https://phytozome.jgi.doe.gov), aligned by ClustalW in MEGA 10.1 software[1] (Kumar et al., 2018) and the tree was reconstructed with PhyML 3.0 algorithm[2] (Guindon et al., 2010) with JTT substitution model and SH-like branch support. Monocot containing clades were compressed in the tree visualization. The groups were named accordingly to *A. thaliana* sequences.

[1] Kumar S *et al.* (2018) *Mol Biol Evol* **35**: 1547-1549.

[2] Guindon S *et al.* (2010) *Syst Biol* **59**: 307-321.

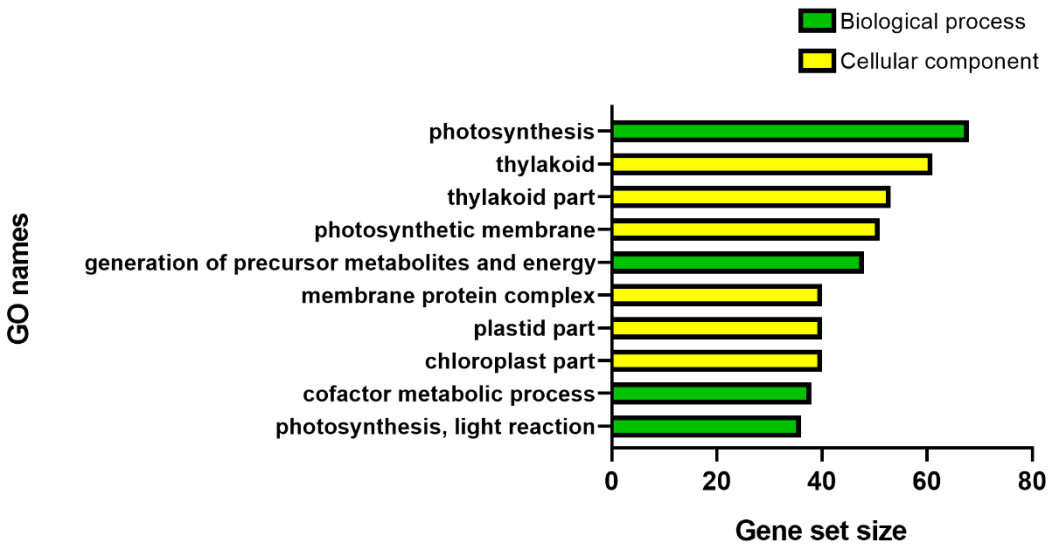

Figure S6. Gene Set Enrichment Analysis (GSEA) of *PPC::B2*^{Y252H} fruits in MG stage compared to wild-type. GO terms are detailed in Table S3.

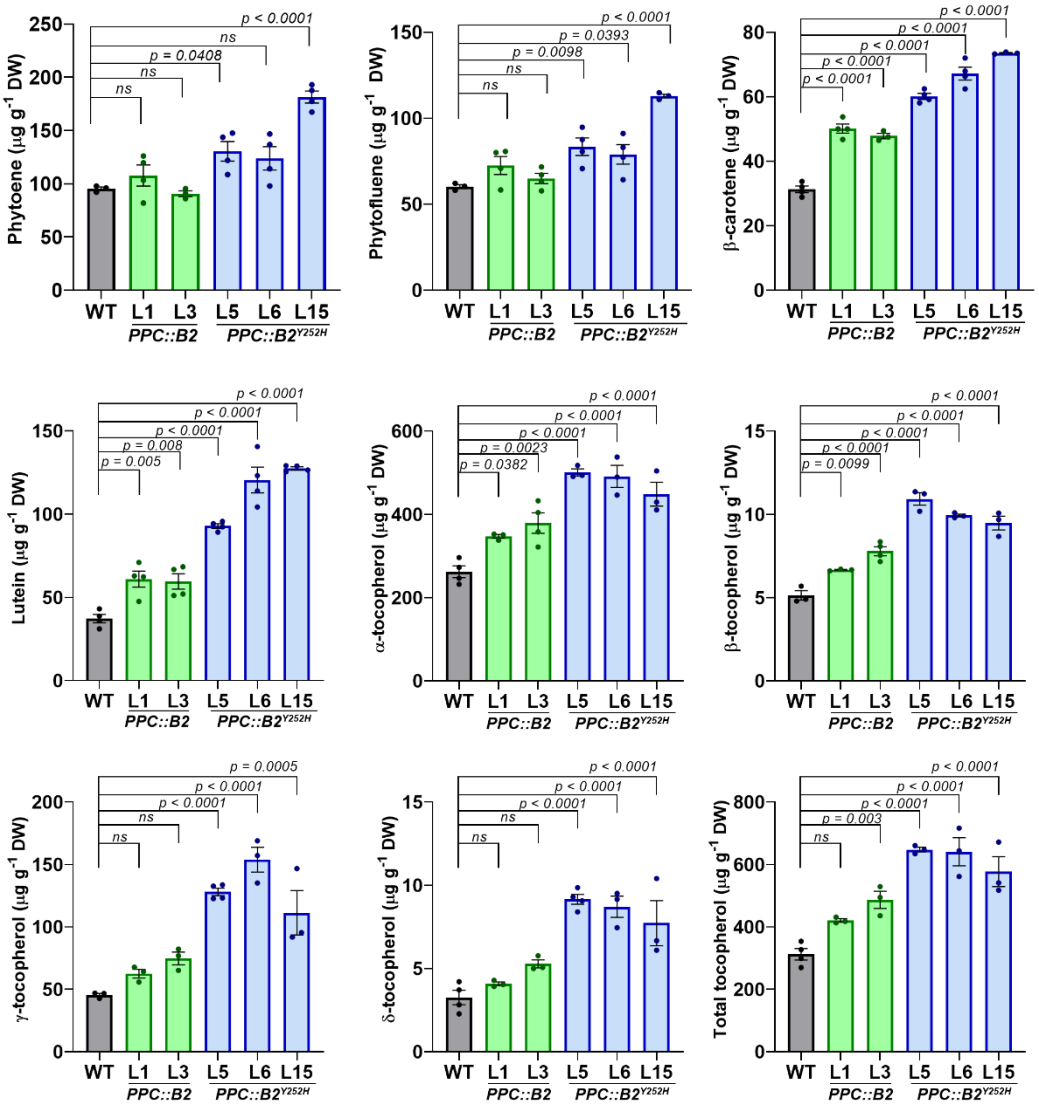

Figure S7. Carotenoid and tocopherol profiles in wild-type and transgenic fruits. Data obtained from red ripe fruits of wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) plants. Data are mean ± SE and dots represent individual values. *p* values are given for each comparison (Dunnett's test with WT as control, α = 0.05). *ns*, non-significant; DW, dry weight.

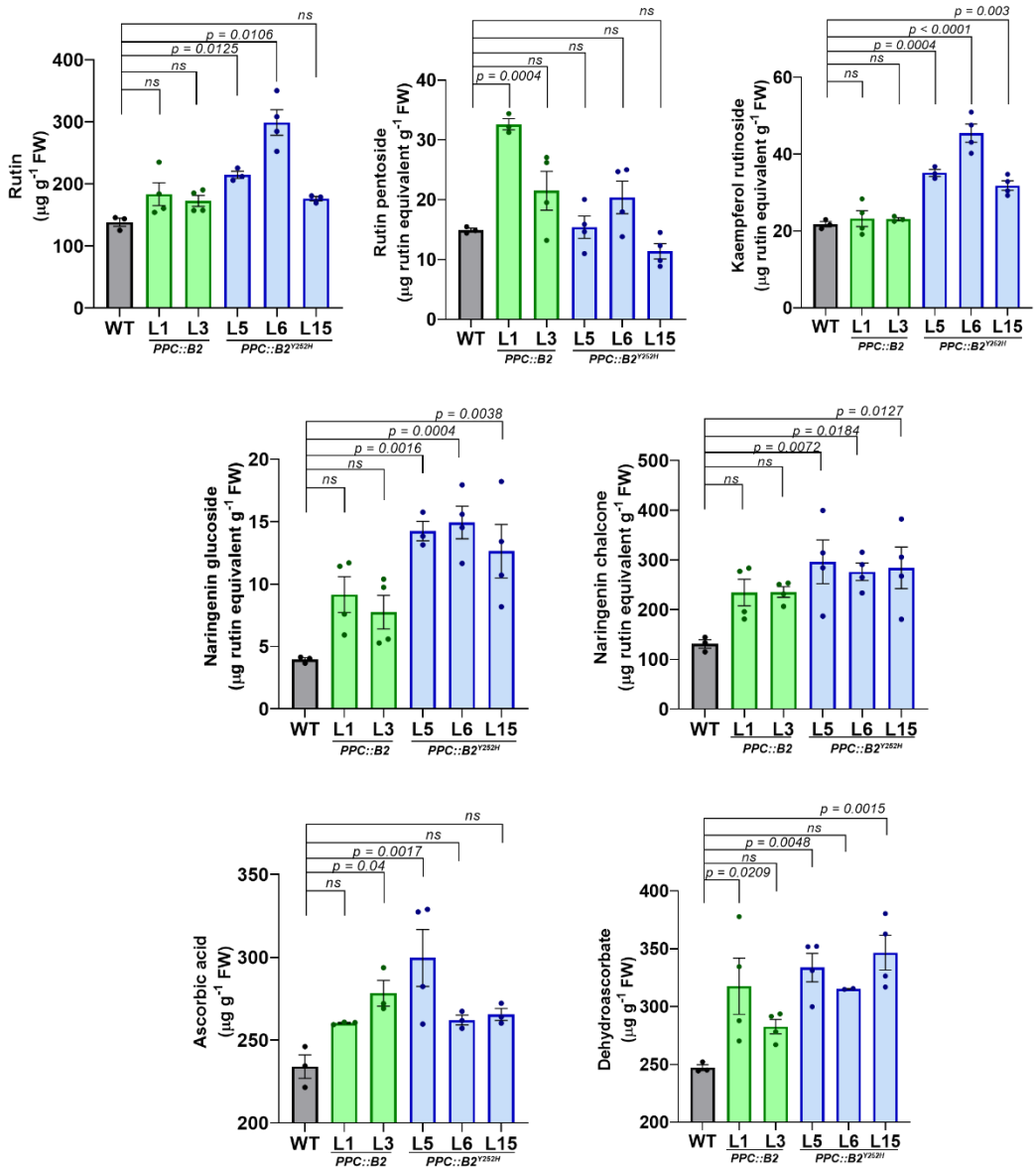

Figure S8. Flavonoid and ascorbate profiles in wild-type and transgenic fruits. Data obtained from red ripe fruits of wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) plants. Data are mean ± SE and dots represent individual values. *p* values are given for each comparison (Dunnett's test with WT as control, α = 0.05). *ns*, non-significant; FW, fresh weight.

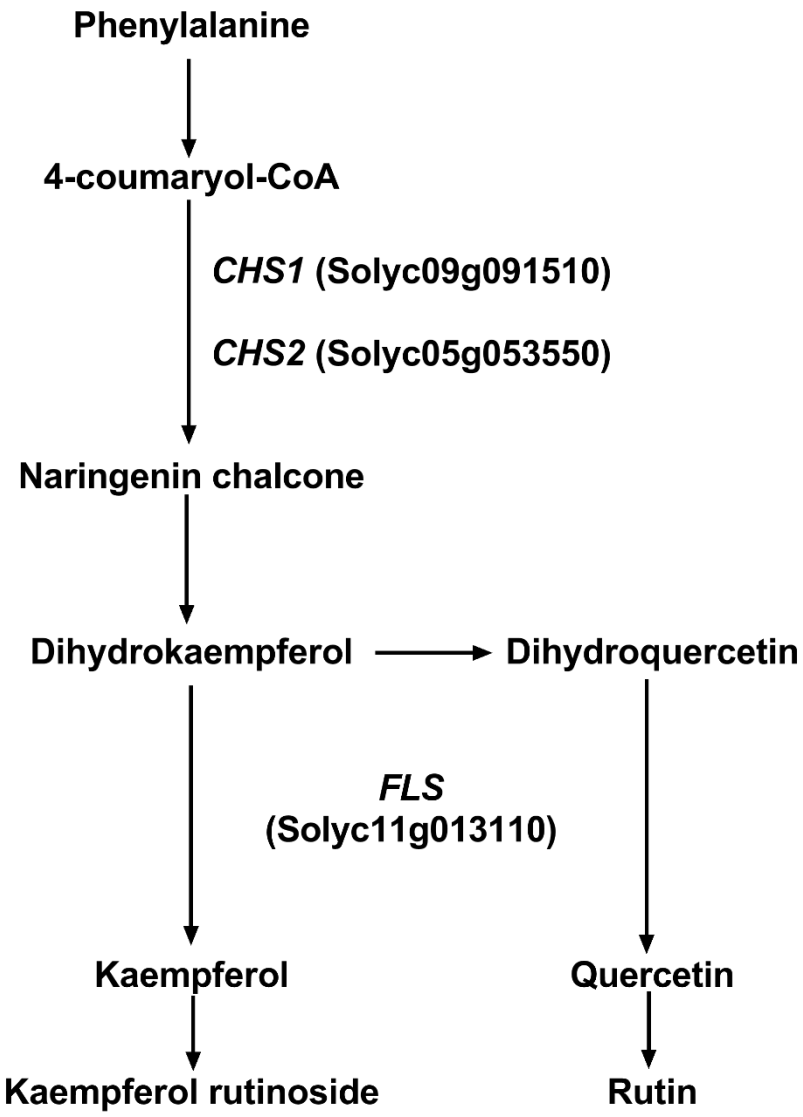

Figure S9. Simplified flavonoid biosynthetic pathway. Intermediate reactions are omitted. Enzyme-coding genes analyzed in transgenic fruits are explicited. Enzymes and corresponding gene IDs according to Sol Genomics Networks iTAG 3.2 annotation are given. Relative transcript values are detailed in Table S9. CHS, chalcone synthase; FLS, flavonol synthase.

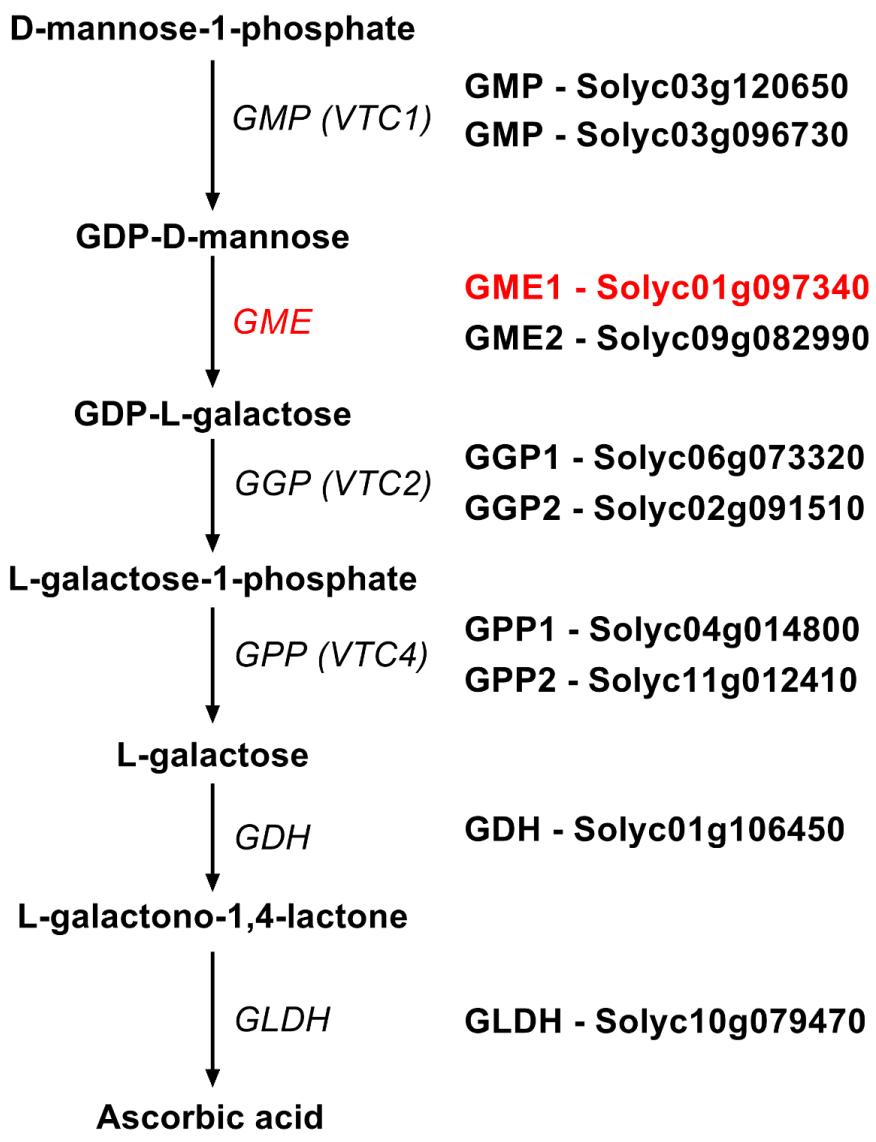

Figure S10. Simplified ascorbate biosynthetic pathway. Enzymes and corresponding gene IDs according to Sol Genomics Networks iTAG 3.2 annotation are given. Up-regulated enzyme-coding gene in *PPC::B2*^{Y252H} fruits compared to the wildtype according to RNASeq analysis (Table S2) is highlighted in red. Gene abbreviations and relative transcript values are detailed in Table S9. GMP, GDP-D-mannose pyrophosphorylase; GME, GDP-D-mannose-3,5-epimerase; GGP, GDP-L-galactose pyrophosphorylase; GPP, L-galactose-1-phosphate phosphatase; GDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase.

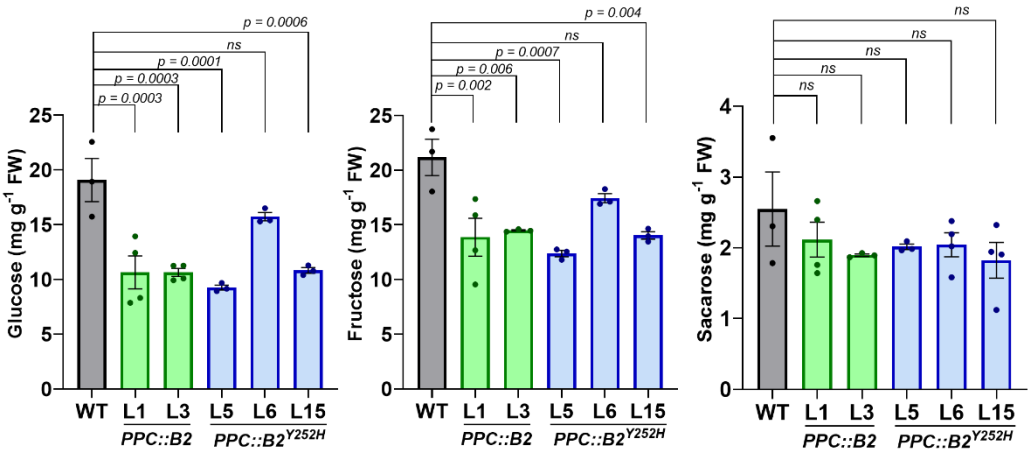

Figure S11. Soluble sugar profiles in wild-type and transgenic fruits. Data obtained from red ripe fruits of wild-type (WT), *PPC2::PHYB2* (*PPC::B2*) and *PPC2::PHYB2*^{Y252H} (*PPC::B2*^{Y252H}) plants. Data are mean ± SE and dots represent individual values. *p* values are given for each comparison (Dunnett's test with WT as control, α = 0.05). *ns*, non-significant; FW, fresh weight.

Table S1. Transcript abundance of phytochrome-encoding genes in wild-type and transgenic fruits.

		Phytochrome-encoding genes				
		PHYA	***PHYB1***	***PHYB2***	***PHYE***	***PHYF***
Tomato fruit stage	**Genotype**	*Phytochrome A*	*Phytochrome B1*	*Phytochrome B2*	*Phytochrome E*	*Phytochrome F*
IMG	WT	1 ± 0.07	1 ± 0.05	1 ± 0.06 a	1 ± 0.14 a	1 ± 0.08 ab
	PPC::B2 - L1	1.13 ± 0.1	0.82 ± 0.07	**3.35 ± 0.15 c**	1.19 ± 0.02 a	1.16 ± 0.09 a
	PPC::B2 - L3	0.68 ± 0.19	0.86 ± 0.05	**2.43 ± 0.04 b**	**0.48 ± 0.05 b**	0.69 ± 0.04 b
	PPC::B2 ^{Y252H} - L5	1.11 ± 0.07	0.97 ± 0	**4.21 ± 0.31 d**	1.1 ± 0.05 a	1.21 ± 0.07 a
	PPC::B2 ^{Y252H} - L6	1.17 ± 0.14	0.87 ± 0.03	**2.01 ± 0.09 b**	1.07 ± 0.04 a	1.04 ± 0.06 a
	PPC::B2 ^{Y252H} - L15	1.18 ± 0.1	0.95 ± 0.03	**4.25 ± 0.19 d**	1.12 ± 0.13 a	1.09 ± 0.02 a
MG	WT	1.42 ± 0.06 ab	1.09 ± 0.03 a	1.3 ± 0.05 a	1.07 ± 0.06 ab	1.51 ± 0.08 ab
	PPC::B2 - L1	1.4 ± 0.03 ab	1.06 ± 0.05 a	**4.2 ± 0.4 b**	1.04 ± 0.02 a	1.69 ± 0.1 a
	PPC::B2 - L3	1.4 ± 0.08 ab	1 ± 0.01 a	**5.67 ± 0.42 b**	0.99 ± 0 a	1.54 ± 0.05 ab
	PPC::B2 ^{Y252H} - L5	1.31 ± 0 a	0.97 ± 0.03 a	**4 ± 0.72 b**	1.01 ± 0.03 a	1.35 ± 0.05 b
	PPC::B2 ^{Y252H} - L6	1.34 ± 0.05 a	0.95 ± 0.01a	**3.94 ± 0.48 b**	0.97 ± 0.04 a	1.47 ± 0.03 ab
	PPC::B2 ^{Y252H} - L15	1.64 ± 0.09 b	1.38 ± 0.1 b	**9.48 ± 0.29 c**	**1.25 ± 0.03 b**	1.31 ± 0 b
BK	WT	3.73 ± 0.66 abc	0.44 ± 0.17 a	0.69 ± 0.06 a	0.41 ± 0.02 ab	1.61 ± 0.06
	PPC::B2 - L1	3.46 ± 0.34 bc	0.65 ± 0.22 a	**1.55 ± 0.05 b**	0.34 ± 0.02 ab	1.75 ± 0.05
	PPC::B2 - L3	3.01 ± 0.31 c	0.27 ± 0.01 a	**1.47 ± 0.17 b**	0.3 ± 0.05 b	1.62 ± 0.18
	PPC::B2 ^{Y252H} - L5	**5.81 ± 0.44 a**	0.33 ± 0 b	**2.51 ± 0.02 cd**	0.53 ± 0.07 a	1.84 ± 0.07
	PPC::B2 ^{Y252H} - L6	2.75 ± 0.06 c	0.29 ± 0.01 ab	**2.37 ± 0.27 c**	0.45 ± 0.06 ab	1.71 ± 0.04
	PPC::B2 ^{Y252H} - L15	5.34 ± 0.78 ab	**0.31 ± 0.01 b**	**3.12 ± 0.18 d**	0.39 ± 0.01 ab	1.7 ± 0.11
RR	WT	1.22 ± 0.17 a	0.18 ± 0.01 ab	0.12 ± 0 a	0.05 ± 0	0.33 ± 0.01
	PPC::B2 - L1	0.83 ± 0.11 a	0.2 ± 0.01 ab	**0.38 ± 0.04 bc**	0.04 ± 0.01	0.35 ± 0
	PPC::B2 - L3	1.41 ± 0.02 ab	0.21 ± 0.01 a	0.24 ± 0 ab	0.05 ± 0.01	0.35 ± 0.01
	PPC::B2 ^{Y252H} - L5	1.66 ± 0.14 ab	**0.21 ± 0 a**	**0.54 ± 0.08 c**	0.05 ± 0	0.34 ± 0.01
	PPC::B2 ^{Y252H} - L6	**3.26 ± 0.28 c**	0.16 ± 0.01 b	0.29 ± 0.02 ab	0.03 ± 0.01	0.38 ± 0.05
	PPC::B2 ^{Y252H} - L15	**2.76 ± 0.56 bc**	**0.2 ± 0 ab**	**0.43 ± 0.07 bc**	0.05 ± 0.01	0.31 ± 0.03

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* ^{Y252H}.

Tables S2 and S3 are available on digital media due to their large size.

Table S4. Transcript abundance of plastid-related genes in wild-type and transgenic fruits.

		Plastid-related genes								
		GLK2	***ARF4***	***APRR2Like***	***BEL11***	***PDV2***	***RBCS2a***	***CURT1a1***	***CURT1a2***	***POR1***
Tomato fruit stage	**Genotype**	*Golden 2-Like 2*	*Auxin Response Factor 4*	*Arabidopsis Pseudo-Response Regulator 2-Like*	*Bel1-Like Homeodomain 11*	*Plastid Division 2*	*RuBisCO small chain 2a*	*Curvature thylakoid 1a-1*	*Curvature thylakoid 1a-2*	*Protochlorophyllide oxidoreductase 1*
IMG	WT	1 ± 0.07 abc	1 ± 0.06 a	1 ± 0.02	1 ± 0.12 ab	1 ± 0.01 a	1 ± 0.03 a	1.00 ± 0.02 a	1 ± 0.06 a	1 ± 0.03 ab
	PPC::B2 - L1	0.76 ± 0.05 bc	1.1 ± 0 a	1.06 ± 0.05	1.29 ± 0.04 b	**1.31 ± 0.02 b**	1.67 ± 0.09 ab	1.01 ± 0.07 ab	1.48 ± 0.1 ab	0.94 ± 0.12 a
	PPC::B2 - L3	0.68 ± 0.15 c	**0.75 ± 0.02 b**	0.74 ± 0.01	0.65 ± 0.18 ab	**1.3 ± 0.03 b**	1.86 ± 0.56 abc	1.05 ± 0.03 ab	1.43 ± 0.15 ab	0.93 ± 0.3 a
	PPC::B2 ^{Y252H} - L5	1.37 ± 0.14 bc	1.18 ± 0.01 a	0.87 ± 0.16	0.85 ± 0.03 a	**1.28 ± 0.07 ab**	**4.02 ± 1.02 c**	**1.50 ± 0.09 c**	**2.21 ± 0.18 b**	**2.02 ± 0.18 c**
	PPC::B2 ^{Y252H} - L6	1.18 ± 0.05 abc	1.06 ± 0.02 a	0.99 ± 0.08	0.96 ± 0.06 ab	1.23 ± 0.04 ab	**3.53 ± 0.27 bc**	**1.26 ± 0.06 bc**	**1.78 ± 0.3 ab**	**1.82 ± 0.01 bc**
	PPC::B2 ^{Y252H} - L15	1.44 ± 0.24 a	0.97 ± 0.06 a	1.16 ± 0.08	0.88 ± 0.06 a	**1.36 ± 0.09 b**	**3.78 ± 0.29 c**	**1.48 ± 0.02 c**	**2.2 ± 0.12 b**	**2.22 ± 0.08 c**
MG	WT	1.06 ± 0.05 a	1.36 ± 0.01	0.62 ± 0.01	2 ± 0.04 a	2.05 ± 0.04	2 ± 0.56	0.17 ± 0.02	0.12 ± 0.01 a	0.37 ± 0.03
	PPC::B2 - L1	0.88 ± 0.04 a	1.5 ± 0.03	0.67 ± 0.1	**1.51 ± 0.15 c**	1.89 ± 0.22	3.56 ± 1.19	0.17 ± 0.02	0.13 ± 0.02 ab	1.12 ± 0.43
	PPC::B2 - L3	0.73 ± 0.06 a	1.4 ± 0.04	0.71 ± 0.11	1.9 ± 0.05 ab	1.99 ± 0.22	1.05 ± 0.06	0.16 ± 0.01	0.12 ± 0.01 a	0.42 ± 0.02
	PPC::B2 ^{Y252H} - L5	1.06 ± 0.21 a	1.22 ± 0.07	0.56 ± 0.01	**1.59 ± 0.06 bc**	1.98 ± 0.01	2.45 ± 0.29	0.21 ± 0.01	0.16 ± 0.01 ab	0.84 ± 0.14
	PPC::B2 ^{Y252H} - L6	1.08 ± 0.01 a	1.21 ± 0.16	0.8 ± 0.06	**1.54 ± 0 bc**	2.14 ± 0.13	4.05 ± 1.17	**0.30 ± 0.05**	0.21 ± 0 ab	2.09 ± 0.69
	PPC::B2 ^{Y252H} - L15	2.18 ± 0.32 b	1.38 ± 0.13	0.67 ± 0.05	2.31 ± 0.02 a	1.61 ± 0.1	4.11 ± 1.03	0.28 ± 0.04	**0.26 ± 0.06 b**	**2.3 ± 0.77**
BK	WT	0.66 ± 0.09 ab	0.09 ± 0	1.05 ± 0.07 a	1.35 ± 0.04	2.04 ± 0.13 a	0.38 ± 0.02 a	0.10 ± 0.00 a	0.11 ± 0.01 a	0.07 ± 0.01 a
	PPC::B2 - L1	**0.38 ± 0.01 bc**	0.09 ± 0	**1.6 ± 0.01 b**	1.27 ± 0.06	**1.64 ± 0.03 b**	0.41 ± 0.05 a	0.10 ± 0.01 a	0.1 ± 0 a	0.13 ± 0.02 a
	PPC::B2 - L3	**0.28 ± 0.02 c**	0.09 ± 0.01	1.39 ± 0.12 ab	1.22 ± 0.03	1.71 ± 0.04 ab	0.36 ± 0.01 a	0.10 ± 0.00 a	0.11 ± 0.01 a	0.12 ± 0.03 a
	PPC::B2 ^{Y252H} - L5	**1.04 ± 0.04 d**	0.11 ± 0	1.25 ± 0 ab	1.33 ± 0.05	1.94 ± 0.07 ab	**1.36 ± 0.01 b**	**0.22 ± 0.02 b**	**0.23 ± 0.02 b**	**0.3 ± 0.03 b**
	PPC::B2 ^{Y252H} - L6	0.77 ± 0 ad	0.09 ± 0.01	1.26 ± 0.04 ab	1.4 ± 0.1	1.97 ± 0.1 ab	**1.04 ± 0.09 bc**	**0.19 ± 0.01 b**	**0.19 ± 0.02 b**	**0.27 ± 0.02 b**
	PPC::B2 ^{Y252H} - L15	0.86 ± 0.09 ad	0.11 ± 0	1.19 ± 0.23 ab	1.51 ± 0.11	1.72 ± 0.04 ab	**0.96 ± 0.08 c**	**0.20 ± 0.01 b**	**0.21 ± 0.02 b**	**0.25 ± 0.03 b**
RR	WT	0.01 ± 0	0.01 ± 0 a	1.15 ± 0.06 a	1 ± 0.08	1.73 ± 0.11 a	0.02 ± 0 a	0.46 ± 0.02	0.02 ± 0 a	0.04 ± 0.02
	PPC::B2 - L1	0.01 ± 0	0.01 ± 0 ab	**2.13 ± 0.2 b**	0.93 ± 0.03	1.99 ± 0.07 ab	0.04 ± 0.01 ab	0.57 ± 0.08	0.02 ± 0 a	0.04 ± 0
	PPC::B2 - L3	0.01 ± 0	0.01 ± 0 ab	**2.03 ± 0.2 b**	0.93 ± 0.09	1.87 ± 0.05 a	0.04 ± 0 ab	0.48 ± 0.01	0.04 ± 0 ab	0.08 ± 0.02
	PPC::B2 ^{Y252H} - L5	0.01 ± 0	0.01 ± 0 ab	1.66 ± 0.03 ab	0.88 ± 0.05	1.8 ± 0.13 a	**0.08 ± 0.01 c**	**0.70 ± 0.10**	0.03 ± 0 a	0.05 ± 0.01
	PPC::B2 ^{Y252H} - L6	0.01 ± 0	**0.01 ± 0 b**	1.68 ± 0.14 ab	0.91 ± 0.08	2.07 ± 0 ab	0.05 ± 0.01 abc	0.47 ± 0.03	0.03 ± 0 a	0.04 ± 0.01
	PPC::B2 ^{Y252H} - L15	0.01 ± 0	0.01 ± 0 ab	**2.51 ± 0.31 b**	1 ± 0.07	**2.39 ± 0.12 b**	**0.06 ± 0.01 bc**	0.58 ± 0.02	**0.05 ± 0.01 b**	0.06 ± 0.02

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* ^{Y252H}.

Table S5. Chlorophyll, carotenoid and tocopherol content in wild-type and transgenic fruits.

Stage	Genotype	Isoprenoid content (µg g^{-1} DW)											
		Chlorophyll a	***Chlorophyll b***	***Phytoene***	***Phytofluene***	***Lycopene***	***β-carotene***	***Lutein***	***α-tocopherol***	***β-tocopherol***	***Y-tocopherol***	***δ-tocopherol***	***Total tocopherol***
IMG	WT	539.83 ± 57.66 a	202.55 ± 12.14 a	0.2 ± 0.1 a	10.6 ± 0.14 a	ND	44.66 ± 0.74 a	89.6 ± 0.7 a	186.07 ± 6.12 a	3.97 ± 0.21	7.17 ± 0.66 a	ND	199.62 ± 7.89 a
	PPC::B2 - L1	691.04 ± 99.72 a	**288.51 ± 8.97 a**	0.64 ± 0.16 ab	12.98 ± 0.17 a	ND	55.43 ± 0.5 a	105.43 ± 1.48 a	206.07 ± 5.16 ab	4.41 ± 0.41	9.67 ± 0.75 ab	ND	220.85 ± 6.70 ab
	PPC::B2 - L3	651.59 ± 93.89 a	**290.17 ± 18.98 a**	0.49 ± 0.01 ab	12.18 ± 0.93 a	ND	51.91 ± 2.86 a	110.26 ± 5.62 a	180.83 ± 6.16 a	3.84 ± 0.42	**11.92 ± 0.99 b**	ND	195.09 ± 7.14 a
	PPC::B2^{Y252H} - L5	**1654.27 ± 115.72 b**	**537.72 ± 27.51 b**	0.68 ± 0.02 ab	**21.64 ± 0.7 b**	ND	**86.3 ± 3.16 b**	**186.29 ± 9.74 b**	**231.70 ± 12.97 b**	4.20 ± 0.18	**11.26 ± 0.86 b**	ND	**252.16 ± 15.81 b**
	PPC::B2^{Y252H} - L6	**1781.69 ± 89.99 b**	**582.23 ± 28.33 b**	**0.88 ± 0.16 b**	**21.17 ± 0.94 b**	ND	**88.08 ± 2.28 b**	**199.67 ± 5.88 b**	214.51 ± 6.77 ab	4.02 ± 0.14	**10.20 ± 0.23 ab**	ND	231.32 ± 9.79 ab
	PPC::B2^{Y252H} - L15	**1599.07 ± 27.59 b**	**549.15 ± 8.93 b**	**0.78 ± 0.01 ab**	**22.09 ± 0.85 b**	ND	**86.35 ± 3.46 b**	**188.57 ± 8.98 b**	**236.75 ± 0.89 b**	4.32 ± 0.02	**10.20 ± 0.3 ab**	ND	**251.35 ± 0.90 b**
MG	WT	301.84 ± 12.27 a	151.31 ± 3.3 a	0.25 ± 0.1	7.05 ± 0.14 a	ND	30.91 ± 0.79 a	61.46 ± 3.51 a	148.20 ± 13.50 a	2.85 ± 0.23 a	7.21 ± 1.26	ND	158.26 ± 14.78 a
	PPC::B2 - L1	595.86 ± 78.63 ab	291.34 ± 17.26 ab	0.36 ± 0.01	11.52 ± 0.92 ab	ND	49.44 ± 0.11 ab	95.61 ± 4.34 a	170.97 ± 10.86 a	3.07 ± 0.18 ab	6.48 ± 0.45	ND	181.51 ± 11.19 a
	PPC::B2 - L3	384.68 ± 12.68 a	197.43 ± 11.69 a	0.29 ± 0.08	8.8 ± 0.39 a	ND	38.55 ± 1.61 a	88.75 ± 9.35 a	167.54 ± 6.07 a	3.47 ± 0.13 ab	6.17 ± 0.52	ND	177.11 ± 6.48 a
	PPC::B2^{Y252H} - L5	**960.19 ± 15.94 bc**	**406.03 ± 19.61 b**	0.26 ± 0.06	**15.97 ± 0.34 b**	ND	**62.92 ± 1.35 b**	**136.62 ± 5.97 b**	**209.43 ± 4.77 b**	3.33 ± 0.05 ab	7.43 ± 0.07	ND	**220.65 ± 4.75 b**
	PPC::B2^{Y252H} - L6	**1519.97 ± 67.27 d**	**727.85 ± 59.82 c**	0.39 ± 0.1	**23.17 ± 1.69 c**	ND	**90.7 ± 6.11 c**	**245.14 ± 7.76 c**	**269.59 ± 8.52 c**	**4.19 ± 0.09 b**	11.60 ± 2.23	ND	**286.36 ± 6.01 c**
	PPC::B2^{Y252H} - L15	**1214.14 ± 149.57 cd**	**453.61 ± 43.81 b**	0.49 ± 0.09	**16.51 ± 1.48 b**	ND	**65.73 ± 4.72 b**	**147.9 ± 13.17 b**	**248.03 ± 2.19 bc**	**4.08 ± 0.03 b**	8.15 ± 0.29	ND	**260.93 ± 2.79 c**
BK	WT	28.04 ± 28.04	56.22 ± 3.15 a	9.75 ± 0.58	4.65 ± 0.32	ND	11.29 ± 0.64 a	19.48 ± 0.68 a	206.30 ± 1.55 a	3.65 ± 0.03 a	31.25 ± 1.23 a	2.03 ± 0.03 a	241.72 ± 0.90 a
	PPC::B2 - L1	92.55 ± 1.59	80.52 ± 4.58 ab	6.26 ± 1.2	3.29 ± 0.59	ND	11.87 ± 0.78 a	35.68 ± 1.61 a	**269.64 ± 4.49 bc**	**4.49 ± 0.06 bc**	**45.17 ± 1.28 ab**	**3.37 ± 0.12 c**	**322.44 ± 5.97 bc**
	PPC::B2 - L3	46.35 ± 26.78	71.43 ± 6.68 a	5.67 ± 1.86	1.95 ± 0.24	ND	15.04 ± 2.01 a	35.72 ± 1.16 a	**266.82 ± 10.3 b**	**4.67 ± 0.04 bc**	38.26 ± 2.80 bc	**2.78 ± 0.23 bc**	**306.53 ± 14.71 b**
	PPC::B2^{Y252H} - L5	**140.43 ± 11.14**	**179.25 ± 12.48 bc**	10.86 ± 2.48	4.97 ± 1.1	ND	**31.42 ± 5.09 b**	**80.65 ± 2.32 b**	**299.35 ± 3.62 cd**	**4.45 ± 0.04 ab**	31.87 ± 2.30 a	**2.57 ± 0.11 ab**	**339.20 ± 4.66 bc**
	PPC::B2^{Y252H} - L6	68.17 ± 34.99	**234.95 ± 52.87 c**	6.65 ± 0.94	3.91 ± 0.85	ND	**38.12 ± 4.72 b**	**126.51 ± 17.03 c**	**334.9 ± 5.70 e**	**4.76 ± 0.05 bc**	**53.27 ± 3.77 c**	**3.39 ± 0.09 c**	**405.43 ± 11.97 d**
	PPC::B2^{Y252H} - L15	**133.62 ± 19.68**	146.01 ± 11.02 abc	6.05 ± 1.09	2.75 ± 0.44	ND	**32.05 ± 1.44 b**	**96.41 ± 3.22 b**	**324.29 ± 6.61 de**	**4.73 ± 0.10 c**	34.31 ± 1.43 ab	2.46 ± 0.11 ab	**365.8 ± 7.77 cd**
RR	WT	0.58 ± 0.34 a	4.19 ± 0.37 a	95.33 ± 1.66 ab	60.22 ± 1.18 a	2398.35 ± 64.63 a	31.28 ± 1.04 a	37.26 ± 2.51 a	261.97 ± 14.21 a	5.13 ± 0.28 a	45.43 ± 1.36 a	3.26 ± 0.43 a	312.32 ± 18.15 a
	PPC::B2 - L1	2.65 ± 0.8 ab	5.71 ± 0.63 ab	107.74 ± 9.87 ab	72.47 ± 5.22 ab	2516.73 ± 138.88 ab	**50.11 ± 1.43 b**	**60.98 ± 4.88 b**	**347.26 ± 4.59 ab**	**6.66 ± 0.03 b**	62.60 ± 3.52 a	4.08 ± 0.11 a	420.60 ± 5.47 ab
	PPC::B2 - L3	2.15 ± 0.18 ab	5.03 ± 0.44 a	90.71 ± 2.71 a	64.93 ± 2.95 ab	2653.52 ± 158.65 abc	**47.8 ± 0.87 b**	**59.6 ± 4.63 b**	**379.25 ± 24.61 bc**	**7.79 ± 0.27 b**	74.77 ± 5.05 ab	5.28 ± 0.25 ab	**486.46 ± 27.30 bc**
	PPC::B2^{Y252H} - L5	**4.1 ± 0.05 bc**	**7.85 ± 0.08 bc**	**130.46 ± 9.2 b**	**83.42 ± 5.12 b**	**3097.53 ± 118 bc**	**60.06 ± 0.95 c**	**92.88 ± 1.39 c**	**501.04 ± 8.40 d**	**10.93 ± 0.37 c**	**128.05 ± 2.98 cd**	**9.16 ± 0.30 c**	**647.25 ± 7.52 d**
	PPC::B2^{Y252H} - L6	**6.47 ± 1.35 c**	**8.56 ± 0.87 c**	123.87 ± 11.04 ab	**78.93 ± 5.66 ab**	**3147.43 ± 184.52 c**	**67.15 ± 1.98 d**	**120.48 ± 7.73 d**	**491.34 ± 26.37 d**	**9.94 ± 0.08 cd**	**153.78 ± 9.90 d**	**8.71 ± 0.63 c**	**640.62 ± 44.78 d**
	PPC::B2^{Y252H} - L15	**5.33 ± 0.26 bc**	**9.88 ± 0.57 c**	**181.37 ± 5.66 c**	**112.92 ± 1.13 c**	**3753.49 ± 45.43 d**	**73.51 ± 0.17 e**	**127.51 ± 0.9 d**	**448.33 ± 28.62 cd**	**9.48 ± 0.42 d**	**111.31 ± 17.73 bc**	**7.73 ± 1.35 bc**	**576.85 ± 47.95 cd**

Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). IMG, immature green; MG, mature green; BK, breaker; RR, red ripe, DW, dry weight; ND, not detected; *PPC::B2, PPC2::PHYB2; PPC::B2*^{Y252H}, *PPC2::PHYB2*^{Y252H}.

Table S6. Enzyme-coding DEGs[1] involved in chlorophyll biosynthesis in tomato *PPC2::PHYB2*^{Y252H} MG fruits.

Enzyme	Abbreviations	iTAG3.2 Locus	logFC L6MGxWTMG	FDR L6MGxWTMG
Glutamate-1-semialdehyde-2,1-aminomutase	GSA-AM, GSA	Solyc04g009200	0.693	0.025
5-Aminolevulinic acid dehydratase	ALAD, HEMB	Solyc08g069030	0.535	0.045
Porphobilinogen deaminase	PBGD, HEMC	Solyc07g066470	0.863	0.000
Uroporphyrinogen III decarboxylase	UROD, HEME	Solyc06g048730 Solyc10g007320	0.670 0.721	0.005 0.006
Coproporphyrinogen III oxidase	CPOX, HEMF	Solyc10g005110	1.054	0.000
Protoporphyrinogen IX oxidase	PPOX, HEMG	Solyc01g079090	0.558	0.030
Magnesium chelatase	CHLH, CHLD, CHLI	Solyc04g015490 Solyc10g008740	0.729 1.172	0.001 0.000
SAM Mg-protoporphyrin IX methyltransferase	CHLM, MgPMT	Solyc03g118240	1.185	0.000
Mg-Proto IX monomethyl ester cyclase	CRD1, MgPMEC	Solyc10g077040	0.985	0.029
Protochlorophyllide oxidoreductase	POR	Solyc12g013710 Solyc10g006900	2.945 2.201	0.000 0.000

[1] DEGs presented here represent a selection from original data available in the Table S2.

Table S7. Transcript abundance of isoprenoid-related genes in wild-type and transgenic fruits.

		DXS1	***GGPS1***	***GGPS2***	***PSY1***	***PDS***	***ZDS***	***ZISO***	***CRTISO***	***εLCY***	***βCYC***	***βLCY***	***GGDR***
Stage	**Genotype**	*1-Deoxy-D-Xylulose-5-Phosphate Synthase 1*	*Geranylgeranyl Diphosphate Synthase 1*	*Geranylgeranyl Diphosphate Synthase 2*	*Phytoene Synthase 1*	*Phytoene Desaturase*	*ζ-Carotene Desaturase*	*ζ-Carotene Isomerase*	*Carotenoid Isomerase*	*e-lycopene cyclase*	*Chromoplast-specific b-lycopene cyclase*	*Chloroplast-specific b-lycopene cyclase*	*Geranylgeranyl Diphosphate Reductase*
IMG	WT	1 ± 0.11	1 ± 0.02	1 ± 0.01 a	1 ± 0.07 ab	1 ± 0.1 ab	1 ± 0.04	1 ± 0.07 a	1 ± 0.09 a	1 ± 0.05 a	1 ± 0.15	1 ± 0.06	1 ± 0.05 a
	PPC::B2 - L1	1.08 ± 0.11	0.87 ± 0.09	0.99 ± 0.07 a	**1.56 ± 0.15 b**	1.16 ± 0.03 a	1.09 ± 0.02	**1.46 ± 0.05 b**	**1.72 ± 0.04 b**	0.79 ± 0.06 a	1.16 ± 0.23	1.06 ± 0.01	0.82 ± 0.09 a
	PPC::B2 - L3	0.75 ± 0.26	1.09 ± 0.16	1.11 ± 0.1 ab	0.98 ± 0.18 a	**0.72 ± 0.02 b**	1.02 ± 0.13	1.37 ± 0.13 ab	**1.59 ± 0.09 b**	0.9 ± 0.01 a	0.83 ± 0.06	1.14 ± 0.08	0.77 ± 0.09 a
	PPC::B2^{Y252H} - L5	1.33 ± 0.28	1.19 ± 0.07	**1.36 ± 0.06 b**	1.5 ± 0.05 ab	**1.3 ± 0.05 a**	1.01 ± 0.06	1.36 ± 0.17 ab	**1.75 ± 0.12 b**	**1.49 ± 0.02 b**	1.17 ± 0.01	1.24 ± 0	**1.5 ± 0.01 b**
	PPC::B2^{Y252H} - L6	1.13 ± 0.09	1.02 ± 0.04	1.08 ± 0.08 ab	1.35 ± 0.11 ab	1.08 ± 0.05 a	1.26 ± 0.06	1.36 ± 0.07 ab	**1.65 ± 0.04 b**	**1.41 ± 0.01 b**	0.98 ± 0.05	1.19 ± 0.11	**1.38 ± 0.09 b**
	PPC::B2^{Y252H} - L15	1.39 ± 0.02	1.03 ± 0.03	1.21 ± 0.06 ab	1.41 ± 0.06 ab	1.09 ± 0.08 a	1.33 ± 0.1	1.32 ± 0.04 ab	**1.66 ± 0.05 b**	**1.56 ± 0.09 b**	0.94 ± 0.13	1.18 ± 0.1	**1.46 ± 0.03 b**
MG	WT	0.12 ± 0.01 a	1.01 ± 0.03 ab	0.58 ± 0 a	1.97 ± 0.02 a	1.73 ± 0.04 a	2.2 ± 0.11 a	1.38 ± 0.08	1.82 ± 0.02	0.34 ± 0.05 a	1.14 ± 0.08 ab	0.81 ± 0.01 a	0.48 ± 0.03 a
	PPC::B2 - L1	0.14 ± 0.01 ab	1.25 ± 0.05 bc	0.61 ± 0.04 ab	1.89 ± 0.06 a	1.81 ± 0.01 ab	2.02 ± 0.11 a	1.65 ± 0.15	2.31 ± 0.4	0.62 ± 0.11 ab	**2.03 ± 0.12 c**	**1.05 ± 0.05 bc**	0.69 ± 0.09 ab
	PPC::B2 - L3	0.13 ± 0 ab	0.92 ± 0.1 a	0.49 ± 0.01 a	2.76 ± 0.38 ab	1.71 ± 0.11 a	2.2 ± 0.28 a	2.21 ± 0.47	2.05 ± 0.19	0.37 ± 0.05 a	1.46 ± 0.16 a	0.9 ± 0.01 ab	0.57 ± 0.04 a
	PPC::B2^{Y252H} - L5	0.12 ± 0.02 a	1.23 ± 0.06 abc	0.68 ± 0.05 ab	1.98 ± 0.01 a	**2.24 ± 0.02 ac**	2.43 ± 0.14 ab	2.2 ± 0.15	2.05 ± 0.1	0.54 ± 0.01 a	1.16 ± 0.09 ab	0.88 ± 0.04 ab	0.53 ± 0.01 a
	PPC::B2^{Y252H} - L6	0.16 ± 0.01 ab	1.27 ± 0.04 bc	**0.78 ± 0.06 b**	2.4 ± 0.19 ab	**2.11 ± 0.05 ac**	**3.16 ± 0.03 b**	2.25 ± 0.12	2.44 ± 0.05	0.7 ± 0.06 ab	1.25 ± 0.01 ab	0.91 ± 0.02 ab	0.64 ± 0.06 ab
	PPC::B2^{Y252H} - L15	**0.2 ± 0 b**	**1.41 ± 0.11 c**	0.65 ± 0.06 ab	**3.46 ± 0.35 b**	**2.36 ± 0.12 c**	2.51 ± 0.13 ab	1.41 ± 0.11	2.17 ± 0.27	**1.13 ± 0.26 b**	0.79 ± 0.02 b	**1.17 ± 0.08 c**	**1.23 ± 0.27 b**
BK	WT	0.1 ± 0.01 a	0.99 ± 0.06	1.2 ± 0.11 ab	70.21 ± 6.67	1.45 ± 0.03 a	5.02 ± 0.84	57.9 ± 16.48	7.91 ± 2.31	0.09 ± 0 a	0.48 ± 0.05	0.47 ± 0.05 a	0.48 ± 0 a
	PPC::B2 - L1	0.14 ± 0 ab	1.02 ± 0.01	1.04 ± 0.03 a	90.31 ± 15.68	1.6 ± 0.07 ab	4.05 ± 0.13	85.75 ± 10.6	6.11 ± 0.3	0.1 ± 0.01 a	0.57 ± 0.02	0.44 ± 0.01 a	0.4 ± 0.04 ab
	PPC::B2 - L3	0.14 ± 0.01 ab	1.15 ± 0.04	1.06 ± 0.06 a	95.74 ± 6.49	1.59 ± 0.11 ab	4.53 ± 0.37	93.65 ± 10.98	6.54 ± 0.1	0.12 ± 0.01 a	0.58 ± 0	0.4 ± 0.01 a	0.33 ± 0.01 b
	PPC::B2^{Y252H} - L5	**0.22 ± 0.02 c**	1.2 ± 0	1.41 ± 0.13 b	72.4 ± 3.35	**1.92 ± 0.07 b**	5.37 ± 0.11	59.54 ± 8.5	7.03 ± 0.1	**0.34 ± 0.01 bc**	0.57 ± 0.08	**0.69 ± 0.02 bc**	**1.09 ± 0.01 c**
	PPC::B2^{Y252H} - L6	**0.17 ± 0.01 bc**	1.07 ± 0.03	1.29 ± 0.03 ab	61.09 ± 9.03	1.68 ± 0.07 ab	5.17 ± 0.43	58.17 ± 3.31	7.21 ± 0.08	**0.25 ± 0.03 b**	0.55 ± 0.05	0.53 ± 0.04 ab	**0.65 ± 0.02 d**
	PPC::B2^{Y252H} - L15	**0.19 ± 0.01 bc**	**1.21 ± 0.08**	1.2 ± 0.02 ab	66.85 ± 6.71	1.82 ± 0.1 ab	4.79 ± 0.35	47.58 ± 3.77	5.29 ± 0.35	**0.41 ± 0.02 c**	0.54 ± 0.07	**0.76 ± 0.06 c**	**1.01 ± 0.01 c**
RR	WT	0.04 ± 0 a	0.38 ± 0.01	1.82 ± 0.23 a	11.25 ± 0.35	0.26 ± 0.02	2.28 ± 0.12 a	34.14 ± 1.64 a	4.15 ± 0.07 a	0.01 ± 0	0.33 ± 0.04 ab	0.11 ± 0	0.03 ± 0 a
	PPC::B2 - L1	0.05 ± 0.01 a	0.42 ± 0	**2.66 ± 0.07 ab**	11.15 ± 0.49	0.28 ± 0.01	2.86 ± 0.21 a	38.48 ± 0.84 a	**6.7 ± 0.49 b**	0.01 ± 0	0.36 ± 0.02 b	0.11 ± 0	0.03 ± 0.01 a
	PPC::B2 - L3	0.04 ± 0.01 a	0.43 ± 0.02	**3.03 ± 0.13 bc**	10.17 ± 0.55	0.32 ± 0	2.82 ± 0.11 a	32.58 ± 0.47 a	5.82 ± 0.39 ab	0.01 ± 0	0.28 ± 0.03 ab	0.11 ± 0.01	0.03 ± 0 a
	PPC::B2^{Y252H} - L5	0.07 ± 0.01 a	0.44 ± 0.01	**2.73 ± 0.11 ab**	12.35 ± 0.53	0.33 ± 0.03	2.45 ± 0.09 a	36 ± 3.71 a	5.24 ± 0.04 ab	0.01 ± 0	**0.21 ± 0.01 b**	0.14 ± 0.01	**0.07 ± 0.01 b**
	PPC::B2^{Y252H} - L6	0.05 ± 0.01 a	0.42 ± 0.02	**3.13 ± 0.21 bc**	14.18 ± 1.11	0.34 ± 0.01	**2.92 ± 0.12 a**	36.96 ± 0.8 a	5.98 ± 0.04 ab	0.01 ± 0	0.28 ± 0.02 ab	0.11 ± 0	0.04 ± 0 ab
	PPC::B2^{Y252H} - L15	**0.1 ± 0.01 b**	0.46 ± 0.03	**3.92 ± 0.25 c**	12.07 ± 1.87	0.33 ± 0.02	**4.11 ± 0.21 b**	**58.24 ± 1.5 b**	**6.91 ± 0.73 b**	0.01 ± 0	0.26 ± 0.02 ab	0.13 ± 0.01	**0.06 ± 0.01 b**

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2*^{Y252H}, *PPC2::PHYB2*^{Y252H}.

Table S7 (cont.). Transcript abundance of isoprenoid-related genes in wild-type and transgenic fruits.

		VTE5	***VTE6***	***VTE2***	***VTE3a***	***VTE3b***	***VTE1***	***VTE4***	***SPS***	***ORR***	***PPH***
Stage	**Genotype**	*Phytol kinase*	*Phytyl phosphate kinase*	*Homogentisate phytyl transferase*	*2,3-dimethyl-5-phytylquinol methyltransferase (1)*	*2,3-dimethyl-5-phytylquinol methyltransferase (2)*	*Tocopherol cyclase*	*Tocopherol C-methyl transferase*	*Solanesyl Diphosphate Synthase*	*Orange Ripening*	*Pheophytinase*
IMG	WT	1.02 ± 0.01 ab	1 ± 0.1 a	1 ± 0.07 ab	1 ± 0.07 ab	1 ± 0.06 ab	1 ± 0.09 ab	1 ± 0.04 abc	1 ± 0.11	1 ± 0.08 a	1 ± 0.06
	PPC::B2 - L1	0.97 ± 0 ab	1.08 ± 0.05 a	0.99 ± 0.08 ab	0.98 ± 0.07 ab	1.09 ± 0.05 abc	1.06 ± 0.1 a	0.89 ± 0.09 bc	1.95 ± 0.17	**1.79 ± 0.13 b**	1.13 ± 0.12
	PPC::B2 - L3	0.85 ± 0.07 a	**0.58 ± 0.06 b**	**0.71 ± 0.11 b**	**0.65 ± 0.14 a**	0.7 ± 0.14 a	**0.56 ± 0.16 b**	**0.64 ± 0.15 c**	1.64 ± 0.12	1.33 ± 0.06 ab	0.98 ± 0.13
	PPC::B2 ^{Y252H} - L5	1.17 ± 0.01 b	1.25 ± 0.04 a	1.16 ± 0.01 a	1.13 ± 0 b	**1.52 ± 0.02 d**	1.24 ± 0.06 a	**1.34 ± 0.08 a**	1.82 ± 0.26	**2.66 ± 0.3 c**	1.13 ± 0.01
	PPC::B2 ^{Y252H} - L6	1.03 ± 0.05 ab	1.22 ± 0.05 a	1.03 ± 0.01 ab	0.96 ± 0.05 ab	**1.36 ± 0.03 bcd**	1.05 ± 0.01 a	1.04 ± 0.05 abc	1.71 ± 0.2	**2.72 ± 0.18 c**	1.06 ± 0.05
	PPC::B2 ^{Y252H} - L15	1.07 ± 0.01 b	1.13 ± 0.07 a	1.07 ± 0.06 a	1.02 ± 0.03 ab	**1.38 ± 0.08 cd**	1.18 ± 0.04 a	1.19 ± 0.01 bc	1.58 ± 0.05	**2.78 ± 0.1 c**	1.34 ± 0.23
MG	WT	1.01 ± 0.03	0.58 ± 0.01 a	0.69 ± 0.03 a	0.89 ± 0.04 ab	1.47 ± 0.06 a	0.44 ± 0.01 ab	0.08 ± 0 a	0.9 ± 0.07	0.61 ± 0.08 a	2.35 ± 0 a
	PPC::B2 - L1	1.19 ± 0.09	0.59 ± 0.03 a	0.68 ± 0.03 ab	0.85 ± 0.01 ab	1.85 ± 0.04 ab	0.53 ± 0.02 ab	0.08 ± 0 ab	1.53 ± 0.02	**1.33 ± 0.04 ab**	1.56 ± 0.07 a
	PPC::B2 - L3	0.95 ± 0.01	0.55 ± 0.02 a	0.63 ± 0.01 ab	0.74 ± 0.02 a	1.4 ± 0.06 a	0.42 ± 0.02 b	0.08 ± 0.01 a	1.2 ± 0.17	1.02 ± 0.16 ab	2.19 ± 0.07 a
	PPC::B2 ^{Y252H} - L5	0.96 ± 0.03	0.62 ± 0.02 a	**0.57 ± 0.02 b**	0.72 ± 0.02 a	1.89 ± 0.05 ab	0.44 ± 0 ab	0.09 ± 0 ab	1.21 ± 0.02	**1.46 ± 0.2 b**	2.13 ± 0.21 a
	PPC::B2 ^{Y252H} - L6	1 ± 0.01	0.61 ± 0 ab	0.69 ± 0 ab	0.76 ± 0.02 ab	**2.15 ± 0.11 b**	0.48 ± 0.01 ab	**0.1 ± 0.01 ab**	1.48 ± 0.22	**1.62 ± 0.15 b**	1.99 ± 0.13 a
	PPC::B2 ^{Y252H} - L15	1.14 ± 0.11	**0.74 ± 0.05 b**	0.73 ± 0.04 a	0.99 ± 0.08 b	**2.32 ± 0.26 b**	**0.62 ± 0.07 a**	**0.11 ± 0.01 b**	1.2 ± 0.02	**1.45 ± 0.07 b**	**3.96 ± 0.33 b**
BK	WT	0.54 ± 0.01 a	1.04 ± 0.05	0.93 ± 0.06	0.84 ± 0.04 ab	0.8 ± 0.11 a	0.31 ± 0.02 a	0.05 ± 0 a	1.06 ± 0.21	0.12 ± 0.02 a	7.78 ± 0.51 ab
	PPC::B2 - L1	0.57 ± 0.01 ab	1.17 ± 0.09	0.91 ± 0.03	0.73 ± 0.02 a	0.74 ± 0.02 a	**0.41 ± 0 c**	0.06 ± 0 a	1.04 ± 0.03	0.19 ± 0.03 a	7.65 ± 0.66 ab
	PPC::B2 - L3	**0.6 ± 0.02 abc**	1.22 ± 0.06	0.87 ± 0.08	0.73 ± 0.03 a	0.77 ± 0.02 a	0.32 ± 0 ab	0.05 ± 0 a	1.08 ± 0.05	0.13 ± 0.03 a	6.58 ± 0.47 a
	PPC::B2 ^{Y252H} - L5	**0.67 ± 0.01 c**	1.22 ± 0.01	1.03 ± 0.04	0.88 ± 0.01 b	**1.08 ± 0.04 b**	**0.4 ± 0 c**	**0.08 ± 0 bc**	2.04 ± 0.21	**0.65 ± 0.08 b**	**10.14 ± 0.19 b**
	PPC::B2 ^{Y252H} - L6	**0.63 ± 0.02 bc**	1.05 ± 0.07	0.93 ± 0.05	0.77 ± 0.04 ab	**0.95 ± 0.05 b**	**0.37 ± 0 abc**	**0.06 ± 0 ab**	1.46 ± 0.03	**0.52 ± 0 b**	8.31 ± 0.8 ab
	PPC::B2 ^{Y252H} - L15	**0.64 ± 0.01 bc**	1.08 ± 0.08	0.94 ± 0.01	0.8 ± 0.02 ab	**0.97 ± 0.05 b**	**0.37 ± 0.02 bc**	**0.08 ± 0 c**	1.68 ± 0.19	**0.63 ± 0.07 b**	9.53 ± 0.08 b
RR	WT	0.12 ± 0 ab	0.21 ± 0	0.14 ± 0	0.13 ± 0.01	0.18 ± 0.01 ab	0.04 ± 0	0 ± 0	0.88 ± 0.01	0.02 ± 0 a	1.28 ± 0.07 a
	PPC::B2 - L1	0.14 ± 0 b	0.2 ± 0.01	0.13 ± 0	0.14 ± 0.01	**0.14 ± 0.01 c**	0.05 ± 0	0 ± 0	1.2 ± 0.05	0.04 ± 0 ab	1.21 ± 0.02 a
	PPC::B2 - L3	0.12 ± 0 ab	0.21 ± 0	0.13 ± 0	0.15 ± 0.01	**0.14 ± 0 bc**	0.04 ± 0	0 ± 0	1.05 ± 0.06	**0.05 ± 0.01 b**	1.25 ± 0 ab
	PPC::B2 ^{Y252H} - L5	0.13 ± 0.01 ab	0.2 ± 0	0.16 ± 0.02	0.13 ± 0.01	0.18 ± 0.01 a	0.05 ± 0	0 ± 0	1.3 ± 0.04	0.03 ± 0 ab	**1.69 ± 0.06 b**
	PPC::B2 ^{Y252H} - L6	0.12 ± 0.01 a	0.19 ± 0.02	0.13 ± 0.01	0.13 ± 0	0.19 ± 0.01 a	0.04 ± 0	0 ± 0	1.64 ± 0.12	0.04 ± 0.01 ab	1.35 ± 0.03 ab
	PPC::B2 ^{Y252H} - L15	0.11 ± 0.01 a	0.21 ± 0.03	0.15 ± 0.02	0.12 ± 0	0.16 ± 0 abc	0.04 ± 0	0 ± 0	1.96 ± 0.16	0.04 ± 0 ab	1.51 ± 0.16 ab

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* ^{Y252H}.

Table S7 (cont.). Transcript abundance of isoprenoid-related genes in wild-type and transgenic fruits.

		PPHL1	***PPHL2***	***CHL1***	***RIN***	***NOR***	***CNR***	***AP2a***	***FUL1***
Stage	**Genotype**	*Pheophytinase-like 1*	*Pheophytinase-like 2*	*Chlorophyllase 1*	*Ripening Inhibitor*	*Non-Ripening*	*Colorless Ripening*	*Apetala2a*	*Fruitfull 1*
IMG	WT	1 ± 0.09 a	1 ± 0.16	1 ± 0.03	1 ± 0.12	1 ± 0.16	1 ± 0.07 ab	1 ± 0.04 ab	1 ± 0.04
	PPC::B2 - L1	**1.71 ± 0.04 ab**	1.28 ± 0.03	1.67 ± 0.14	1.05 ± 0.12	1.65 ± 0.28	1.4 ± 0.05 a	**1.49 ± 0.03 c**	1.81 ± 0.07
	PPC::B2 - L3	1.14 ± 0.12 ab	1.08 ± 0.15	2.1 ± 0.49	2.1 ± 0.86	2.13 ± 0.17	1.13 ± 0.18 ab	1.23 ± 0.1 bc	1.45 ± 0.16
	PPC::B2 ^{Y252H} - L5	1.22 ± 0.35 ab	0.99 ± 0.34	1.81 ± 0.27	0.48 ± 0.05	1.32 ± 0.3	0.9 ± 0.12 b	0.85 ± 0.06 a	1.73 ± 0.34
	PPC::B2 ^{Y252H} - L6	1.3 ± 0.02 ab	1.02 ± 0.01	1.31 ± 0.1	1 ± 0.24	2.72 ± 1.09	0.83 ± 0.02 b	0.78 ± 0.01 a	1.29 ± 0.04
	PPC::B2 ^{Y252H} - L15	**1.85 ± 0.08 b**	1.29 ± 0.07	1.7 ± 0.13	1.21 ± 0.37	0.79 ± 0.15	0.79 ± 0.08 b	0.97 ± 0.11 ab	1.46 ± 0.17
MG	WT	2.94 ± 0.37 a	0.69 ± 0.13 a	4.02 ± 0.22 a	2.57 ± 0.44	11.35 ± 0.46	2.98 ± 0.22 ab	3.19 ± 0.47 ab	37.59 ± 2.1
	PPC::B2 - L1	4.45 ± 0.02 ab	0.69 ± 0.01 a	**7.12 ± 0.98 ab**	1.48 ± 0.7	10.24 ± 3.61	2.71 ± 0.33 abc	1.73 ± 0.23 b	37.54 ± 7.85
	PPC::B2 - L3	3.57 ± 0.08 a	0.76 ± 0 ab	5.97 ± 1.1 ab	1.82 ± 0.54	15.57 ± 4.58	3.43 ± 0.05 a	2.64 ± 0.1 ab	45.16 ± 4.52
	PPC::B2 ^{Y252H} - L5	3.13 ± 0.56 a	0.67 ± 0.05 a	**8.65 ± 0.63 b**	1.69 ± 0.17	14.8 ± 0.5	2.2 ± 0.08 bc	2.43 ± 0.52 b	41.67 ± 4.5
	PPC::B2 ^{Y252H} - L6	3.58 ± 0.39 a	0.66 ± 0.09 a	6.67 ± 0.38 ab	2.72 ± 1.23	14.28 ± 4.85	**1.94 ± 0.12 c**	2.04 ± 0.27 b	51.32 ± 1.23
	PPC::B2 ^{Y252H} - L15	**6 ± 0.41 b**	**1.12 ± 0.08 b**	4.87 ± 0.21 a	6.25 ± 2.29	19 ± 5.53	2.97 ± 0.05 ab	4.42 ± 0.64 a	54.33 ± 2.71
BK	WT	0.87 ± 0.24 a	0.44 ± 0.11	1.3 ± 0.27 ab	316.34 ± 52.68	200.88 ± 36.45	4.15 ± 0.24 a	22.49 ± 3.5	26.82 ± 2.63
	PPC::B2 - L1	1.29 ± 0.08 ab	0.5 ± 0.01	0.88 ± 0.08 b	412.56 ± 21.48	215.23 ± 0.8	**6.83 ± 0.08 b**	23.09 ± 0.99	25.1 ± 0.37
	PPC::B2 - L3	1.28 ± 0.07 ab	0.66 ± 0.01	1.13 ± 0.01 ab	390.41 ± 42.24	184.54 ± 7.9	5.67 ± 0.36 ab	21.99 ± 3.18	25.82 ± 0.5
	PPC::B2 ^{Y252H} - L5	**2.28 ± 0.23 c**	0.69 ± 0.09	1.83 ± 0.13 a	319.34 ± 11.88	205.73 ± 16.67	4.85 ± 0.61 a	19.71 ± 0.8	29.51 ± 0.52
	PPC::B2 ^{Y252H} - L6	**1.9 ± 0.14 bc**	0.66 ± 0.13	1.47 ± 0.08 ab	322.36 ± 21.3	183.86 ± 6.14	5.2 ± 0.06 ab	17.86 ± 1.14	27.95 ± 1.76
	PPC::B2 ^{Y252H} - L15	**2.37 ± 0.27 c**	0.74 ± 0.06	1.54 ± 0.26 ab	324.27 ± 6.12	167.2 ± 7.5	4.45 ± 0.18 a	16.99 ± 1.68	24.99 ± 1.24
RR	WT	0.3 ± 0.02 a	0.16 ± 0.05 ab	4.18 ± 0.12	409.91 ± 26.93	101.55 ± 0.39	3.44 ± 0.06 abc	11.25 ± 0.04 a	23.33 ± 0.57 a
	PPC::B2 - L1	0.39 ± 0.02 a	0.18 ± 0.03 ab	6.68 ± 0.3	521.5 ± 26.52	127.12 ± 6.66	4.43 ± 0.34 bcd	10.19 ± 0.72 a	28.05 ± 2.35 a
	PPC::B2 - L3	**0.42 ± 0.01 a**	0.32 ± 0.08 ab	6.16 ± 1.87	390.43 ± 40.22	113.32 ± 11.26	3.11 ± 0.36 ab	10.62 ± 0.11 a	27.79 ± 0.52 a
	PPC::B2 ^{Y252H} - L5	0.39 ± 0 a	0.19 ± 0.03 ab	5.47 ± 0.19	400.49 ± 34.06	140.23 ± 19.79	2.44 ± 0.28 a	11.06 ± 1.03 a	27.53 ± 1.86 a
	PPC::B2 ^{Y252H} - L6	0.34 ± 0.03 a	0.11 ± 0.02 a	6.77 ± 1.01	521.22 ± 9.36	109.94 ± 11.26	4.51 ± 0.22 cd	12.48 ± 1.45 a	28.85 ± 2.13 a
	PPC::B2 ^{Y252H} - L15	**0.59 ± 0.03 b**	0.33 ± 0.07 b	**9.05 ± 0.85**	553.74 ± 13.04	134.77 ± 11.39	**5.22 ± 0.31 d**	**16.74 ± 0.74 b**	**38.65 ± 0.07 b**

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* ^{Y252H}.

Table S8. Antioxidant capacity and flavonoid contents in wild-type and transgenic fruits.

		DPPH assay	***Rutin***	***Rutin pentoside***	***Kaempferol rutinoside***	***Naringenin glucoside***	***Naringenin chalcone***
Stage	**Genotype**	*µM TEAC mg^{-1} FW*	*µg g^{-1} FW*	*µg g^{-1} FW*	*µg g^{-1} FW*	*µg g^{-1} FW*	*µg g^{-1} FW*
IMG	WT	64.58 ± 2.13 a	59.71 ± 8.99	7.4 ± 0.46	12.27 ± 1.96 a	ND	ND
	PPC::B2 - L1	71.58 ± 1.6 ab	71.16 ± 1.49	11.6 ± 1.66	**8.06 ± 0.27 bc**	ND	ND
	PPC::B2 - L3	78.45 ± 1.77 ab	60.22 ± 1.74	11.66 ± 0.41	**7.29 ± 0.13 c**	ND	ND
	PPC::B2^{Y252H} - L5	92.25 ± 5.55 ab	56.3 ± 4.51	7.54 ± 0.6	9.85 ± 0.2 abc	ND	ND
	PPC::B2^{Y252H} - L6	**101.38 ± 12.42 b**	52.66 ± 3.46	9.96 ± 0.5	8.86 ± 0.38 abc	ND	ND
	PPC::B2^{Y252H} - L15	94.36 ± 0.63 ab	68.43 ± 6.56	9.5 ± 1.55	11.33 ± 0.47 ab	ND	ND
MG	WT	81.45 ± 1.1 ab	46.1 ± 2.5 ab	12.13 ± 0.24 ab	13.85 ± 0.79 ab	ND	ND
	PPC::B2 - L1	69.17 ± 7.83 b	**64.86 ± 3.53 c**	16.94 ± 1.18 ab	**9.79 ± 0.13 c**	ND	ND
	PPC::B2 - L3	72.23 ± 3.98 b	57.23 ± 5.14 bc	12.51 ± 2.3 ab	**10.19 ± 0.29 bc**	ND	ND
	PPC::B2^{Y252H} - L5	70.42 ± 1.61 b	38.81 ± 1.95 a	8.07 ± 0.54 b	15.52 ± 1.15 a	ND	ND
	PPC::B2^{Y252H} - L6	78.98 ± 6.47 ab	54.59 ± 0.62 abc	18.15 ± 2.6 a	**10.42 ± 0.26 bc**	ND	ND
	PPC::B2^{Y252H} - L15	101.1 ± 4.47 a	**62.04 ± 0.95 bc**	14.56 ± 2.06 ab	14.37 ± 1.13 a	ND	ND
BK	WT	134.64 ± 4.18	92.93 ± 5.7 a	14.25 ± 2.24	22.81 ± 0.65 abc	ND	1056.25 ± 193.26
	PPC::B2 - L1	146.27 ± 10.07	119 ± 14.85 ab	18.3 ± 0.5	18.58 ± 0.18 bc	ND	950 ± 55.72
	PPC::B2 - L3	139.3 ± 0.11	96.51 ± 5.59 a	18.76 ± 2.11	**15.94 ± 0.51 c**	ND	899.35 ± 86.48
	PPC::B2^{Y252H} - L5	159.34 ± 4.52	119.85 ± 5.83 ab	15.83 ± 0.88	27.85 ± 1.05 a	ND	1197.89 ± 143.66
	PPC::B2^{Y252H} - L6	154.37 ± 5.97	**138.71 ± 2.78 ab**	17.78 ± 2.72	25.31 ± 2.72 ab	ND	1387.12 ± 124.34
	PPC::B2^{Y252H} - L15	159.9 ± 8.48	**141.97 ± 2.84 b**	16.04 ± 1.38	20.99 ± 1.52 abc	ND	1226.4 ± 53.52
RR	WT	146 ± 1.61 a	138.29 ± 6.51 a	14.94 ± 0.31 ab	21.78 ± 0.7 a	3.95 ± 0.15 a	131.06 ± 8.59 a
	PPC::B2 - L1	**167.04 ± 6.77 b**	183.46 ± 18.34 ab	**32.63 ± 0.93 c**	23.26 ± 2.07 a	9.16 ± 1.43 abc	234.34 ± 26.68 ab
	PPC::B2 - L3	162.49 ± 4.99 ab	172.58 ± 8.84 ab	21.52 ± 3.24 b	23.13 ± 0.4 a	7.75 ± 1.35 ab	235.48 ± 10.81 ab
	PPC::B2^{Y252H} - L5	**181.25 ± 4.48 bc**	**214.51 ± 5.85 b**	15.41 ± 1.87 ab	**35.16 ± 0.91 b**	**14.25 ± 0.78 bc**	**296.16 ± 43.86 b**
	PPC::B2^{Y252H} - L6	**193.2 ± 3.67 c**	**298.94 ± 20.6 c**	20.4 ± 2.72 ab	**45.5 ± 2.37 c**	**14.93 ± 1.3 c**	**276.06 ± 17.56 b**
	PPC::B2^{Y252H} - L15	**174.75 ± 3.93 bc**	176.38 ± 3.5 ab	11.4 ± 1.27 a	**31.86 ± 1.23 b**	**12.63 ± 2.15 bc**	**283.99 ± 41.9 b**

Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). IMG, immature green; MG, mature green; BK, breaker; RR, red ripe, DW, dry weight; ND, not detected; NA, not analyzed; *PPC::B2, PPC2::PHYB2; PPC::B2^{Y252H}, PPC2::PHYB2^{Y252H}*.

Table S9. Transcript abundance of flavonoid and ascorbate-related genes in wild-type and transgenic fruits.

		CHS1	***CHS2***	***FLS***	***GME1***
Tomato fruit stage	**Genotype**	*Chalcone Synthase 1*	*Chalcone Synthase 2*	*Flavonol Synthase*	*GDP-mannose-3,5-epimerase*
IMG	WT	1 ± 0.07	1 ± 0.04	1 ± 0.03 a	1 ± 0.01
	PPC::B2 - L1	1.07 ± 0.12	1.36 ± 0.08	**1.61 ± 0.1 b**	1.45 ± 0.18
	PPC::B2 - L3	1.15 ± 0.07	1.45 ± 0.17	1.43 ± 0.08 ab	1.26 ± 0.11
	PPC::B2 ^{Y252H} - L5	1.07 ± 0.19	0.8 ± 0.02	0.96 ± 0.05 a	1.04 ± 0.33
	PPC::B2 ^{Y252H} - L6	0.78 ± 0.02	1.12 ± 0.19	1.11 ± 0.17 a	1.19 ± 0.02
	PPC::B2 ^{Y252H} - L15	1.3 ± 0	0.87 ± 0.13	1.05 ± 0.1 a	1.56 ± 0.01
MG	WT	0.44 ± 0.08 a	5.08 ± 0.26	3.02 ± 0.21	0.38 ± 0.04
	PPC::B2 - L1	0.49 ± 0.11 a	4.39 ± 0.78	3.31 ± 0.31	0.46 ± 0.07
	PPC::B2 - L3	0.39 ± 0.03 a	4.85 ± 0.38	3.54 ± 0.29	0.42 ± 0
	PPC::B2 ^{Y252H} - L5	0.34 ± 0.04 a	3.44 ± 0.21	2.45 ± 0.04	0.32 ± 0.02
	PPC::B2 ^{Y252H} - L6	0.62 ± 0.14 ab	3.64 ± 0.13	2.8 ± 0.09	0.38 ± 0.02
	PPC::B2 ^{Y252H} - L15	**0.95 ± 0.12 b**	5.15 ± 0.53	3.13 ± 0.21	0.59 ± 0.12
BK	WT	16.19 ± 1.33	4.05 ± 0.25 ab	30.23 ± 4.86 a	0.18 ± 0.01 a
	PPC::B2 - L1	18.25 ± 0.54	3.43 ± 0.11 b	34.44 ± 0.44 ab	0.13 ± 0.01 a
	PPC::B2 - L3	17.86 ± 3.24	4.43 ± 0.51 ab	33.22 ± 4.13 ab	0.15 ± 0.01 a
	PPC::B2 ^{Y252H} - L5	21.78 ± 0.42	**5.4 ± 0.2 a**	**50.16 ± 0.89 b**	**0.25 ± 0.01 b**
	PPC::B2 ^{Y252H} - L6	20.81 ± 1.3	4.18 ± 0.26 ab	**45.1 ± 3.14 ab**	**0.26 ± 0.01 b**
	PPC::B2 ^{Y252H} - L15	16.88 ± 0.7	3.78 ± 0.04 b	**48.82 ± 2.22 b**	**0.24 ± 0 b**
RR	WT	0.57 ± 0.02 a	0.83 ± 0.04 a	0.84 ± 0.02	0.08 ± 0.01 a
	PPC::B2 - L1	0.83 ± 0.09 ab	**1.06 ± 0.03 bc**	1.11 ± 0.11	**0.18 ± 0 bc**
	PPC::B2 - L3	**1.06 ± 0.1 b**	**1.18 ± 0.03 cd**	1.24 ± 0.12	0.11 ± 0 ab
	PPC::B2 ^{Y252H} - L5	0.79 ± 0.12 ab	0.89 ± 0.03 ab	0.85 ± 0.03	**0.18 ± 0.02 bc**
	PPC::B2 ^{Y252H} - L6	0.89 ± 0.07 ab	0.96 ± 0.02 ab	0.95 ± 0.05	**0.15 ± 0.01 b**
	PPC::B2 ^{Y252H} - L15	**1.1 ± 0.05 b**	**1.28 ± 0.05 d**	1.16 ± 0.17	**0.24 ± 0.02 c**

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* ^{Y252H}.

Table S10. Relative metabolite abundance registered via non-targeted profiling in red ripe transgenic fruits.

Metabolite name	Lineage				
	PPC2::PHYB2		*PPC2::PHYB2* ^{Y252H}		
	L1	L3	L5	L6	L15
Acetic acid	**-0,95138**	**-1,39020**	**-1,40147**	**-2,22833**	**-2,61518**
Benzoic acid	**-0,84664**	**-1,51986**	**-1,53710**	**-1,52841**	**-1,94230**
Butyric acid	-0,55754	-0,74164	0,43350	0,36473	0,15856
Citric acid	0,02955	-0,03019	0,01600	0,05600	0,14833
Lactic acid	**-0,84428**	**-1,60346**	**-1,50681**	**-2,16040**	**-2,46342**
Oxalic acid	**0,68097**	**0,61569**	0,33492	**0,76510**	0,38908
Succinic acid	-0,71567	**-1,15494**	-0,74330	**-0,96810**	**-1,19115**
Tricarballylic acid	0,28992	0,20480	0,23032	0,30980	-0,09336
Glycerol	0,69421	0,59934	-0,23106	-0,30419	0,28382
Thiourea	-0,29806	-0,50019	0,76337	0,40463	-0,09002
Alanine	0,00167	**-0,76969**	-0,47460	-0,22128	-0,53422
Aspartic acid	**1,10075**	**0,82239**	0,71564	**1,27921**	0,50161
Glutamic acid	0,52979	-0,16170	**-1,01423**	-0,82031	**-1,51250**
Isoleucine	0,13410	**-1,35521**	-0,55749	-0,83196	**-1,29619**
Proline	-0,01784	0,09897	**0,76512**	**1,03914**	0,42770
Serine	-0,67083	-0,96292	-0,25862	0,19959	-0,41474
Valine	-0,87770	**-1,12159**	-0,13285	-0,34607	**-1,28131**
Arachidic acid	**0,69549**	**1,11891**	**1,52263**	**1,60996**	**1,82371**
Caproic acid	-0,25266	-0,16948	-0,25120	-0,23769	-0,02165
Caprylic acid	0,53631	-0,39911	**0,85001**	0,15612	0,36125
Lauric acid	**0,75120**	**0,95087**	**1,36354**	**0,74327**	**1,25748**
Lignoceric acid	**1,19715**	**1,40498**	**1,76876**	**1,60065**	**1,72129**
Linoleic acid	0,16671	0,43612	0,22606	0,37314	0,46953
Linolenic acid	0,08527	0,20655	**0,41517**	**0,36106**	**0,38191**
Myristic acid	**-0,20795**	0,00000	-0,10407	**-0,20812**	-0,10702
Oleic acid	0,11857	0,12244	1,30958	0,61591	1,38013
Palmitic acid	0,13436	**0,18924**	**0,33952**	**0,15833**	**0,27866**
Palmitoleic acid	-0,02346	-0,02004	**0,78481**	0,17924	0,40660
Stearic acid	0,40685	0,47774	**0,81339**	0,30503	**0,75729**
Sitosterol	0,02233	0,15536	0,44233	0,42670	**1,87828**
Stigmasterol	-0,06997	0,76990	0,71493	0,27739	0,06141

Values given are of metabolite abundance relative to wild-type in generalized logarithm. Data are mean ± SE of at least three biological replicates. Statistical differences are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05).

Table S11. Starch, sugar and acid contents in wild-type and transgenic fruits.

		Starch	***Glucose***	***Fructose***	***Sucrose***	***Citrate***
Stage	**Genotype**	*mg glucose g^{-1} DW*	*mg g^{-1} FW*	*mg g^{-1} FW*	*mg g^{-1} FW*	*mg g^{-1} FW*
IMG	WT	283.58 ± 17.59 a	9.47 ± 0.41 a	8.65 ± 0.34 ab	4.65 ± 0.19	2.93 ± 0.17
	PPC::B2 - L1	226.29 ± 3.4 ab	8.59 ± 0.62 ab	9.28 ± 0.58 ab	3.83 ± 0.34	2.4 ± 0.33
	PPC::B2 - L3	**185.93 ± 9.93 bc**	9.52 ± 0.22 ab	10.43 ± 0.31 a	4.41 ± 0.52	2.54 ± 0.36
	PPC::B2^{Y252H} - L5	**154.52 ± 22.19 c**	**6.92 ± 0.49 b**	7.66 ± 0.55 b	3.68 ± 0.35	2.04 ± 0.28
	PPC::B2^{Y252H} - L6	**156.37 ± 22.77 bc**	7.65 ± 0.81 ab	8.7 ± 0.65 ab	4.19 ± 0.53	2.73 ± 0.51
	PPC::B2^{Y252H} - L15	**178.54 ± 8.83 bc**	7.92 ± 0.5 ab	9.29 ± 0.33 ab	4.27 ± 0.6	2.36 ± 0.69
MG	WT	157.61 ± 7.15 a	9.91 ± 0.45	10.11 ± 0.37 a	2.66 ± 0.42	2.9 ± 0.19
	PPC::B2 - L1	**96.04 ± 7.69 bc**	8.82 ± 0.76	9.32 ± 0.77 ab	2.26 ± 0.31	3.13 ± 0.64
	PPC::B2 - L3	116 ± 16.3 ab	9.66 ± 0.5	9.1 ± 0.5 ab	2.98 ± 0.41	4.28 ± 0.07
	PPC::B2^{Y252H} - L5	**81.62 ± 14.7 bc**	7.69 ± 0.35	7.42 ± 0.35 ab	2.36 ± 0.56	3.38 ± 0.38
	PPC::B2^{Y252H} - L6	**38.08 ± 8.39 c**	**6.2 ± 0.12**	**6.12 ± 0.09 b**	1.95 ± 0.05	3.13 ± 0.45
	PPC::B2^{Y252H} - L15	**98.53 ± 17.75 b**	9.58 ± 1.53	9.23 ± 1.4 ab	3.32 ± 0.5	3.32 ± 0.86
BK	WT	88.79 ± 1.27 a	13.12 ± 0.19 a	12.42 ± 0.24 a	3.95 ± 0.15	6.15 ± 0.43 ab
	PPC::B2 - L1	**47.48 ± 0.92 b**	**10.5 ± 0.48 ab**	11.13 ± 0.28 ab	3.24 ± 0.27	7.06 ± 0.15 a
	PPC::B2 - L3	**26.64 ± 1.41 c**	**8.22 ± 0.76 b**	**9.37 ± 0.65 b**	**2.93 ± 0.28**	5.74 ± 0.08 b
	PPC::B2^{Y252H} - L5	**42.23 ± 3.32 bd**	**10.02 ± 0.56 b**	10.79 ± 0.52 ab	3.81 ± 0.13	6.87 ± 0.18 a
	PPC::B2^{Y252H} - L6	**32.87 ± 2.14 cd**	**8.29 ± 0.25 b**	11.22 ± 0.7 ab	3.1 ± 0.03	6.2 ± 0.12 ab
	PPC::B2^{Y252H} - L15	**66.45 ± 6.4 e**	10.9 ± 1.15 ab	12.87 ± 1.27 a	3.72 ± 0.42	6.06 ± 0.28 ab
RR	WT	ND	19.07 ± 1.97 a	21.16 ± 1.67 a	2.55 ± 0.52	6.37 ± 0.27
	PPC::B2 - L1	ND	**10.63 ± 1.5 b**	**13.86 ± 1.74 b**	2.11 ± 0.25	6.5 ± 0.17
	PPC::B2 - L3	ND	**10.63 ± 0.36 b**	**14.48 ± 0.07 b**	1.89 ± 0.02	6.24 ± 0.31
	PPC::B2^{Y252H} - L5	ND	**9.25 ± 0.22 b**	**12.37 ± 0.29 b**	2.01 ± 0.04	6.44 ± 0.18
	PPC::B2^{Y252H} - L6	ND	15.73 ± 0.39 ac	17.43 ± 0.42 ab	2.04 ± 0.17	6.62 ± 0.06
	PPC::B2^{Y252H} - L15	ND	**10.81 ± 0.25 bc**	**14.02 ± 0.34 b**	1.82 ± 0.25	7.06 ± 0.42

Data are mean ± SE of at least three biological replicates. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). IMG, immature green; MG, mature green; BK, breaker; RR, red ripe; DW, dry weight; FW, fresh weight; ND, not detected; *PPC::B2, PPC2::PHYB2; PPC::B2^{Y252H}, PPC2::PHYB2^{Y252H}*.

Table S12. Photosynthetic parameters, chlorophyll and starch contents in leaves of wild-type and transgenic plants.

		A	*gs*	*E*	*Ci/Ca*	*Fv/Fm*	*Fq'/Fm'*	*NPQ*	*ETR*	*Chlorophyll a*	*Chlorophyll b*	*Starch*
	Genotype	*µmol CO_2 m^{-2} s^{-1}*	*mol H_2O m^{-2} s^{-1}*	*mmol H_2O m^{-2} s^{-1}*						*mg g^{-1} FW*	*mg g^{-1} FW*	*mg g^{-1} DW*
Leaves	WT	12.00 ± 0.21	0.37 ± 0.02	5.07 ± 0.26	0.82 ± 0.01	0.78 ± 0.0013	0.73 ± 0.0022	0.23 ± 0.0050	306.49 ± 0.9315	2.10 ± 0.185	0.71 ± 0.071	43.37 ± 1.87
	PPC::B2 - L1	10.52 ± 0.22	0.30 ± 0.03	4.22 ± 0.35	0.78 ± 0.02	0.78 ± 0.0008	0.73 ± 0.0020	0.18 ± 0.0094	306.55 ± 0.8386	2.18 ± 0.181	0.80 ± 0.077	46.40 ± 0.60
	PPC::B2 - L3	11.81 ± 0.10	0.31 ± 0.01	4.33 ± 0.16	0.81 ± 0.01	0.78 ± 0.0005	0.73 ± 0.0018	0.19 ± 0.0067	308.53 ± 0.7559	2.52 ± 0.079	0.89 ± 0.027	43.85 ± 1.75
	PPC::B2 ^{Y252H} - L5	11.41 ± 0.38	0.36 ± 0.02	4.64 ± 0.19	0.83 ± 0.01	0.78 ± 0.0016	0.73 ± 0.0011	0.20 ± 0.0074	306.84 ± 0.4462	2.27 ± 0.093	0.80 ± 0.032	37.33 ± 1.47
	PPC::B2 ^{Y252H} - L6	11.72 ± 0.28	0.30 ± 0.01	4.22 ± 0.16	0.81 ± 0.01	0.78 ± 0.0003	0.73 ± 0.0014	0.18 ± 0.0084	305.72 ± 0.5932	2.16 ± 0.059	0.76 ± 0.019	39.22 ± 2.73
	PPC::B2 ^{Y252H} - L15	11.22 ± 0.08	0.36 ± 0.03	4.78 ± 0.29	0.82 ± 0.01	0.78 ± 0.0006	0.74 ± 0.0015	0.16 ± 0.0138	308.90 ± 0.6426	2.11 ± 0.063	0.74 ± 0.023	40.72 ± 3.19

Data are mean ± SE of at least three biological replicates. No statistical differences determined by ANOVA were reported for any variable; WT, wild-type; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* $^{Y252H;}$ *A* , net photosynthesis rate; *gs* , stomatal conductance; *E* , transpiration rate; Ci/Ca, internal carbon:external carbon ratio; Fv/Fm, photosystem II potential quantum efficiency; Fq'/Fm', photosystem II effective quantum efficiency; NPQ, non-photochemical quenching; ETR, electron transport rate.

Table S13. Transcript abundance of sugar-related genes in wild-type and transgenic fruits.

		Sugar-related genes						
		AGPaseL1	***AGPaseL2***	***AGPaseL3***	***AGPaseS1***	***LIN5***	***LIN6***	***TIV1***
Tomato fruit stage	**Genotype**	*ADP-glucose pyrophosphorylase Large subunit 1*	*ADP-glucose pyrophosphorylase Large subunit 2*	*ADP-glucose pyrophosphorylase Large subunit 3*	*ADP-glucose pyrophosphorylase Small subunit 1*	*Lycopersicum Invertase 5*	*Lycopersicum Invertase 6*	*Tonoplast Invertase 1*
IMG	WT	1 ± 0.05 a	1 ± 0.02	1 ± 0.11 a	1 ± 0.05	1 ± 0.09 ab	1 ± 0.17	1 ± 0.1 a
	PPC::B2 - L1	**0.6 ± 0.07 b**	1.3 ± 0.14	1.12 ± 0.04 a	0.81 ± 0.05	**0.48 ± 0.06 c**	1.14 ± 0.28	1.44 ± 0.18 ab
	PPC::B2 - L3	**0.66 ± 0.07 b**	1.33 ± 0.1	0.93 ± 0.11 a	0.77 ± 0.1	0.74 ± 0.06 bc	1.04 ± 0.11	1.19 ± 0.06 ab
	PPC::B2 ^{Y252H} - L5	0.82 ± 0.01 ab	1.31 ± 0.05	**2.02 ± 0.07 bc**	1.08 ± 0.03	1.08 ± 0.02 a	1.22 ± 0.04	1.36 ± 0 ab
	PPC::B2 ^{Y252H} - L6	**0.6 ± 0.05 b**	1.09 ± 0.03	1.45 ± 0.02 ab	0.84 ± 0.13	0.91 ± 0.07 ab	1.19 ± 0.08	**1.7 ± 0.06 b**
	PPC::B2 ^{Y252H} - L15	**0.7 ± 0 b**	1.11 ± 0.04	**2.37 ± 0.31 c**	0.83 ± 0.02	0.94 ± 0.07 ab	0.86 ± 0.23	1.2 ± 0.11 ab
MG	WT	0.2 ± 0.04	2.14 ± 0.08 ab	0.97 ± 0.09 ab	0.51 ± 0.03	0.82 ± 0.05 a	0.57 ± 0.12 a	0.67 ± 0.1
	PPC::B2 - L1	0.25 ± 0.03	2.68 ± 0.21 a	1.42 ± 0.25 ab	0.61 ± 0.1	**0.44 ± 0.08 b**	1.03 ± 0.28 ab	0.48 ± 0.03
	PPC::B2 - L3	0.2 ± 0.01	2.06 ± 0.04 b	0.62 ± 0.06 a	0.46 ± 0.03	0.58 ± 0.09 ab	0.61 ± 0.18 ab	0.53 ± 0.04
	PPC::B2 ^{Y252H} - L5	0.15 ± 0.02	2.23 ± 0.03 ab	1.17 ± 0.06 ab	0.46 ± 0.05	0.62 ± 0.13 ab	1.38 ± 0.29 ab	0.56 ± 0.03
	PPC::B2 ^{Y252H} - L6	0.15 ± 0.01	2.06 ± 0.16 b	1.78 ± 0 b	0.56 ± 0.04	0.72 ± 0.09 ab	**2.79 ± 1.03 b**	0.49 ± 0.05
	PPC::B2 ^{Y252H} - L15	0.31 ± 0.11	2.53 ± 0.04 ab	**1.71 ± 0.24 b**	0.71 ± 0.13	**0.49 ± 0.07 ab**	1.42 ± 0.16 ab	0.59 ± 0
BK	WT	0.01 ± 0 ab	0.85 ± 0.04	0.51 ± 0.05 a	0.12 ± 0.01 a	0.42 ± 0.05	1.02 ± 0.2	3.74 ± 0.58 a
	PPC::B2 - L1	0.01 ± 0 b	0.93 ± 0.03	0.6 ± 0.08 a	**0.07 ± 0 b**	0.33 ± 0.04	2.48 ± 0.65	**1.58 ± 0.16 b**
	PPC::B2 - L3	0.01 ± 0 b	0.89 ± 0.07	0.77 ± 0.05 ab	**0.08 ± 0 b**	0.34 ± 0.04	3.65 ± 0.89	**1.18 ± 0.1 b**
	PPC::B2 ^{Y252H} - L5	0.02 ± 0 a	1 ± 0	**1.39 ± 0.14 c**	0.14 ± 0.01 a	0.45 ± 0.02	2.9 ± 1.45	2.21 ± 0.19 ab
	PPC::B2 ^{Y252H} - L6	0.01 ± 0 ab	0.86 ± 0.02	**1.23 ± 0.13 bc**	0.14 ± 0 a	0.49 ± 0.06	3.95 ± 1.27	2.39 ± 0.63 ab
	PPC::B2 ^{Y252H} - L15	0.02 ± 0 ab	0.97 ± 0.02	**1.27 ± 0.21 bc**	0.14 ± 0.01 a	0.48 ± 0.01	3.7 ± 1.7	**1.48 ± 0.12 b**
RR	WT	0.02 ± 0	0.23 ± 0.01 a	0.22 ± 0.03	0.03 ± 0	0.03 ± 0 ab	0.25 ± 0.1	54.04 ± 9.45 a
	PPC::B2 - L1	0.02 ± 0	**0.29 ± 0 ab**	0.29 ± 0.01	0.03 ± 0	0.01 ± 0 b	0.25 ± 0.01	37.19 ± 3.71 ab
	PPC::B2 - L3	0.01 ± 0	0.26 ± 0.01 ab	0.27 ± 0.03	0.02 ± 0	0.01 ± 0 b	0.4 ± 0.03	**18.74 ± 4.44 b**
	PPC::B2 ^{Y252H} - L5	0.01 ± 0	**0.29 ± 0.01 ab**	0.32 ± 0.03	0.04 ± 0.01	0.06 ± 0 a	0.5 ± 0.05	35.32 ± 2.65 ab
	PPC::B2 ^{Y252H} - L6	0.01 ± 0	0.24 ± 0.02 ab	0.3 ± 0.01	0.02 ± 0	0.03 ± 0.01 ab	0.57 ± 0.14	49.68 ± 1.76 a
	PPC::B2 ^{Y252H} - L15	0.02 ± 0.01	**0.29 ± 0.02 b**	0.33 ± 0.04	0.04 ± 0.01	0.04 ± 0.01 ab	0.51 ± 0.01	**32.85 ± 3.36 ab**

Transcript abundance was normalized against the immature green (IMG) stage of the wild-type (WT) genotype. Statistical differences within each stage are given by bold numbers (Dunnett's Test with WT as control group, α = 0.05) or different letters (Tukey's Test, α = 0.05). MG, mature green; BK, breaker; RR, red ripe; *PPC::B2, PPC2::PHYB2; PPC::B2* ^{Y252H}, *PPC2::PHYB2* ^{Y252H}.

Table S14. Oligonucleotides used in the study.				
Primer name	**iTAG3.2 Locus**	**Commentaries**	**Primer sequence**	
CAC	Solyc08g006960	Reference genes for RT-qPCR	F	CATCCAAGCATGTAGATAAGCC
			R	TTTAAGCCCAACTTCAGATCAG
EXPRESSED	Solyc07g025390	Reference genes for RT-qPCR	F	GCTAAGAACGCTGGACCTAATG
			R	TGGGTGTGCCTTTCTGAATG
PHYB2 CLONING	Solyc05g053410	Cloning of *PHYB2* sequence for insertion into pDONR221 entry vector	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCTTCTGGGAGTGGAAG
			R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTGAATCAGCATTGGTAGTTGAAGG
PHYB2_Y252H	Solyc05g053410	T-to-C base change insertion in *PPC2::PHYB2* vector	F	Phos – GATCGGGTGATGGTGCATAAGTTTCATGATG
			R	Phos – CATCATGAAACTTATGCACCATCACCCGATC
NPTII	-	Selection of positive transgenic plants	F	GAACAAGATGGATTGCACGC
			R	GAAGAACTCGTCAAGAAGGC
PHYA	Solyc10g044670	RT-qPCR	F	CACTCTCGTGGAGGATTCAT
			R	GAGCCATAAAACACACACCC
PHYB1	Solyc01g059870	RT-qPCR	F	ACTTCTGTTCGGTCCATTCC
			R	TCTCAGACAACTGTGATGCC
PHYB2	Solyc05g053410	RT-qPCR	F	GTGAGGGTTATTCAGGATGA
			R	TGACCATATACTGAGGGTGAC
PHYE	Solyc02g071260	RT-qPCR	F	CGCTATTGAGGAACCCACTT
			R	GCATCAACACCAATCAGACC
PHYF	Solyc07g045480	RT-qPCR	F	ACTAGCCAAGATCATTGACG
			R	CTCCAAGATTGAACTCACAAG
GLK2	Solyc10g008160	RT-qPCR	F	ATGCTTGCTCTATCTTCATCATTG
			R	GATTACCAAAATACCCCCAACT
ARF4	Solyc11g069190	RT-qPCR	F	TGAAAGCCATCAACTCTCGG
			R	ATCCCATCTGACCATCAAGCATC
APRR2Like	Solyc08g077230	RT-qPCR	F	GAGTTCCTTCAGAAACCA
			R	TTTGTCATCTGCTTCACC
BEL11	Solyc11g068950	RT-qPCR	F	CTACAACAATTAGGGATGATGC
			R	CAATTTGATACCTGGCTCCT
PDV2	Solyc01g109260	RT-qPCR	F	TCTTGACTTTGGCTGGGTTC
			R	TTCACGACGCACTGAGTTTC
RBCS2a	Solyc03g034220	RT-qPCR	F	GACACTCTCATACCTTCCTG
			R	ACCTTCCATCATAGTATCCTG
CURT1a1	Solyc01g095430	RT-qPCR	F	CGGTCATAGTTTATGGAGG
			R	TTGAAGAGAAGGTAGCGG
CURT1a2	Solyc10g011770	RT-qPCR	F	GCCATCAACTCAGTTCCT
			R	TCAATTCCTCAATGTCTTCC
POR1	Solyc12g013710	RT-qPCR	F	GACAACAGGGCTATTCAG
			R	GACAACTGGTTCTCAAAGG
DXS1	Solyc01g067890	RT-qPCR	F	CAGGACTGGTGTGGTTTCAG
			R	GGGATAGTTCACAGTGTCC
GGPS1	Solyc09g008920	RT-qPCR	F	GCTGTTGGTGTCTTATATCGTG
			R	CTTCTCAATGCCATAAACGCTG
GGPS2	Solyc04g079960	RT-qPCR	F	TTTGCCTTGTATGGATGAC
			R	CAGATTTCGCCAATTCGG
PSY1	Solyc03g031860	RT-qPCR	F	CGATGGTGCTTTGTCCGATAC
			R	CTCATCAACCCAACCGTACC

Table S14. Oligonucleotides used in the study.				
PDS	Solyc03g123760	RT-qPCR	F	CGTTCCGTGCTTCTCCGC
			R	CTAGAACATCCCTTGCCTCCAG
ZDS	Solyc01g097810	RT-qPCR	F	AGTGGTTTCTGTCTAAAGGTGG
			R	ACCGAGCACTCATGTTATCAC
ZISO	Solyc12g097810	RT-qPCR	F	CCTTCTTCTTCCTATACCCGTCG
			R	AGCGTGTGAGCTAAGCACCA
CRTISO	Solyc10g081650	RT-qPCR	F	AAGACCCACAGACGATACCT
			R	ATCGCCAACACAATATAGACCA
εLCY	Solyc12g008980	RT-qPCR	F	CTTACCAGTTCAAGTATCCCGAG
			R	GCAATATCAGAGCCAGTCCA
βLCY	Solyc06g074240	RT-qPCR	F	CGGGTTATATGGTAGCAAGGA
			R	CAGATGCCGATAACTCATTACC
βCYC	Solyc04g040190	RT-qPCR	F	GCACCCACATCAAAGCCAGAG
			R	GCCACATGGAGAGTGGTGAAG
GGDR	Solyc03g115980	RT-qPCR	F	CAGAGACGCTCGCTAAGG
			R	GCTTCAGAGTCTGTCCGATATC
VTE5	Solyc03g071720	RT-qPCR	F	CGTATCAGGACGGGCTCGC
			R	TCACCACCACACATCATTGCTAATG
VTE6	Solyc07g062180	RT-qPCR	F	AGCACAAGCATCAGTGTCTG
			R	AAGAAAGCAGCCGCAATACC
VTE2	Solyc07g017770	RT-qPCR	F	CAATTCCAGTTCCTGCTGAG
			R	CCTCCAACATGCTCTTGCGTG
VTE3a	Solyc09g065730	RT-qPCR	F	CTTGACCAATCTCCTCATC
			R	GCACGCCTTTCCTCCAGG
VTE3b	Solyc03g005230	RT-qPCR	F	GCTAAGGCTAGGCAGAAGGAG
			R	CAGGCAACCCCACCTATGG
VTE1	Solyc08g068570	RT-qPCR	F	CGAACTCCTCATAGCGGGTATC
			R	CACGCCAGTAAACCGAGGC
VTE4	Solyc08g076360	RT-qPCR	F	CAGATCATCGTGCTGCTCAG
			R	CCTCTCTGCTTGTACAGGAC
SPS	Solyc07g061990	RT-qPCR	F	GAACTCACCACAAATCACAG
			R	GAATAGTCTCCTTTCCTCTTCG
ORR	Solyc04g057980	RT-qPCR	F	ACCCTACTGATGAATTTGTG
			R	GTGCTCTATGTCTGAACC
PPH	Solyc01g088090	RT-qPCR	F	TATGGAGGGAGCAAGTACGC
			R	TGGAGGGCAGAGGAAAAGTAC
PPHL1	Solyc02g062610	RT-qPCR	F	GATTTGGTGCTTCTGCCTTTC
			R	GCTGTTTCTTCAGTTCCTTC
PPHL2	Solyc12g098660	RT-qPCR	F	AGGGCTATTCCATCCGATACC
			R	GTCTACCAAACCAAGGCTG
RIN	Solyc05g012020	RT-qPCR	F	TCAAACATCATGGCATTGTGGTG
			R	TGCATTTTCGGGTTGTACATTATCG
NOR	Solyc10g006880	RT-qPCR	F	TAATGATGGGGTCGTCTTTCG
			R	ATTTTACAGGGCTAACTATTTTTTGC
CNR	Solyc02g077920	RT-qPCR	F	GCCAAATCAAGCAATGATGA
			R	TCGCAACCATACAGACCATT
AP2a	Solyc03g044300	RT-qPCR	F	AACGGACCACAATCTTGAC
			R	CTGCTCGGAGTCTGAACC
FUL1	Solyc06g069430	RT-qPCR	F	GTTTTGCCACAACAACTGGACTC
			R	CTTGCTGCTGTGAAGAACTACC

Table S14. Oligonucleotides used in the study.				
CHS1	Solyc09g091510	RT-qPCR	F	GGCTTACATTCCACTTACTC
			R	GAGAGTTCCAGTCAGATATACC
CHS2	Solyc05g053550	RT-qPCR	F	TTAGCTGAGAACAACAAGGG
			R	CCAACCATACTATCCAAATGAG
FLS	Solyc11g013110	RT-qPCR	F	CTTATCACTTGGGCTTGG
			R	CTTGGACTTCATTTGGGAC
GME1	Solyc01g097340	RT-qPCR	F	CGGATAAGTTTGAAATGTGGG
			R	CATCTCATTCATGCTGACCA
AGPaseL1	Solyc01g109790	RT-qPCR	F	AGCAGACTACTACCAAACAG
			R	ATTCCAATCGGTACTTTCC
AGPaseL2	Solyc07g019440	RT-qPCR	F	CGATCCCGTATAGGCACCAA
			R	CTGAGGATCTGTCTGCTTC
AGPaseL3	Solyc01g079790	RT-qPCR	F	CGCGCTACTTCGTAATAACC
			R	CCATCAATTCTCCATTGCA
AGPaseS1	Solyc07g056140	RT-qPCR	F	TGTAAGATTCACCATTCCGT
			R	TCTTCTATAATTGCTCCCTCTG
LIN5	Solyc09g010080	RT-qPCR	F	TTGGAAGGGATTGAGAATCG
			R	AATTCCAGCCCATCCTTTCT
LIN6	Solyc10g083290	RT-qPCR	F	AACCCGCTATCTACCCGTCT
			R	GGGCTTGATCCACTTACGAA
TIV1	Solyc03g083910	RT-qPCR	F	ACTTGGGAAAGAACAAATGG
			R	GTCGTAAAGCACTGTCCT

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CONSIDERAÇÕES FINAIS

O conhecimento dos mecanismos moleculares, bioquímicos e fisiológicos pelos quais as plantas respondem aos sinais luminosos nos permite identificar possíveis alvos de manipulação biotecnológica visando especialmente a modificação de traços agronômicos de interesse, tanto para facilitação do cultivo e colheita quanto para a melhoria da saúde e nutrição humana. Nesse sentido, os resultados obtidos pela presente tese contribuem com a demonstração de que a manipulação da atividade de fotorreceptores consiste numa interessante estratégia biotecnológica para a biofortificação de frutos.

Através da sobre-expressão fruto-específica da forma nativa do fotorreceptor de luz vermelha/vermelha-distante *PHYB2* e de uma forma mutada constitutivamente ativa *PHYB2*^{Y252H} em tomateiro, verificamos aumentos significativos em diversas classes de compostos bioativos considerados antioxidantes benéficos para a saúde humana, como carotenoides, flavonoides e vitaminas C e E. Entre os resultados mais interessantes, comprovamos pela primeira vez que a manipulação da atividade dos fotorreceptores é uma estratégia fotobiotecnológica para a biofortificação de frutos carnosos, uma vez que o acúmulo de antioxidantes foi significativamente intensificado pela sobre-expressão de PHYB2^{Y252H} em comparação à forma nativa do fotorreceptor.

Grande parte dos impactos positivos observados puderam ser explicados pela influência de PHYB2 e PHYB2^{Y252H} sobre o desenvolvimento de plastídios, organelas relacionadas com a síntese e a capacidade de armazenamento de vários compostos bioativos, e sobre a modulação diferencial de genes envolvidos com a biossíntese de antioxidantes.

A mutação resultante da ativação constitutiva independente de luz do fitocromo foi originalmente observada em PHYB de *Arabidopsis thaliana* e, nesta tese, constatamos que a mesma estratégia pôde ser utilizada em outra espécie como o tomateiro, uma vez que a estrutura e a função de diversos elementos da cadeia de transdução do sinal luminoso é conservada entre os vegetais. Dessa forma,

hipotetizamos que a modulação da atividade dos fotorreceptores como estratégia para biofortificação pode ser aplicada com sucesso em outros frutos carnosos.

Algumas novas interessantes perguntas podem ser formuladas a partir dos achados registrados nesta tese. Como se dá a interação do fitocromo constitutivamente ativo com seus fatores intermediários bem como o seu *turnover* ao nível de proteína? Seria possível potencializar os efeitos observados pela introdução de novas mutações de ganho-de-função além da Tyr^{252}-para-His ou pela combinação de outros fitocromos mutados para ganho-de-função? Qual seria o limite de capacidade de estocagem de compostos bioativos no fruto através da modulação fruto-específica? Como se comportaria o fruto transgênico fora da planta mãe em condições de pós-colheita? Quais seriam os impactos da incorporação da mutação Tyr^{252}-para-His via estratégias de edição genômica (e.g., sistema CRISPR-Cas9) sobre o desenvolvimento e produtividade da planta, bem como sobre a qualidade dos frutos?

Espero, ainda, que a presente tese tenha contribuído para ressaltar a importância de investimentos para a Educação, Ciência e Tecnologia brasileiras, uma vez que todos os resultados e avanços científicos aqui apresentados somente foram possíveis graças ao apoio financeiro e suporte institucional de órgãos públicos.

PRODUÇÃO CIENTÍFICA E ACADÊMICA NO PERÍODO

Co-autoria em artigos completos publicados em periódicos

Lira BS, Gramegna G, Trench BA, **Alves FRR**, Silva EM, Silva GFF, Thirumalaikumar VP, Lupi ACD, Demarco D, Purgatto E, Nogueira FTS, Balazadeh S, Freschi L, Rossi M (2017) Manipulation of a senescence-associated gene improves fleshy fruit yield. *Plant Physiology* **175**: 77-91.

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Organização de eventos

Silva FN, **Alves FRR**, Ferrari RC, Gobara BNK, Sacramento GN, Rosado D, Maximo EP, Silva LNNS, Martins NT, Della AP, Cabral A. VIII Botânica no Inverno. 2018.