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Caracterização funcional dos fatores de transcrição PIF (*Phytochrome Interacting Factors*) em tomateiro: papel na produtividade e qualidade nutricional de frutos carnosos

Functional characterization of tomato PIF (Phytochrome Interacting Factors): role in fleshy fruit yield and nutritional quality

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Comissão Julgadora

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Dedico esta tese à minha mãe,
que sempre incentivou a busca por
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Sumário

Resumo

O tomate tem grande importância econômica e nutricional. Durante o amadurecimento do fruto, os cloroplastos são convertidos em cromoplastos, a clorofila é degradada e metabólitos secundários de importância nutracêutica, como carotenoides e vitamina E (VTE), são acumulados. A luz tem papel fundamental na regulação da atividade plastidial e, portanto, na determinação da qualidade nutricional dos frutos. Por exemplo, o silenciamento do gene *DE-ETIOLATED 1*, um efetor negativo na via de transdução de sinal luminoso, leva ao aumento do número de cloroplastos e ao maior acúmulo de clorofila, carotenoides e VTE em frutos tomate. Ainda, a luz tem papel fundamental na determinação dos ritmos de crescimento e no tempo de floração em diversas espécies. Em *Arabidopsis thaliana*, na presença de luz, os fitocromos (PHYs) induzem a degradação dos fatores de interação a PHYs (PHY-Interacting Factors, PIFs). As AtPIFs são fatores de transcrição que atuam como reguladores negativos da fotomorfogênese e participam de uma extensa rede regulatória que controla diversos processos do desenvolvimento desde a germinação até a senescência. Diretamente, as AtPIF controlam genes relacionados à biogênese e manutenção de cloroplastos, síntese e degradação de clorofila, carotenogênese, floração e crescimento. Assim, as PIF surgem como potenciais alvos de manipulação para melhoramento da qualidade nutricional e da produtividade. Neste contexto, este projeto objetivou caracterizar funcionalmente as PIFs de tomateiro. Desta maneira, no Capítulo I, foram identificados os genes *SlPIF*, os quais foram estudados no contexto de diversidade, história evolutiva e perfil de expressão. No Capítulo II, foi avaliado o efeito da luz sobre o metabolismo de VTE e a participação das SlPIFs como fatores regulatórios. No Capítulo III, foi avaliado o impacto do silenciamento do gene *SlPIF4* sobre a produtividade e qualidade nutricional de frutos. Assim, este trabalho traz novas informações que auxiliam na compreensão do papel desempenhado pelas proteínas SlPIFs na regulação de importantes processos metabólicos e do desenvolvimento, que, por fim, afetam caracteres de interesse agronômico e nutricional do tomate.

Abstract

Tomato is a species of great economical and nutritional importance. During fruit ripening, chloroplasts are converted into chromoplasts, chlorophylls are degraded and secondary metabolites of nutraceutical importance, such as carotenoids and vitamin E (VTE), accumulate. Light plays a key role in the regulation of plastid activity and, therefore, of fruit nutritional quality. For instance, silencing of *DE-ETIOLATED 1*, a negative effector on light signaling, leads to increased chloroplast number and higher levels of chlorophyll, carotenoids and vitamin E in tomato fruits. On the other hand, light affects growth rates and flowering time in various species. In *Arabidopsis thaliana*, light-activated phytochromes (PHYs) induce the degradation of PHY-interacting factors (PIFs). PIFs as transcription factors acting as negative regulators of photomorphogenesis, part of a complex regulatory network that controls many developmental processes, from seed germination to senescence. AtPIFs directly regulate genes involved in plastid biogenesis and maintenance, chlorophyll breakdown, carotenogenesis, as well as pathways regulating growth and flowering. Therefore, PIFs emerge as potential targets of genetic manipulation towards improving fruit yield and quality. In this context, this work aimed to functionally characterize tomato PIFs. In Chapter I, *SlPIFs* were identified, and their diversity, evolutionary history and expression profile were studied. In Chapter II, the impacts of light on vitamin E biosynthesis and the role of SlPIFs as regulators of this process were investigated. In Chapter III, the effects of *SlPIF4* constitutive silencing were evaluated regarding mainly fruit production and nutritional quality. Overall, this work brings new information that help understanding the role of SlPIFs in the regulation of important developmental and metabolic process, that ultimately affect agronomic and nutraceutical characteristic of the tomato fruit.

INTRODUÇÃO GERAL

"Of the many intricate and beautiful control mechanisms living organisms have evolved to optimize their survival in a variable and changing environment, none is more elegant than the phytochrome system of plants."

Warren L. Butler

1. Considerações iniciais

Há milhares de anos, a busca por cultivares de alta produtividade e crescimento acelerado conduz a domesticação das plantas. De modo empírico, a humanidade modificou recursos vegetais por meio de cruzamentos e seleção artificial, finalmente, obtendo cultivares adaptados a crescer melhor em determinada época, região e clima. Esse tipo de melhoramento clássico levou ao surgimento de tomateiros de hábito semi-determinado e frutos grandes, de cereais com espigas altas e número aumentado de sementes que passaram a ser cultivados em regiões muito diferentes daquelas habitadas por seus ancestrais selvagens (Salamini et al., 2002; Meyer and Purugganan, 2013; Bergougnoux, 2014).

Embora a domesticação tenha trazido benefícios, a falta de conhecimento sobre a fisiologia ainda representava uma barreira para o melhoramento direcionado e o próprio processo produtivo. Por exemplo, agricultores ainda eram limitados pela sazonalidade particular de muitos cultivares para germinação, crescimento e floração. Não obstante, as bases fisiológicas do efeito das estações sobre o desenvolvimento só começaram a ser compreendidas no último século. Garner and Allard (1920), demonstraram que o tempo de exposição diária à luz é o fator ambiental mais importante na determinação da fase reprodutiva em tabaco e soja. Trabalhos subsequentes mostraram que existem variáveis graus de sensibilidade à luz: determinadas plantas só florescem quando expostas a um período mínimo de noite ininterrupta, enquanto outras permanecem em estado vegetativo se o comprimento da noite ultrapassar um período máximo, ainda há plantas indiferentes a essa variável.

Nas décadas seguintes, mostrou-se que outros processos são regulados pela luz, como a germinação, o alongamento de internós, a expansão foliar e a pigmentação. Foi proposto por Borthwick et al. (1952) que a germinação de sementes de alface dependia da ativação de um "pigmento" por luz na faixa do vermelho; e que a inativação desse "pigmento" por luz vermelho-extrema ou escuro seriam responsáveis por inibir esse processo. Interessantemente, descobriu-se que outros fenômenos controlados por fotoperíodo também estavam sob controle dessa reação fotorreversível (Liverman, 1960). Somente em 1959, o fitocromo foi isolado e reconhecido como molécula responsável por mediar estas respostas (Butler et al., 1959; Borthwick and Hendricks, 1960). Em 1985, o grupo de Peter Quail publicou a primeira sequência de um gene codificante

para um fitocromo de aveia (Hershey et al., 1985). Desde então, as bases moleculares das respostas mediadas por fitocromos são amplamente estudadas.

Hoje, o conhecimento sobre fotobiologia permite o cultivo de plantas fora de época, por exemplo, por meio da suplementação artificial - da qualidade, quantidade e duração - da luz necessária para induzir a floração e aumentar a produtividade (Dorais, 2003; Moe et al., 2005). Ainda, a engenharia das redes regulatórias controladas por fitocromos tem grande potencial para o melhoramento vegetal pois elas controlam inúmeros processos fisiológicos que determinam diversos caracteres de importância agronômica. Não obstante, o conhecimento acumulado nos últimos 30 anos se concentra majoritariamente na espécie modelo *Arabidopsis thaliana*, sendo muito escasso para espécies de interesse comercial.

Trabalhos recentes sugerem que mutações selecionadas durante o processo de domesticação do tomateiro causaram uma atenuação do relógio circadiano e a perda de sensibilidade ao comprimento do dia para a floração (Müller et al., 2016; Soyk et al., 2017; Müller et al., 2018). Em ambos os casos, foi demonstrada uma interação genética dos *loci* mutados com o fitocromo B1 - embora a natureza molecular dessas interações não seja bem conhecida - ressaltando a importância da sinalização luminosa mediada por fitocromos para a adaptação ambiental (Cao et al., 2016; Cao et al., 2018; Müller et al., 2018). Especula-se que essas mutações foram importantes para permitir o plantio em regiões diferentes das habitadas pelos tomateiros ancestrais andinos. Neste contexto, o objetivo principal do presente trabalho é aumentar a compreensão dos processos regulados por fitocromos em tomateiro, especialmente aqueles mediados pelos fatores de transcrição *PHYTOCROME-INTERACTING FACTORS* (*PIFs*), e investigar como a manipulação da resposta à luz pode afetar a produtividade e qualidade nutricional de frutos carnosos.

2. PIFs e a atividade plastidial

Além de energia para as reações de fotossíntese, a luz também provê sinais ambientais que regulam os ritmos circadianos, a fotomorfogênese e até mesmo o movimento de órgãos e organelas (McDonald, 2003). Assim, o desenvolvimento das plantas, seres sésseis e foto-autotróficos, decorre justamente da capacidade de perceber e se adaptar, entre outros fatores ambientais, às

condições de luminosidade. As cascatas de transdução do sinal luminoso mediadas pelos fotorreceptores permitem que a planta responda a diferentes qualidades e intensidades de luz, adequando o metabolismo e os ritmos de crescimento ao ambiente em constante mudança (Kami et al., 2010).

Os fitocromos (PHYs) são fotorreceptores responsáveis pela percepção da luz vermelha, vermelha-extrema e azul e, portanto, componentes fundamentais da regulação do ritmo circadiano e da fotomorfogênese. Existem duas formas interconversíveis dos PHYs, sensíveis ao espectro vermelho (Fv) e vermelho-extremo (Fve), consistindo nas formas inativa e ativa do fotorreceptor, respectivamente. A exposição à luz solar leva à ativação dos PHYs, enquanto a manutenção das plantas no escuro, leva à conversão lenta e espontânea de Fve para Fv (revisado em Mathews, 2006). Os PHYs em sua forma ativa migram do citosol para o núcleo, onde interagem com diversos fatores e induzem modificações no padrão de produção e processamento de transcritos em resposta à luz (Castillon et al., 2007; Leivar and Quail, 2011; Park et al., 2012; Shikata et al., 2014).

Dentre os fatores que intermediam essa via de sinalização, destacam-se as proteínas de interação aos PHYs (PIF – *phytochrome-interacting factor*). As PIFs são fatores de transcrição do tipo *basic helix loop helix* (bHLH) parte de uma família multigênica de seis membros em *A. thaliana* (*i.e. AtPIF1*, *AtPIF3*, *AtPIF4*, *AtPIF5*, *AtPIF7* e *AtPIF8*). As proteínas PIF se acumulam no escuro, induzindo respostas à ausência de luz. Já na presença de luz, PHYs interagem com as PIFs no núcleo, impedindo a ligação a seus alvos transcricionais e levando à degradação destas proteínas e, em alguns casos, do próprio fotorreceptor (Castillon et al., 2007; Shen et al., 2007; Leivar and Quail, 2011; Park et al., 2012; Ni et al., 2014).

A interação antagônica descrita acima coloca as PIFs como componentes centrais na regulação de importantes processos fisiológicos das plantas. Em plântulas de *A. thaliana* recém-germinadas, AtPIF1 e AtPIF3 inibem o acúmulo de clorofila (Chl) e a abertura dos cotilédones no escuro, enquanto AtPIF3, AtPIF4 e AtPIF5 promovem o alongamento do hipocótilo. Durante o estiolamento, as AtPIF1 e AtPIF3 inibem o desenvolvimento de cloroplastos e a biossíntese de Chl, reprimindo a síntese de anéis tetrapirrólicos e o acúmulo de protoclorofilide (Huq et al., 2004; De Lucas et al., 2008; Moon et al., 2008; Shin et al., 2009; Stephenson et al., 2009; Chen et al., 2013; Liu et al., 2013). Esta inibição é particularmente importante durante o início do processo de desetiolamento, quando a produção rápida e excessiva de precursores de Chl poderia levar a danos

oxidativos (Chen et al., 2013; Lockhart, 2013). Contraditoriamente, esta mesma inibição é importante para o correto esverdeamento do tecido. Plantas sobre-expressando AtPIF1 e AtPIF3 crescidas no escuro apresentam maiores taxas de esverdeamento quando expostas à alta luz em comparação com plantas selvagens (Chen et al., 2013).

Além do anel tetrapirrólico da clorofila, a biossíntese de outros metabólitos de origem plastidial é regulada diretamente pelas proteínas PIF; como os carotenoides. Estudos demonstram que a luz exerce controle sobre diversos passos da biossíntese desses compostos. Neste contexto, as PIFs atuam reprimindo a expressão da primeira enzima responsável pela biossíntese de carotenoides, a FITOENO SINTASE (PSY), tanto em *A. thaliana* quanto em tomateiro (Toledo-Ortiz et al., 2010; Llorente et al., 2016). No entanto, o controle sobre a síntese destes compostos ocorre também a montante da PSY, pela inibição de genes da via do metileritritol fosfato (MEP) que fornecem os precursores necessários para a carotenogênese (Chenge-Espinosa et al., 2018).

A diferenciação e atividade plastidial estão estritamente controladas pela luz por meio de uma complexa cascata de sinalização da qual as PIFs participam como reguladores negativos. Em contraposição, os genes *GOLDEN2-LIKE* (*GLKs*) codificam fatores de transcrição que induzem o desenvolvimento plastidial e a expressão de transcritos nucleares necessários à fotossíntese (Waters *et al.*, 2008) na presença de luz. Foi descrito em *A. thaliana* que os promotores destes genes possuem motivos G-box, os quais são reconhecidos alternativamente pelas AtPIFs ou pela proteína LONG HYPOCOTYL 5 (AtHY5) (Song et al., 2014; Toledo-Ortiz et al., 2014). No escuro, em ausência de AtHY5, as AtPIFs se ligam a estes promotores inibindo a transcrição dos *AtGLKs*. Na luz, a degradação das AtPIFs e indução de AtHY5 pelos PHYs ativados levam à produção de AtGLKs e à diferenciação dos cloroplastos (Figura 1).

Devido à importância da luz na biologia dos cloroplastos, faz-se necessário um ajuste fino da expressão gênica nuclear e do metabolismo plastidial de acordo com o estágio de desenvolvimento e as condições de luminosidade (Biswal et al., 2013). Um trabalho de 2013 demonstrou a existência de um sinal retrógrado do cloroplasto que modula o *splicing* de diversos fatores nucleares em resposta à luz (Petrillo et al., 2014), evidenciando a importância de cloroplastos funcionais para respostas fisiológicas a diferentes condições de luminosidade. Por exemplo, em situações de excesso de luminosidade, um sinal retrógrado do cloroplasto reprime a expressão de *AtGLK1* por um mecanismo independente de AtPIFs, ainda não completamente

elucidado (Martín et al., 2016; Hernández-Verdeja and Strand, 2018). Esse fato é fundamental para a proteção contra estresse oxidativo provocado por luz. Ainda neste contexto, AtPIF1 e AtPIF3 atuam antagonicamente a AtHY5 na regulação de genes responsivos a ROS. As AtPIFs reprimem diretamente genes relacionados à estresse e, desta maneira, inibem a morte celular durante o desestiolamento. Pelo contrário, mutantes *Atpifs* produzem mais $^1O_2$ e apresentam mais sintomas de morte celular durante esse processo (Chen et al., 2013).

3. PIFs e o crescimento

Inúmeros estudos demonstram que a atividade da proteína e os níveis de transcritos de *AtPIF4* são regulados por diferentes estímulos além da luz, como a temperatura, o ritmo circadiano, o conteúdo de açúcares e hormônios. Neste contexto, AtPIF4 funciona como um fator integrador da complexa rede regulatória que controla o crescimento em resposta a condições ambientais e endógenas (Choi and Oh, 2016).

Após a germinação, por exemplo, plântulas são capazes de otimizar o uso de recursos para promover o crescimento dentro da terra em busca de luz: enquanto AtPIF1 e AtPIF3 inibem a diferenciação plastidial e a expansão dos cotilédones, AtPIF4 e AtPIF5 induzem o alongamento do hipocótilo no escuro. Ao atingir a luz, AtPHYs degradam AtPIFs, inibem o crescimento do hipocótilo e induzem a fotomorfogênese. A partir deste momento, AtPIF4 e AtPIF5 atuam na promoção do crescimento diário durante o período escuro, por meio da ativação direta de genes que estimulam o crescimento e por ativação das vias de biossíntese e de resposta à auxina. Em *A. thaliana*, viu-se que a taxa de crescimento está associada à interação entre a duração do período noturno e o relógio circadiano (Nozue et al., 2007; Niwa et al., 2009; Franklin et al., 2011; Kunihiro et al., 2011; Nusinow et al., 2011; Nomoto et al., 2012; Sun et al., 2012; De Montaigu et al., 2015).

Altas temperaturas induzem o acúmulo e atividade de AtPIF4, intensificando o alongamento de hipocótilos, folhas e caules. Esse efeito é conhecido há pelo menos uma década e é modulado pelo relógio circadiano e por fatores hormonais (Koini et al., 2009; Stavang et al., 2009; Franklin et al., 2011; Sun et al., 2012; Nieto et al., 2015; Gangappa and Kumar, 2017; Ibañez et al., 2018; Martínez et al., 2018). Não obstante, apenas recentemente o mecanismo molecular da

resposta à temperatura foi desvendado. Demonstrou-se que altas temperaturas aceleram a taxa de fotorreversão de PHYB de Fve para Fv, explicando ao menos parcialmente como temperatura e luminosidade interagem na regulação dos processos morfogenéticos (Jung et al., 2016; Legris et al., 2016; Huang et al., 2019; Qiu et al., 2019).

Em plantas adultas, as AtPIFs regulam ainda o tempo de floração e a senescência induzida por escuro e idade (Kumar et al., 2012; Sakuraba et al., 2014; Song et al., 2014). Durante a senescência foliar, as AtPIFs participam da sinalização que culmina na degradação das Chls e no desmantelamento dos cloroplastos visando a remobilização de nutrientes. Esta atuação será discutida na seção a seguir.

4. PIFs e a regulação da senescência foliar

A indução da senescência foliar é desencadeada por fatores como a idade do órgão, estresse salino ou hídrico, privação de nutrientes e ausência de luz. Estes sinais são transduzidos por uma complexa rede regulatória, da qual participam fitormônios (Khan et al., 2014) e que objetiva a remobilização de nutrientes para outras regiões da planta (*e.g.* folhas jovens, estruturas reprodutivas, órgãos de reserva e frutos) (Buchanan-Wollaston, 1997). Como resultado da expressão dos *SENESCENCE-ASSOCIATED GENES* (*SAGs*), ocorrem diversas modificações bioquímicas e estruturais que levam ao declínio da capacidade fotossintética e a degradação de Chls, de macromoléculas e de membranas (Sarwat et al., 2013).

Foi descrita a relação das AtPIF3, AtPIF4 e AtPIF5 com a senescência foliar em *A. thaliana* (Sakuraba et al., 2014; Song et al., 2014). Viu-se que estes fatores são expressos no início da senescência induzida por escuro e idade e levam à expressão de diversas proteínas SAGs. Mutantes de perda de função destas AtPIFs apresentam maiores conteúdos de Chls, taxas de fotossíntese e longevidade foliar. Além disso, a proteína AtPIF4 induz a produção de etileno, um dos hormônios responsáveis pela indução da senescência e do amadurecimento de frutos carnosos (Bleecker and Kende, 2000; Song et al., 2014).

Em *A. thaliana*, o gene *ORESARA1* (*AtORE1*), da família de fatores de transcrição NAC (NAM, ATAF, e CUC), controla diversos genes envolvidos na senescência, dentre eles os *AtSAGs*.

A proteína codificada por *AtORE1* interage com os AtGLKs formando um heterodímero, bloqueando a atividade desta última e resultando no desmantelamento dos cloroplastos. Adicionalmente, a expressão de *AtORE1* é induzida pelo envelhecimento de forma dependente de ETHYLENE INSENSITIVE 2 (EIN2), proteína da via de sinalização de etileno, e reprimida pelo miR164. Por sua vez, o nível de miR164 decresce com o envelhecimento via o efeito inibitório de EIN2 (Kim et al., 2009; Khan et al., 2014). Recentemente, foi verificado que AtPIF4 e AtPIF5 controlam os níveis de *AtORE1* em *A. thaliana*. Por meio da ativação de genes das vias de etileno (ETHYLENE INSENSITIVE 3) e de ABA (ABA-INSENSITIVE 5 e ENHANCED EM LEVEL), essas PIFs induzem a expressão de *AtORE1* e diversos *AtSAGs*, incluindo enzimas do catabolismo de Chl (Sakuraba et al., 2014) e levam direta (*via* repressão transcricional) e indiretamente (*via* AtORE1) à repressão dos *AtGLKs* (Rauf et al., 2013) (Figura 1).

A manipulação da senescência é uma estratégia importante para o melhoramento vegetal. Trabalhos realizados por nosso grupo de pesquisa revelam que a manipulação da senescência em tomateiro pode ter um efeito na produtividade e na qualidade nutricional dos frutos. Por exemplo, o silenciamento do gene que codifica a enzima FEOFITINASE (PPH), envolvida na degradação da Chl durante a senescência, afeta diferentes parâmetros de interesse comercial em tomateiro (Lira et al., 2016). Isso porque o silenciamento desse gene afeta a produção de carotenoides, vitamina E e o metabolismo de carbono em folhas e frutos. Ainda, foi demonstrado que o gene ortólogo ao *AtORE1* em tomateiro, *SlORE1S02*, é regulado também pelo miR164 e interage fisicamente com as SlGLKs, inibindo sua ação e desencadeando a senescência. O silenciamento do gene *SlORE1S02* resultou no retardo na senescência foliar mantendo a fotossíntese ativa por mais tempo resultando no aumento na produtividade e qualidade dos frutos (Lira et al., 2017). No entanto, o papel das SlPIFs neste processo não foi caracterizado até o momento em tomateiro.

5. PIFs e o controle da floração

O controle da floração está associado à adaptação de espécies vegetais ao ambiente, de maneira a otimizar o *fitness*. Diversos fatores endógenos e exógenos podem regular o tempo de floração, como o comprimento do dia, a temperatura e fitohormônios (Srikanth and Schmid, 2011). Em A. *thaliana*, o controle da floração é realizado pelo módulo CONSTANS (AtCO) –

FLOWERING LOCUS T (AtFT). Em dias curtos, o fator de transcrição AtCO induz a expressão de AtFT em folhas. Após a tradução, a proteína AtFT é translocada pelo floema para o meristema apical (*shoot apical meristem* – SAM), onde induz a transição do meristema vegetativo para floral (Golembeski et al., 2014).

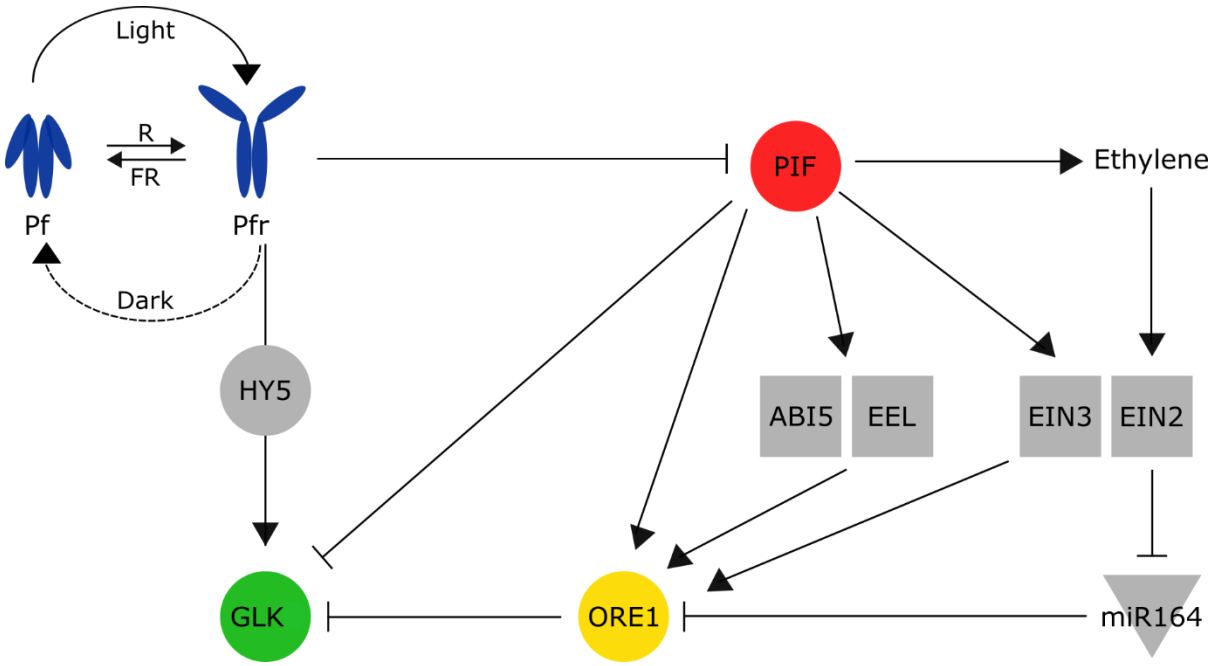


Figura 1: Esquema da regulação da senescência descrito para *A. thaliana*. Adaptado de Rauf *et al*. (2013) e Sakuraba *et al*. (2014). FR: vermelho-extremo; R: vermelho; Pr: fitocromo inativo; Pfr: fitocromo ativo; PIF: PHYTOCHROME INTERACTING FACTOR; HY5: LONG HYPOCOTYL 5; GLK: GOLDEN2-LIKE; ORE1: ORESARA1; EIN2 e EIN3: ETHYLENE INSENSITIVE 2 e 3, proteínas da rota de sinalização de etileno; ABI5 e EEL: ABA-INSENSITIVE e ENHANCED EM LEVEL, proteínas da rota de sinalização de ácido abscísico (ABA); miR164: microRNA 164.

Um segundo fator importante que regula a floração é a temperatura. Em *A. thaliana*, foi descrito recentemente que PHYB além de receptor luminoso, é também um termosensor, porque a reversão para a forma inativa é induzida por altas temperaturas, de modo que este fotorreceptor

atua na integração desses dois sinais ambientais (Legris et al., 2016). Assim, sob altas temperaturas, o fator BRASSINAZOLE RESISTANT 1 (AtBZR1) induz a expressão de *AtPIF4* que, associada à inativação de PHYB, leva a um acúmulo da proteína AtPIF4 (Ibañez et al., 2018). AtPIF4, por sua vez, interage diretamente com o promotor de *AtFT*, induzindo a expressão deste gene (Kumar et al., 2012). Ainda, AtPIF4 reprime a expressão do miR156, responsável por silenciar o gene *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3* (*AtSPL3*), um indutor da floração (Kim et al., 2012; Xie et al., 2017). Como resultado dessas interações, há um aumento na expressão de *AtFT* e indução da floração sob altas temperaturas.

Uma diferença notável entre *A. thaliana* e *S. lycopersicum* é o fato de que o tomateiro é uma planta de dia-neutro, ou seja, indiferente ao comprimento do dia para a floração. Desta maneira, embora o módulo CO-FT seja conservado em angiospermas, o florescimento em tomateiro é induzido pelo homólogo de FT, SINGLE FLOWER TRUSS (SlSFT), de maneira independente de CO (Molinero-Rosales et al., 2004; Lifschitz and Eshed, 2006; Srikanth and Schmid, 2011). Adicionalmente, a transição floral provoca o fim do crescimento vegetativo em *A. thaliana*, pois o SAM se diferencia em meristema reprodutivo, gerando uma flor terminal; este é o chamado crescimento determinado. Em tomateiro, no entanto, o SAM se diferencia para produzir um ramo floral e a gema lateral imediatamente abaixo adquire dominância e passa a produzir novas folhas. Isso ocorre sucessivamente, de modo que ocorra crescimento simpodial. A porção da planta entre cada nova folha e a terminação floral é denominada unidade simpodial (*sympodial unit* - SU). Dois genes são os principais responsáveis por este tipo de crescimento, o SlSFT e o fator antagônico SELF PRUNNING (SlSP). *SlSP* é expresso no SAM no início do desenvolvimento e é responsável pela manutenção do crescimento vegetativo. Ao longo do tempo, a expressão de SP diminuiu e, concomitantemente, a expressão de *SlSFT* nas folhas aumenta levando à floração. Contraditoriamente, *SlSFT* induz indiretamente a expressão de *SlSP* na gema lateral, dando origem à próxima SU (Lifschitz and Eshed, 2006; Lifschitz et al., 2006). Existem também variedades com crescimento determinado como consequência da mutação *sp* (Pnueli et al., 1998). Finalmente, diferentes combinações alélicas entre SlSP e SlSFT, assim como outros genes envolvidos na morfogênese (*i.e.* CENTRORADIALIS/TERMINALFLOWER1/SELF-PRUNING – CETS gene Family) regulam a arquitetura da planta produzindo plantas semi-determinadas afetando a produtividade (Krieger et al., 2010; Jiang et al., 2013; Vicente et al., 2015). Embora o efeito da

relação entre *SlSP* e *SlSFT* sobre o crescimento e produtividade em tomateiro tenha sido extensamente estudado, não há relatos da relação das SlPIFs com a floração nesta espécie.

6. *Solanum lycopersicum* como espécie modelo

O tomateiro, *Solanum lycopersicum* L., pertence à família Solanaceae, a mesma de diversas outras espécies de importância agronômica como a batata (*Solanum tuberosum*), o tabaco (*Nicotiana tabacum*) e a berinjela (*Solanum melongena*). O fruto desta planta é altamente consumido em todo o mundo tanto em sua forma *in natura*, como processada. O Brasil é 10º maior produtor mundial desta *commodity* e em 2017 foi responsável por 4,2 milhões das 182,3 milhões de toneladas produzidas no mesmo ano (FAOSTAT, acessado em 12/05/2019).

S. lycopersicum é uma espécie autógama, diploide, cujo genoma de 900 Mb compreendido em 12 cromossomos foi completamente sequenciado (Consortium, 2012). A existência de diversas espécies selvagens filogeneticamente próximas, com as quais é possível obter híbridos, constitui uma importante fonte de variabilidade para o melhoramento genético (Schauer et al., 2006). Exemplo disto são as diversas populações de linhagens introgredidas interespecíficas estabelecidas (Eshed and Zamir, 1995). Finalmente, eficientes técnicas de transformação estável (Pino et al., 2010) e transiente (Orzaez et al., 2006; Quadrana et al., 2011) fazem do tomateiro uma espécie modelo alternativa a *A. thaliana*.

Particularmente, pela importância para a nutrição humana e a biologia dos frutos, *S. lycopersicum* se consolidou como o modelo para o estudo dos processos bioquímicos envolvidos no desenvolvimento e amadurecimento de frutos carnosos (Carrari and Fernie, 2006). Ao longo do desenvolvimento do fruto, distinguem-se quatro fases, que podem ser observadas na Figura 2: (i) o desenvolvimento da flor, desde a iniciação floral até antese; (ii) o período de intensa divisão celular que começa após fertilização (estágios verde-imaturos, *immature green* - IG); (iii) o período de expansão celular, que se estende desde o fim da fase de divisão até o início do amadurecimento, quando o fruto atinge o tamanho máximo e se torna responsivo ao etileno (estágio verde-maduro, *mature green* – MG); e (iv) o amadurecimento (estágios *breaker* – BR), marcado por alterações bioquímicas, incluindo o acúmulo de açúcares, ácidos, pigmentos e

compostos voláteis, que afetam a aparência, a textura e o teor nutricional atraindo organismos dispersores de sementes (Gillaspy et al., 1993; Giovannoni, 2004).

Figura 2: Desenvolvimento de flores e frutos de tomateiro MicroTom. F: flor em desenvolvimento, FA: flor em antese; IGs: estágios verde-imaturos; MG: estágio verde maduro; BR: breaker, BR1-BR12: 1-12 dias após início da mudança de cor.

Apesar dos frutos de tomateiro serem órgãos dreno, necessitando mais fotoassimilados do que conseguem produzir, durante as fases de divisão e expansão celular, a maquinaria fotossintética local é responsável por até 20% do total de carbono no fruto maduro (Carrara et al., 2001; Cocaliadis et al., 2014). Durante o amadurecimento do fruto, os cloroplastos fotossinteticamente ativos são convertidos em cromoplastos, sendo esse processo marcado pela desorganização do sistema interno de membranas e intensa alteração metabólica. Este processo é acompanhado da degradação de clorofila (Chl) e do acúmulo de diversos compostos nutracêuticos antioxidantes, tais como tocoferóis e carotenoides, dentre os quais predomina o licopeno, pigmento que determina a mudança de cor do fruto iniciada no estágio BR (Figura 2) (Egea et al., 2010; Klee and Giovannoni, 2011). A maturação do tomate, um fruto climatérico, coincide com a maturação da semente e está intimamente relacionada ao aumento da respiração e biossíntese de etileno, desencadeando a reprogramação da expressão gênica que regula importantes rotas metabólicas (Carrari and Fernie, 2006; Renato et al., 2014). Sob a perspectiva agronômica, o valor nutricional, o sabor, o aroma, as características de processamento e o tempo de prateleira determinam a qualidade do fruto (Osorio et al., 2013).

7. Plastídios e qualidade dos frutos

O fruto maduro de tomate contém quantidades significativas de açúcares e de compostos com atividade antioxidante, como carotenoides, flavonoides, fenilpropanoides e tocoferóis derivados do metabolismo secundário (Abushita et al., 2000; Giovannucci et al., 2002). As rotas que produzem os precursores para a biossíntese desses compostos são plastidiais. Neste sentido, genes que controlam o metabolismo plastidial tem demonstrado afetar a qualidade nutricional e industrial dos frutos (Cocaliadis et al., 2014). Por exemplo, o produto do gene *SlDET1* (*DE-ETIOLATED1*) é um regulador negativo da resposta à luz que controla a expressão gênica por meio do remodelamento da cromatina. A perda de função de *DET1* em frutos leva ao aumento do número e do volume dos cloroplastos por célula resultando em frutos com maior quantidade de carotenoides, flavonoides, fenilpropanoides e tocoferóis (Davuluri et al., 2004; Enfissi et al., 2010).

Em *S. lycopersicum*, os genes *SlGLK1* e *SlGLK2* são expressos nas folhas, sendo que apenas o transcrito de *SlGLK2* é detectado nos frutos. O padrão de expressão de *SlGLK2* nos frutos está distribuído em um gradiente latitudinal que gera o chamado "ombro verde", local de maior densidade de cloroplastos e conteúdo de Chl (Powell et al., 2012). A sobre-expressão de *SlGLK2* resulta em frutos imaturos com maior número de cloroplastos que apresentam maior empilhamento dos grana (Lupi et al., 2019). Após amadurecimento, esses frutos possuem maiores conteúdos de açúcares solúveis, carotenoides e tocoferóis, evidenciando que o aumento em número e a manutenção de cloroplastos ativos nos frutos verdes são importantes para o acúmulo de nutrientes nos frutos maduros (Nguyen et al., 2014; Lira et al., 2017; Lupi et al., 2019). O alelo selvagem funcional de *SlGLK2* (*U*) foi perdido em tomateiros cultivados pela progressiva seleção de frutos com amadurecimento uniforme (*u/u - uniform ripening*). O alelo mutado *Slglk2* gera uma proteína truncada inativa pela inserção de uma base na sua região codificante. Em decorrência da falta de SlGLK2 ativa, há diminuição dos níveis de carotenoides e sólidos solúveis (*brix*), evidenciando que o processo de domesticação dos tomateiros levou a perdas significativas da qualidade nutricional dos tomates (Powell et al., 2012; Nguyen et al., 2014).

Um trabalho publicado por nosso grupo de pesquisa (Almeida et al., 2015) demonstrou que mutantes de tomateiro deficientes no amadurecimento e na degradação da Chl apresentam alterações no acúmulo de carotenoides e tocoferóis em frutos. Finalmente, foi descrito que

mutantes de tomateiro deficientes em PHYs apresentam alteração no conteúdo de carotenoides nos frutos, assim como alteração na progressão entre as diferentes fases de desenvolvimento e amadurecimento (Gupta et al., 2014). Recentemente, demonstrou-se que o silenciamento fruto-específico dos genes SlPHYA e SlPHYB2 afeta a divisão e diferenciação plastidial, bem como o metabolismo dos plastídios nesse órgão (Bianchetti et al., 2018). E que, portanto, a expressão alterada de genes relacionados à força do dreno (metabolismo de açúcares) e biossíntese de carotenoides nos próprios frutos levaram à redução de açúcares solúveis e de carotenoides nessas plantas.

Desta forma, pelo papel fundamental da luz no desenvolvimento e atividade plastidial, e a participação das PIFs na sinalização luminosa, espera-se que estas proteínas afetem o padrão de amadurecimento e qualidade dos frutos maduros.

8. Vitamina E

Diversos radicais livres, como as espécies reativas de oxigênio (ROS), são gerados naturalmente em função do metabolismo celular. Algumas condições abióticas, como estresse luminoso, temperaturas extremas, alta salinidade e seca; e também bióticas, como ataque de patógenos, podem levar a uma produção excessiva de ROS. São conhecidas algumas funções sinalizadoras para essas moléculas, que estão relacionadas a respostas ao ambiente (Choudhury et al., 2017). Entretanto, um desbalanço entre a produção e a capacidade celular de mitigar a ação desses radicais livres pode ocasionar o chamado estresse oxidativo, que tem como consequências danos a ácidos graxos poli-insaturados (PUFAs), proteínas, ácidos nucléicos e, em casos extremos, morte celular. Por este motivo, diversos mecanismos enzimáticos e não-enzimáticos evoluíram em plantas e outros organismos para a manutenção da homeostase através da detoxificação de ROS (Tripathy and Oelmüller, 2012; Barbosa et al., 2014).

Em plantas, os cloroplastos, as mitocôndrias e os peroxissomos são as principais fontes de ROS nos tecidos verdes (Janků et al., 2019). Nos cloroplastos, a produção constante de oxigênio molecular (O_2) em função da fotólise da água origina um ambiente propício para a formação de ROS. Sob condições normais e acentuadamente sob luminosidade excessiva, a transferência ineficiente de energia da Chl para outros componentes da cadeia de transporte de elétrons causa a

conversão dessa molécula para o estado triplet (Chl*), que por sua vez interage com O_2 levando à formação de oxigênio singleto ($^1O_2$), uma espécie altamente reativa (Tripathy and Oelmüller, 2012). Desta maneira, a própria atividade fotossintética gera radicais danosos aos fotossistemas (PSI e PSII).

Para lidar com a produção de ROS nos cloroplastos, as plantas contam com um conjunto de moléculas antioxidantes não-enzimáticas, como carotenoides, ascorbato e tocoferóis. Estes últimos são moléculas lipossolúveis anfipáticas com grande poder antioxidante que, conjuntamente com os tocotrienóis, são chamados de vitamina E (VTE). Presentes no PSII, são especialmente importantes sob condições de alta luminosidade, pois eliminam $^1O_2$ e inibem a propagação da peroxidação lipídica, de modo a prevenir danos à maquinaria fotossintética protegendo as membranas dos tilacóides (Miret and Munné-Bosch, 2015). Estas funções foram comprovadas em diversos estudos. Por exemplo, em *Chlamydomonas reinhardtii*, foi verificado que o bloqueio na biossíntese de VTE por herbicidas leva à perda de atividade do PSII (Trebst et al., 2004). Em *A. thaliana*, mutantes deficientes na produção de VTE são mais suscetíveis à peroxidação lipídica e a danos fotooxidativos (Havaux et al., 2005).

Do ponto de vista da nutrição humana, os tocoferóis têm grande importância. Primeiramente, porque os danos oxidativos estão relacionados ao envelhecimento e a diversas doenças, como câncer e afecções cardíacas (Organization, 2004; Mathur et al., 2015; Grimm et al., 2016; Peh et al., 2016). Neste contexto, os tocoferóis são os principais antioxidantes lipossolúveis no sistema de defesa humano a ROS. Obtidos exclusivamente da dieta, os tocoferóis agem, de modo análogo às plantas, inibindo a peroxidação lipídica de PUFAs e outros componentes de membranas celulares. Os problemas ocasionados por deficiência de VTE decorrem, no geral, do extravasamento dos conteúdos das células pelo rompimento de membranas. Assim, o diagnóstico dessa deficiência pode ser feito pela detecção de enzimas musculares e altos níveis de produtos de peroxidação lipídica no plasma sanguíneo (Organization, 2004). Dentre as afeções causadas pela deficiência de VTE estão síndromes neuromusculares e neurodegenerativas como a ataxia (Guggenheim et al., 1982; Ouahchi et al., 1995), anemia hemolítica (Oski and Barness, 1967), e doenças coronárias (Bellizzi et al., 1994).

A VTE é sintetizada exclusivamente por organismos fotossintetizantes. A síntese ocorre nos plastídios, a partir da condensação de dois precursores: uma cauda apolar - o fitil-difosfato

(PDP) - e uma cabeça polar - o homogentisato -, derivados das vias do metil-eritritol fosfato (MEP) e do ácido chiquímico (SK), respectivamente. A biossíntese de tocoferol está intimamente relacionada ao metabolismo de Chl e de carotenoides. Isso porque a via do MEP fornece precursores isoprenóides também para a síntese dessas substâncias, o PDP e o geranilgeranil difosfato, respectivamente. O PDP pode ser ainda originado por meio da reciclagem da cauda de fitol liberado na degradação da Chl, em complementação à síntese *de novo* via MEP (Almeida et al., 2015) (Figura 4).

Os tocoferóis existem em quatro diferentes formas que variam na quantidade e posição dos radicais metila (CH_3-) no anel aromático da cabeça polar (Figura 5). Dentre estas formas, o α-tocoferol possui a maior atividade biológica de VTE, isto é, se apresenta como a forma com maior atividade e a mais absorvida em humanos (Bjørneboe et al., 1990; DellaPenna, 2005; Azzi, 2018). Ainda, o α-tocoferol é a forma mais abundante em folhas e frutos, inclusive em tomateiro (Almeida et al., 2011).

Nos últimos anos, o grupo do Laboratório de Genética Molecular de Plantas do Instituto de Biociências da USP tem estudado a biossíntese de tocoferóis em tomateiro. Neste contexto, foram identificados e caracterizados todos os genes que codificam enzimas participantes da biossíntese destas substâncias em *Solanum lycopersicum* (Almeida et al., 2011). Trabalhos do grupo demonstraram que a transcrição dos genes da biossíntese de VTE é regulada espaço-temporalmente e que diversos genes co-regulados em tecidos fotossintéticos e em frutos durante o amadurecimento apresentam os mesmos motivos *cis*-regulatórios nos seus promotores, sugerindo a regulação por fatores de transcrição comuns. Ainda, análises de redes de co-regulação revelaram que os perfis de expressão de alguns dos genes da rota de tocoferol correlacionam com os conteúdos de Chl (Quadrana et al., 2013).

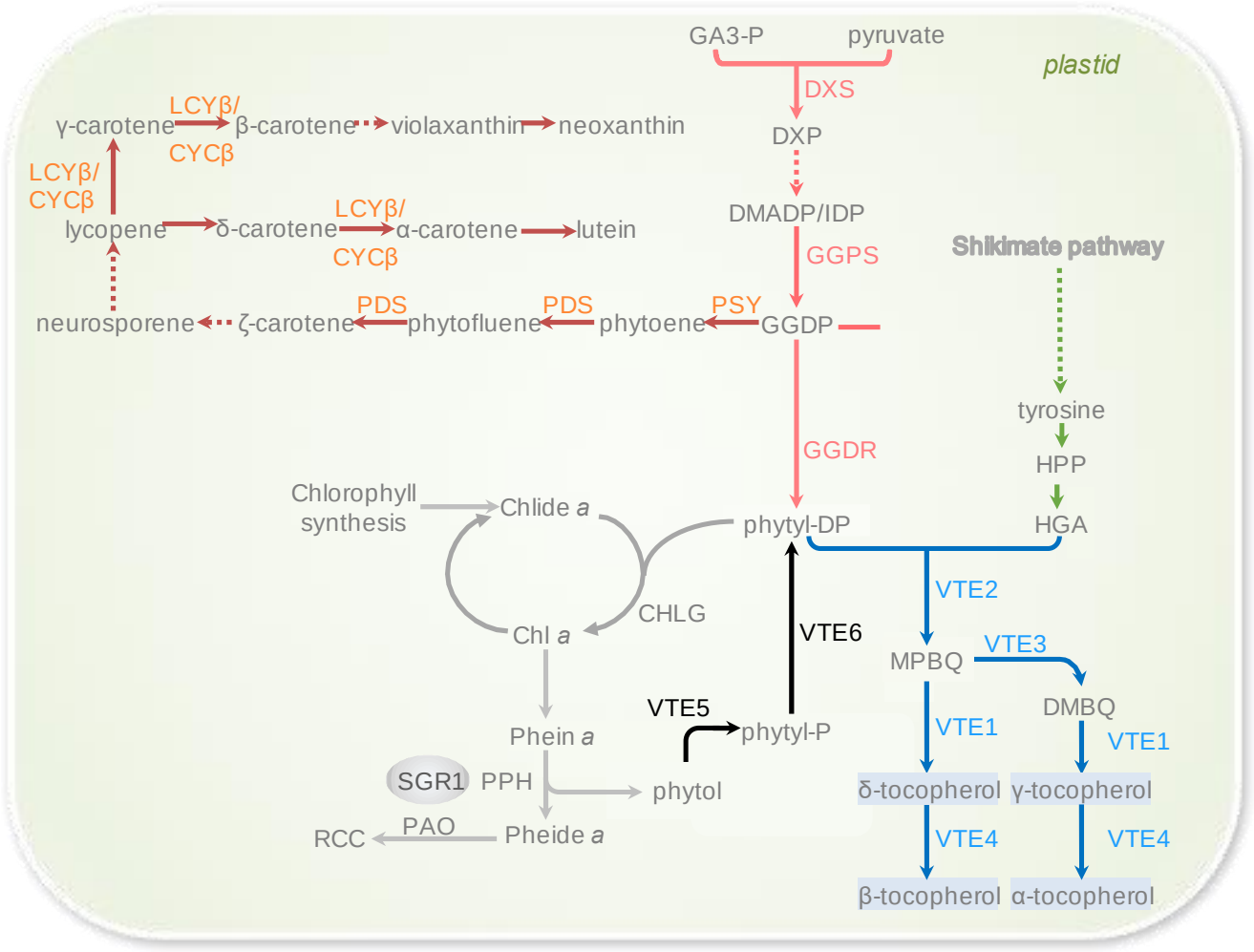
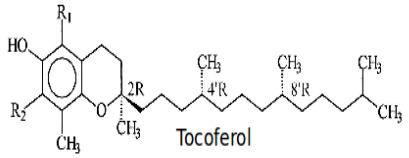


Figura 4: Biossíntese de tocoferóis, carotenoides e Chl nos plastídios. As enzimas estão indicadas de acordo às seguintes abreviaturas: DXS, 1-deoxi-D-xilulose-5-P sintase; GGDR, geranilgeranil difosfato redutase; VTE2, homogentisato fitil transferase; VTE3, 2,3-dimethil-5-fitilquinol metiltransferase; VTE1, tocoferol ciclase; VTE4, γ-tocoferol-C-methil transferase; PSY, fitoeno sintase; PDS, fitoeno desaturase; LCYβ, β-licopeno ciclase cloroplástica; CYCβ, β-licopeno ciclase cromoplástica; CHLG, clorofila sintase; SGR1, *staygreen 1*; PPH, feofitinase; PAO, feoforbide a oxigenase; VTE5, fitol quinase; VTE6, fitil fosfato quinase. Os metabólitos estão indicados de acordo às seguintes abreviaturas: GA3-P, gliceraldeido 3-P; DXP, 1-deoxi-D-xilulose-5-P; IDP, isopentenil difosfato; DMADP, dimetilalil difosfato; GGDP, geranilgeranil-difosfate; HPP, HGA, hidroxifenilpiruvato; homogentisato; Chlide a, clorofilide a; Phein a, feofitina a; Pheide a, feoforbide a; RCC, catabolito vermelho da clorofila; MPBQ, 2-metil-6-geranilgeranilbenzoquinol; DMBQ, 2,3-dimetil-6-geranilgeranilbenzoquinol. Adaptado de Almeida et al. (2015).

O estudo da regulação transcricional também expôs que a disponibilidade de PDP é um fator limitante para o acúmulo de VTE (Quadrana et al., 2013), hipótese que foi reforçada pelo estudo dos perfis bioquímicos e de expressão de mutantes deficientes no amadurecimento e degradacão de Chl (Almeida et al., 2015). Durante o amadurecimento dos frutos, o precursor geranilgeranil difosfato é canalizado para a produção de carotenoides, pela redução da transcrição de *GERANILGERANIL DIFOSFATO REDUTASE* (*GGDR*) (Figura 4), de modo que o fitol proveniente da degradação de Chl tem papel fundamental na manutenção dos níveis de tocoferol em frutos. O silenciamento do gene *VTE5* (*FITOL QUINASE*), que codifica a enzima responsável pela primeira etapa de fosforilação do fitol durante sua reciclagem, reduz em 80% a produção de VTE tanto em folhas quanto em frutos de tomateiro. Ainda, a deficiência de VTE resulta na queda da taxa fotossintética e afeta o particionamento de carbono, diminuindo a produtividade (Almeida et al., 2015). Mais recentemente, foi demostrado que as plantas silenciadas para *VTE5* apresentam hipersensibilidade a condições de alta irradiância e alta temperatura (Spicher et al., 2017). Desta forma, os dados obtidos demonstram a importância agronômica do tocoferol e expõem claramente a relação entre a luz, a Chl e a VTE. Neste contexto, as proteínas PIF emergem como possíveis reguladores dos conteúdos de VTE, dada a relação com o metabolismo isoprenóide discutido nas seções anteriores.



Atividade *versus* α-tocoferol

Forma	R1	R2	Atividade vitamina E (%)
α-tocoferol	CH_3	CH_3	100
β-tocoferol	CH_3	H	25-50
γ-tocoferol	H	CH_3	8-19
δ-tocoferol	H	H	<3

Figura 5: Estrutura dos tocoferóis. A atividade de vitamina E está representada em porcentagem relativizada com a de α-tocoferol (Adaptado de DellaPenna, 2005).

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OBJETIVOS

Considerando-se que as proteínas PIF são fatores chave da sinalização luminosa, da importância da sinalização luminosa para a atividade plastidial e desta para a determinação da produtividade e qualidade nutricional, este trabalho tem como hipótese que a modulação da expressão de PIFs terá um impacto sobre a produtividade e qualidade nutricional. Assim, o objetivo geral do projeto é caracterizar funcionalmente as PIFs de tomateiro. Para isto, propõem-se os seguintes objetivos específicos: (I) estudar a diversidade, história evolutiva e perfil de expressão dos genes *SlPIFs*; (II) avaliar o efeito da luz sobre o metabolismo de VTE e a participação das SlPIFs como fatores regulatórios; (III) avaliar o impacto do silenciamento do gene *SlPIF4* sobre a produtividade e qualidade nutricional. Assim, o trabalho está organizado em três capítulos que compreendem os objetivos mencionados:

Capítulo I: Diversidade, História Evolutiva e Perfil de Expressão de *SlPIFs*

1) Estudo filogenético e evolutivo dos genes *PIF* em Viridiplantae.
2) Caracterização do perfil de transcritos de *SlPIFs* durante:
    a. o desestiolamento de plântulas;
    b. ao longo da senescência induzida por escuro;
    c. ao longo do fotoperíodo e;
    d. o amadurecimento de frutos.

Capítulo II: Efeito da luz sobre o metabolismo de VTE e a participação das SlPIFs como fatores regulatórios

1) Estudar o efeito da luz sobre o acúmulo de VTE;
2) Estudar o efeito da luz sobre a expressão dos genes da rota biossintética de VTE e;
3) Estudar a regulação direta por SlPIFs dos genes responsivos à luz.

Capítulo III: Impacto do silenciamento do gene *SlPIF4* sobre a produtividade e qualidade nutricional

1) Obtenção de linhagens de tomateiro silenciadas para o gene *SlPIF4.*
2) Avaliação fenotípica de linhagens silenciadas para o gene *SlPIF4.*

CAPÍTULO I

Diversidade, história evolutiva e perfil de expressão de *SlPIFs*

"[…] from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved."

Charles Darwin

This chapter is organized as published in the scientific journal PLOS ONE:

Rosado D, Gramegna G, Cruz A, Lira BS, Freschi L, de Setta N, Rossi M (2016) Phytochrome Interacting Factors (PIFs) in *Solanum lycopersicum*: Diversity, evolutionary history and expression profiling during different developmental processes. PloS one **11**: e0165929.

ABSTRACT

Although the importance of light for tomato plant yield and edible fruit quality is well known, the PHYTOCHROME INTERACTING FACTORS (PIFs), main components of phytochrome-mediated light signal transduction, have been studied almost exclusively in *Arabidopsis thaliana*. Here, the diversity, evolution and expression profile of *PIF* gene subfamily in *Solanum lycopersicum* was characterized. Eight tomato *PIF* loci were identified, named *SlPIF1a*, *SlPIF1b*, *SlPIF3*, *SlPIF4*, *SlPIF7a*, *SlPIF7b*, *SlPIF8a* and *SlPIF8b*. The duplication of *SlPIF1*, *SlPIF7* and *SlPIF8* genes were dated and temporally coincided with the whole genome triplication event that preceded tomato and potato divergence. Different patterns of mRNA accumulation in response to light treatments were observed during seedling deetiolation, dark-induced senescence, diel cycle and fruit ripening. *SlPIF4* showed similar expression profile as that reported for *A. thaliana* homologs, indicating an evolutionary conserved function of PIF4 clade. A comprehensive analysis of the evolutionary and transcriptional data allowed proposing that duplicated *SlPIFs* have undergone sub- and neofunctionalization at mRNA level, pinpointing the importance of transcriptional regulation for the maintenance of duplicated genes. Altogether, the results indicate that genome polyploidization and functional divergence have played a major role in diversification of the *Solanum PIF* gene subfamily.

INTRODUCTION

Every aspect of plant physiology is influenced by light. Right after germination, etiolated growth (skotomorphogenesis) allows seedlings to seek for light at the soil surface and, upon light exposure, signal transduction initiates photomorphogenic development (deetiolation), characterized by chloroplast differentiation and initiation of photosynthetic activity. During autotrophic vegetative development, light provides the energy that fuels plant growth, designs architecture of mature plant and regulates flowering. Furthermore, light deprivation is na important senescence inducer in lower leaves shaded by upper leaves for nutrient remobilization. The capability to adjust to environmental light conditions is mediated by photoreceptors, which perceive and transduce light signals to the downstream transcriptional network that triggers adaptive responses [1].

Solanum lycopersicum, a fleshy fruit bearing species, is an excellent model for deciphering light signal transduction network. Firstly, because tomato plant yield and edible fruit quality are determined by plastid biogenesis and activity that, in turn, are highly dependent on light perception and transduction. *High pigment* tomato mutants, *hp1* and *hp2*, are deficient in the negative regulators of light signal transduction *DAMAGE DNA BINDING PROTEIN 1* (*DDB1*) and *DE-ETIOLATED* (*DET1*), respectively. The fruits of these plants show increased levels of chlorophyll and higher levels of the nutraceutical carotenoids, flavonoids and tocopherols in immature and mature stages, respectively [2,3]. Light-grown seedlings of tomato transgenic lines silenced for *ELONGATED HYPOCOTYL 5* (*HY5*), a positive regulator of light signaling involved in plastid biogenesis, displayed etiolated phenotype and adult plants showed over 30% reduction in leaf and immature fruit chlorophyll accumulation. Moreover, total carotenoid levels in ripe fruits of HY5-deficient plants were significantly decreased compared to wild type controls [4]. Secondly, *Solanum* lineage have been affected by two whole-genome triplications; the first occurred before the divergence between *Arabidopsis* and *Solanum* more than 120 MYA, while the second preceded the divergence between tomato and potato estimated at 71 (± 19.4) MYA [5]. Polyploidization events provide the basis for the evolution of novel functions and, in particular, the expansion of genes encoding transcription factors correlates with the evolutionary gain of morphological complexity [6]. In this sense, it has been proposed that these genome triplications contributed with

fruit-specific functions in tomato, such as the ripening master transcription factor *RIPENING INHIBITOR* (*RIN*) and phytochrome (PHYs) photoreceptors that influence fruit quality [5].

PHYs are major photoreceptors that perceive red (R)/far-red (FR)-light. Five PHYs *loci* have been identified in tomato genome designated *PHYA*, *PHYB1*, *PHYB2*, *PHYE* and *PHYF* in accordance to the *A. thaliana PHYA* to *PHYE* homologs [7]. The role of the tomato *PHYs* in vegetative development has been explored by the characterization of mutants [8] and overexpressing [9] plants for *PHYA*, *PHYB1* and *PHYB2*. Increasing *PHYA* and *PHYB1* expression rendered mild effects on anthocyanin levels and on seedling and adult plant development. On the contrary, transgenic plants with high levels of *PHYB2* showed an acute inhibition of elongation, enhancement of anthocyanin accumulation, and strong amplification of the red light high irradiance response [9]. By using single, double or triple mutants (*phyA*, *phyB1*, *phyB2*, *phyB1B2*, *phyAB1* and *phyAB1B2*), a recent report evaluated the participation of different phytochrome species in the regulation of fruit development and ripening. The results showed that the impairment in distinct *PHYs* differentially influences the time intervals among fruit developmental stages as well as the carotenoid content [10].

PHYs exist in two different forms, the R-absorbing Pr form and the FR-absorbing Pfr form. R triggers activation of PHYs by converting the Pr form to the Pfr form, whereas FR inactivates Pfr converting it back to the Pr form. Active PHYs Pfr form is translocated to the cell nucleus where it physically interacts with the PHYTOCHROME-INTERACTING FACTORS (PIFs). PIFs are basic helix–loop–helix (bHLH) transcription factors that play a key role in PHY-mediated light signal transduction being part of the regulatory network of a wide range of developmental processes, from seed germination towards senescence. However, with few exceptions [11–14], *PIFs* have been only studied in *A. thaliana*. PIF proteins have an Active Phytochrome B-binding (APB) and a DNA-binding bHLH domain. The canonical PIFs, *i.e*. PIF1, PIF3, PIF4, PIF5 and PIF7, physically interact with PHYB; while PIF1 and PIF3 also interact with PHYA through an Active Phytochrome A-binding (APA) domain. Pfr-PIF interaction triggers phosphorylation and subsequent proteasomal degradation of PIFs, which leads to physiological responses. A notable exception to this dynamic behavior is PIF7, which despite interacting with PHYB shows no detectable light-induced degradation [1]. Several target genes for *A. thaliana* PIF proteins have been identified. PIF3 mediates the initial phases of seedling light induced chloroplast development

during deetiolation through the regulation of nuclear genes involved in photosynthesis and chloroplast biogenesis [15]. ChIP–PCR experiment confirmed that PIF4 binds to the E-box motifs of the promoters of both chloroplast activity maintainer genes *GOLDEN 2-LIKE 1* (*GLK1*) and *GLK2*, repressing their expression [16]. Additionally, PIF4 and PIF5 act as transcriptional activators of the master senescence transcription factor *ORESARA 1* (*ORE1*) and chlorophyll degrading enzyme encoding genes, such as *STAY GREEN 1* (*SGR1*) and *NON-YELLOW COLORING 1* (*NYC1*), during dark-induced senescence by direct interaction with the G-box motifs on the corresponding promoter regions [16–18]. Finally, PIF1 has been shown to directly bind the G-box motif of the promoter of the chlorophyll and carotenoid biosynthetic genes *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE* and *PHYTOENE SYNTHASE* (*PSY*), inducing and inhibiting their transcription, respectively [19,20]. Only one tomato *PIF* gene has been characterized so far, *PIF1a*, and, in agreement with its *Arabidopsis* ortholog showed to modulate carotenoid biosynthesis during fruit ripening. During green stages of fruit development, as a consequence of self-shading, Chl reduces R/ FR ratio stabilizing PIF1a, which, in turn, represses the expression of the fruit-specific*PSY1*. After the onset of ripening, degreening allows the activation of Pfr and the consequent PIF1a degradation releases *PSY1* transcription, enhancing carotenogenesis [12, 21].

Considering the importance of light perception and signaling for plant development and fruit quality and, the poorly available knowledge about *PIF* genes in tomato; here we performed a comprehensive characterization of this gene subfamily in *S. lycopersicum*. By surveying the tomato genome, we identified eight *PIF* homolog sequences. The phylogenetic, divergence time estimation and selective pressure evaluation analyses allowed us to reconstruct the evolutionary history of *PIF* genes in *S. lycopersicum* and closely related Solanaceae species, the wild tomato *S. pennellii* and *S. tuberosum*. We further explored the transcriptional profile in four different developmental contexts, deetiolation, dark-induced senescence, daily cycle and fruit ripening, and identified expression patterns that suggest functional specificity. The data were discussed in the context of tomato genome evolution.

RESULTS

Phylogenetic and Evolutionary Analysis of *PIF* loci

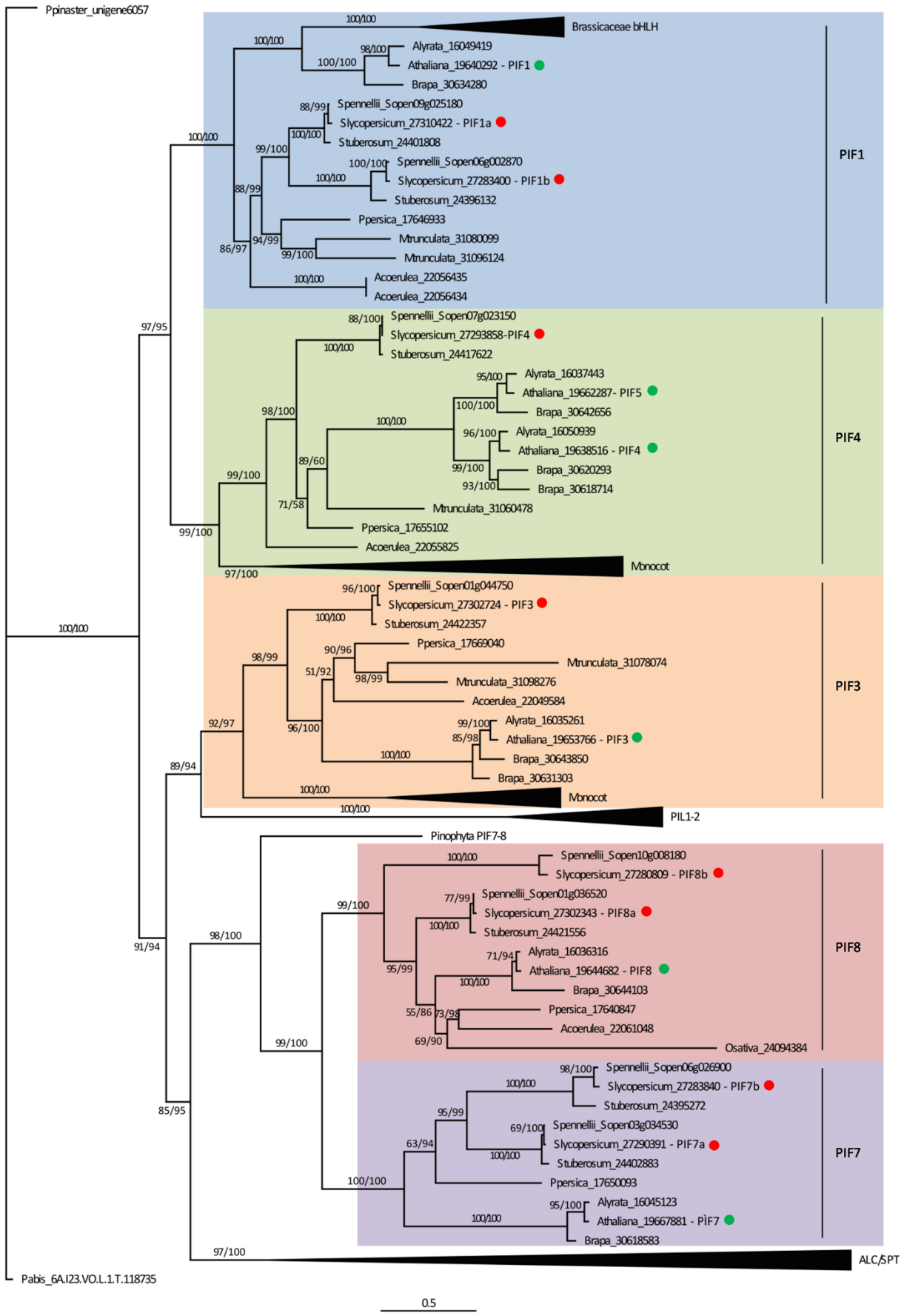
By performing a BLAST search against fully sequenced genome databases using *A. thaliana* canonical PIF sequences as queries, 119 sequences from 16 species were retrieved including sequences of the bHLH superfamily that do not belong to the PIF subfamily [1] (see Material and Methods, S1 Table). In agreement with previous report, no PIF homologs were found in chlorophytes [22]. In the basal land plants *Marchantia polymorpha* (liverworth), *Physcomitrella patens* (moss) and *Selaginella moellendorffii* (lycophyte), one, four and three *PIF* homologs were identified, respectively. Spermatophyte species harbor several gene copies that, based on the phylogenetic reconstruction, are mainly divided in two super clades named according to the corresponding *A. thaliana* homolog representative. The first contains PIF1 and PIF4 sequences and, the second encompasses PIF3, PIF3-like 1 and 2 (PIL1/2) [23], PIF8, PIF7, *ALCATRAZ* (*ALC*) and *SPATULA* (*SPT*) [24] sequences. In the second clade, PIF3 and PIL1/2, PIF7 and PIF8 and, ALC and SPT clustered together, respectively (Fig 1, S1 Fig, S1 Text). Whereas *Arabidopsis* has six PIF encoding genes, henceforth named *AtPIFs*, eight *loci* were identified in *S. lycopersicum* genome, corresponding to the following accessions in Sol Genomics Network database [25]: *SlPIF1a*: Solyc09g063010, *SlPIF1b*: Solyc06g008030, *SlPIF3*: Solyc01g102300, *SlPIF4*: Solyc07g043580, *SlPIF7a*: Solyc03g115540, *SlPIF7b*: Solyc06g069600, *SlPIF8a*: Solyc01g090790, *SlPIF8b*: Solyc10g018510 (Fig 1). Aminoacid pairwise sequence alignments indicated that *Arabidopsis* and tomato homologs share 27–51% identity (S2 Table). Despite this low identity score, the APB-binding and bHLH domains were found in all tomato protein sequences, reinforcing their identity as PIF proteins. However, it is worth mentioning that tomato SlPIF1b, SlPIF4 and SlPIF8b display an amino acid substitution in the APB-binding domain that alters the conserved Q residue to G, E and E, respectively [26]. On the contrary, APA-binding domain was exclusively identified in *SlPIF1s* and *SlPIF3* (S2 Fig). Interestingly, the tree topology clearly showed that *Arabidopsis AtPIF4* and *AtPIF5* genes were originated by a Brassicaceae exclusive duplication, explaining the existence of a single gene in tomato genome within the clade PIF4. No differences in gene copy number were observed between *S. lycopersicum* and the most distantly related species within Lycopersicon section (*i.e*. tomatoes), *S. pennellii*. For PIF1, PIF7 and PIF8 clades, the analyzed tomato species harbor two gene copies, while for PIF3 and PIF4 a

single copy was identified. *S. tuberosum* has a similar *PIF* gene copy number, excepting for a single *PIF8 locus* (Fig 1).

To gain insight on the evolutionary history of *PIF* gene family, we estimated the divergence time of *PIFs* using molecular clock [27]. The duplication of *PIF1*, *PIF7* and *PIF8* was estimated in a range of time from 59.2 to 91.2 MYA (millions of years ago). As expected [28,29], our data indicated that tomato and potato *PIF* genes diverged around the species splitting event (Fig 2) estimated about 5.1 to 7.3 MYA [30]; excepting *PIF7b*, for which an estimate of 22.5–23.8 MYA was retrieved. Similarly, the divergence of *S. lycopersicum* and *S. pennellii PIF* genes dates close to the estimated age of the most recent common ancestors within the species, 2.2–3.1 MYA [27], with the exception of *PIF8b*, for which a value of 6.2 MYA was obtained. The high divergence times observed for *PIF7b* and *PIF8b* are consequence of high synonymous substitution values (*dS*). Aiming to test whether the high *dS* values were consequence of positive selection or neutral evolution, we evaluated the selective constraints under which *PIF* gene are evolving (Table 1). Indeed, *PIF7b* showed signatures of positive selection, particularly in threonine 343 and serine 369 (BEB test $P>95\%$). The rest of the *PIF* clades showed to be evolving under purifying selection. Unfortunately, we were unable to perform the test for *PIF8b* because it is absent in *S. tuberosum*.

PIF Transcript Profile along Seedling Deetiolation, Daily Cycle, Dark-Induced Senescence and Fruit Ripening

A. thaliana PIF proteins are known regulators of seedling deetiolation and dark-induced senescence, and are modulated by the circadian clock [1,17]. Particularly in *S. lycopersicum*, recently, the role of *SlPIF1a* in ripening-associated carotenogenesis was also been demonstrated [12]. To evaluate the functional diversity of tomato *PIF* genes, a comprehensive mRNA accumulation profiling was performed during seedling deetiolation, dark-induced senescence, diel cycle and fruit ripening. *SlPIF8a* and *SlPIF8b* were not considered for functional analyses because there are no publications demonstrating *A. thaliana* PIF8 and PHYs interaction, therefore, this clade was not considered a canonical PIF.


Ppinaster_unigene6057
Brassicaceae bHLH
Alyrata_16049419
Athaliana_19640292 - PIF1
Brapa_30634280
Spennellii_Sopen09g025180
Slycopersicum_27310422 - PIF1a
Stuberosum_24401808
Spennellii_Sopen06g002870
Slycopersicum_27283400 - PIF1b
Stuberosum_24396132
Ppersica_17646933
Mtrunculata_31080099
Mtrunculata_31096124
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PIF1
Spennellii_Sopen07g023150
Slycopersicum_27293858-PIF4
Stuberosum_24417622
Alyrata_16037443
Athaliana_19662287- PIF5
Brapa_30642656
Alyrata_16050939
Athaliana_19638516 - PIF4
Brapa_30620293
Brapa_30618714
Mtrunculata_31060478
Ppersica_17655102
Acoerulea_22055825
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Mtrunculata_31098276
Acoerulea_22049584
Alyrata_16035261
Athaliana_19653766 - PIF3
Brapa_30643850
Brapa_30631303
Monocot
PIL1-2
PIF3
Pinophyta PIF7-8
Spennellii_Sopen10g008180
Slycopersicum_27280809 - PIF8b
Spennellii_Sopen01g036520
Slycopersicum_27302343 - PIF8a
Stuberosum_24421556
Alyrata_16036316
Athaliana_19644682 - PIF8
Brapa_30644103
Ppersica_17640847
Acoerulea_22061048
Osativa_24094384
PIF8
Spennellii_Sopen06g026900
Slycopersicum_27283840 - PIF7b
Stuberosum_24395272
Spennellii_Sopen03g034530
Slycopersicum_27290391 - PIF7a
Stuberosum_24402883
Ppersica_17650093
Alyrata_16045123
Athaliana_19667881 - PÌF7
Brapa_30618583
PIF7
ALC/SPT
Pabis_6A.I23.VO.L.1.T.118735
0.5

Fig 1. Phylogenetic reconstruction of PIF protein family. Phylogenetic analysis of PIF protein subfamily in Viridiplantae performed with 112 sequences from 13 species. Accession numbers of all sequences are detailed in S1 Table. Compacted clades encompassing more than one sequence are indicated by black triangles. Arabidopsis thaliana and Solanum lycopersicum sequences are indicated with green and red circles, respectively. PIF clades are highlighted with colored squares. Numbers at nodes represent bootstrap/ approximate likelihood-ratio test (aLRT) values.

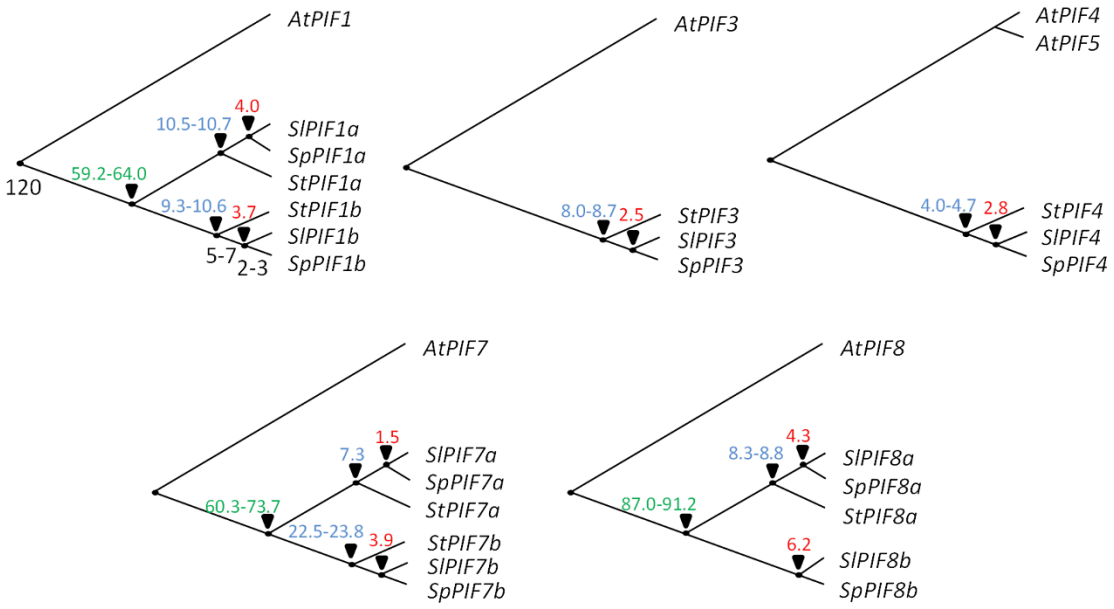

Fig 2. Divergence time estimations for *PIF* genes. The divergence times between the duplicated *PIF* genes in Solanaceae are shown in green. The divergence times between tomatoes (*S. lycopersicum* and *S. pennellii*) and *S. tuberosum* and, *S. lycopersicum* and *S. pennellii* homologs are indicated in blue and red, respectively. Species divergence times are shown in black (*Arabidopsis thaliana*-Solanaceae [34], Solanum tuberosum- *Solanum lycopersicum* [30], *S. pennelli-S. lycopersicum* [27]). Values are expressed in million years ago.

The expression profile of *SlPIF* genes during deetiolation was analyzed in 4 day-old darkgrown seedlings exposed to 24, 48 and 72 h of constant light or dark conditions. The

darktreated seedlings exhibited typical skotomorphogenic phenotype presenting long hypocotyls as well as closed, small and chlorotic cotyledons. Seedlings exposed to constant light underwent photomorphogenesis and showed shorter hypocotyls, opened apical hooks, expanded and green cotyledons and anthocyanin accumulation (S3A Fig). Cotyledon chlorophyll accumulation (S3B Fig) and mRNA levels of the chloroplast activity maintainer gene *SlGLK1* [31] (Fig 3) confirmed the skotomorphogenetic and the photomorphogenetic growth of the dark and lighttreated seedlings, respectively. Light induced the expression of *SlPIF1a*, *SlPIF4* and *SlPIF7a*, whereas *SlPIF1b* and *SlPIF3* mRNA levels were significantly reduced upon light exposure. Interestingly, *SlPIF1* paralogs showed contrasting patterns of light regulation displaying an increase up to 5-fold for *SlPIF1a* and 4-fold for *SlPIF1b* after 72 h of light and dark treatment, respectively. No significant transcript levels of *SlPIF7b* were detected in either treatment. A similar expression pattern of *SlPIF* genes was observed in the hypocotyls of light- and darktreated seedlings (S3C Fig). It is worth noting that in terms of relative expression, *SlPIF1b* was the most abundantly *PIF* gene expressed in seedlings, both in cotyledons and hypocotyls. In cotyledons, *SlPIF4* showed intermediate mRNA levels followed by *SlPIF1a*, *SlPIF3* and *SlPIF7a*. In hypocotyls, *SlPIF4*, *SlPIF1a* and *SlPIF3* displayed similar intermediate amounts of transcript, while *SlPIF7a* was the least abundantly expressed (S3 Table).

The expression pattern of tomato *PIFs* during 24 h under 12/12 light/dark photoperiod was analyzed in 3-week-old plants (Fig 4). *SlPIF1a*, *SlPIF3* and *SlPIF7a* showed similar oscillation patterns, characterized by lowest transcript abundance at the end of the light period followed by a progressive increase during the dark period and maximum levels 4 h after dawn. *SlPIF4* mRNA abundance was significantly reduced during the afternoon achieving the lowest level at dusk and progressively increasing over the night to reach the maximum level 8 h after dawn. *SlPIF7b* mRNA levels were high and constant during the light period, progressively decreasing during the night. *SlPIF1a* and its paralog *SlPIF1b* exhibited distinct diel expression patterns since during the night period *SlPIF1a* and *SlPIF1b* mRNA levels progressively increased and decreased, respectively. Interestingly, *SlPIF1* genes were the most copiously expressed *PIF*s at beginning of the light period displaying over 2-fold more transcripts in leaves than the other *PIF* genes (S3 Table).

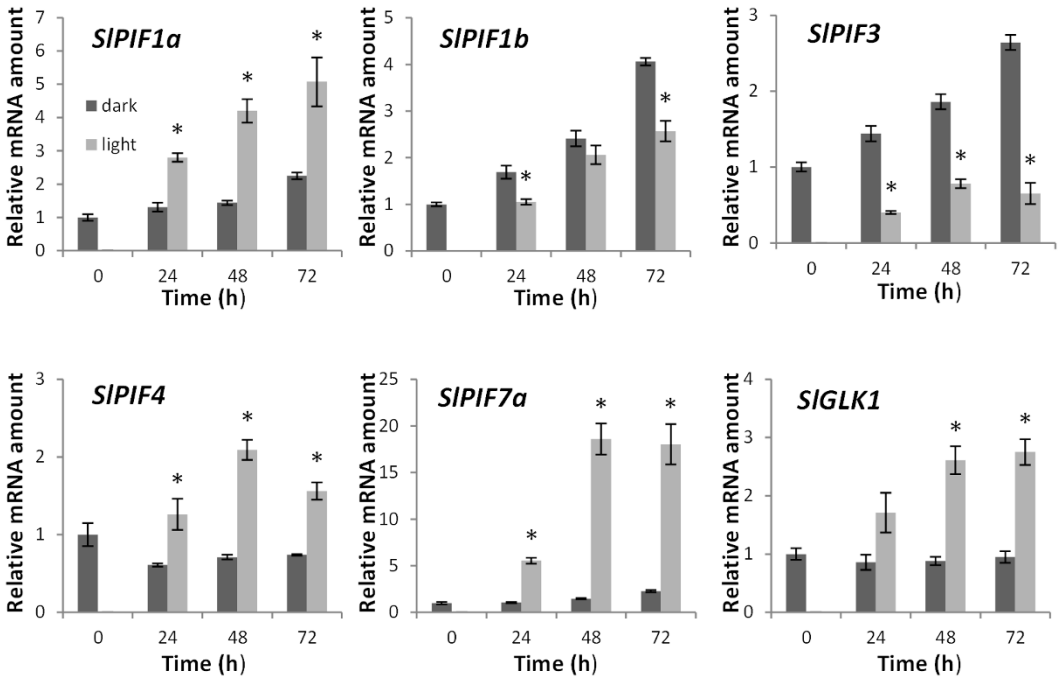

Fig 3. Expression profile of *PIF* genes in cotyledons under contrasting light conditions. Seedlings were grown in dark for 4 days and were either kept in darkness or transferred to continuous white light treatment. Significant differences (P<0,05) among treatments are indicated by asterisks. Values shown are means ± SE of at least three biological replicates.

Furthermore, we explored the transcriptional profile of *S. lycopersicum PIF* genes in leaves sampled from 3-week-old-plants maintained in darkness for 0, 1, 2, 3 and 7 days. The leaves showed clear signs of senescence as evidenced by the reduction in chlorophyll content at the seventh day (S4A Fig). The degreening was explained by the increment in *PHEOPHYTINASE* expression, the enzyme responsible for chlorophyll dephytylation in tomato leaves [32] and accompanied by a reduction in *SlGLK1* transcripts. Additionally, the induction of senescence was confirmed by the mRNA accumulation of the senescence marker *SENESCENCE-ASSOCIATED GENE 12* (*SlSAG12*, [33]) and *A. thaliana ORE1* homologs *SlORE1S02*, *SlORE1S03* and *SlORE1S06* (S4B Fig). These data allowed us to conclude that after 7 days of dark treatment, the plants underwent dark-induced senescence. Transcriptional profiling revealed that *SlPIF1a*, *SlPIF3*, *SlPIF7a* and *SlPIF7b* were downregulated whereas *SlPIF1b* and *SlPIF4* were upregulated

upon darkness exposure, suggesting that probably the formers are involved in dark-induced senescence signaling (Fig 5).

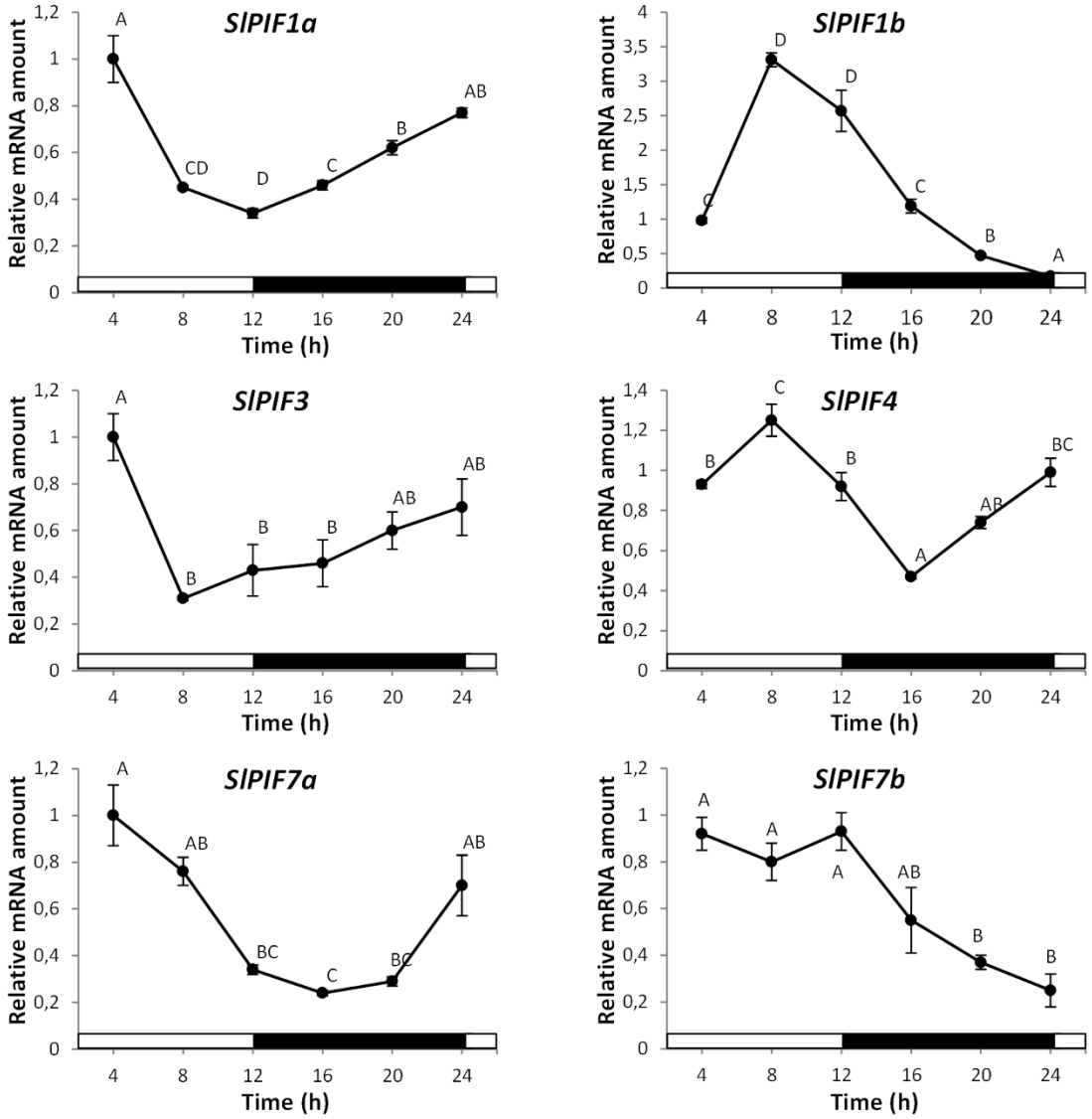

Fig 4. Expression profile of PIF genes during daily cycle. 3-week-old plants were grown under 12 h/12h light/dark photoperiod. The second fully expanded leaves were harvested every 4 h. White and black bars represent light and dark periods, respectively. Different letters indicate statistical differences ($P<0.05$). Values shown are means ± SE of at least three biological replicates.

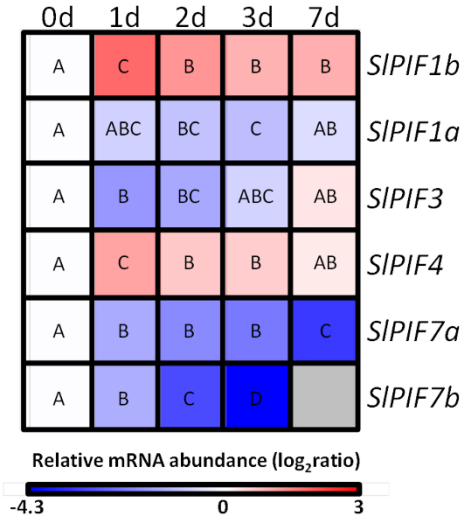

Fig 5. Expression profile of PIF genes during dark-induced senescence. 3-week-old plants were grown under 12 h/12 h light/dark photoperiod and transferred to constant darkness during 7 d and the second fully expanded leaves was sampled every day 4 h after the beginning of the light period. Heatmap representation of the relative mRNA abundance compared to day 0. Different letters indicate statistical differences ($P<0.05$). Values shown are means ± SE of at least three biological replicates.

To address the transcriptional behavior of all six *SlPIF* genes during tomato fruit ripening and evaluate their possible involvement in the light-dependent regulation of this developmental process, fruits at mature-green (MG) stage were harvested and left ripen under constant light or dark conditions. Total chlorophyll and carotenoids levels were measured and, as expected a concomitant reduction in total chlorophylls temporally coincided with the accumulation of the main carotenoids typically found in tomato fruits, thereby demonstrating that the detached fruits were undergoing normal ripening (S5 Fig). The levels of transcripts for both *SlPIF7* paralogs were undetectable in all fruit stages analyzed. Under dark conditions, *SlPIF1a*, *SlPIF1b*, *SlPIF3* and *SlPIF4* mRNA levels peaked 2 days after the start of the treatment, when the fruits were still in MG stage, followed by a reduction at the breaker (BR) stage (Fig 6). During the progression of ripening, *SlPIF1a* showed to be transcriptionally induced and significantly higher in the presence of light, whereas the mRNA levels of its paralog, *SlPIF1b*, were clearly lower in light- than in dark-treated fruits and did not respond to ripening. Finally, *SlPIF3* and *SlPIF4* mRNA levels were relatively constantly low along ripening and did not show clear patterns of regulation by light and dark treatments. In terms of relative expression, the most abundantly expressed *PIF* gene in fruits was *SlPIF3*, with 17-, 6- and 1.7-fold more mRNA amount than *SlPIF4*, *SlPIF1a* and *SlPIF1b*, respectively (S3 Table).

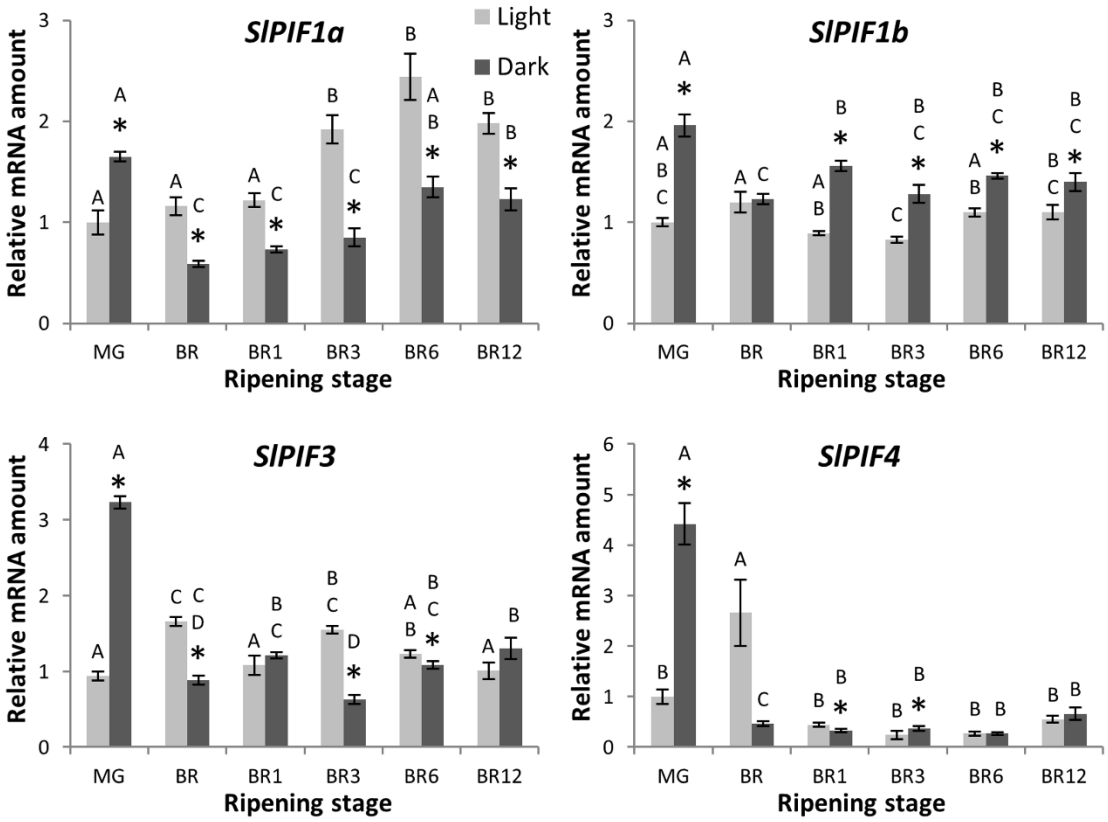

Fig 6. Expression profile of *PIF* genes during ripening under contrasting light conditions. Fruits were harvested at MG (mature-green) stage and left to ripen under constant light or dark conditions. Pericarp samples were harvested at MG (two days after the beginning of treatment), BR (breaker), BR1 (1 day after BR), BR3, BR6 and BR12 stages. Asterisks and letters represent significant ($P<0.05$) differences between treatments and stages, respectively. Values shown are means ± SE of at least three biological replicates.

DISCUSSION

The key role played by light signaling on tomato plant development and fruit nutritional value has been widely studied by the use of mutants and transgenic approaches [3,4]. However, *PIF* genes have been lagged behind and almost exclusively studied in *A. thaliana* [11–14]. Besides the well described PHY-mediated proteasomal degradation mentioned above, *PIF* genes are under tight transcriptional regulation as indicated by database and genome-wide binding site analyses for several *A. thaliana* transcription factors. Moreover, it has been suggested that AtPIFs regulate their

own expression by a complex autoregulatory mechanism [34], pinpointing the importance of studies approaching the expression regulation at transcriptional level. Aiming to gain insights on the function of these genes in tomato, here we explored their genetic diversity and expression profiling along different physiological contexts.

PIF is one of the 26 gene subfamilies of the monophyletic bHLH superfamily of plant transcription factors [22]. By using the five well described PIF proteins sequences from *A. thaliana* as baits (*i.e.* AtPIF1/3/4/5/7), we retrieved 119 homologs from 16 species and performed a phylogenetic analysis that allowed the identification of eight clades in Spermatophyte species (Fig 1). A comparative study between tomato and grape genomes proposed that a wholegenome triplication affecting Rosids, which includes *Arabidopsis*, and Euasterids, which includes *Solanum*, occurred in a common eudicot ancestor more than 120 MYA [5,35]. Interestingly, the monocot representatives of our phylogenetic reconstruction, *Oryza sativa* and *Sorghum bicolor*, did not show *PIF* genes in all the eight identified clades (Fig 1, S1 Table). Further, another triplication estimated at 71 (± 19.4) MYA occurred in the *Solanum* lineage followed by widespread gene loss that predates the 7.3 MYA tomato–potato divergence [5,30]. This second event was likely the origin of the duplications within PIF1, PIF7 and PIF8 clades since the estimated divergence time between the duplicated genes coincided with the date of the whole-genome triplication (Fig 2). To confirm this hypothesis, the gene collinearity was analyzed along the flanking genomic regions of the duplicated genes. As demonstrated for Solanaceae *PSY* genes [5], the *SlPIF1*, *SlPIF7* and *SlPIF8* paralog regions showed recognizable small scale synteny (S6 Fig). Thus, these polyploidization events may represent the foundation of the PIF subfamily diversification.

The evolutionary history of a genome is the result of the interdependent diversification of different genetic features like regulatory sequences, mobile elements and coding regions. Although, it is expectable that the gene divergence time approximately coincides with the corresponding species splitting date, heterogeneity in the nucleotide substitution rates among genetic features within the genome can defy the molecular clock approach [36]. In this sense, the estimated divergence time for tomato and potato *PIF7b* genes significantly predated the splitting date between species; while a similar situation was observed for *S. lycopersicum* and *S. pennellii PIF8b*. Interestingly, the evolutionary analysis for *PIF7b* demonstrated signatures of positive

selection, which can be associated to functional divergence. The absence of *PIF8b* in *S. tuberosum* might be attributed to stochastic gene loss; consequently, no evolutionary analysis was performed.

To explore the functional diversification of tomato *PIF* genes, a comprehensive expression profile was carried out under various physiological processes induced or regulated by light, such as deetiolation, daily cycle, senescence and fruit ripening. Interestingly, *SlPIF* genes displayed differential mRNA accumulation pattern at least along one of the analyzed contexts, suggesting that these genes have undergone functional specification. Little and fragmented information is currently available about the transcriptional regulation of *PIF* genes and only punctual similarities with our experimental conditions were found in literature.

The transcription of *AtPIF4* and *AtPIF5* has shown to be upregulated in *Arabidopsis* seedlings upon white light exposure [37]. This result is consistent with our observation that tomato *SlPIF4* mRNA levels increase during deetiolation (Fig 3). Tomato *SlPIF4* and *SlPIF7a* transcript accumulation patterns during diel cycle resemble those observed for *Arabidopsis AtPIF4*, *AtPIF5 and AtPIF7*, whose mRNA levels are regulated by the circadian clock [38,39]. However, while *AtPIF1* and *AtPIF3* mRNA levels in *Arabidopsis* remained relatively constant along the diel cycle [37], the tomato *SlPIF1a*, *SlPIF1b* and *SlPIF3* oscillated under 12 h/12 h light/dark photoperiod. *SlPIF7b* transcript levels also fluctuated during the diel cycle, suggesting that all tomato *PIFs* are transcriptionally regulated by the circadian clock (Fig 4). Besides the diel cycling, *SlPIF7s* have shown to be exclusively expressed in true leaves (S3 Table), reinforcing their role in circadian response regulation as demonstrated for *AtPIF7* ortholog [40]. Similarities were also found with *Arabidopsis* during dark-induced senescence. In *Arabidopsis*, *AtPIF4* and *AtPIF5* exhibited a peak of transcript accumulation in leaves one day after dark treatment [16] triggering senescence through the activation of the master transcription factor *AtORE1* [17]. Accordingly, tomato *SlPIF4* mRNA reached the highest levels one day after the start of the constant dark treatment (Fig 4). Interestingly, tomato *ORE1* homologs, *SlORE1S02*, *SlORE1S03* and *SlORE1S06* were also induced by the dark treatment, suggesting that a similar functional link between *PIF4* and *ORE* genes regulates dark-induced senescence in both *Arabidopsis* and tomato (S4B Fig).

A recent publication functionally characterized tomato *SlPIF1a* demonstrating its involvement as a negative regulator of fruit carotenogenesis [12]. Llorente et al. (2016) reported that *SlPIF1a* expression is induced along ripening and *SlPIF1b* is not expressed in fruits. Our data

also showed that *SlPIF1a* transcripts do accumulate during ripening, however, the amount of *SlPIF1b* mRNA at MG stage was 3-fold higher than *SlPIF1a* (S3 Table, Fig 6). These apparent contrasting data might be the results of differences in experimental design, since the transcriptional profile showed here was performed from fruits ripened off vine and under constant light/dark treatments. Moreover, *SlPIF1a* transcript accumulated at higher levels in fruits ripened under light, while *SlPIF1b* transcription was repressed by this treatment. This opposite pattern of light response between *SlPIF1* duplicated genes was also observed in the other physiological contexts analyzed in this work and might be the result from differences in transcriptional promoter activities. Therefore, we surveyed a fragment of 2 kb upstream the translation initiation site of these genes by a *de novo* search for *cis*-regulatory elements. Motifs recognized by *A. thaliana* transcription factors involved in light signaling, such as PIFs and HY5, were found in both sequences. *SlPIF1a* promoter showed PIF and HY5 binding-motifs, PBE-box and CA-hybrid, respectively [41,42]. Additionally, CArG motifs, which are recognized by the ripening inducer transcription factor RIN [43], were also found in *SlPIF1a* promoter region. CA-hybrid and CArG motifs were also identified in *SlPIF1b* regulatory region together with the HY5-binding ACE-motif (S7A Fig). The presence of gene-specific motifs and, different number and distribution of shared motifs might, at least in part, explain the different transcriptional behavior of *SlPIF1* duplicated genes. In particular, *SlPIF1a* might be target of HY5-mediated light-induction and of the above mentioned PIF autoinhibitory mechanism.

The comparison of the mRNA profiles of *SlPIF7a* and *SlPIF7b* duplicated genes was only possible in leaves because in other organs *PIF7* mRNA levels were near or below the detection threshold. *SlPIF7a* and *SlPIF7b* mRNA levels displayed opposite accumulation pattern during diel cycle, while both genes were downregulated by dark-induced senescence, *SlPIF7a* mRNA levels being 10-fold higher than those detected for *SlPIF7b* (Fig 4, Fig 5, S3 Table). In this case, the analysis of the promoter sequences showed also differential number and distribution of PIF and HY5 binding motifs: PBE-box, ACE-motif and CG-hybrid in *SlPIF7a* and; PBE-box and ACE-motif in *SlPIF7b* regulatory region (S7B Fig). To further evaluate differentially selected motifs between these pairs of duplicated genes, a promoter phylogenetic analysis was performed. Resembling the topology of the tree obtained from amino acid sequences, regulatory fragments also revealed that gene duplication predates species divergence (S8 Fig). Interestingly, none of the motifs identified in *SlPIF1s* and *SlPIF7s* is conserved between paralogs, being either *S.*

lycopersicum exclusive or shared with *S. tuberosum* and *S. pennellii* orthologs (S9 Fig). These data reinforced that the duplicated genes have undergone functional divergence in the Solanaceae common ancestral.

According to Force et al. (1999) [44], the loss of regulatory subfunctions in the promoter region by mutation and genetic drift is the main process by which duplicated genes are preserved, as long as they retain the complete set of subfunctions from the ancestral gene. In this context, it is expected that the duplicated *loci* should complement each other and show differences at the regulatory region. This model postulates that duplicated *loci* can undergo three different fates: nonfunctionalization, neofunctionalization and subfunctionalization. The first occurs when one copy acquires disabling mutations at the promoter region, leading to gene expression loss, while the other copy remains intact. The second takes place when a copy acquires new regulatory motifs, which confers a new regulatory function to this gene. The last is caused by degenerative mutations at both *loci* leading to loss or reduction of subfunctions. Our observations suggest that *SlPIF1* genes suffered qualitative subfunctionalization, as evidenced by their opposite responsiveness to light, and neofunctionalization, since *SlPIF1a* acquired a regulatory function during fruit ripening. Whilst, *SlPIF7* duplicated *loci* appeared to have undergone quantitative subfunctionalization possibly caused by fixed reduction-ofexpression mutations, which resulted in lowered expression of both copies. Moreover, it has been proposed that quantitative subfunctionalization is a transitory state to eventual neofunctionalization [45]. This seems to be the case of *SlPIF7b* gene for which, besides the reduced expression levels described above, positive selection has been also verified. A very interesting mechanism of neofunctionalization of duplicated genes in *Solanum* lineage has been recently described in tomato [46]. While in photosynthetic tissue, a CHLOROPLAST-SPECIFIC LYCOPENE β-CYCLASE (LCYβ) mediates the conversion of lycopene to β-carotene, in chromoplast, this reaction is executed by the product of the *CHROMOPLAST-SPECIFIC LYCOPENE β-CYCLASE* gene (*CYCβ*), a *LCYβ* paralog. Sequence analysis of *CYCβ* gene from a repository of tomato and wild relative accessions showed that *CYCβ* undergoes purifying selection in tomato clade. However, the abundant and diverse variations in the promoter region are likely related to regulatory neofunctionalization that played a key role in fruit color development in tomato.

The data presented here bring evidences that *SlPIF* duplicated genes (*e.g*. *SlPIF1a* and *SlPIF1b*), originated during *Solanum* lineage specific whole-genome triplication, have undergone sub- and neofunctionalization most likely due to variations in promoter region than in the coding region, disclosing the impact of polyploidization events during the evolution of *PIF* gene subfamily.

CONCLUSIONS

Solanum lycopersicum genome harbors eight *PIF* encoding *loci*, *SlPIF1a*, *SlPIF1b*, *SlPIF3*, *SlPIF4*, *SlPIF7a*, *SlPIF7b*, *SlPIF8a* and *SlPIF8b*. *SlPIF1*, *SlPIF7* and *SlPIF8* duplications occurred during the *Solanum* lineage polyploidization event 71 (± 19.4) MYA, prior to the divergence between tomato and potato species. Transcriptional profiling revealed coincident expression patterns between tomato *SlPIF4* and Arabidopsis *AtPIF4* and *AtPIF5*, highlighting the evolutionary conserved function of PIF4 clade. Combined evolutionary analysis and transcriptional profile data indicated that *SlPIF7a* and *SlPIF7b* may have suffered quantitative subfunctionalization that reduced their expression level, followed by neofuctionalization process, supported by the differential pattern of light responsive motifs and the positive selection signatures observed. Finally, *SlPIF1a* and *SlPIF1b* promoter regions showed differential pattern of light and fruit ripening transcriptional factor binding motifs, providing also evidences for regulatory sub- and neofunctionalization. In summary, our data underlined the importance of polyploidization events on *PIF* subfamily diversification.

MATERIALS AND METHODS

Phylogenetic, Gene Divergence Time and Evolutionary Analyses

The amino acid sequences of the five *A. thaliana* canonical PIF proteins (S1 Table) were used as queries to perform a BLAST search against Viridiplantae in Phytozome [47], DNA Data Bank in Japan [48], Dendrome [49], SustainPine [50], Sol Genomics [25] databases. 119 sequences with complete bHLH domain from 16 species, representing liverworts, mosses, lycophytes, gymnosperms and flowering plants, were retrieved. T-Coffee Structural-Alignment algorithm [51]

was used to perform an alignment of gymnosperms and flowering plants sequences. The phylogenetic reconstruction was performed by maximum-likelihood method using JTT substitution model and validated by approximate Likelihood Ratio Test (aLRT) with the Shimodaira-Hasegawa-like (SH-like) and 100 bootstrap replicates procedures available at PhyML Interface [52].

Gene divergence time was estimated using $T = dS/2K$ equation, where T is the divergence time, dS is the pairwise synonymous distance calculated in the MEGA 6 software using the corrected Nei-Gojobori method (Jukes-Cantor) [53] and, K is the mean substitution rate estimated for 27 *loci* belonging to three different chromosomes of *S. pennellii* and *S. lycopersicum* ([28]; 4.38x10-9 substitutions per site per year).

Evolutionary analysis was conducted individually for *PIF1a*, *PIF1b*, *PIF3*, *PIF4*, *PIF7a*, *PIF7b* and *PIF8a* genes using the sequences of *S. lycopersicum*, *S. pennellii* and *S. tuberosum*. Non-synonymous (*dN*) and synonymous (*dS*) distances and their SE values were estimated with MEGA 6. In order to preserve the reading frames, the alignment gaps were deleted prior to estimation of *dS* and *dN*. Codon bias was determined by the effective number of codons (Nc) value computed in the CodonW program [54]. Nc varies between 21 for maximum codon bias, when only one codon is used per amino acid, and 61 for minimum codon bias, when synonymous codons for each amino acid are used at similar frequencies. Three evolutionary models were evaluated using the Codeml program implemented in the PAML4.8a package and the graphical interface PAMLX 1.3.1 [55]. Phylogenetic trees were constructed using manually adjusted alignments of the coding sequences and neighbor-joining method with the optimal model of nucleotide substitution estimated by "Find Best DNA/Protein Model" using MEGA 6 software. To test for neutral evolution, the nearly neutral model (M1a) was compared with the null hypothesis, one ratio model (M0). To test positive selection, the model M2a was compared with M1a. The M0 model assumes that all codons across the sequences have the same level of dN/dS. The model M1a proposes that there two classes of codon, some with $0 < dN/dS < 1$ and the remainder with $dN/dS = 1$. Finally, model M2a divides codons into three classes: those with $0 < dN/dS < 1$, $dN/dS = 1$, and $dN/dS > 1$. The fit of model M1a versus M0 or M2a versus M1a is evaluated by a likelihood ratio test (LRT) comparing twice the difference in log likelihoods with a $\chi 2$ distribution [56]. In M1a versus M0 and M2a versus M1a the degrees of freedom (df) are 1 and 2, respectively. Bayes

empirical Bayes (BEB) analyses were performed to identify positively selected residues with a BEB posterior probability 95%.

Gene collinearity was addressed by BLASTN search against tomato genome [25] using the CDS sequences within a window of 100 Kb upstream and downstream the *SlPIF1*, *SlPIF7* and *SlPIF8* duplicated genes as queries.

Plant Material

All the experiments were performed with *Solanum lycopersicum* (cv. Micro-Tom). For deetiolation, diel cycle and dark-induced leaf senescence experiments, tomato seeds were surface sterilized and directly sown *in vitro* as described by Lira *et al*. [57]. After 120 h pre-germination in absolute darkness, seedlings were transferred to specific treatment conditions as described below. For deetiolation experiment, seedlings were either transferred to continuous white light (~100 μmol m-2 s-1) or maintained in absolute darkness for 0, 24, 48 and 72 h, after which cotyledons and hypocotyls were separately harvested. For daily cycle experiment, plants were grown under 12 h/12 h light/dark (~300 μmol m-2 s-1) photoperiod for three weeks and the second fully expanded leaves were harvested every 4 hours, for 24 hours. For dark-induced senescence, plants were grown in the same conditions as the daily cycle experiment and subsequently the plants were transferred to darkness for 0, 1, 2, 3 and 7 days for inducing leaf senescence. The second fully expanded leaves were harvested 4 h after the beginning of the light period since this day point has been shown to exhibits the highest mRNA levels of most tomato *PIF* genes. All experiments were conducted at 25 ± 1°C.

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Seedling, plant and fruit tissues were harvested either under the specific light conditions used for the treatments or under dim green light (~0.01 μmol m-2 s-1), as appropriate. All samples were harvested and immediately frozen in liquid nitrogen, powdered and stored at -80°C.

Chlorophyll and Carotenoid Measurement

Chlorophyll and carotenoid extraction and analysis were carried out as described by Lira et al. [58]. When a data set showed homoscedasticity, an ANOVA test followed by a Tukey test ($P < 0.05$) was used to compare genotypes and fruit developmental stages. In the absence of homoscedasticity, a non-parametric ANOVA test was performed by applying the Kruskal–Wallis test ($P < 0.05$).

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR) Analysis

RNA extraction, cDNA synthesis and qPCR reactions were performed as described by Quadrana et al. [59]. The primers used for qPCR are listed in S4 Table. All reactions were performed with two technical replicates and at least three biological replicates. mRNA levels were quantified using a 7500 Real-Time PCR system (Applied Biosystem) and SYBR Green Master Mix (Applied Biosystem). Absolute fluorescence data were analyzed with LinRegPCR software [60] to obtain Ct values and to calculate primer efficiency. Expression values were normalized to the mean of two constitutively expressed genes: GAGA and CAC for seedlings [61], TIP41 and EXPRESSED for leaves and CAC and EXPRESSED for fruits [59]. A permutation test lacking sample distribution assumptions [62] was applied to detect statistical differences ($P < 0.05$) in

expression levels between mutants and the control using the algorithms in the fgStatistics software package [63]. For senescence analysis, the normalized expression pattern was presented by a heat map constructed with GENE-E program [64].

Promoter Analysis

A 2 Kb fragment of the promoter sequences of *PIF1s* and *PIF7s* were retrieved from Sol Genomics Network [25]. The presence of transcription factor binding motifs was analyzed in *S. lycopersicum* sequences using PlantPAN 2.0 platform [65]. The promoter regions were aligned using T-Coffee Structural-Alignment algorithm [51] and the Neighbor-Joining tree was reconstructed with 100 bootstrap replicates and p-distance implemented MEGA 6 [53].

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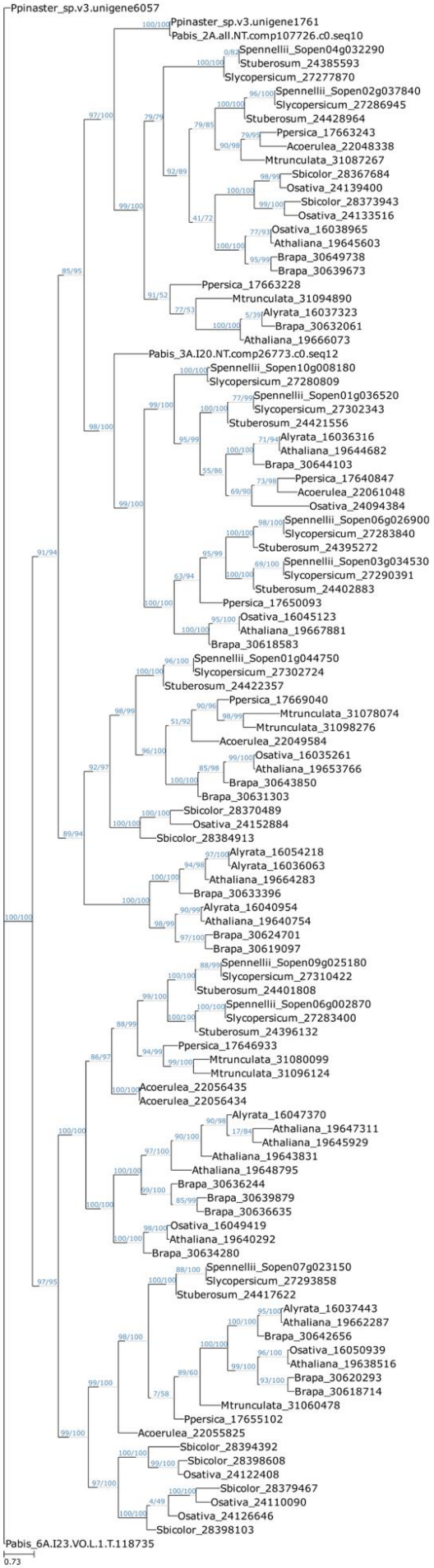
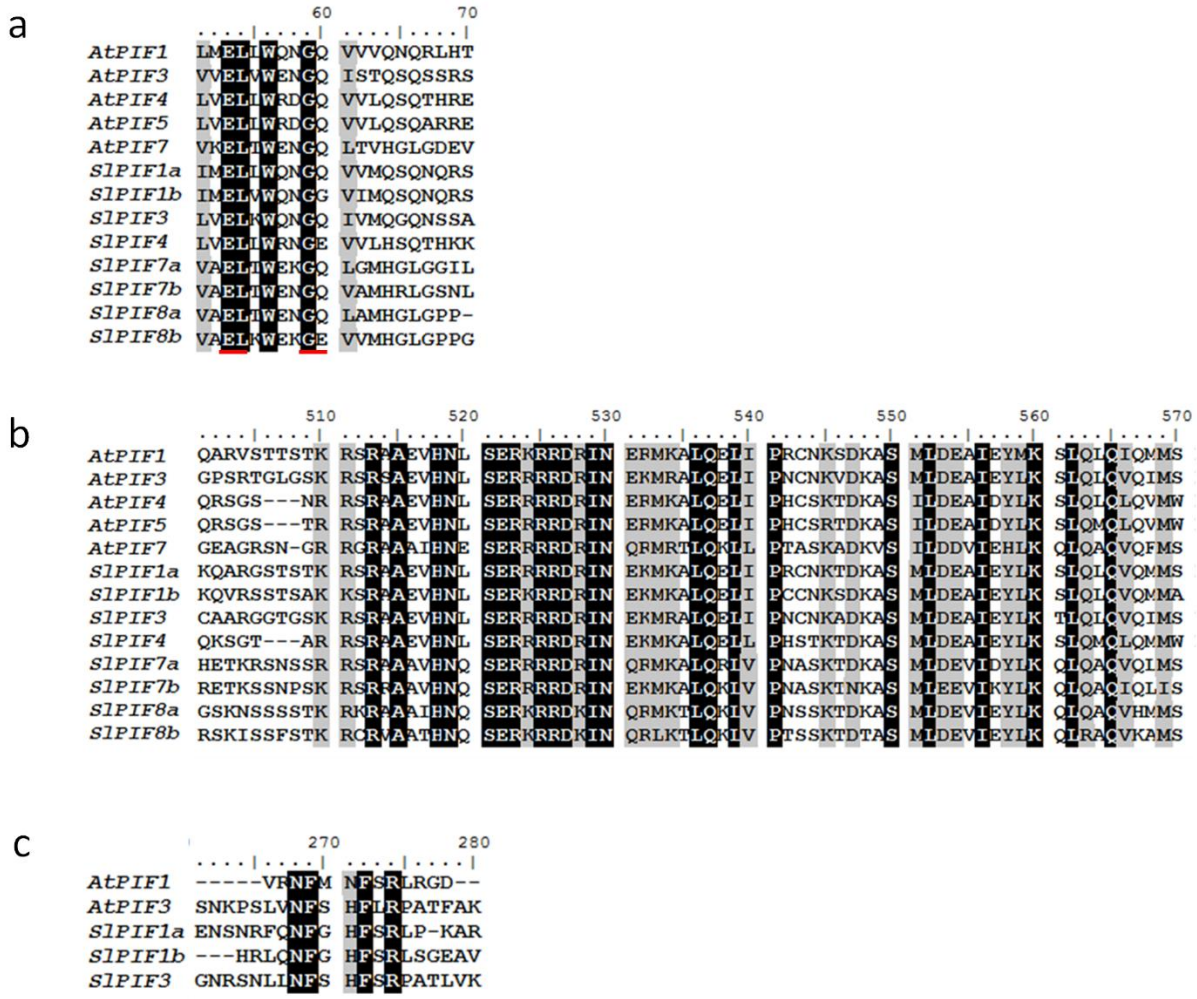
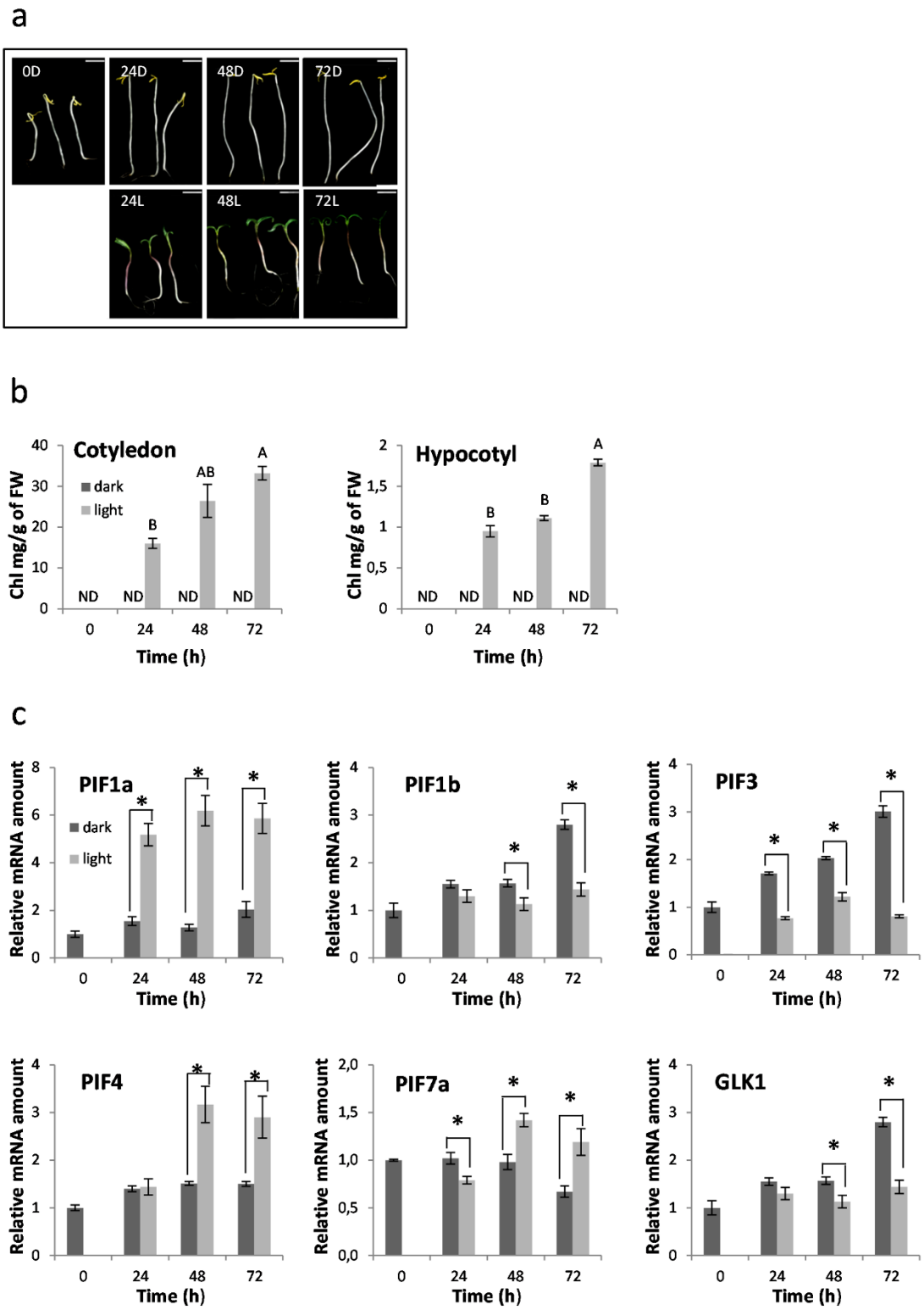


S1 Fig. Phylogenetic reconstruction of PIF protein family. Phylogenetic analysis of PIF protein subfamily in Viridiplantae performed with 112 sequences from 13 species. Accession numbers of all sequences are detailed in S1 Table. Numbers at nodes represent bootstrap/approximate likelihood-ratio test (aLRT) values.

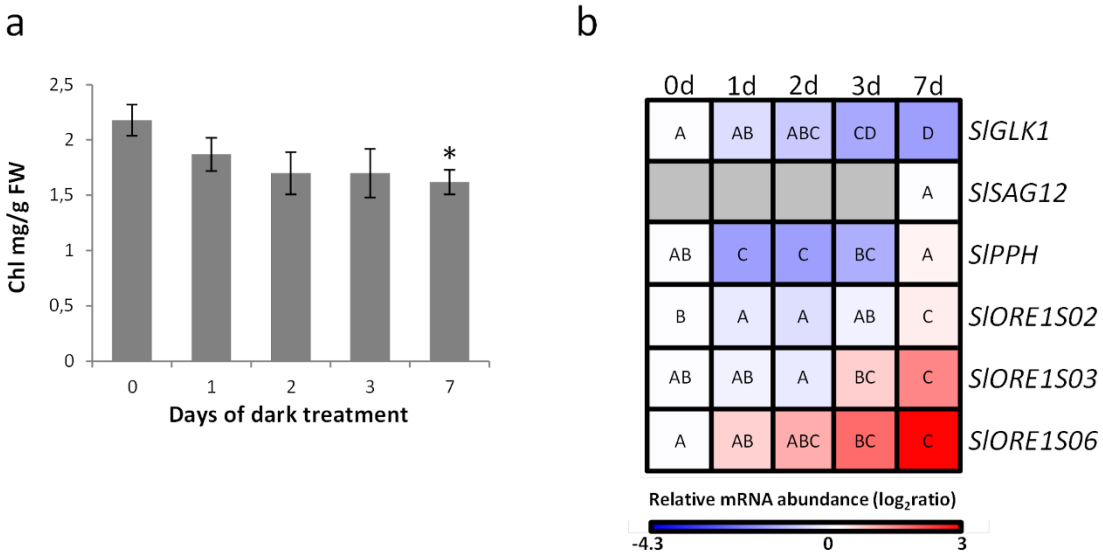


S2 Fig. PIF functional domains. Alignment of PIF amino acid sequences from Arabidopsis thaliana and Solanum lycopersicum showing the conserved domains [25]. (a) Active phytochrome B-binding (APB) domain. Residues highlighted in red are required for APB function in A. thaliana. (b) Basic helix-loop-helix (bHLH) DNA-binding domain. (c) Active phytochrome A-binding (APA) domain.

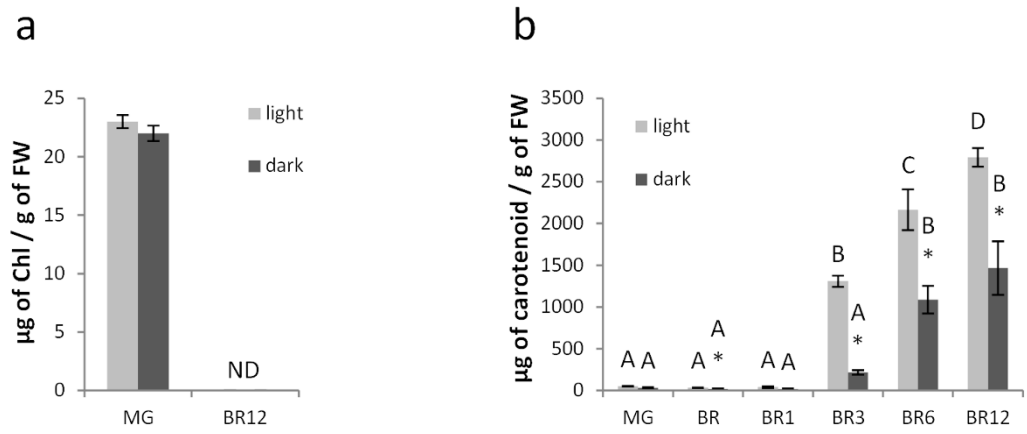


S3 Fig. Expression profile of *SlPIF* genes in seedling in response to light conditions. (a) Phenotype of 4-day-old dark-grown seedlings (0D) and after 24, 48 and 72 h maintained in constant light (24L, 48L and

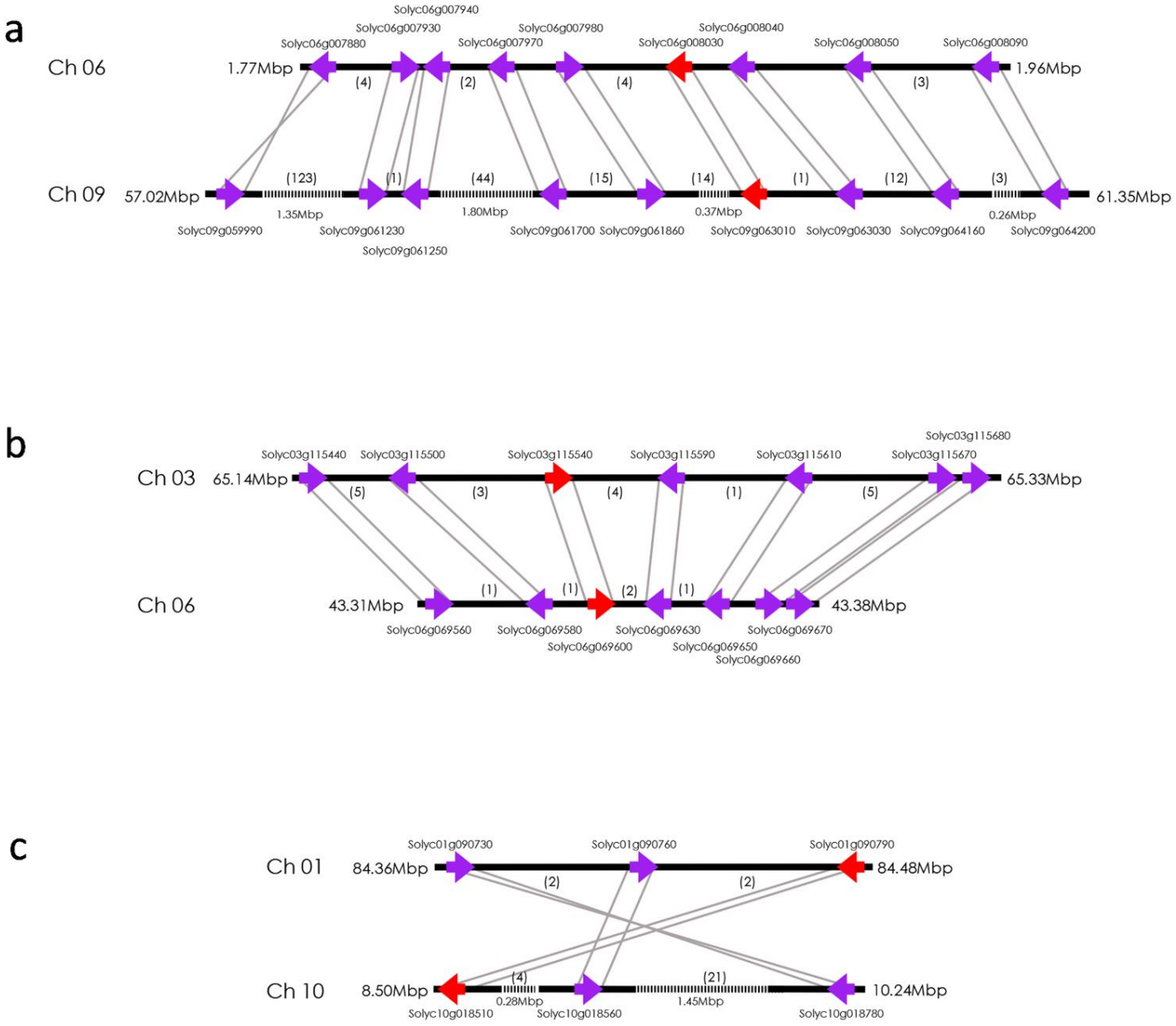
72L) or dark (24D, 48D and 72D) conditions. Bars: 1 cm. (b) Chlorophyll content in cotyledons and hypocotyls. Different letters indicate significant differences (P<0.05) within treatments. (c) SlPIF expression profile in hypocotyls. Significant differences (P<0.05) among treatments are indicated by asterisks. Values shown are means ± SE of at least three biological replicates. ND: not detected.



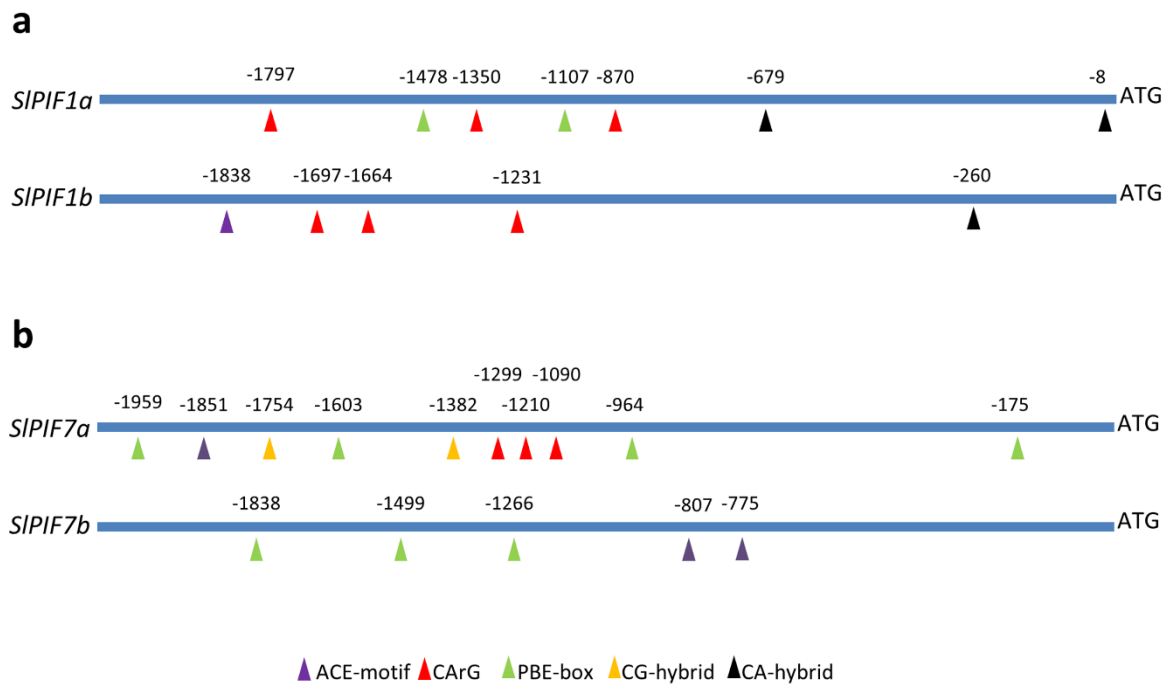
S4 Fig. Chlorophyll degradation and expression profile of senescence-related genes during dark-induced senescence. 3-week-old plants grown under 12 h/12 h light/dark photoperiod were transferred to constant darkness during 7 days and the second fully expanded leaves was sampled every day 4 h after the beginning of the light period. (a) Chlorophyll content along dark treatment. Significant differences (P<0.05) among treatments are indicated by asterisks. (b) Expression profile of *GOLDEN 2-LIKE 1* (*SlGLK1*, involved in chloroplast development, [65]), *SENESCENCE-ASSOCIATED GENE 12* (*SlSAG12*, late senescence marker, [32]), *PHEOPHYTINASE* (*SlPPH*, involved in leaf chlorophyll degradation, [56]) and, three genes tomato genes homologs to the Arabidopsis thaliana *ORESARA 1* (*SlORE1S02*, *SlORE1S03* and *SlORE1S06*, senescence-related transcription factor). Heatmap representation of the relative mRNA abundance compared to day 0. Different letters indicate statistical differences (P<0.05) among sampling times. Values shown are means ± SE of at least three biological replicates.



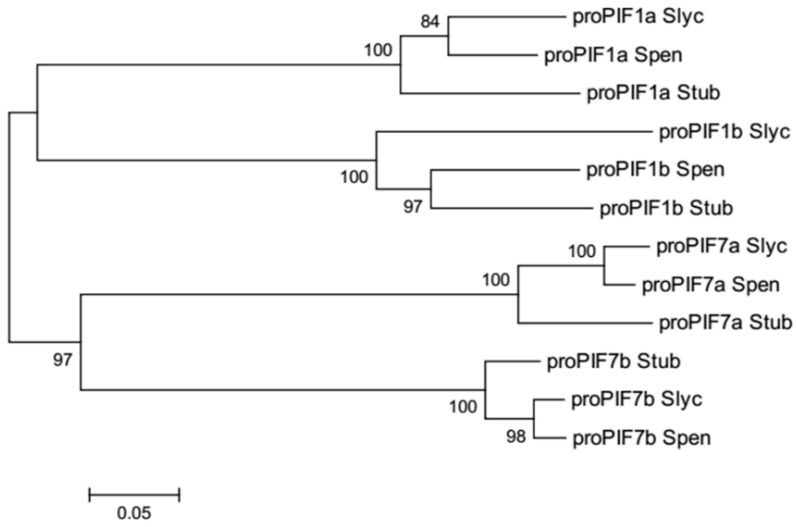
S5 Fig. Off-vine treated fruits undergo normal ripening process. Total Chlorophyll (a) and total carotenoid (b) levels were measured spectrophotometrically. Fruits were harvested at MG (mature-green) stage and left to ripen under constant light or dark conditions. Pericarp samples were harvested at MG (two days after the beginning of treatment), BR (breaker), BR1 (1 day after BR), BR3, BR6 and BR12 stages. Asterisks and letters represent significant ($P<0.05$) differences between treatments and stages, respectively. Values shown are means ± SE of at least three biological replicates.



S6 Fig. Microsynteny along the genomic regions flanking duplicated genes. Gene collinearity was addressed within a window of 100 Kb upstream and downstream the *SlPIF1* (a), *SlPIF7* (b) and *SlPIF8* (c) duplicated genes. *SlPIF1b* (Solyc06g008030), *SlPIF1a* (Solyc09g063010), *SlPIF7a* (Solyc03g115540), *SlPIF1b* (Solyc06g069600), *SlPIF8a* (Solyc01g090790) and *SlPIF8b* (Solyc10g018510) are highlighted in red. Collinear loci are indicated by arrows. The number of predicted genes within the intervals are indicated between parentheses.

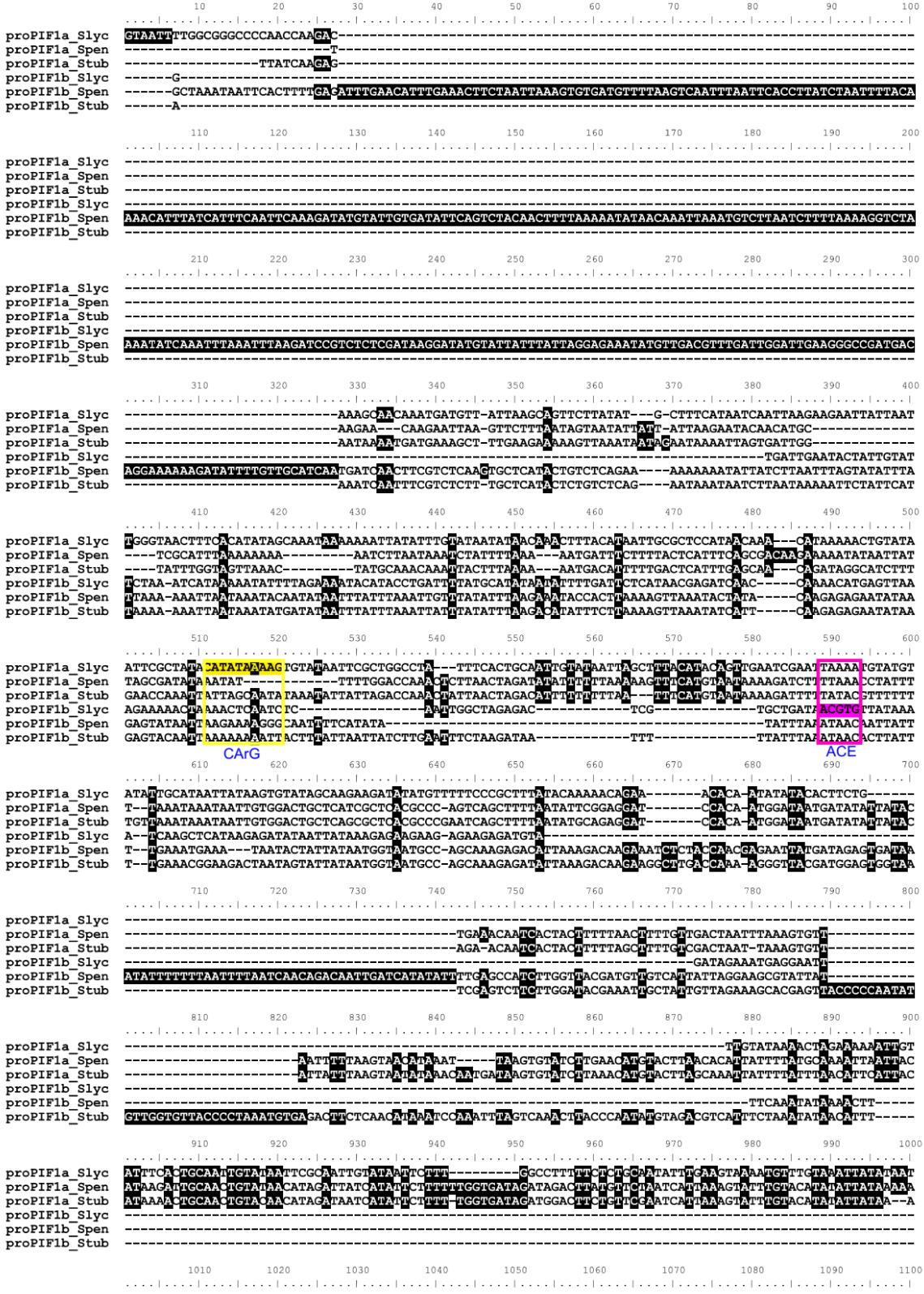


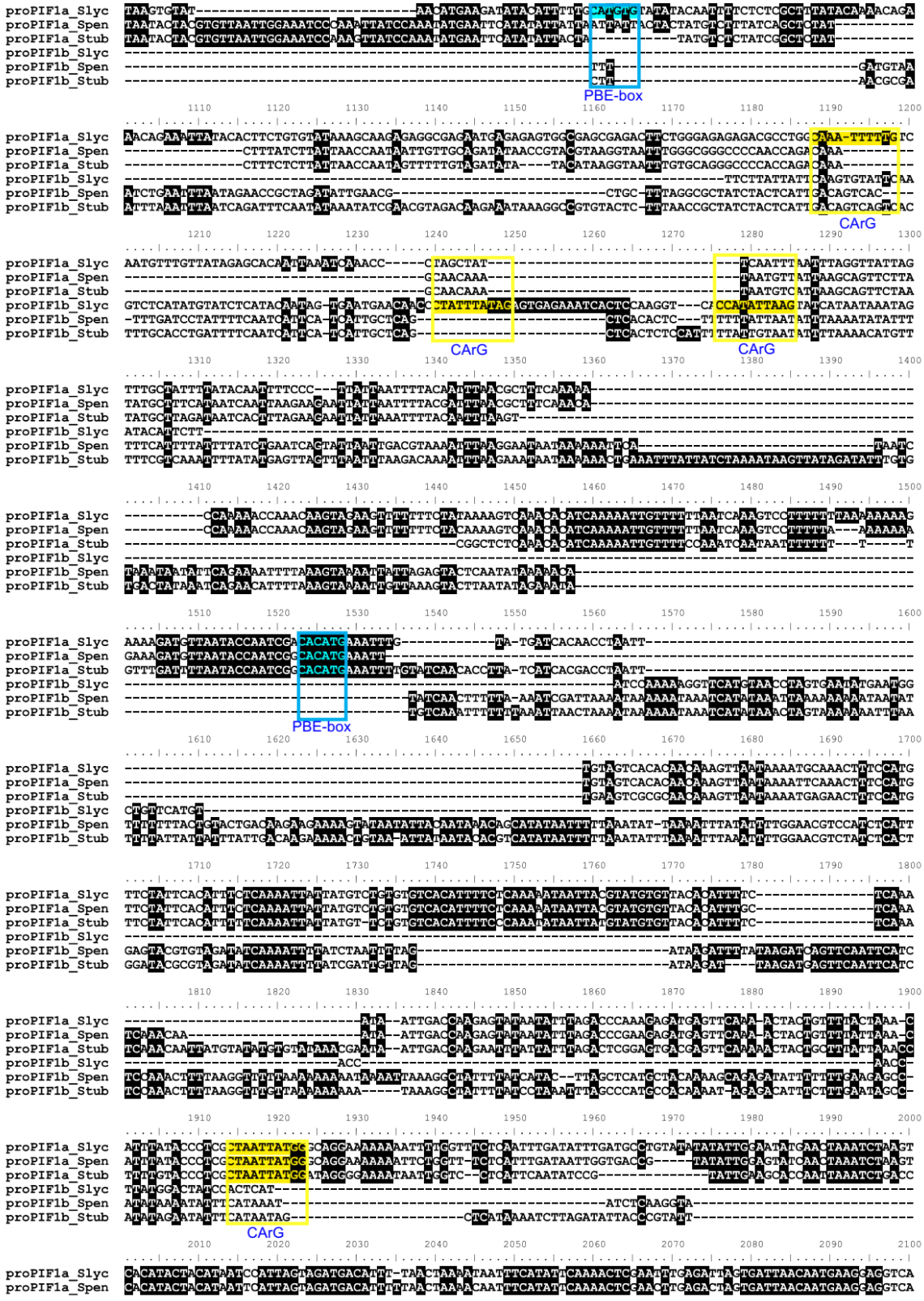
S7 Fig. Motifs identified in *SlPIF* gene promoter region. Fragments of 2 kb upstream the translation initiation site of *SlPIF1a* and *SlPIF1b* (a) and, *SlPIF7a* and *SlPIF7b* (b) genes are represented by a blue line. Motif positions are indicated by triangles. CArG [42], PBE-box [40], CA-hybrid, CG-hybrid and ACE-motif [41].

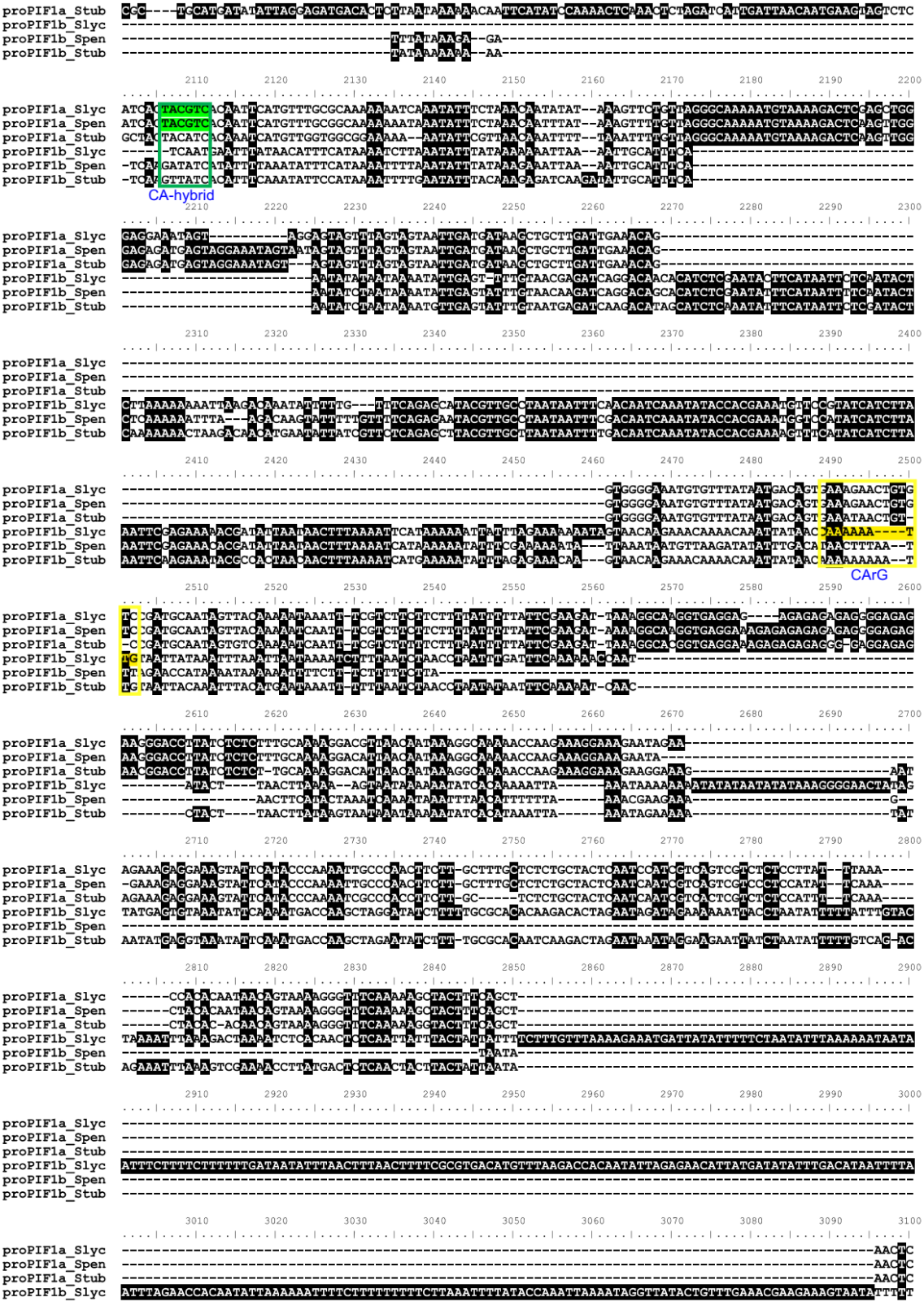


S8 Fig. Phylogenetic analysis of duplicated gene promoter sequences.

a


proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
CArG
ACE


PBE-box
CArG
CArG
CArG
PBE-box
CArG


proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
proPIF1a_Slyc
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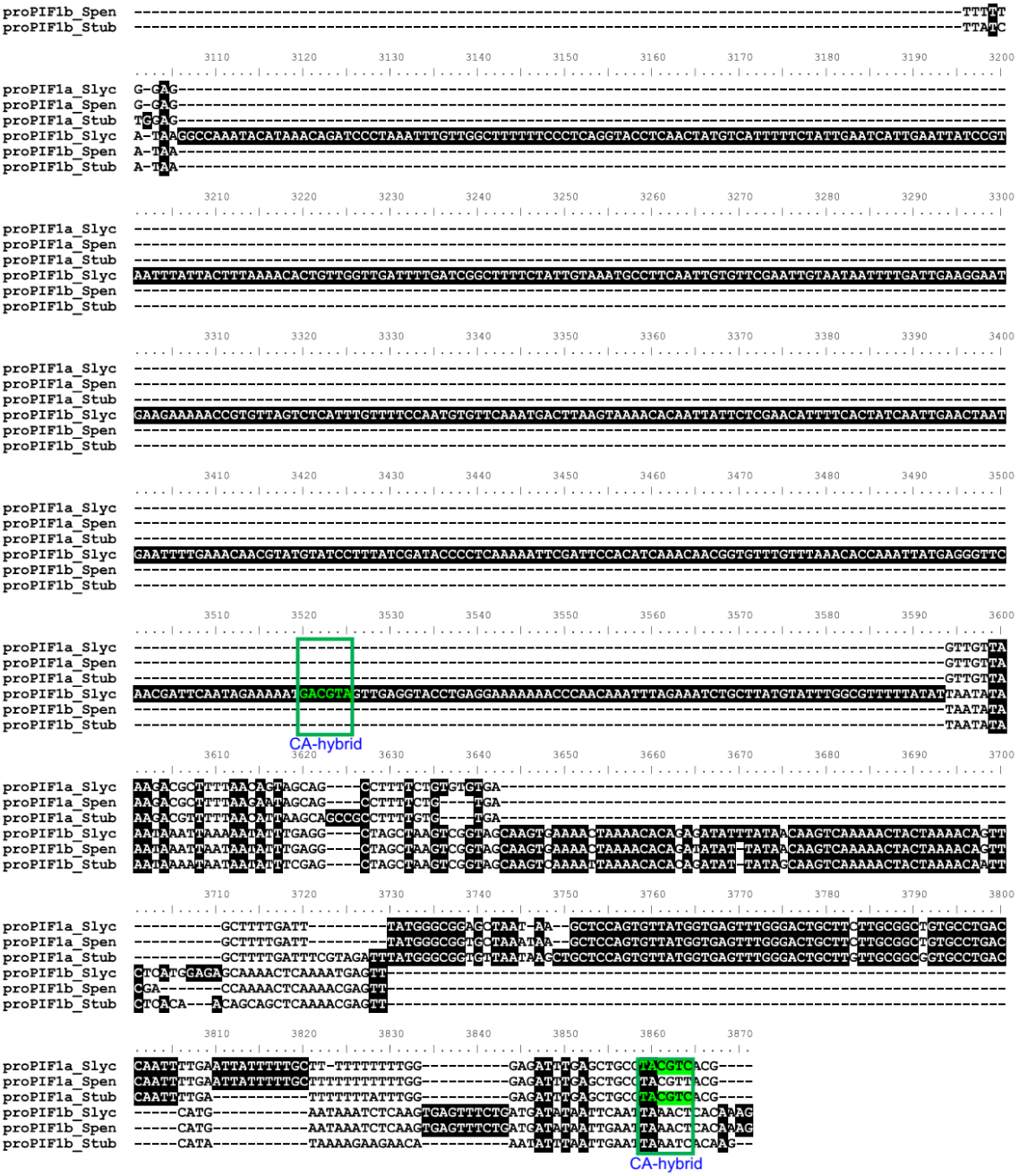
proPIF1b_Stub
CA-hybrid
CArG


proPIF1b_Spen
proPIF1b_Stub
3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
3410 3420 3430 3440 3450 3460 3470 3480 3490 3500
proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
proPIF1b_Slyc
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proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
CA-hybrid
3610 3620 3630 3640 3650 3660 3670 3680 3690 3700
proPIF1a_Slyc
proPIF1a_Spen
proPIF1a_Stub
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proPIF1b_Spen
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proPIF1a_Slyc
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proPIF1a_Stub
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
3810 3820 3830 3840 3850 3860 3870
proPIF1a_Slyc
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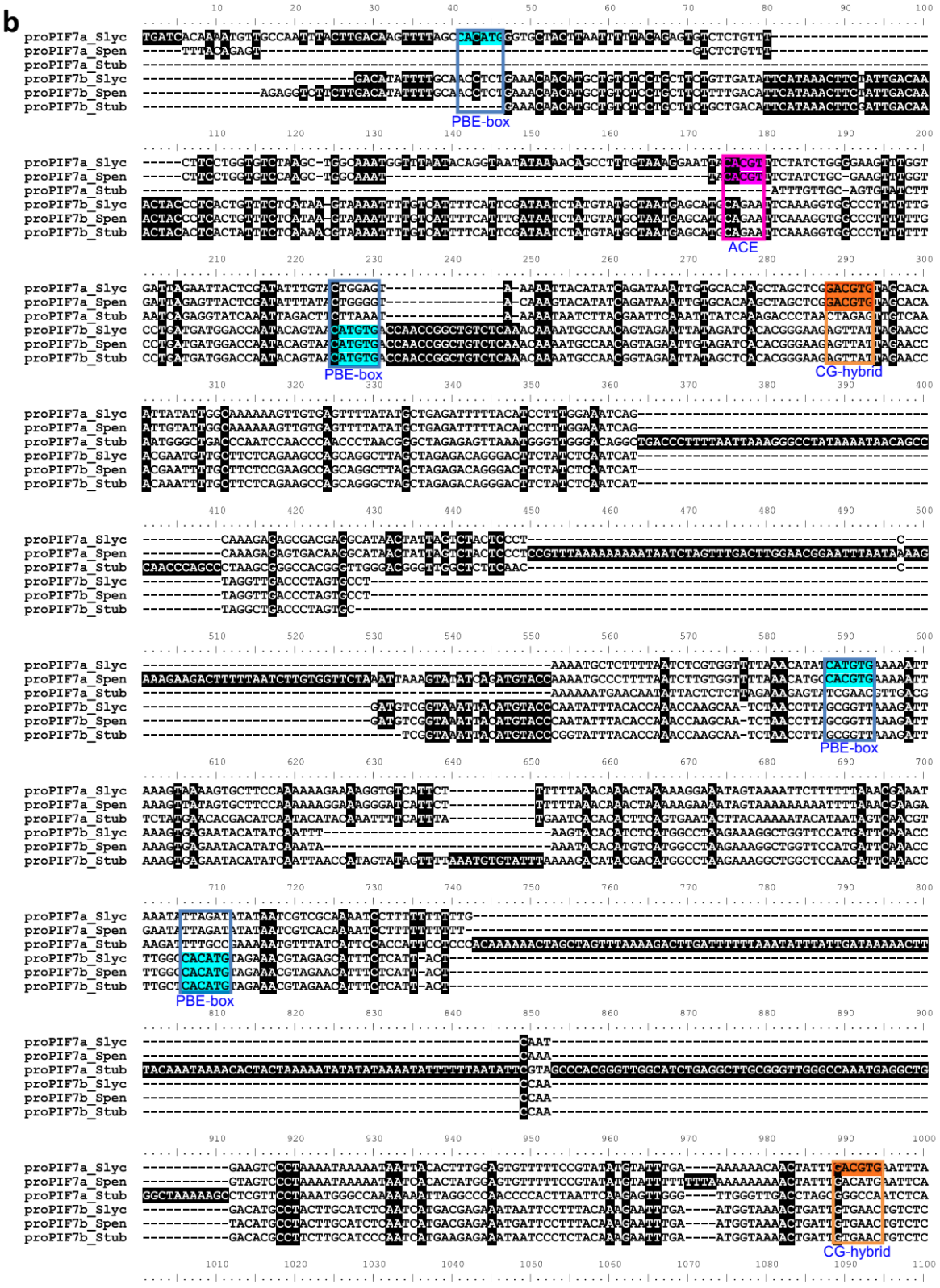
proPIF1b_Slyc
proPIF1b_Spen
proPIF1b_Stub
CA-hybrid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ACE
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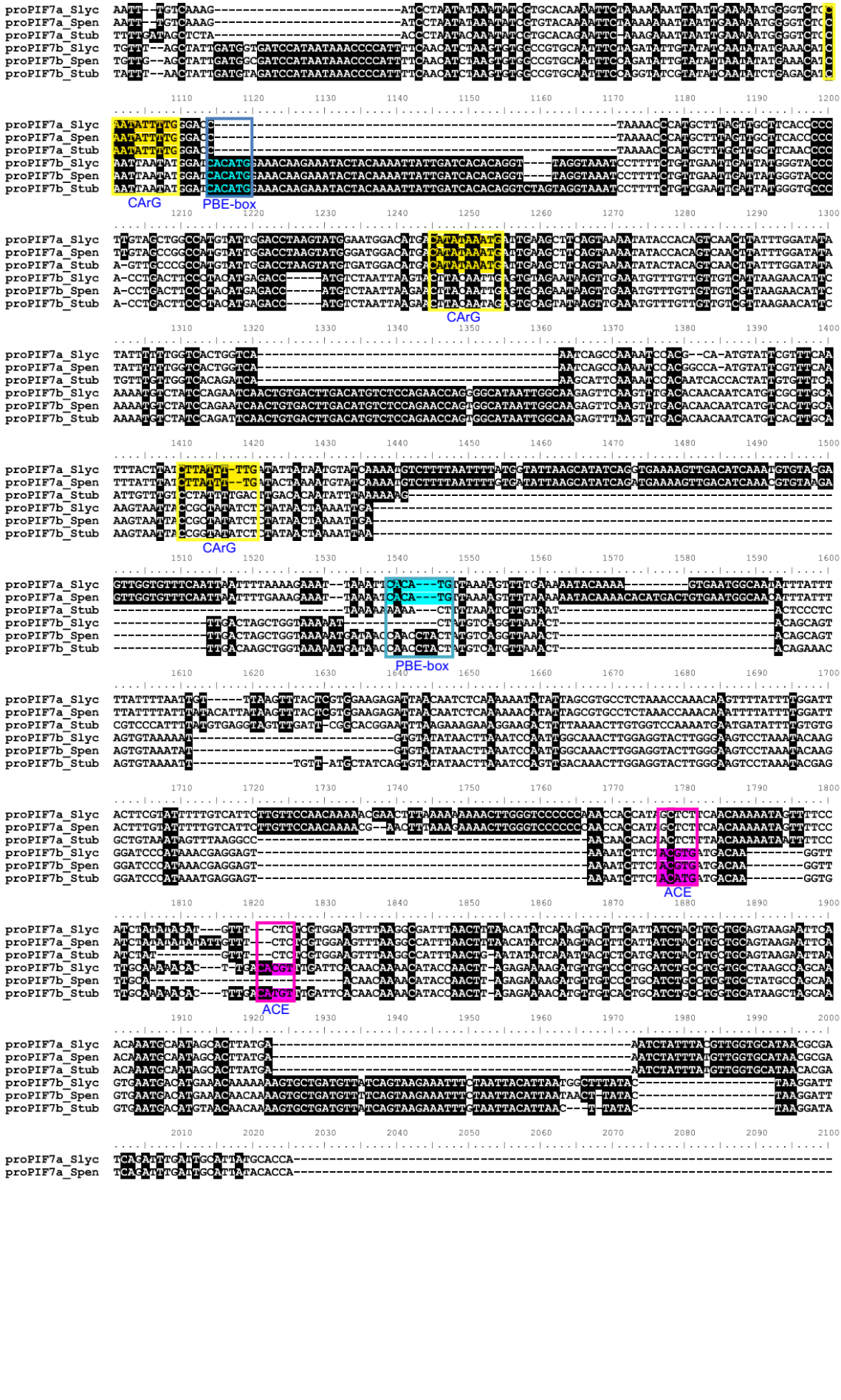
PBE-box
PBE-box
CG-hybrid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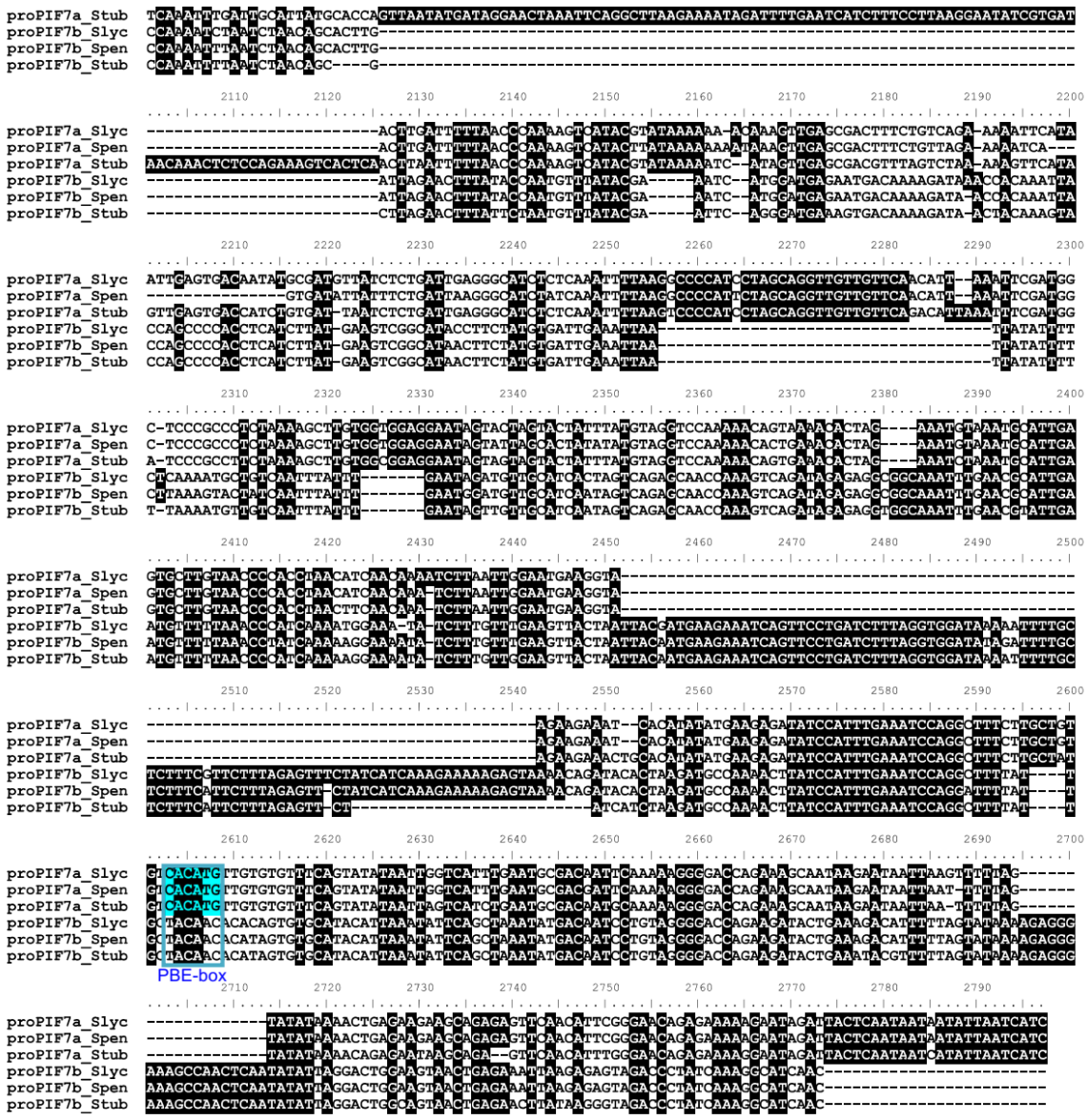
PBE-box
CArG
CArG
PBE-box
ACE
ACE



S9 Fig. Motif conservation in duplicated gene promoter sequences. The motifs identified in S7 Fig are highlighted in yellow (CArG [42]), blue (PBE-box [40]), green (CA-hybrid [41]), orange (CG-hybrid [41]) and pink (ACE-motif [41]).

S1 Table. Sequences used in phylogenetic reconstruction of PIF protein subfamily.

Clade[a]	Group	Species	Accession[b]
PIF1	Eudicot (Rosid)	*Arabidopsis thaliana*	19648795
PIF1	Eudicot (Rosid)	*Arabidopsis thaliana*	19643831
PIF1	Eudicot (Rosid)	*Arabidopsis lyrata*	16047370
PIF1	Eudicot (Rosid)	*Arabidopsis thaliana*	19645929
PIF1	Eudicot (Rosid)	*Arabidopsis thaliana*	19647311
PIF1	Eudicot (Rosid)	*Brassica rapa*	30636244
PIF1	Eudicot (Rosid)	*Brassica rapa*	30639879
PIF1	Eudicot (Rosid)	*Brassica rapa*	30636635
PIF1	Eudicot (Rosid)	*Brassica rapa*	30634280
PIF1	Eudicot (Rosid)	*Arabidopsis lyrata*	16049419
PIF1	Eudicot (Rosid)	*Arabidopsis thaliana*	19640292
PIF1	Eudicot (Rosid)	*Medicago trunculata*	31096124
PIF1	Eudicot (Rosid)	*Medicago trunculata*	31080099
PIF1	Eudicot (Rosid)	*Prunus persica*	17646933
PIF1	Eudicot (Euasterid)	*Solanum pennellii*	Sopen09g025180
PIF1	Eudicot (Euasterid)	*Solanum lycopersicum*	27310422
PIF1	Eudicot (Euasterid)	*Solanum tuberosum*	24401808
PIF1	Eudicot (Euasterid)	*Solanum lycopersicum*	27283400
PIF1	Eudicot (Euasterid)	*Solanum pennellii*	Sopen06g002870
PIF1	Eudicot (Euasterid)	*Solanum tuberosum*	24396132
PIF1	Eudicot (basal)	*Aquilegia coerulea*	22056434
PIF4	Eudicot (Rosid)	*Brassica rapa*	30642656
PIF4	Eudicot (Rosid)	*Arabidopsis lyrata*	16037443
PIF4	Eudicot (Rosid)	*Arabidopsis thaliana*	19662287
PIF4	Eudicot (Rosid)	*Arabidopsis lyrata*	16050939
PIF4	Eudicot (Rosid)	*Arabidopsis thaliana*	19638516
PIF4	Eudicot (Rosid)	*Brassica rapa*	30620293
PIF4	Eudicot (Rosid)	*Brassica rapa*	30618714
PIF4	Eudicot (Euasterid)	*Solanum tuberosum*	24417622
PIF4	Eudicot (Euasterid)	*Solanum lycopersicum*	27293858
PIF4	Eudicot (Euasterid)	*Solanum pennellii*	Sopen07g023150
PIF4	Eudicot (Rosid)	*Prunus persica*	17655102
PIF4	Eudicot (Rosid)	*Medicago trunculata*	31060478
PIF4	Eudicot (basal)	*Aquilegia coerulea*	22055825
PIF4	Monocot	*Sorghum bicolor*	28398103
PIF4	Monocot	*Oryza sativa*	24126646
PIF4	Monocot	*Sorghum bicolor*	28379467
PIF4	Monocot	*Oryza sativa*	24110090
PIF4	Monocot	*Sorghum bicolor*	28394392
PIF4	Monocot	*Sorghum bicolor*	28398608

PIF4	Monocot	*Oryza sativa*	24122408
PIF1-4	Pinophyta	*Picea abies*	6A I23 VO L 1 T 118735
PIF1-4	Pinophyta	*Pinus pinaster*	unigene6057
PIL1-2	Eudicot (Rosid)	*Arabidopsis thaliana*	19664283
PIL1-2	Eudicot (Rosid)	*Arabidopsis lyrata*	16036063
PIL1-2	Eudicot (Rosid)	*Arabidopsis lyrata*	16054218
PIL1-2	Eudicot (Rosid)	*Brassica rapa*	30633396
PIL1-2	Eudicot (Rosid)	*Arabidopsis thaliana*	19640754
PIL1-2	Eudicot (Rosid)	*Arabidopsis lyrata*	16040954
PIL1-2	Eudicot (Rosid)	*Brassica rapa*	30624701
PIL1-2	Eudicot (Rosid)	*Brassica rapa*	30619097
PIF3	Eudicot (Rosid)	*Brassica rapa*	30631303
PIF3	Eudicot (Rosid)	*Brassica rapa*	30643850
PIF3	Eudicot (Rosid)	*Arabidopsis lyrata*	16035261
PIF3	Eudicot (Rosid)	*Arabidopsis thaliana*	19653766
PIF3	Eudicot (basal)	*Aquilegia coerulea*	22049584
PIF3	Eudicot (Rosid)	*Prunus persica*	17669040
PIF3	Eudicot (Rosid)	*Medicago trunculata*	31098276
PIF3	Eudicot (Rosid)	*Medicago trunculata*	31078074
PIF3	Eudicot (Euasterid)	*Solanum lycopersicum*	27302724
PIF3	Eudicot (Euasterid)	*Solanum pennellii*	Sopen01g044750
PIF3	Eudicot (Euasterid)	*Solanum tuberosum*	24422357
PIF3	Monocot	*Oryza sativa*	24152884
PIF3	Monocot	*Sorghum bicolor*	28370489
PIF3	Monocot	*Sorghum bicolor*	28384913
PIF7	Eudicot (Rosid)	*Brassica rapa*	30618583
PIF7	Eudicot (Rosid)	*Arabidopsis lyrata*	16045123
PIF7	Eudicot (Rosid)	*Arabidopsis thaliana*	19667881
PIF7	Eudicot (Rosid)	*Prunus persica*	17650093
PIF7	Eudicot (Euasterid)	*Solanum tuberosum*	24402883
PIF7	Eudicot (Euasterid)	*Solanum lycopersicum*	27290391
PIF7	Eudicot (Euasterid)	*Solanum pennellii*	Sopen03g034530
PIF7	Eudicot (Euasterid)	*Solanum tuberosum*	24395272
PIF7	Eudicot (Euasterid)	*Solanum pennellii*	Sopen06g026900
PIF7	Eudicot (Euasterid)	*Solanum lycopersicum*	27283840
PIF8	Eudicot (Euasterid)	*Solanum pennellii*	Sopen10g008180
PIF8	Eudicot (Euasterid)	*Solanum lycopersicum*	27280809
PIF8	Eudicot (Euasterid)	*Solanum tuberosum*	24421556
PIF8	Eudicot (Euasterid)	*Solanum lycopersicum*	27302343
PIF8	Eudicot (Euasterid)	*Solanum pennellii*	Sopen01g036520
PIF8	Eudicot (Rosid)	*Brassica rapa*	30644103
PIF8	Eudicot (Rosid)	*Arabidopsis lyrata*	16036316
PIF8	Eudicot (Rosid)	*Arabidopsis thaliana*	19644682

PIF8	Monocot	*Oryza sativa*	24094384
PIF8	Eudicot (Rosid)	*Prunus persica*	17640847
PIF8	Eudicot (basal)	*Aquilegia coerulea*	22061048
PIF7/8	Pinophyta	*Picea abies*	3A I20 NT comp26773 c0 seq12
ALC/SPT	Eudicot (Rosid)	*Prunus persica*	17663228
ALC/SPT	Eudicot (Rosid)	*Medicago trunculata*	31094890
ALC/SPT	Eudicot (Rosid)	*Arabidopsis thaliana*	19666073
ALC/SPT	Eudicot (Rosid)	*Brassica rapa*	30632061
ALC/SPT	Eudicot (Rosid)	*Arabidopsis lyrata*	16037323
ALC/SPT	Eudicot (Euasterid)	*Solanum lycopersicum*	27277870
ALC/SPT	Eudicot (Euasterid)	*Solanum tuberosum*	24385593
ALC/SPT	Eudicot (Euasterid)	*Solanum pennellii*	Sopen04g032290
ALC/SPT	Eudicot (Euasterid)	*Solanum tuberosum*	24428964
ALC/SPT	Eudicot (Euasterid)	*Solanum pennellii*	Sopen02g037840
ALC/SPT	Eudicot (Euasterid)	*Solanum lycopersicum*	27286945
ALC/SPT	Eudicot (Rosid)	*Medicago trunculata*	31087267
ALC/SPT	Eudicot (basal)	*Aquilegia coerulea*	22048338
ALC/SPT	Eudicot (Rosid)	*Prunus persica*	17663243
ALC/SPT	Eudicot (Rosid)	*Arabidopsis lyrata*	16038965
ALC/SPT	Eudicot (Rosid)	*Arabidopsis thaliana*	19645603
ALC/SPT	Eudicot (Rosid)	*Brassica rapa*	30649738
ALC/SPT	Eudicot (Rosid)	*Brassica rapa*	30639673
ALC/SPT	Monocot	*Sorghum bicolor*	28367684
ALC/SPT	Monocot	*Oryza sativa*	24139400
ALC/SPT	Monocot	*Sorghum bicolor*	28373943
ALC/SPT	Monocot	*Oryza sativa*	24133516
ALC/SPT	Pinophyta	*Pinus pinaster*	unigene1761
ALC/SPT	Pinophyta	*Picea abies*	2A all NT comp107726 c0 seq10
Smoellendorfii	Lycopodiophyta	*Selaginella moellendorffii*	15412311
Smoellendorfii	Lycopodiophyta	*Selaginella moellendorffii*	15416909
Smoellendorfii	Lycopodiophyta	*Selaginella moellendorffii*	15415765
Ppatens	Bryophyta	*Physcomitrella patens*	28269126
Ppatens	Bryophyta	*Physcomitrella patens*	28257180
Ppatens	Bryophyta	*Physcomitrella patens*	28236070
Ppatens	Bryophyta	*Physcomitrella patens*	28247325
Mpolymorpha	Marchanthiophyta	*Marchanthia polymorpha*	LC093265

[a] Clades are named and ordered as they appear in the phylogenetic recontruction (Figure 1).

[b] Accession numbers of the sequences retrieved from Phytozome (https://phytozome.jgi.doe.gov/), with the exeption of *Marchanthia polymorpha*, *Picea abies*, *Pinus pinaster* and *Solanum pennellii* that were obtained from DDBJ (DNA Database in Japan, http://www.ddbj.nig.ac.jp/), Dendrome (http://dendrome.ucdavis.edu), SustainPine (http://www.scbi.uma.es/sustainpine/) and Sol Genomics (https://solgenomics.net/), respectively.

S2 Table. Percentage of identity between *Arabidopsis thaliana* and tomato homologs.

Genes	Coding Sequence (aminoacid)	Promoter[a] (nucleotide)
AtPIF1 vs *SlPIF1a*	41	nd
AtPIF1 vs *SlPIF1b*	41	nd
AtPIF3 vs *SlPIF3*	29	nd
AtPIF4 vs *SlPIF4*	33	nd
AtPIF5 vs *SlPIF4*	33	nd
AtPIF7 vs *SlPIF7a*	32	nd
AtPIF7 vs *SlPIF7b*	27	nd
AtPIF8 vs *SlPIF8a*	51	nd
AtPIF8 vs *SlPIF8b*	40	nd
SlPIF1a vs *SlPIF1b*	54	45
SlPIF7a vs *SlPIF7b*	45	50

[a] Fragments of 2Kb upstream the translation initiation site were aligned. nd: not determined.

S3 Table. Relative expression of *SlPIF* genes in the tested organs.

	Cotyledon[a]	Hypocotyl[b]	Leaf[c]	Fruit[d]
SlPIF1a	25.99 ± 2.59 c	5.78 ± 0.18 bc	8.05 ± 0.58 b	2.95 ± 0,36 [a]
SlPIF1b	70.19 ± 2.83 a	19.80 ± 2.54 a	9.48 ± 0.41 a	9.83 ± 0,39 [b]
SlPIF3	16.41 ± 1.10 c	8.51 ± 0.80 [b]	1.43 ± 0.09 cd	17.25 ± 0,94 c
SlPIF4	41.22 ± 6.19 b	4.23 ± 0.21 bc	2.72 ± 0.07 c	1,00 ± 0,14 [a]
SlPIF7a	1.00 ± 0.13 [d]	1.00 ± 0.07 [c]	1.00 ± 0.08 d	ND
SlPIF7b	ND	ND	0.10 ± 0.01 d	ND

[a] cotyledons of 4-day-old tomato dark-grown seedlings.
[b] hypocotyls of 4-day-old tomato dark-grown seedlings.
[c] leaves of 3-week-old plants grown under 12h/12h light/dark photoperiod harvested after 4 h of the begining of light.
[d] Fruits at mature-green stage after 48 h under constant light.
Values represent mean ± standard error of at least three biological replicates. Letters represent significant differences (P<0,05) between genes in the same organ.

S4 Table. Primers used por qPCR analysis.

Gene	Locus	Sequence
Actin	Solyc01g104770	F TCCGGGCATCTGAACCTCT
		R TTGACATTTTCTTGATTGCCC
CAC	Solyc08g006960	F CCTCCGTTGTGATGTAACTGG
		R ATTGGTGGAAAGTAACATCATCG
Expressed	Solyc07g025390	F GCTAAGAACGCTGGACCTAATG
		R TGGGTGTGCCTTTCTGAATG
GAGA	Solyc04g008380	F GTAGTTTATTGATGGATGACGACG
		R GGTGCTTCTGGGATGATAG
SlGLK1	Solyc07g053630	F GCTGTAGAGCAACTAGGTGTAGATAAGG
		R CAACTCGCTGCCTCCACTTC
SlORE1S02	Solyc02g088180	F ACAACAGCGAGAAGTAGTGG
		R GCATCAATCCAGAATCTCCATAC
SlORE1S03	Solyc03g115850	F ACATTTCAGGGCTTGTGAGA
		R AGGTGAATTGTTGAAGGAATTGAT
SlORE1S06	Solyc06g069710	F GATTCTGCTACTGCTACTGCTT
		R GGATCTTGAACCCCAAATGAAG
SlPIF1a	Solyc09g063010	F AACTTCTTGCTTTGCTCTCTG
		R GCTCCGCCCATAAATCA
SlPIF1b	Solyc06g008030	F TAGTATGGCAAAATGGTGGAG
		R CGGCGTCACAACTCGGTG
SlPIF3	Solyc01g102300	F AAGGCTTCCCAATAATGC
		R CCATCAGACCAAACTTCCC
SlPIF4	Solyc07g043580	F GGCTTAGGTTCACATACAG
		R TGATGGTGTCGTTGTCTC
SlPIF7a	Solyc03g115540	F CCTCACCTACATAACCAGCA
		R ATACAGCACCCCAGTTTTCA
SlPIF7b	Solyc06g069600	F GTACTCCACCACAACCTATT
		R TTGACATTTTCTTGATTGCCC
SlSAG12	Solyc02g076910	F ATGTCCTCCTCAAAGCCAAA
		R TTTCAGTTGGTGTAGCCCTT
TIP41	Solyc10g049850	F ATGGAGTTTTTGAGTCTTCTGC
		R GCTGCGTTTCTGGCTTAGG

Additional Supplemental material (S1 Text – Fasta alignment) may be found at: https://doi.org/10.1371/journal.pone.0165929

CAPÍTULO II

Efeito da luz sobre o metabolismo de vitamina E a participação das SlPIFs como fatores regulatórios

"[...] la luz

se parte

en dos

mitades

de tomate […]"

Pablo Neruda

This chapter is organized as published in the scientific journal Plant, Cell & Environment:

Gramegna G*, Rosado D*, Sánchez Carranza AP, Cruz AB, Simon-Moya M, Llorente B, Rodríguez-Concepcíon M, Freschi L, Rossi M (2019) Phytochrome-Interacting Factor 3 mediates light-dependent induction of tocopherol biosynthesis during tomato fruit ripening. Plant, cell & environment **42**: 1328-1339. *both authors contributed equally.

ABSTRACT

Tocopherols are important antioxidants exclusively produced in plastids that protect the photosynthetic apparatus from oxidative stress. These compounds with vitamin E activity are also essential dietary nutrients for humans. Although the tocopherol biosynthetic pathway has been elucidated, the mechanisms that regulate tocopherol production and accumulation remain elusive. Here, we investigated the regulatory mechanism underlying tocopherol biosynthesis during ripening in tomato fruits, which are an important source of vitamin E. Our results show that ripening under light conditions increases tocopherol fruit content in a phytochrome-dependent manner by the transcriptional regulation of biosynthetic genes. Moreover, we show that light-controlled expression of the *GERANYLGERANYL DIPHOSPHATE REDUCTASE* (*SlGGDR*) gene, responsible for the synthesis of the central tocopherol precursor phytyl diphosphate (PDP), is mediated by PHYTOCHROME-INTERACTING FACTOR 3 (SlPIF3). In the absence of light, SlPIF3 physically interacts with the promoter of *SlGGDR*, downregulating its expression. By contrast, light activation of phytochromes prevents the interaction between SlPIF3 and the *SlGGDR* promoter, leading to transcriptional derepression and higher availability of the PDP precursor for tocopherol biosynthesis. The unraveled mechanism provides a new strategy to manipulate fruit metabolism toward improving tomato nutritional quality.

KEYWORDS: *Solanum lycopersicum*; geranylgeranyl diphosphate reductase; tocopherol; light; phytochrome interacting factor; methylerythritol 4-phosphate pathway.

INTRODUCTION

Tomato, *Solanum lycopersicum*, is an important crop species both in terms of economic and nutraceutical value of its fruits (FAOSTAT 2014; WHO 2005). It is also a model for the study of fleshy-fruit development and ripening (Carrari & Fernie, 2006). During ripening, the differentiation of chloroplasts into chromoplasts is accompanied by metabolic changes that include de-greening due to chlorophyll (Chl) catabolism, cell wall degradation leading to softening, and the accumulation of pigments, sugars, acids and volatiles for disperser attraction. These biochemical processes result not only in organoleptic changes in flavor, texture and color that influence the consumption appeal, but also determine the nutritional composition of the edible fruits (Gapper, McQuinn & Giovannoni, 2013). Regarding nutraceutical compounds, besides the extensively studied carotenoids (Liu, Shao, Zhang & Wang, 2015), tomato fruits are relevant source of tocopherols (Quadrana et al., 2013). Tocopherols are non-enzymatic lipid-soluble antioxidants exclusively synthesized in the plastids of photosynthetic organisms. They exist in four forms named α-, β-, γ- and δ-tocopherol, differing in the number and position of the methyl radicals in the polar head (Figure 1). α-tocopherol is especially important from the nutritional perspective as it displays the highest vitamin E (VTE) activity in mammals (DellaPenna & Pogson, 2006). Tocopherols prevent neuromuscular, neurodegenerative and cardiac disorders by avoiding oxidative damage to human cells (Bellizzi, Franklin, Duthie & James, 1994; Copp et al., 1999; Guggenheim, Ringel, Silverman & Grabert, 1982; Ouahchi et al., 1995). Besides its nutritional value, tocopherols are essential protective substances in the chloroplasts, participating in the scavenging of reactive oxygen species and inhibition of lipid peroxidation (Krieger-Liszkay & Trebst, 2006; Miret & Munné-Bosch, 2015). As such, these compounds are an essential part of the photosynthetic machinery, affecting plant adaptability to light conditions and tomato fruit productivity (Almeida et al., 2016; Munné-Bosch, 2005; Spicher et al., 2017).

Tocopherols are formed by the condensation of two precursors: homogentisic acid (HGA), donor of a chromanol ring derived from the shikimate pathway, and phytyl diphosphate (PDP), a prenyl side-chain originated from the methylerythritol-4-phosphate (MEP) pathway (Figure 1) (Mène-Saffrané, 2018). The enzyme responsible for the first committed step is the HOMOGENTISATE PHYTYL TRANSFERASE, encoded by the *VTE2* gene. Further, TOCOPHEROL CYCLASE (VTE1), DIMETHYL-PHYTYLQUINOL METHYL

TRANSFERASE (VTE3) and γ-TOCOPHEROL C-METHYL TRANSFERASE (VTE4) are responsible for the balance between α, β, γ and δ forms. Tocopherol biosynthesis is tightly linked to chlorophyll (Chl) and carotenoid metabolisms. The MEP intermediate geranylgeranyl diphosphate (GGDP) is the precursor for the production of carotenoids and PDP, which is further used for both, Chl and tocopherol biosynthesis (Figure 1). Conversion of GGDP into PDP is catalyzed by the enzyme GERANYLGERANYL DIPHOSPHATE REDUCTASE (GGDR) (Almeida et al., 2015; DellaPenna & Pogson, 2006; Quadrana et al., 2013). Additionally, besides the *de novo* synthesis from GGDP, PDP can be produced from the recycling of Chl degradation-derived phytol catalyzed by PHYTOL KINASE (VTE5) and PHYTYL PHOSPHATE KINASE (VTE6) (Ischebeck, Zbierzak, Kanwischer & Dörmann, 2006; vom Dorp et al., 2015) (Figure 1).

Tocopherol biosynthesis has been well described during tomato fruit development and ripening (Almeida et al., 2011, 2015). A network analysis based on a dedicated transcriptional and metabolite data showed that tocopherol biosynthesis is transcriptionally regulated, pinpointed the link between Chl and tocopherol metabolism, and revealed that the supply of the prenyl donor is limiting for VTE accumulation at later stages of fruit development (Quadrana et al., 2013). These hypotheses were further confirmed by metabolic and transcript profiling of fruits from Chl-breakdown and ripening-impaired mutants (Almeida et al., 2015). During green stages, tomato GGDR (SlGGDR) produces *de novo* PDP for Chl biosynthesis and Chl turnover feeds tocopherol pathway via phytol recycling (Almeida et al., 2016). From the onset of ripening onwards, Chl synthesis ceases and the precursor GGDP is channeled towards carotenoid production by the transcriptional inhibition of the *SlGGDR* gene (Quadrana et al., 2013) and activation of the *PHYTOENE SYNTHASE 1* (*SlPSY1*) gene, which encodes a fruit-specific enzyme diverting GGDP into the carotenoid pathway (Llorente et al., 2016). Chl degradation releases phytol, which is, in part, incorporated into tocopherol, leading to the increment of VTE content during ripening.

Although several advances have been achieved in characterizing VTE biosynthesis and accumulation pattern in tomato fruits, little is known about the molecular mechanisms directly regulating the enzyme-encoding genes. The network analysis mentioned above, including mRNA and metabolites quantification data in different organs and developmental stages, revealed a spatiotemporal coordination in the expression of some tocopherol biosynthetic genes. Moreover,

the identification of common *cis*-regulatory elements in VTE biosynthetic pathway gene promoters suggested these genes could be controlled by the same transcription factors (Quadrana et al., 2013).

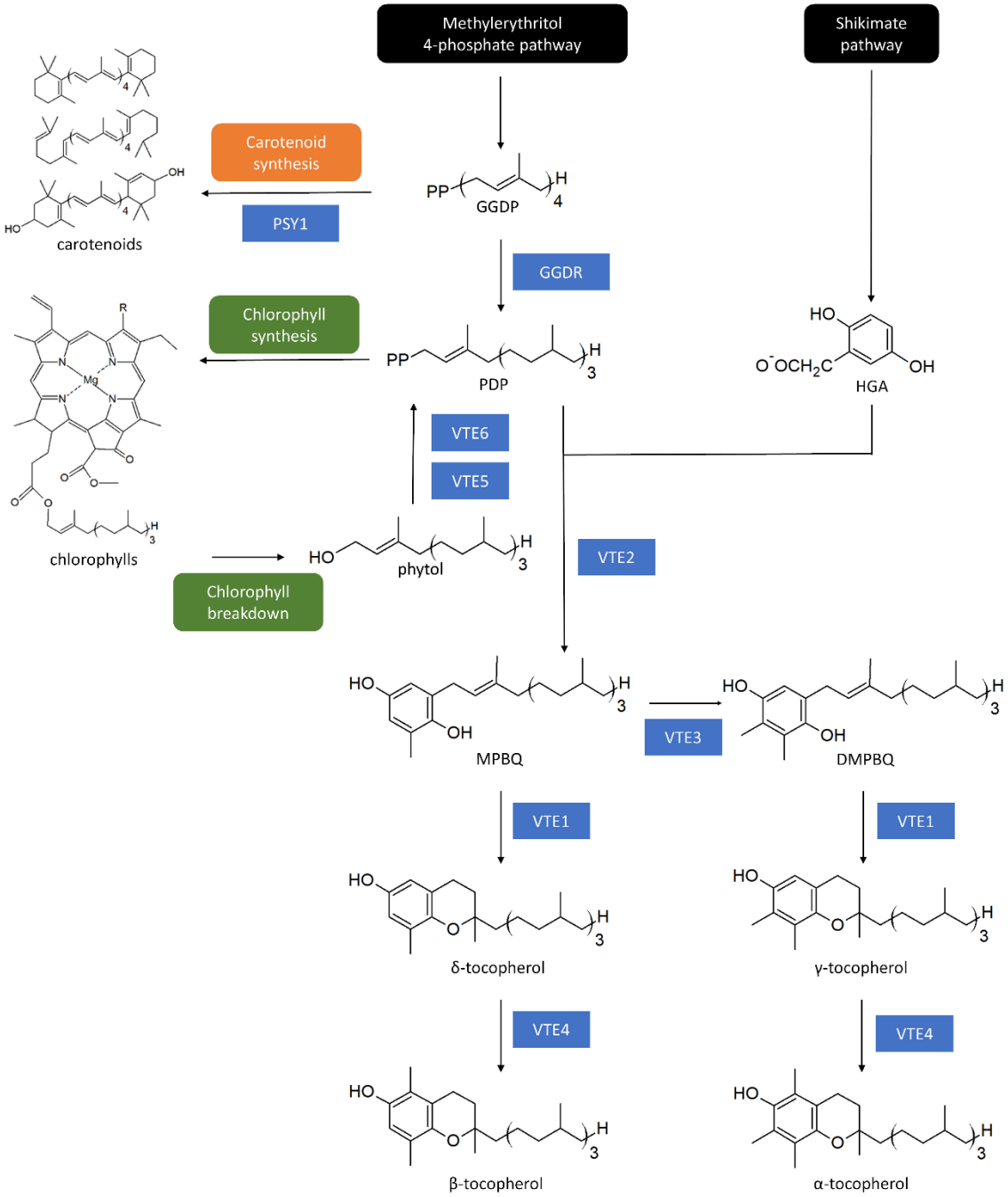


Figure 1 Schematic view of the tocopherol biosynthetic pathway. Enzymes and metabolites are denoted accordingly to the following abbreviations: PHYTOENE SYNTHASE 1 (PSY1); GERANYLGERANYL DIPHOSPHATE REDUCTASE (GGDR); HOMOGENTISATE PHYTYL TRANSFERASE (VTE2); 2,3-DIMETHYL-5-PHYTHYLKINOL METHYLTRANSFERASE (VTE3); TOCOPHEROL CYCLASE (VTE1); γ-TOCOPHEROL-C-METHYL TRANSFERASE (VTE4); PHYTOL KINASE (VTE5); PHYTYL PHOSPHATE KINASE (VTE6); geranylgeranyl-diphosphate (GGDP); hydroxyphenylpyruvate (HPP); phytyl diphosphate (PDP); homogentisate (HGA); 2-methyl-6-geranylgeranylbenzokinol (MPBQ); 2,3-dimethyl-6-geranylgeraniybenzokinol (DMBQ).

Recently, WRINKLED1 (WRI1) was characterized in *Arabidopsis thaliana* as a protein that directly interacts with the promoter of the ACETYL COA CARBOXYLASE (ACC), the first committed step in plastidial fatty acid biosynthesis, inducing its transcription (Pellaud et al., 2018). Although *wri1* mutant displayed conspicuous accumulation in total VTE content in seeds, this effect is not due to the direct regulation of VTE-related genes by WRI1, but caused by competition between VTE and lipid metabolisms for MEP pathway precursors (Mène-Saffrané, 2018; Pellaud et al., 2018). Hence, no transcription factors directly targeting the promoters of the tocopherol biosynthetic enzyme encoding gene have been reported, so far.

Considering that light has a central role in regulating chloroplast activity and differentiation during tomato fruit ripening (Cocaliadis, Fernández-Muñoz, Pons, Orzaez & Granell, 2014) and that tocopherols are photosynthesis-related compounds synthesized in the plastids, it is expected that light also affects the accumulation of fruit tocopherols, as already demonstrated for carotenoids (Llorente et al., 2016). The red/far-red light perception via PHYTOCHROMES (PHYs) plays a central role in controlling fruit development and ripening (Bianchetti et al., 2017, 2018; Gupta et al., 2014). Functional PHYs are homodimers with each polypeptide (apoprotein) associated with the linear tetrapyrrole chromophore phytochromobilin (PϕB). In the absence of light, PHYs are inactive in the cytoplasm and, upon red light exposure, an isomeric alteration of the PϕB leads to a rearrangement of the apoprotein structure that exposes the nuclear signaling domain, leading to PHY translocation into the nucleus (Bae & Choi, 2008). In the nucleus, PHYs promote the phosphorylation and further degradation of PHYTOCHROME INTERACTING FACTORS (PIFs), negative regulators of light signaling. PHYs and other

photoreceptors also promote the accumulation of positive regulators, such as ELONGATED HYPOCOTYL 5 (HY5), by downregulating the negative effectors in light signal transduction pathway CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), DETIOLATED1 (DET1), DAMAGE DNA BINDING1 (DDB1) and CULLIN4 (CUL4) (Leivar & Quail, 2011; Lau & Deng, 2012). *DET1* silenced fruits displayed higher levels of tocopherol providing evidences of the effect of light signaling over VTE metabolism in tomato (Enfissi et al., 2010); however, no consistent upregulation of the biosynthetic enzyme encoding genes was observed. Thus, the increment in tocopherol can be explained as the consequence of the higher number of chloroplasts and Chl accumulation in green fruits, rather than a direct effect of light signaling disturbance.

To better understand the interplay between light and VTE accumulation during tomato fruit ripening, we investigated whether this environmental cue regulates tocopherol biosynthesis and the molecular mechanism underneath. Our results demonstrated that PHY-dependent light perception positively regulates tocopherol production during tomato fruit ripening. Moreover, light-dependent tocopherol accumulation during fruit ripening is mediated by the physical interaction of SlPIF3 transcription factor with the promoter region of *SlGGDR*, resulting in its transcriptional upregulation and boosting the *de novo* biosynthesis of PDP precursor.

MATERIALS AND METHODS

Plant material, growth conditions and sampling

Solanum lycopersicum (cv. MicroTom and Moneymaker), *Nicotiana benthamiana*, and *Nicotiana tabacum* plants were grown under standard greenhouse conditions (14h light at 27 ± 1 °C and 10h dark at 22 ± 1 °C). Seeds of *aurea* and *hp2* mutants, in cv. MicroTom background, were donated by Dr. Lázaro Eustáquio Pereira Peres (University of Sao Paulo, Brazil). Seeds of single and multiple *phya*, *phyb1* and *phyb2* mutants, in Moneymaker background, were provided by Dr. Rameshwar Sharma (University of Hyderabad, India). In *aurea*, a mutation on PHYTOCHROMOBILIN SYNTHASE encoding gene prevents the correct synthesis of the PHY chromophore PϕB, leading to a global deficiency in functional PHYs (Kendrick., Kerckhoffs, Tuinen & Koornneef, 1997; Muramoto et al., 2005; Parks et al., 1987). The *hp2* mutant is deficient for DET1 transcription factor, a negative regulator of light signal transduction (Soressi et al., 1975).

phya, *phyb1*, *phyb2*, *phyab1*, *phyb1b2* and *phyab1b2* are loss-of-function mutants initially characterized by Kerckhoffs et al., 1996; Kerckhoffs, Schreuder, Van Tuinen, Koornneef M. & Kendrick, 1997; Kerckhoffs et al., 1999; Lazarova et al., 1998a; Lazarova et al., 1998b; and Weller, Schreuder, Smith, Koornneef & Kendrick, 2000.

For fruit ripening experiments, fruits at mature green (MG) stage were harvested about 30 days after anthesis (dpa) and were transferred to continuous white light (400 to 800 nm, approximately 50 μmol m^{-2} s^{-1}) or maintained under absolute darkness until reaching distinct ripening stages in a temperature-controlled growth chamber maintained at 25 ± 2 °C and air relative humidity at 80 ± 5%. Top and bottom illumination was applied to homogenize the light environment surrounding the fruits. Pericarp samples (without placenta and locule walls) were harvested at MG (displaying jelly placenta 2 days after harvesting), breaker (BR, 34 dpa displaying the first external yellow color signals), one day after BR (BR1), three days after BR (BR3), six days after BR (BR6), and twelve days after BR (BR12) stages.

Gene expression analysis

RNA extraction, cDNA synthesis, and qPCR (quantitative polymerase chain reaction) procedures were performed as described by Quadrana et al. (2013). The primers used for qPCR are listed in Table S1. All reactions were performed with two technical replicates and at least three biological replicates. Experiments were performed in a 7500 Real-Time PCR system (Applied Biosystem) using Power SYBR Green Master Mix (Thermo Fischer Scientific). Absolute fluorescence data was analyzed with LinRegPCR software (Ruijter et al., 2009) to obtain Ct values and to calculate primer efficiency. Relative mRNA abundance was calculated and normalized with the ΔΔCt method using two reference genes as in Quadrana et al. (2013).

Tocopherol and chlorophyll quantification

Tocopherols were extracted from approximately 25 mg dry weight as described by Lira et al. (2016). Chl extraction was carried out as described in Porra, Thompson & Kriedemann (1989). A 1 ml aliquot of dimethylformamide (DMF) was added to 200 mg of fresh weight fruit samples.

After sonication for 5 min at 42 kHz and further centrifugation at 13,000 *g* for 5 min, the supernatant was collected. The procedure was repeated twice until total removal of tissue green color and the supernatants were combined. Spectrophotometer measurements were performed at 664 and 647 nm. Chlorophyll a content was estimated as (12*Abs 664)-(3,11*Abs 647), while chlorophyll b was calculated as (20,78*Abs 647)-(4,88*Abs 664).

Promoter analysis

Approximately 3 Kb fragments of the promoter sequences of the *SlGGDR*, *SlVTE2* and *SlVTE5* genes were retrieved from Sol Genomics Network (Fernandez-Pozo et al., 2015). To gain evidences about the eventual role of SlPIFs in the regulation of these genes, the presence of PIF-binding motifs was analyzed using PlantPAN 2.0 platform (Chow et al., 2015).

Transactivation assay

Full-length cDNAs encoding SlPIF1a (Solyc09g063010), SlPIF1b (Solyc06g008030), SlPIF3 (Solyc06g008030) and SlRIN (Solyc05g012020) were amplified with the primers listed in Table S1. The fragments were cloned into pENTR/DTOPO vector using Gateway technology (Invitrogen). The entry plasmids were recombined into pK7WG2D (Karimi, Inzé & Depicker, 2002) using LR Clonase (Invitrogen) to produce 35S::SlPIFs/SlRIN effector constructs. Fragments of 2838 bp for *SlPSY1* (Solyc03g031860), 3123 bp for *SlVTE2* (Solyc08g068570), and 2648 bp for *SlGGDR* (Solyc01g067890) upstream the ATG starting codon were amplified from genomic DNA, using the primers listed in Table S1. The amplified fragments were digested with XhoI and BamHI restriction enzymes and cloned into the multicloning site of pGreenII 0800 LUC (Hellens et al., 2005) to produce the target constructs. All the constructs were sequenced and introduced into *Agrobacterium tumefaciens* (GV3101). For transient expression in *N. tabacum* leaves, *A. tumefaciens* cells carrying the different constructs were grown at 28 °C for 48 h in YEP medium (Sambrook, Fritsch & Maniatis, 1989) with appropriate antibiotics. The cells were harvested, washed twice and resuspended in infiltration buffer (50 mM MES pH 5.6, 2 mM sodium phosphate buffer pH 7, 0.5% glucose and 200 µM acetosyringone).

Leaves of 4-week-old plants were co-infiltrated with a mix of equal volumes of effector and target cultures, both at a final OD_{600} of 0.05. After three days, Firefly Luciferase and Renillia Luciferase activity were assayed using the Dual-Luciferase Reporter Assay System (Promega) as described by Hellens et al. (2005), with slight modifications. Two leaf discs of 2 cm were harvested and grounded in 500 µl of Passive Lysis Buffer. Ten µl of this crude extract were assayed in 40 µl of Luciferase Assay Reagent and the chemiluminescence was measured. Then, 40 µl of Stop and Glow™ Reagent was added and a second chemiluminescence measurement was made. Absolute relative light units (RLU) were measured by Synergy H1 (Biotek) luminometer, with a 5 s delay and 15 s measurement. Data was collected as Luciferase/Renilla activity ratio and subsequently normalized relative to the control condition (leaves infiltrated with *A. tumefaciens* harboring pK7WG2D empty vector).

Subcellular localization and chromatin immunoprecipitation assay

Full-length cDNA encoding *SlPIF1a*, *SlPIF1b*, *SlPIF3* and *SlHY5* without the stop codon were amplified with the primers listed in Table S1. The fragments were cloned into pENTR/DTOPO using Gateway technology (Invitrogen). The entry plasmids were recombined into pK7FWG2 (Karimi et al., 2002) using LR Clonase (Invitrogen) to produce 35S::SlPIF/SlHY5-GFP fusion proteins. The constructs obtained were introduced into *A. tumefaciens* (GV3101) for further subcellular localization assay and for chromatin immunoprecipitation (ChIP). For subcellular localization, 35S::SlPIF/SlHY5-GFP fusion proteins were agroinfiltrated in *N. benthamiana* leaves and 3 days after the infiltration the fluorescence was detected using a Leica TCS SP5 confocal laser-scanning microscope. Excitation filter of 450–490 nm was used for detection of GFP fluorescence.

ChIP assay followed by qPCR was performed as described in Ricardi, González & Iusem (2010) with some modifications. Briefly, MG fruits were agroinfiltrated with 35S::SlPIF3-GFP construct, kept for three days under light or dark conditions and fixed with formaldehyde to promote the crosslinking between DNA and proteins. Following nuclei enrichment with a Percoll (GE Healthcare) gradient, the chromatin was fragmented by sonication (10 s on/20 s off, amplitude 70, during 10 min using QSonica700 device) and then incubated with Dynabeads Protein-A

(Invitrogen) with either anti-GFP or anti-HA antibodies (Invitrogen). Next, the immunoprecipitated DNA was purified by phenol:chloroform:isoamyl alcohol extraction and used as template for qPCR analysis. Specific primer pairs flanking the predicted transcription factor binding motifs for each promoter region and the coding region of *SlACTIN4* (Fujisawa, Nakano & Ito, 2011) as normalizer (Table S1) were used.

Fruit transient overexpression

For transient SlPIF3 overexpression assay, MG fruits were agroinfiltrated (Orzaez, Mirabel, Wieland & Granell, 2006) with the 35S::SlPIF3 construct used for transactivation experiments and, after three days in the dark, the gene expression of *SlGGDR* and *SlPIF3* was addressed by qPCR as previously described using the primers listed in Table S1.

Data analyses

Differences in parameters were analyzed in Infostat software version 17/06/2015 (Di Rienzo et al., 2011). When the data set showed homoscedasticity, *t*-test ($P<0.05$) was performed to compare genotypes or treatments. In the absence of homoscedasticity, a non-parametric comparison was performed by applying Mann-Whitney test ($P<0.05$). All values represent the mean of at least three biological replicates.

RESULTS

Tocopherol accumulation is transcriptionally regulated by light during fruit ripening

To investigate whether light regulates VTE biosynthesis during fruit ripening, tocopherol levels were quantified in fruits from wild type (WT) plants and two light-related mutants, the light-hyposensitive PϕB deficient *aurea* and the light-hypersensitive DET1 deficient *high-pigment 2* (*hp2*). Fruits at the mature green (MG) stage were detached from the plants and let to ripen under constant white light or darkness (Figure 2a). As previously reported (Almeida et al., 2015), the

tocopherol levels increased during ripening in WT fruits. However, the accumulation was higher when the fruits were maintained under constant white light, indicating that this stimulus promotes tocopherol biosynthesis. Interestingly, this light-associated increase in tocopherol contents was not observed in the *aurea* mutant (Figure 2a), strongly suggesting a PHY-mediated effect. Consistently, fruits from the light hypersensitive mutant *hp2* displayed a pronounced increment of total tocopherols when ripened in the light, reaching higher absolute levels than those observed from fruits ripened in the darkness (Table S2).

The transcript levels of the genes involved in VTE biosynthesis were next profiled in WT fruits along ripening under constant light or dark conditions (Figure 2b and Figure S1). We observed that *SlGGDR, SlVTE1*, *SlVTE2*, *SlVTE4*, and *SlVTE5* mRNA levels were lower when the fruits were incubated in the dark. Particularly interesting were the reductions in *SlGGDR*, *SlVTE5*, and *SlVTE2* mRNA levels (Figure 2b). The two formers are responsible for PDP production either *de novo* (*SlGGDR*) or from Chl degradation (*SlVTE5*), while *SlVTE2* encodes the enzyme responsible for the condensation of PDP and HGA in the first committed step of tocopherol biosynthesis (Figure 1).

Together, these results revealed that tocopherol accumulation in tomato fruits is induced by light, which might be explained, at least in part, by the transcriptional regulation of tocopherol biosynthetic genes.

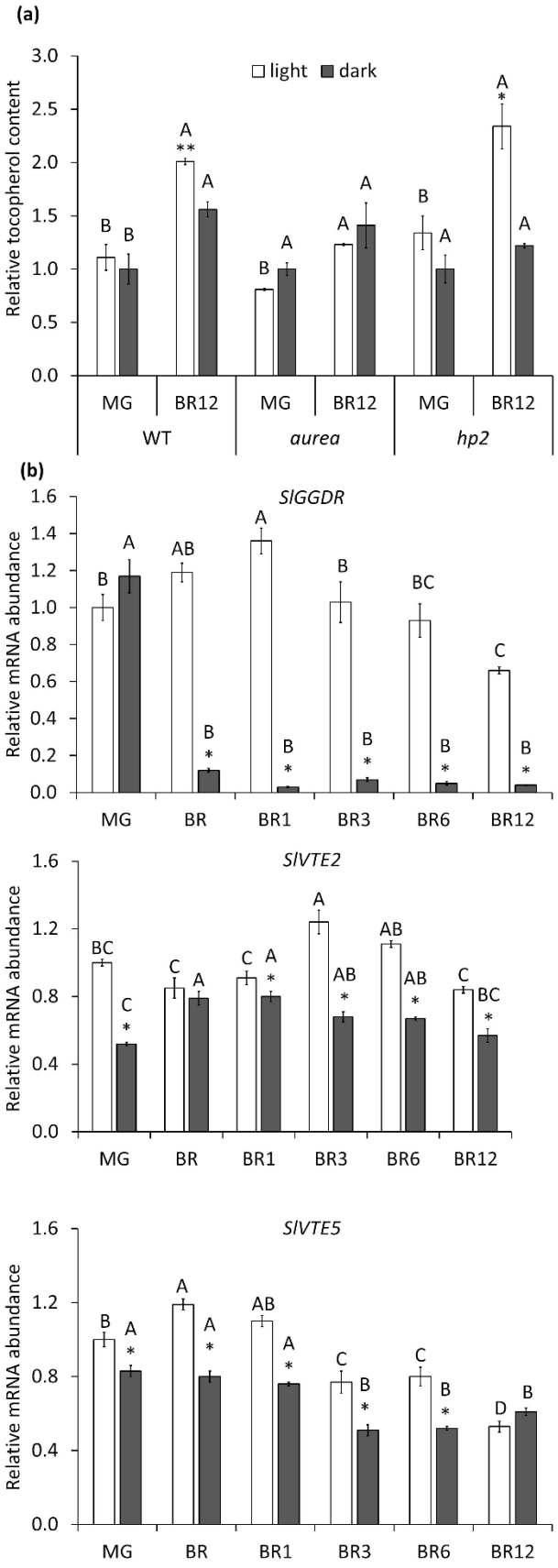


Figure 2 Effect of light on tocopherol metabolism during fruit ripening. (a) Tocopherol content in fruits from different genotypes. Mature green (MG) fruits were detached from the plants and maintained in constant white light (white bars) or darkness (black bars). Tocopherol levels were quantified 2 days after the beginning of the light treatment (MG) and 12 days after breaker stage (BR12). Total tocopherol (summed values of α, β, γ and δ forms) was expressed relative to MG fruits maintained in the darkness. WT: wild type; *hp2*: *high pigment2* mutant. The absolute amounts are detailed in Table S2. (b) Transcript profile of VTE biosynthetic genes in response to light. Relative mRNA abundance in WT fruits along ripening, from MG to BR12, under constant light (white bars) or darkness (black bars) treatments. Values are represented as means of at least three biological replicates. The complete set of data is detailed in Table S3. Asterisks denote significant differences (* $p<0.05$; ** $p<0.01$) between treatments. Different letters indicate statistically significant differences between stages ($p<0.05$) within the same treatment and genotype.

PHY-mediated light signal transduction enhances tocopherol accumulation

To test whether the observed positive effect of light on the regulation of fruit tocopherol content is mediated by PHYs, we analyzed the tocopherol accumulation in fruits from WT and six loss-of-function PHY mutants (*phya*, *phyb1*, *phyb2*, *phyab1*, *phyb1b2*, *phyab1b2*). Interestingly, only the double *phyab1* and triple *phyab1b2* mutants displayed a reduction in tocopherol content in ripe fruits (Figure 3a). These results reinforce our previous conclusion that light induces tocopherol accumulation during ripening in a PHY-mediated manner. They further reveal redundancy of individual phytochrome functions in the regulation of VTE biosynthesis. Quantification of tocopherol biosynthetic gene expression in WT and *phyab1b2* mutant fruits showed reduced levels of *SlGGDR*, *SlVTE2*, and *SlVTE5* (but not *SlVTE6*) transcripts in the PHY-defective triple mutant (Figure 3b), hence confirming that the PHY-dependent regulation of fruit tocopherol content is mediated by the transcriptional regulation of key tocopherol biosynthetic genes.

SlPIF3 represses *SlGGDR* transcription by physically interacting with its promoter

As PIFs are major transcription factors acting downstream of PHY-mediated light signaling; we investigated the eventual role of SlPIFs in the PHY-dependent regulation of tocopherol accumulation in tomato fruits. *SlPIF loci* were previously identified and transcriptionally characterized, being *SlPIF1a*, *SlPIF1b* and *SlPIF3* the most abundantly expressed in fruits (Rosado et al., 2016). Thus, we addressed whether the light treatment (Figure 2a) or the PHY deficiency (Figure 3a) affected the expression of these *SlPIFs*. Interestingly, these genes were found to be up-regulated in WT fruits maintained in darkness (Figure S2a), as well as in the triple mutant *phyab1b2* fruits (Figure S2b), reinforcing their candidature as mediators of the PHY-dependent regulation of tocopherol accumulation. As an additional step in their functional characterization, we aimed to confirm their expected nuclear localization. In order to do so, the coding region of these genes was fused to the *Green Fluorescence Protein* (*GFP*) and transiently expressed in *Nicotiana benthamiana* leaves. As reported for *A. thaliana* PIFs (Leivar & Monte, 2014), SlPIF1a, SlPIF1b and SlPIF3 proteins localized as nuclear speckles (Figure 4a). Such nuclear speckles, also referred to as photobodies, have been associated to the interaction of PHYs

with photolabile PIFs for their phosphorylation and further degradation of these transcription factors (Al-Sady, Ni, Kircher, Schäfer & Quail, 2006). Indeed, a GFP-tagged version of SlHY5, a transcription factor that does not directly interact with PHYs, showed a homogeneous distribution in the nucleus (Figure S3). Together, these results strongly support that, as demonstrated for SlPIF1a (Llorente et al., 2016), SlPIF1b and SlPIF3 are true PIFs, *i.e.* PHY-interacting factors.

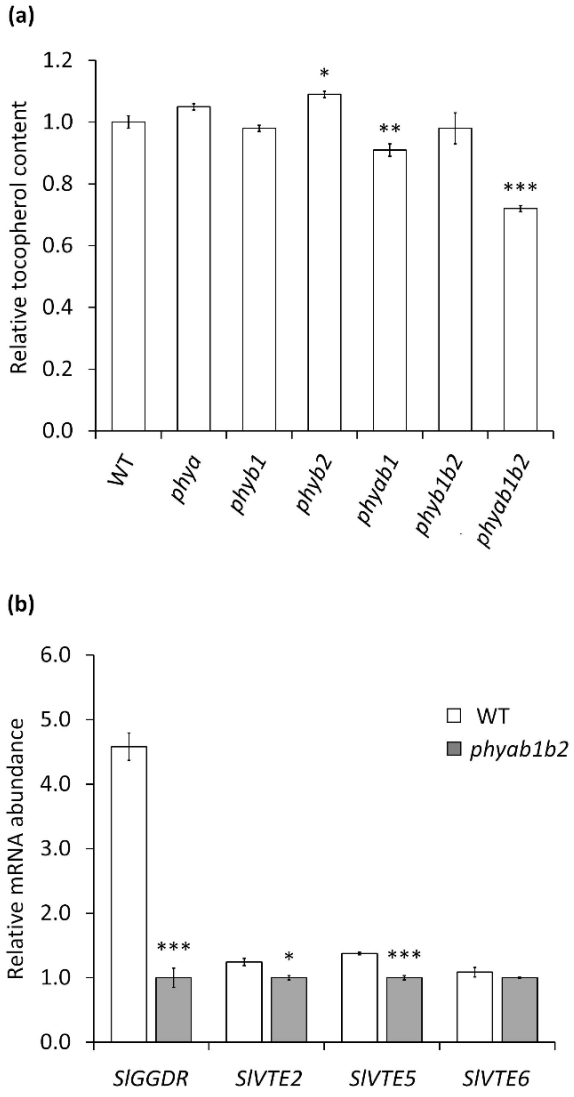


Figure 3 Effect of PHY-mediated light perception on tocopherol metabolism during fruit ripening. (a) Tocopherol accumulation in PHY mutants (*phy*). Mature green fruits were detached from the plants and maintained in constant white light from mature green until 12 days after breaker (BR12) stage. Tocopherol levels were quantified and expressed relative to WT fruits. The absolute amounts are detailed in Table S4. (b) Relative mRNA abundance of *SlGGDR*, *SlVTE2*, *SlVTE5*, and *SlVTE6* in BR12 fruits from WT and *phyab1b2* triple mutant genotypes. The complete set of data is detailed in Table S5. Values are represented as means of at least three biological replicates. Asterisks denote significant differences compared to the WT genotype (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Being demonstrated that PHY-mediated light perception affected *SlPIF* expression in fruits and that SlPIFs localized in speckles, which is indicative of their light-induced PHY-mediated degradation, we investigated whether SlPIFs are responsible for the light-regulated expression of tocopherol biosynthetic genes. A *de novo* search for putative PIF-binding motifs in 3000 bp fragments upstream the ATG starting codon of *SlGGDR*, *SlVTE2* and *SlVTE5* retrieved from the Heinz reference tomato genome (Sol Genomics Network, Fernandez-Pozo et al., 2015) revealed the presence of G-boxes and PBE-boxes (Song et al., 2008; Chen et al., 2013; Zhang et al., 2013; Toledo-Ortiz et al., 2014) in the promoter regions of *SlGGDR* and *SlVTE2* (data not shown). To test the functionality of these putative PIF-binding motifs, transient transactivation assays were performed in *Nicotiana tabacum* (Figure 4). Promoter regions of 2648 bp for *SlGGDR* and 3123 bp for *SlVTE2* were cloned from MicroTom genotype and sequenced (Figure S4). Polymorphisms were identified compared to the reference Heinz sequence, which do not alter the previously identified motifs on *SlGGDR* but reduce to 4 the number of PBE-boxes identified on *SlVTE2* (Figure 4b). As positive control, the previously reported inductive effect of the transcription factor RIPENING INHIBITOR (SlRIN) on the *SlPSY1* promoter (Martel, Vrebalov, Tafelmeyer & Giovannoni, 2011) was also tested (Figure S5). The activity of the *SlGGDR* promoter was found to be downregulated in the presence of SlPIF1a, SlPIF1b, and SlPIF3, whereas none of these SlPIFs affected the activity of the *SlVTE2* promoter (Figure 4c).

The direct interaction between SlPIF3, the most highly expressed *SlPIF* gene in tomato fruits (Rosado et al., 2016), and the *SlGGDR* promoter was further addressed by chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR). MG fruits were infiltrated with *Agrobacterium tumefaciens* harboring a 35S::SlPIF3-GFP construct and maintained in constant dark or light conditions for 3 days. After chromatin purification, an enrichment in *SlGGDR* promoter sequences harboring PBE boxes (Figure 4b) was observed in anti-GFP immunoprecipitated samples, *i.e.* those containing SlPIF3-GFP, in comparison with anti-HA immunoprecipitated control in those fruits maintained in the darkness. This enrichment was not observed when the fruits were kept in the light, hence indicating that SlPIF3 physically interacts with the *SlGGDR* promoter preferably in the darkness (Figure 4d).

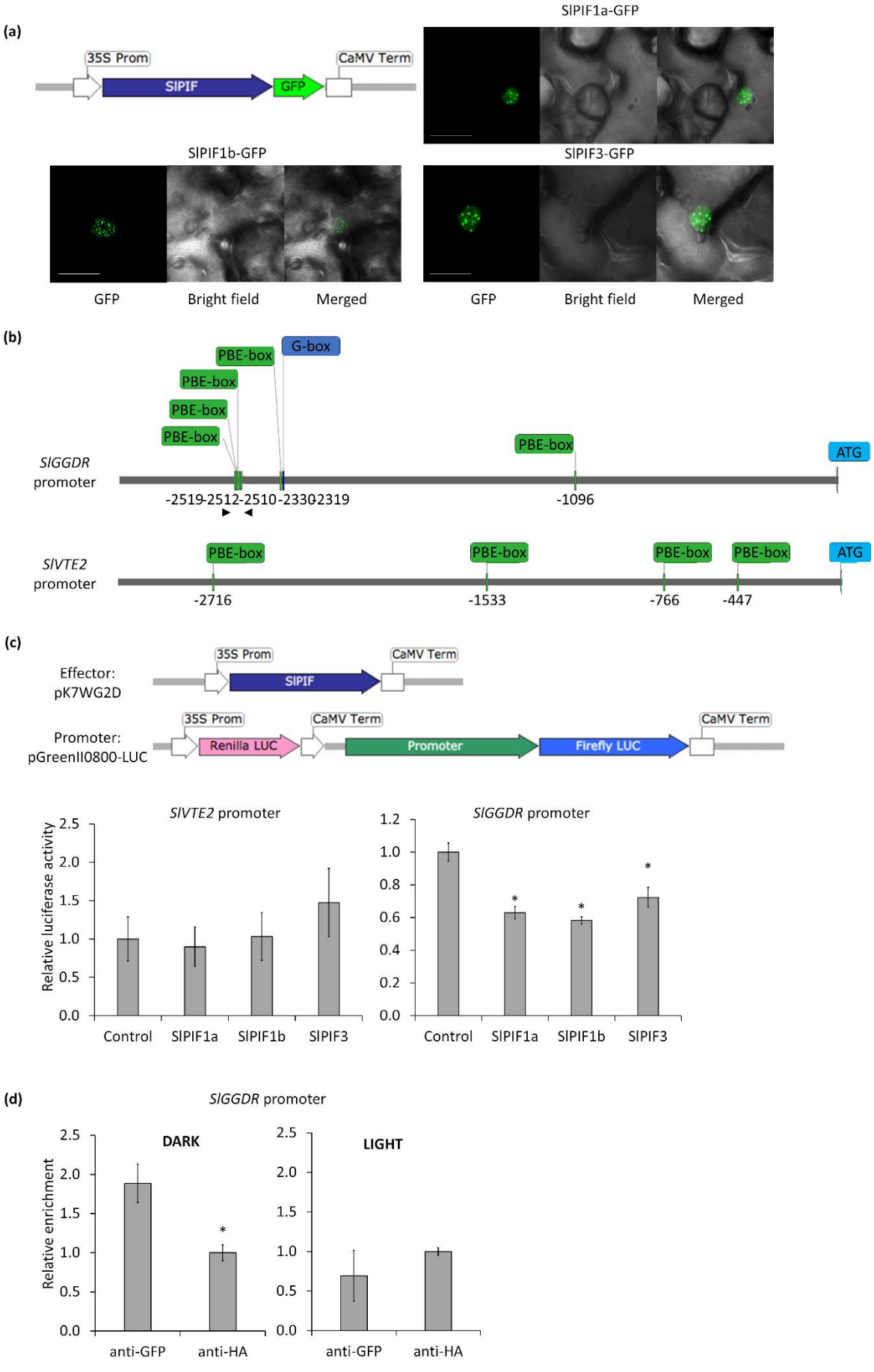

(a)
35S Prom
SlPIF
GFP
CaMV Term
SlPIF1a-GFP
SlPIF1b-GFP
SlPIF3-GFP
GFP
Bright field
Merged
GFP
Bright field
Merged
(b)
PBE-box
G-box
PBE-box
PBE-box
PBE-box
PBE-box
ATG
SlGGDR promoter
-2519
-2512
-2510
-2330
-2319
-1096
SlVTE2 promoter
PBE-box
PBE-box
PBE-box
PBE-box
ATG
-2716
-1533
-766
-447
(c)
Effector: pK7WG2D
35S Prom
SlPIF
CaMV Term
Promoter: pGreenII0800-LUC
35S Prom
Renilla LUC
CaMV Term
Promoter
Firefly LUC
CaMV Term
SlVTE2 promoter
Relative luciferase activity
2.5
2.0
1.5
1.0
0.5
0.0
Control
SlPIF1a
SlPIF1b
SlPIF3
SlGGDR promoter
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Control
SlPIF1a
SlPIF1b
SlPIF3
(d)
SlGGDR promoter
Relative enrichment
DARK
LIGHT
2.5
2.0
1.5
1.0
0.5
0.0
*
anti-GFP
anti-HA
anti-GFP
anti-HA

Figure 4 SlPIF3 regulates *SlGGDR* expression. (a) Subcellular localization of SlPIF1a, SlPIF1b, and SlPIF3 proteins. Confocal microscopy images of *N. benthamiana* leaves infiltrated with 35S::SlPIF-GFP constructs. Scale bars = 20 μm. (b) PIF-binding motifs in the promoter region of *SlGGDR* and *SlVTE2* genes. The arrowheads on *SlGGDR* promoter indicate the primers used for ChIP-qPCR assay. (c) Transactivation of *SlVTE2* and *SlGGDR* promoters. A schematic representation of effector and target constructs used for LUCIFERASE transient assay in *N. tabacum* leaves is shown. Relative luciferase activity is expressed as LUCIFERASE/RENILLA activity ratio relative to the pK7WG2D empty vector (control). Values are expressed as means of two independent experiments with at least 6 biological replicates each. Asterisks denote significant differences ($p<0.05$) relative to the control. (d) ChIP-qPCR experiment performed in tomato fruits transiently expressing 35S::SlPIF3-GFP under continuous dark or light conditions using anti-GFP and anti-HA (as negative control) antibodies and the primers for *SlGGDR* indicated in panel (b). Values are expressed as means of two independent experiments, each with three biological replicates. Asterisk denote significant difference ($p<0.05$) relative to anti-GFP.

Transient SlPIF3 overexpression reduces the expression of SlGGDR

The fact that *SlPIF3* is the most abundantly expressed *SlPIF* gene in MG tomato fruits (Rosado et al., 2016), together with the results obtained by transactivation and ChIP-qPCR assays, strongly pointed this gene as the most evident candidate for downregulating *SlGGDR* expression in darkness. To test this hypothesis, we transiently overexpressed *SlPIF3* in MG tomato fruits by agroinfiltration and analyzed the impact of increasing SlPIF3 levels on the expression level of *SlGGDR*. A decrease in *SlGGDR* mRNA levels was verified (Figure 5a) that inversely correlated with the level of *SlPIF3* transcripts (Figure 5b). Altogether, the data indicate that SlPIF3 mediates the PHY-dependent regulation of VTE biosynthesis via the transcriptional inhibition of *SlGGDR* expression in tomato fruit.

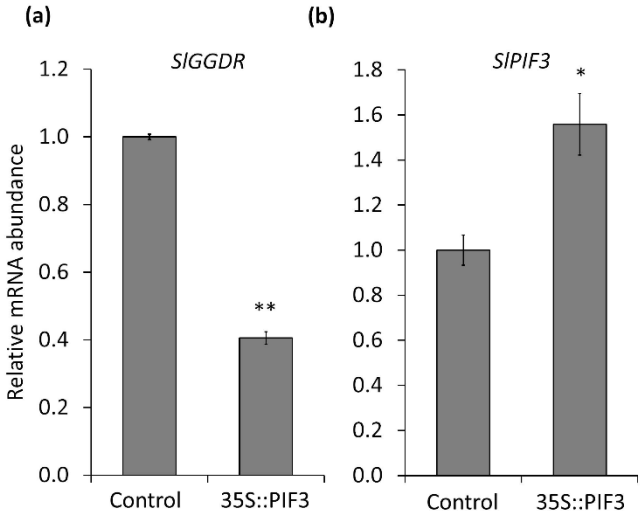


Figure 5 Overexpression of *SlPIF3* downregulates *SlGGDR* expression. Relative *SlGGDR* (a) and *SlPIF3* (b) mRNA abundance in mature green WT fruits agroinfiltrated with 35S::SlPIF3. Values are expressed as means of two independent experiments with 3 biological replicates each. Asterisks indicate significant difference against control sample fruits infiltrated with pK7WG2D empty vector (control) (*p <0.05; ** p<0.001)

DISCUSSION

Manipulation of tomato genes involved in light signaling has been shown to impact the nutritional quality of tomato fruits (Azari et al., 2010; Liu et al., 2004; Llorente, Martinez-Garcia, Stange & Rodriguez-Concepcion, 2017). Downregulation of the photomorphogenic transcription factor SlHY5 resulted in thylakoid deficient chloroplasts with larger plastoglobules at green stages and reduced carotenoid levels in ripe fruits (Liu et al., 2004). On the contrary, silencing of the negative regulators of light signaling *SlDDB1* and *SlCUL4* led to a significant increment in the number of plastids that resulted in enhanced carotenoid and flavonoid accumulation during ripening (Wang et al., 2008). In particular, a positive role of PHY-dependent light response cascade in fruit carotenogenesis has been demonstrated (Alba, Cordonnier-Pratt, & Pratt, 2000; Bianchetti et al 2018; Llorente et al., 2016).

Compared to the well-studied effect of light on carotenoid accumulation in fruits, little is known about the role of this environmental stimulus on the regulation of tocopherol biosynthesis, another important family of antioxidant health-promoting compounds. To address this issue, here we analyzed tocopherol accumulation along ripening in fruits from the PHY chromophore deficient *aurea*, the light hyperresponsive *hp2*, single and multiple *phya*, *phyb1* and *phyb2* mutants, and corresponding control genotypes.

Tocopherol biosynthesis is highly dependent on Chl degradation-derived phytol (Almeida et al., 2016) and, consequently, the rising of tocopherol content from MG to the ripe stage of fruit development correlates with the amount of Chl right before the onset of ripening. In agreement, the more sensitive to light the genotype is, the higher the level of Chl at the MG stage (Figure S6) and the higher the tocopherol accumulation in ripe fruits. Interestingly, our results showed that ripening under constant light conditions boosts (over 20 %) tocopherol production in WT genotype from the last green stage of tomato fruit (*i.e.* MG stage) onwards, once there is no more Chl synthesis. This increment was not observed in the *aurea* mutant, indicating PHY-mediated modulation of tocopherol biosynthesis. The observed light effect on tocopherol accumulation can be explained by the light-triggered changes in the expression profile of the biosynthetic genes. Light not only upregulated tocopherol core pathway genes but also *SlGGDR* and *SlVTE5*, which produce the limiting precursor PDP. The PHY-mediated induction of tocopherol biosynthesis was reinforced by the analysis of *phyab1b2* triple mutant. Although the reduced levels of tocopherol could be, at least in part, due to the reduced amount of Chl (Weller et al., 2000), the expression profiles observed in ripe fruits from *phyab1b2* mimicked those from WT fruits ripened in the darkness, thus demonstrating that this effect on gene expression is mediated by PHYs. In agreement with our results, regulation of tocopherol biosynthesis by light has been recently reported in vegetative tissues of *A. thaliana*. Leaves from plants maintained in the dark displayed lower levels of tocopherol than those from plants exposed to light, which correlated with the downregulation of *AtVTE1*, *AtVTE2*, *AtVTE3*, and *AtVTE4* genes (Tanaka et al., 2015).

Being demonstrated that PHY-mediated light perception controls tocopherol accumulation during fruit ripening, PIF proteins, hub players of light response (Leivar & Quail, 2011), appeared as the most likely transcription factors involved in this process. Indeed, the fruit most abundantly expressed *SlPIFs* showed higher levels of mRNA accumulation in response to darkness or PHY deficiency. Moreover, SlPIF localization as nuclear speckles is directly involved in their light-dependent degradation as demonstrated for AtPIF3 in *A. thaliana* (Al-Sady et al., 2006). The two PIF-binding motifs described so far, PBE- and G-boxes, are present in the promoter regions of photosynthesis-related genes. In *A. thaliana*, AtPIF1 directly binds the G-box motifs located in the promoters of the chlorophyll and carotenoid biosynthetic genes *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE* (*AtPORC*) and *PHYTOENE SYNTHASE* (*AtPSY*), inducing and inhibiting their transcription, respectively (Moon, Zhu, Shen & Huq, 2008;

Toledo-Ortiz, Huq & Rodríguez-Concepción, 2010). Moreover, AtPIF4 and AtPIF5 interact with the G-box motifs of the senescence transcription factor *ORESARA 1* (*AtORE1*) and chlorophyll degrading enzyme encoding genes, such as *STAY GREEN 1* (*AtSGR1*) and *NON-YELLOW COLORING 1* (*AtNYC1*), upregulating them during dark-induced senescence (Sakuraba et al., 2014; Song et al., 2014; Zhang, Liu, Chen, He & Bi, 2015). Regarding tomato, it was discovered recently that SlPIF1a modulates carotenoid biosynthesis during fruit development, by binding to the PBE-box motifs of *SlPSY1* gene leading to its repression (Llorente et al., 2016). It was proposed that the presence of Chl in the chloroplasts of green (*e.g.* MG) fruits results in a "self-shading" effect that leads to PHY deactivation and subsequent accumulation of PIFs in the inner layers of the pericarp (Llorente et al., 2016). High SlPIF1a levels in MG fruit result in the repression of *SlPSY1* expression. As Chl degrades during ripening, PHYs are activated degrading PIFs, which in turn release *SlPSY1* gene expression and carotenoid biosynthesis.

Here, our data obtained from transactivation and ChIP assays, demonstrated the light-dependent direct interaction of SlPIF3 with a PBE-box rich region of the *SlGGDR* promoter leading to the downregulation of its expression. Based on our results, we propose a model that describes PHY- and SlPIF3-mediated transcriptional control of *SlGGDR* that regulates the influx of the PDP precursor for the light-dependent production of VTE during fruit ripening (Figure 6). While Chl degrades during ripening, the recycling of Chl breakdown derived phytol enhances the availability of PDP for tocopherol biosynthesis (Almeida et al., 2016), increasing tocopherol content in the ripe fruits, both in the darkness and under light conditions. However, when fruits ripen in the light, PHY-mediated SlPIF3 degradation and the consequent increment in *SlGGDR* expression provide an extra input of PDP, from the *de novo* synthesis, resulting in higher tocopherol levels.

Our study, together with that from Llorente et al. (2016), reveals that the regulation of SlPIF levels in tomato fruits could serve as a mechanism to coordinate the production of carotenoids (pro-vitamin A) and tocopherols (vitamin E). Manipulation of these underlying mechanisms in plants therefore appears as an effective strategy to increase fruit nutraceutical value, highlighting the importance of light signaling modulation as a biotechnological approach to improve functional properties in crops.

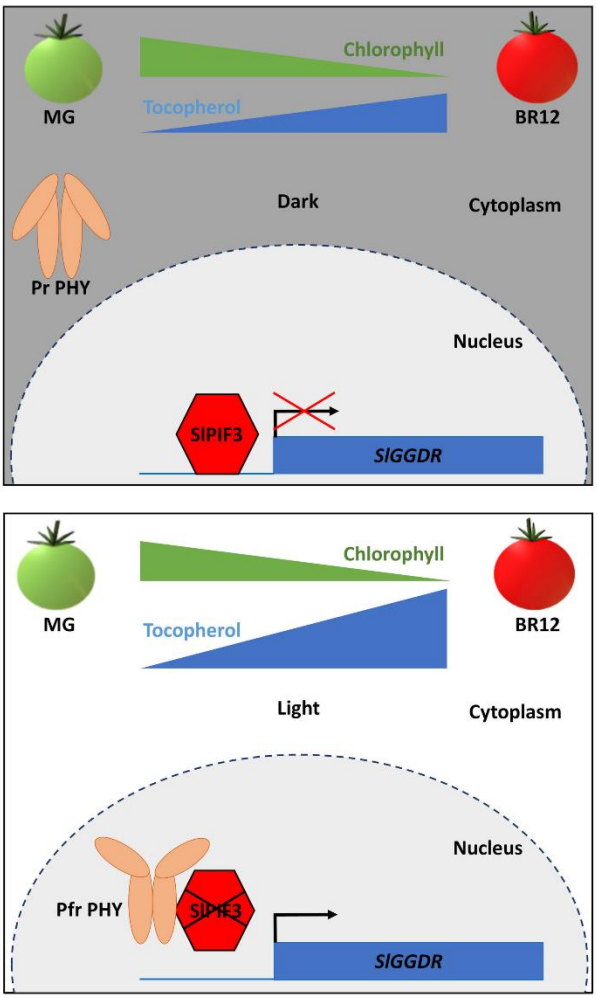


Figure 6 Model for tocopherol biosynthesis regulation during ripening. In the absence of light, from the onset of ripening onwards, the recycling of the Chl degradation-derived phytol provides phytyl diphosphate (PDP) for tocopherol production. The biologically inactive Pr form of phytochrome is localized in the cytosol and the nuclear-localized SlPIF3 transcription factor binds to the promoter of *SlGGDR*, repressing its expression. In the light, the biologically active Pfr form of phytochrome translocates into the nucleus and interacts with SlPIF3, leading to *SlGGDR* transcriptional derepression. Consequently, increased *SlGGDR* expression leads to increased production of PDP, ultimately resulting in a higher availability of this precursor for VTE synthesis. MG: mature green; BR12: 12 days after breaker.

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SUPPORTING INFORMATION

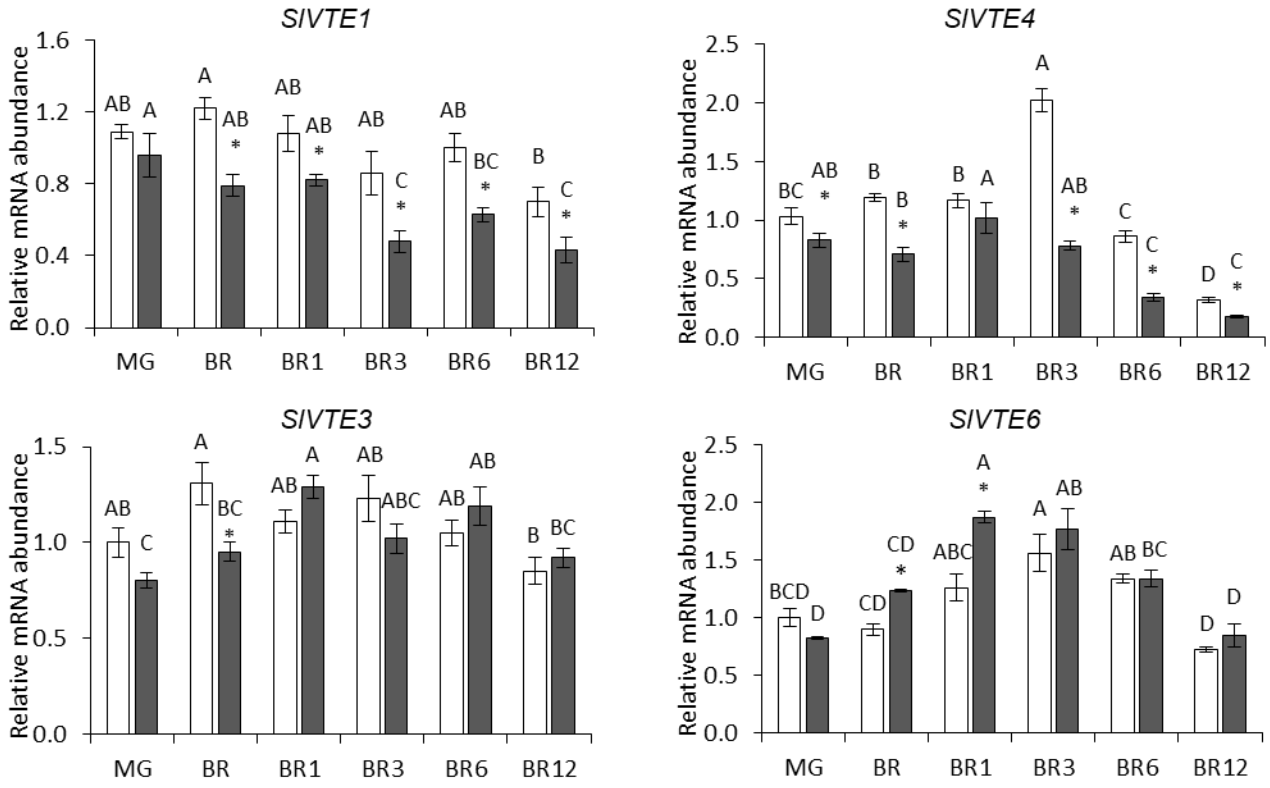

Figure S1. Transcript profile of VTE biosynthetic genes in response to light. Relative mRNA abundance in wild-type fruits along ripening, from mature green (MG) to 12 days after breaker (BR12), under constant light (white bars) or darkness (black bars) treatments. Values are represented as means of at least three biological replicates. The complete set of data are detailed in Table S3. Asterisks denote significant differences ($p<0.05$) between treatments. Letters denote significant differences between stages ($p<0.05$) within the same treatment.

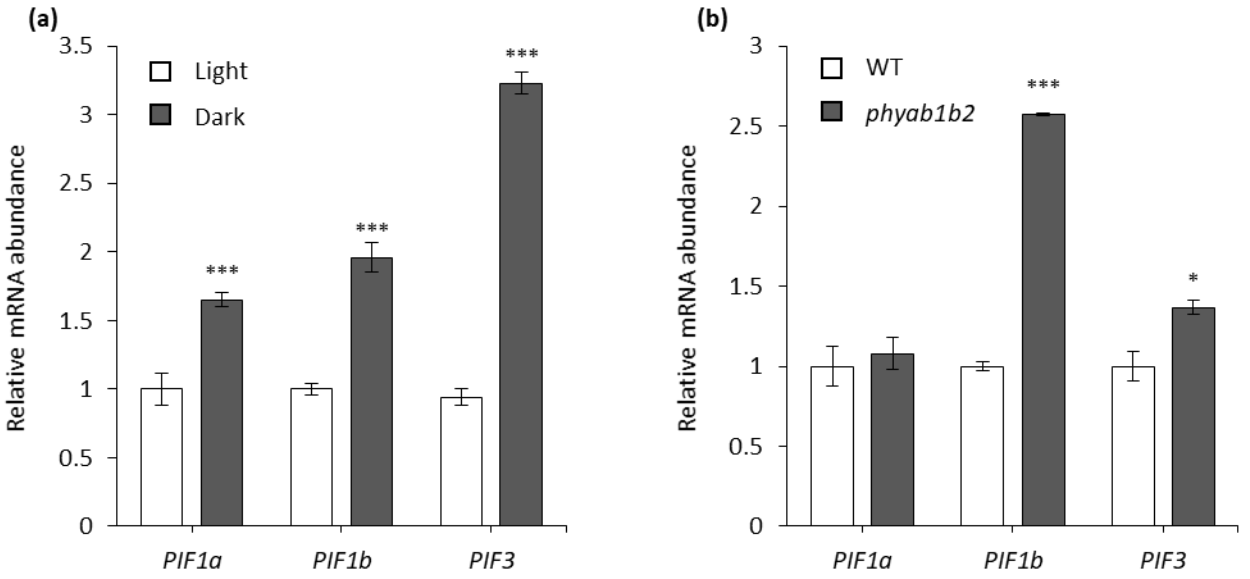

Figure S2. Transcript profile of *SlPIFs* in fruits. (a) Relative mRNA abundance of *SlPIF1a*, *SlPIF1b* and *SlPIF3* in mature green fruits. Fruits were detached from the plants and maintained in constant white light (white bars) or darkness (black bars) for 2 days (Figure 2a) . (b) Relative mRNA abundance of *SlPIF1a*, *SlPIF1b* and *SlPIF3* in breaker+12day fruits from WT and *phyab1b2* triple mutant genotypes (Figure 3a). Asterisks denote significant differences (* $p<0.05$; *** $p<0.001$) between treatments.

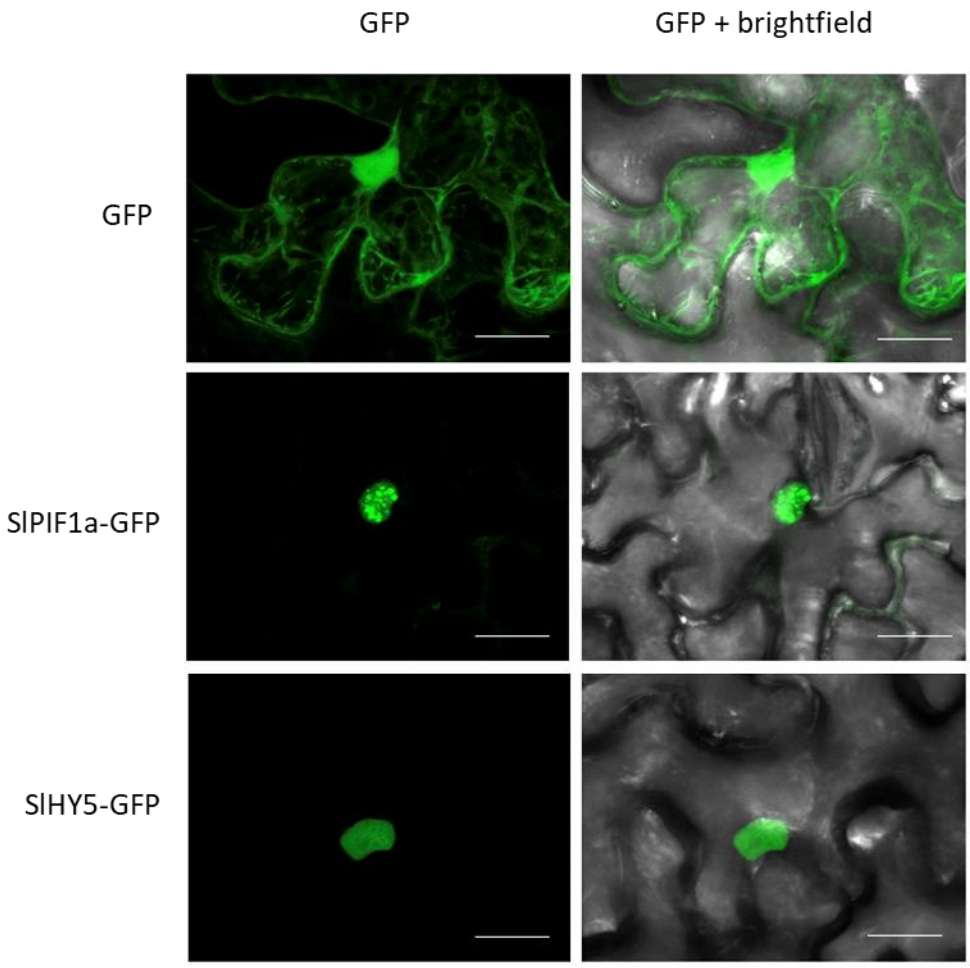

Figure S3. Subcellular localization of tomato GFP-tagged SlPIF1a and SlHY5 proteins. Confocal microscopy images of *N. benthamiana* leaves infiltrated with GFP (control), SlPIF1a-GFP and SlHY5-GFP. Scale bars = 20 μm.

> *SlGGDR* Promoter

CCACTTTGAAACCCAGCGACAAAGAAAAATGTGAAACCTTAGCCGTGAAATGAGGGAGAAGGGGTGAAATCATTCTGAAAAATAATGAAATGAGG
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>*SlVTE2* Promoter

GACTACTAAACAAGGACCACTCACAAACCATTTATAGCTTATAATTTTAAACTTATTTTAAGCTTAAAAATAAACATACTAAATACATTTTTCATTTCTG
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GTGGAGTTCAGTGCTGATTTAAAAATCAAAA**ATG**

Figure S4. Sequences of *SlGGDR* and *SlVTE2* gene promoter regions cloned from the MicroTom genotype. PBE- and G-boxes are highlighted in green and blue, respectively. A PBE-box on the minus strand of the *SlGGDR* promoter is underlined. Translation initiation sites are indicated in bold.

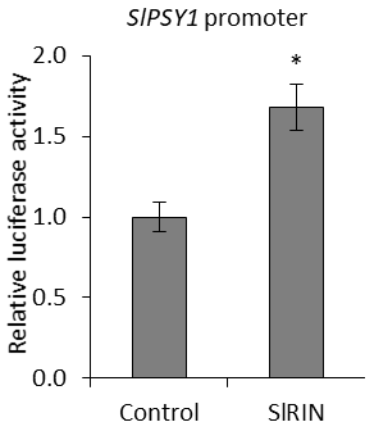

Figure S5. Transactivation control assay. Transactivation of *SlPSY1* promoter by SlRIN transcription factor. Relative luciferase activity is expressed as LUCIFERASE/RENILLA activity ratio relative to the pK7WG2 empty vector (control). Values are expressed as means of two independent experiments with at least 6 biological replicates each. Asterisk denotes significant difference ($p<0.05$) relative to the control.

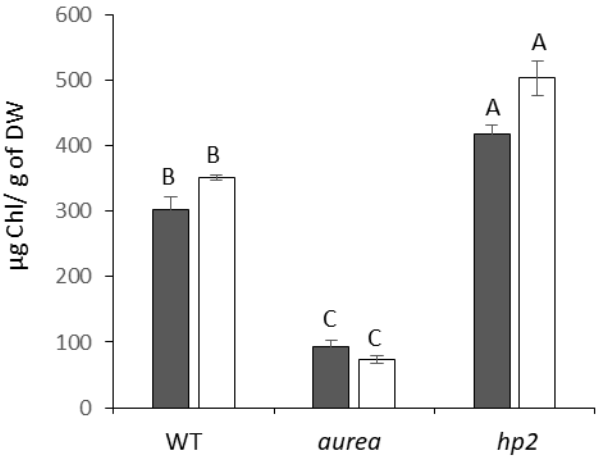

Figure S6. Total chlorophyll levels in fruits from different genotypes. Mature green fruits were detached from the plants and maintained in constant white light (white bars) or darkness (black bars) for 2 days. Chlorophyll levels were quantified 2 days after the beginning of the light treatment. WT: wild-type; *hp2*: *high pigment2* mutant. Values shown are means ± SE of at least three biological replicates. Letters denote significant differences between samples ($p<0.05$).

Table S1. Primers used.

Gene	Accession number	Experiment		Primer sequence
Expressed	Solyc07g025390	qPCR	F	GCTAAGAACGCTGGACCTAATG
			R	TGGGTGTGCCTTTCTGAATG
SlTIP41	Solyc10g049850	qPCR	F	ATGGAGTTTTTGAGTCTTCTGC
			R	GCTGCGTTTCTGGCTTAGG
SlPIF1a	Solyc09g063010	localization cloning	F	CACCATGAATCATTCTGTTCCTGATT
			R	ACCAGATTGATGATTGCCTGGATTTC
		transactivation cloning	F	CACCATGAATCATTCTGTTCCTGATT
			R	TTAACCAGATTGATGATTGCCTGGATTTC
SlPIF1b	Solyc06g008030	localization cloning	F	CACCATGAATTACTGTGTTGTTCCTG
			R	AATAGTATGCTCACCAGATTGATGATTC
		transactivation cloning	F	CACCATGAATTACTGTGTTGTTCCTG
			R	CTAAATAGTATGCTCACCAGATTGATGATTC
SlPIF3	Solyc01g102300	qPCR	F	AAGGCTTCCCAATAATGC
			R	CCATCAGACCAAACTTCCC
		qPCR (infiltration)	F	GGATGGGATTTGGGTTGGGT
			R	TGGATAAGCGGTGGAAGCAG
		localization/ ChIP cloning	F	CACCATGCCTCTCTCTGAGTTTTTGAAGATG
			R	CAAACTGGGACCAGCTTCATTTCC
		transactivation cloning	F	CACCATGCCTCTCTCTGAGTTTTTGAAGATG
			R	CTACAAACTGGGACCAGCTTCATTTCC
SlRIN	Solyc05g012020	transactivation cloning	F	CACCATGGGTAGAGGGAAAGTAGAATTG
			R	TCAAAGCATCCATCCAGGTAC
SlHY5	Solyc08g061130	localization cloning	F	CACCATGCAAGAGCAAGCGA
			R	CTACTTCCTCCCTTCCTGACC
SlVTE1	Solyc08g068570	qPCR	F	CGAACTCCTCATAGCGGGTATC
			R	CACGCCAGTAAACCGAGGC
SlVTE2	Solyc07g017770	qPCR	F	CAATTCCAGTTCCTGCTGAG
			R	CCTCCAACATGCTCTTGCGTG
		promoter cloning	F	TATAACTCGAGGACTACTAAACAAGGACCACTCAC
			R	ATATAGGATCCGAACTCCACTCTACTTTGAACTACCTG
SlVTE3(1)	Solyc09g065730	qPCR	F	CTTGACCAATCTCCTCATC
			R	GCACGCCTTTCCTCCAGG
SlVTE4	Solyc08g076360	qPCR	F	CAGATCATCGTGCTGCTCAG
			R	CCTCTCTGCTTGTACAGGAC
SlVTE5	Solyc03g071720	qPCR	F	CGTATCAGGACGGGCTCGC
			R	TCACCACCACACATCATTGCTAATG
SlVTE6	Solyc07g062180	qPCR	F	AGCACAAGCATCAGTGTCTG
			R	AAGAAAGCAGCCGCAATACC
SlGGDR	Solyc03g115980	qPCR	F	CAGAGACGCTCGCTAAGG
			R	GCTTCAGAGTCTGTCCGATATC
		qPCR (ChIP)	F	TGAAACCCAGCGACAAAGAA
			R	GCTCAAACCTATCCTTCCCG
		promoter cloning	F	TATAACTCGAGCCACTTTGAAACCCAGCGAC
			R	GCATAGGATCCGTTGTGTAGTGTGTGAGAACTG
SlACTIN4	Solyc04g011500	qPCR (ChIP)	F	CCTTCCACATGCCATTCTCC
			R	CCACGCTCGGTCAGGATCT
SlPSY1	Solyc03g031860	promoter cloning	F	AATAACTCGAGCACCCACTTTTCACCATCAC
			R	ATATAGGATCCTCTGAGCAAGAAAACCTTGGTTGG

Table S2. Tocopherol content in fruits from different genotypes ripened under distinct light conditions.

		Darkness		White light	
		MG	**BR+12**	**MG**	**BR+12**
α-tocopherol	**WT**	115.92 ± 16.64	**178.08 ± 8.29**	127.25 ± 14.3	**233.5 ± 3.63**
	aurea	93.27 ± 6.16	133.23 ± 20.61	75.57 ± 1.17	**115.73 ± 1.69**
	hp2	181.65 ± 23.31	218.64 ± 3.35	241.33 ± 29.28	**424.4 ± 38.11**
β-tocopherol	**WT**	2.83 ± 0.27	**3.92 ± 0.1**	2.71 ± 0.09	**5.69 ± 0.19**
	aurea	2.05 ± 0.1	2.36 ± 0.25	1.85 ± 0.01	**2.3 ± 0.03**
	hp2	4.27 ± 0.59	5.38 ± 0.31	6.14 ± 0.64	**16.2 ± 1.06**
γ-tocopherol	**WT**	6.16 ± 0.77	**11.65 ± 0.56**	8.52 ± 0.76	11.54 ± 1.12
	aurea	3.67 ± 0.1	4.53 ± 0.29	3.31 ± 0.24	3.64 ± 0.21
	hp2	6.67 ± 0.7	**11.82 ± 0.65**	10.03 ± 1.29	11.13 ± 2.05
Total-tocoherol	**WT**	124.91 ± 17.67	**194.56 ± 9.02**	138.48 ± 15.13	**250.73 ± 3.13**
	aurea	99.35 ± 6.3	140.12 ± 21.09	80.59 ± 1.3	**121.86 ± 1.71**
	hp2	192.59 ± 24.44	235.84 ± 3.91	257.49 ± 30.89	**458.73 ± 40.13**

Statistically significant differences between MG and BR+12 stages within the same genotype and light treatment are indicated in bold (t-test, $P < 0.05$). Values represent means from at least three biological replicates and are expressed in µg/g dry weight. δ-tocopherol was not detected.

Table S3. Transcript profile of VTE biosynthetic genes in response to light.

	Darkness					
	MG	**BR**	**BR+1**	**BR+3**	**BR+6**	**BR+12**
SlVTE1	1.02 ± 0.15	0.73 ± 0.06	0.76 ± 0.03	**0.45 ± 0.05**	0.58 ± 0.03	**0.40 ± 0.06**
SlVTE2	0.52 ± 0.01	**0.79 ± 0.04**	**0.8 ± 0.03**	**0.68 ± 0.03**	**0.67 ± 0.01**	0.57 ± 0.04
SlVTE3	0.8 ± 0.04	0.95 ± 0.05	**1.29 ± 0.06**	1.02 ± 0.08	**1.19 ± 0.1**	0.92 ± 0.05
SlVTE4	0.78 ± 0.06	0.68 ± 0.06	0.99 ± 0.12	0.76 ± 0.04	**0.33 ± 0.03**	**0.17 ± 0.01**
SlVTE5	0.83 ± 0.03	0.8 ± 0.03	0.76 ± 0.01	**0.51 ± 0.03**	**0.52 ± 0.01**	**0.61 ± 0.02**
SlVTE6	0.82 ± 0.01	1.23 ± 0.01	**1.87 ± 0.05**	**1.77 ± 0.18**	**1.34 ± 0.07**	0.85 ± 0.1
SlGGDR	1.17 ± 0.09	**0.12 ± 0.01**	**0.03 ± 0.0048**	**0.07 ± 0.01**	**0.05 ± 0.01**	**0.04 ± 0.0018**
	White Light					
	MG	**BR**	**BR+1**	**BR+3**	**BR+6**	**BR+12**
SlVTE1	1.00 ± 0.04	1.12 ± 0.05	0.99 ± 0.09	0.79 ± 0.11	0.92 ± 0.08	**0.64 ± 0.08**
SlVTE2	1.00 ± 0.02	0.85 ± 0.06	0.91 ± 0.04	**1.24 ± 0.07**	**1.11 ± 0.02**	0.84 ± 0.02
SlVTE3	1.00 ± 0.08	1.31 ± 0.11	1.11 ± 0.06	1.23 ± 0.12	1.05 ± 0.07	0.85 ± 0.07
SlVTE4	1.00 ± 0.06	1.15 ± 0.03	1.13 ± 0.06	**1.96 ± 0.10**	0.83 ± 0.05	**0.31 ± 0.02**
SlVTE5	1.00 ± 0.04	**1.19 ± 0.03**	1.1 ± 0.03	**0.77 ± 0.06**	**0.8 ± 0.05**	**0.53 ± 0.03**
SlVTE6	1.00 ± 0.08	0.9 ± 0.05	1.26 ± 0.12	**1.56 ± 0.16**	1.34 ± 0.04	0.72 ± 0.02
SlGGDR	1.00 ± 0.07	1.19 ± 0.05	**1.36 ± 0.07**	1.03 ± 0.11	0.93 ± 0.09	**0.66 ± 0.02**

Statistically significant differences against MG stage within the same light treatment are indicated in bold (t-test, $P < 0.05$). Values represent means from at least three biological replicates and are expressed as relative transcript levels.

Table S4. Tocopherol content in fruits from phy mutants ripened under light conditions.

	α-tocopherol	β-tocopherol	γ-tocopherol	Total-tocoherol
WT	97.83 ± 1.07	1.4 ± 0.06	2.88 ± 0.71	102.4 ± 2.02
phya	100.07 ± 2.19	1.63 ± 0.09	5.77 ± 1.17	107.7 ± 0.81
phyb1	**85.7 ± 1.52**	1.37 ± 0.09	**11.85 ± 1.49**	100.5 ± 0.84
phyb2	102.4 ± 0.37	**2.1 ± 0.19**	7.13 ± 0.63	111.33 ± 0.74
phyab1	**86.13 ± 0.95**	1.28 ± 0.19	5.7 ± 1.26	93.63 ± 1.84
phyb1b2	94.95 ± 4.37	0.8 ± 0.06	3.73 ± 0.33	100.13 ± 4.9
phyab1b2	**70.5 ± 0.72**	**0.03 ± 0.03**	3.1 ± 0.1	**73.63 ± 0.77**

Statistically significant differences between WT and mutants are indicated in bold (t-test, $P < 0.05$). Values represent means from at least three biological replicates and are expressed in µg/g dry weight. δ-tocopherol was not detected.

Table S5. Transcript profile of VTE biosynthetic genes from *phy* mutants ripened under light conditions.

	SlVTE2	***SlVTE5***	***SlVTE6***	***SlGGDR***
WT	1.25 ± 0.06	1.38 ± 0.02	1.09 ± 0.07	4.58 ± 0.21
phyab1b2	**1.00 ± 0.03**	**1.00 ± 0.03**	1.00 ± 0.01	**1.00 ± 0.15**

Statistically significant differences between mutants and WT control are indicated in bold (t-test, $P < 0.05$). Values represent means from at least three biological replicates and are expressed as relative transcript levels.

CAPÍTULO III

Impacto do silenciamento do gene *SlPIF4* sobre a produtividade e a qualidade nutricional

"I submit that all these remarkable findings make sense in the light of evolution; they are nonsense otherwise."

Theodosius Dobzhansky

This chapter is organized as a manuscript submitted to the scientific journal Plant Physiology:

Rosado D, Trench B, Bianchetti R, Zuccarelli R, Alves FRR, Purgatto E, FLoh EIS, Nogueira FTS, Freschi L, Rossi M. Downregulation of PHYTOCHROME-INTERACTING FACTOR 4 impacts plant development and fruit production.

ABSTRACT

Plant development is highly dependent on the ability to perceive and cope with environmental changes. In this context, PIF proteins are key players in the cellular hub controlling responses to light and temperature conditions. Reports in *Arabidopsis thaliana* and switchgrass show that manipulation of PIF4 levels affects important agronomical traits. In tomato, SlPIF1a and SlPIF3 regulate the nutraceutical composition of fruits. However, the potential of this protein family for the improvement of other traits has not been explored. Here we report the effects of constitutive silencing of tomato *SlPIF4* in whole plant physiology and development. Ripening anticipation and higher carotenoid levels observed in silenced fruits revealed a redundant role of SlPIF4 in the accumulation of nutraceutical compounds. Furthermore, silencing triggered a significant reduction in plant size, flowering, fruit yield and fruit size. This phenotype was most likely caused by reduced auxin levels and altered carbon partitioning. Impaired thermomorphogenesis and delayed leaf senescence were also observed in silenced plants, highlighting the functional conservation of PIF4 homologs in Angiosperms. Overall, this work contributes with new information that help to understand the role of PIF proteins – and light signaling – in metabolic and developmental processes that affect yield and composition of fleshy fruits.

KEYWORDS: *Solanum lycopersicum*, tomato, PHYTOCHROME INTERACTING FACTORS, ripening, flowering, senescence, yield, thermosensing.

INTRODUCTION

Light is one of the most critical ambient factors controlling plant development, providing energy for photosynthesis reactions and information about an environment in constant change (McDonald, 2003). The ability to sense and adapt growth rhythms and metabolism to light conditions is paramount for plant survival (Kami et al., 2010). Phytochromes (PHYs) are red/far-red light photoreceptors, activated by light and deactivated by dark and high temperature (Wang and Deng, 2004; Jung et al., 2016; Legris et al., 2016). Upon light exposure, PHYs are translocated into the nucleus, where they interact with PHY-INTERACTING FACTORS (PIFs) and induce the degradation of these transcription factors. PIFs, in turn, act downstream of PHYs, repressing photomorphogenic responses in the dark. This interaction module regulates many developmental and physiological responses such as deetiolation, growth, flowering and senescence (Castillon et al., 2007; Leivar and Monte, 2014; Pham et al., 2018).

In tomato, *Solanum lycopersicum*, PHY-mediated light perception and PIF-dependent light signal transduction have been described to regulate fruit development, nutritional quality and ripening time (Azari et al., 2010; Cruz et al., 2018; Gramegna et al., 2019). For example, mutations and fruit-specific silencing of *SlPHYA*, *SlPHYB1* and *SlPHYB2* alter carbohydrate metabolism, sink activity and carotenoid biosynthesis, ultimately affecting the nutritional composition of ripe fruits (Alba et al., 2000; Gupta et al., 2014; Bianchetti et al., 2018). In addition, the down-regulation of PHY-signaling repressors, such as CONSTITUTIVE PHOTOMORPHOGENESIS 1 (SlCOP1), DEETIOLATED 1 (SlDET1) and SlPIF1a, has the opposite effect on ripe fruit pigmentation (Davuluri et al., 2004; Liu et al., 2004; Enfissi et al., 2010; Llorente et al., 2016). In line with these observations, SlPIF3 has been recently shown to repress tocopherol biosynthesis in tomato (Gramegna et al., 2019).

The study of functional conservation among PIFs has the potential to bring new tools for plant breeding, considering that these proteins control traits of agronomical importance. For instance, natural variation of *Arabidopsis thaliana* AtPIF4 is associated with quantitative traits such as internode length, flowering time and fruit setting (Brock et al., 2010). Additionally, variation of *AtPIF4* gene expression is associated with heterosis. In this species, hybrid vigor correlates with increased expression of *AtPIF4*. It was proposed that this protein, at least in part, regulates hybrid vigor by inducing auxin biosynthesis and action, resulting in larger rosettes and increased biomass (Wang et al., 2017). Although manipulation of light signals bear a great

potential to influence fruit yield, so far, PIF studies in tomato have been limited to impacts on isoprenoid metabolism in fruits (Llorente et al., 2016; Gramegna et al., 2019).

Among the multiple PIF-encoding genes in tomato genome, *SlPIF1a*, *SlPIF1b*, *SlPIF3* and *SlPIF4* showed the highest expression level in seedlings, leaves and fruits (Rosado et al., 2016). Based on phylogenetic and transcriptional analyses, it has been proposed that SlPIF4 might have similar functions to *A. thaliana* orthologs AtPIF4 and AtPIF5 (Rosado et al., 2016). Therefore, it has the potential to regulate hypocotyl elongation, plant growth, flowering and leaf senescence in response to light and temperature (Kunihiro et al., 2011; Kumar et al., 2012; Sun et al., 2012; Sakuraba et al., 2014; Xie et al., 2017). Here we show that these functions are indeed shared, further strengthening the idea of the functional conservation of PIF4 clade within Angiosperm and; demonstrating that manipulation of *SlPIF4* levels has pleiotropic effects in tomato plant physiology, ultimately affecting yield and quality of the edible fruit.

RESULTS

Constitutive silencing of tomato *SlPIF4*

To investigate the role of PIF4 in tomato, we first addressed *SlPIF4* expression under regular cultivation conditions (Figure 1A). Highest mRNA levels were observed in leaves, while in fruits, *SlPIF4* expression dramatically decreased upon ripening, confirming previous observations in detached fruits (Rosado et al., 2016). Considering this broad expression profile and the well-described role of AtPIF4 in several distinct physiological processes, we decided to generate constitutively silenced lines for *SlPIF4* by RNAi-mediated knockdown. In order to avoid co-silencing of other *SlPIFs*, a fragment of 180 bp of the 3'-untranslated region of *SlPIF4* was used to expressed a hairpin loop mRNA (Figure 1B). Constitutive silencing with a reduction of at least 60% in transcript abundance in leaves and green fruits was confirmed by qPCR in three independent lines: 35S::SlPIF4-RNAi L6, 35S::SlPIF4-RNAi L17 and 35S::SlPIF4-RNAi L20, hereafter named L6, L17 and L20 (Figure 1C). No co-silencing of *SlPIF1a*, *SlPIF1b* or *SlPIF3* was observed, although punctual reductions in expression were detected (Figure 1C).

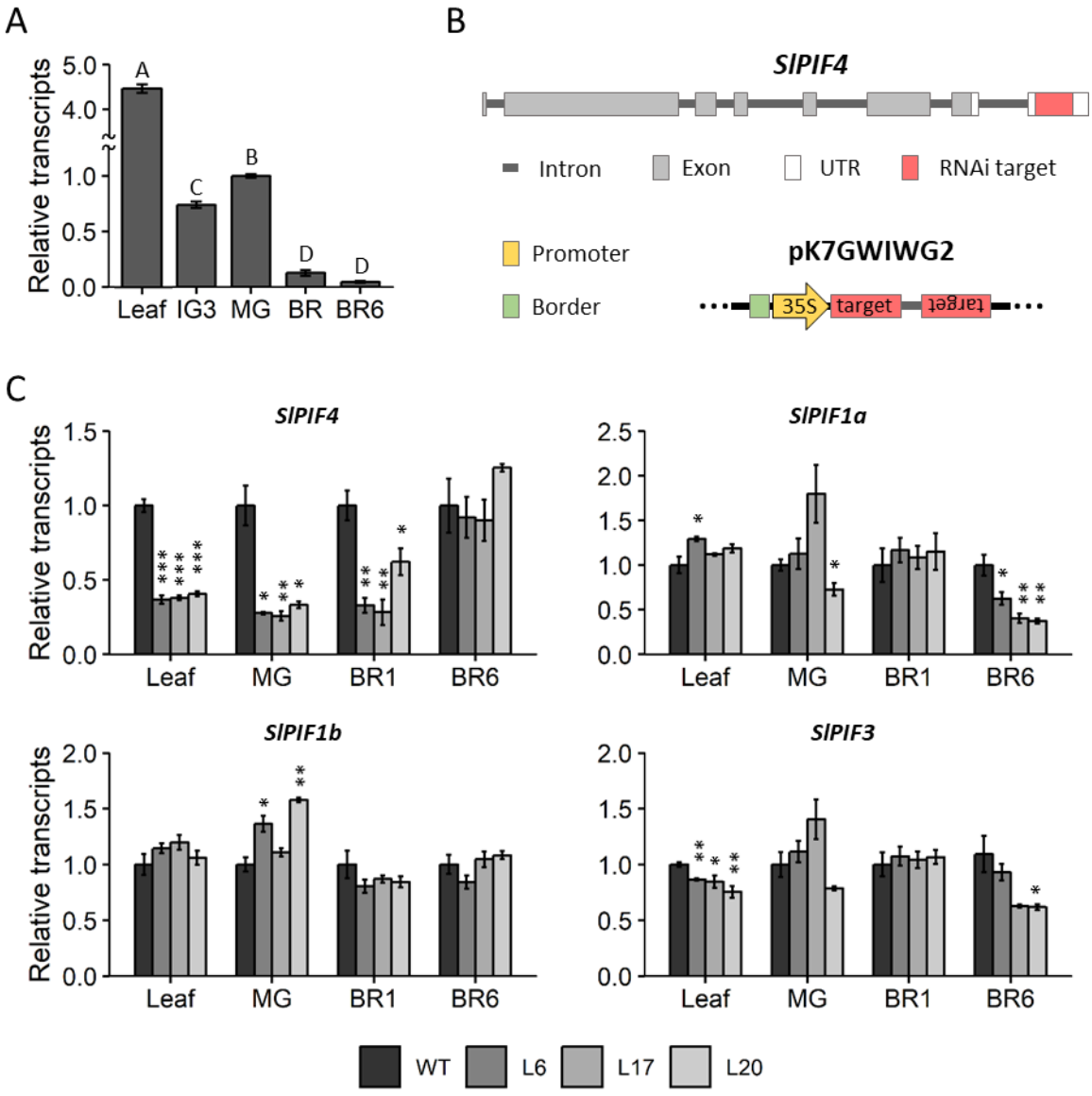

Figure 1. Expression profile of *SlPIF4* in wild type genotype and *SlPIF* genes in *SlPIF4*-silenced plants. A, Transcript profile of *SlPIF4* in wild type genotype. B, *SlPIF4* gene structure showing the RNAi target sequence on the 3' UTR in red. Construct used for silencing in pK7GWIWG2 vector. C, mRNA abundance of *SlPIF* genes in *SlPIF4*-silenced plants. Data shown are mean ± SE of at least three biological replicates (each composed of 4 fruits or 2 leaves) normalized against the MG stage (A) or the wild type control (C). Significant differences with wild type control are denoted by letters (ANOVA followed by Fisher's LSD test) and asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: IG3, immature-green; MG, mature-green; BR, breaker stage; BR1, one day after BR stage; BR6: 6 days after BR stage; WT, wild type; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20.

SlPIF4 regulates fruit ripening and quality

Two previous studies in tomato (Llorente et al., 2016; Gramegna et al., 2019) showed a role of SlPIF1a and SlPIF3 in inhibiting the accumulation of nutraceutical compounds during ripening, in particular carotenoids and tocopherols, respectively. To test whether this is a conserved function among tomato PIFs, we evaluated the levels of these isoprenoid-derived compounds, as well as total soluble solids (°BRIX) in ripe fruits (12 days after breaker, BR, *i.e.* fully red ripe fruits). Carotenoid levels were up to two-fold higher in two of the transgenic lines (L17 and L20) than in the wild type (WT) counterparts (Figure 2A). In contrast, no significant changes in tocopherol and °BRIX were detected between the transgenic and WT fruits (Figure 2B,C).

Interestingly, fruits not only accumulated more carotenoids, but also ripened faster than control considering the time from anthesis to BR stage (Figure 2D). We further confirmed this phenotype by analyzing colorimetric parameters of detached fruits throughout ripening (Supplemental Figure S1). In accordance with the observed advance, the color change was initially faster from BR to BR2 (2 days after BR) in fruits from L20 homozygous silenced plants. In accordance with their higher lycopene content, silenced ripe fruits showed stronger red color in comparison to the WT (Supplemental Table S1). Higher transcript abundance of the ripening master regulator *RIPENING INHIBITOR* (*SlRIN*) and the key genes involved in carotenoid biosynthesis, namely *GERANYL GERANYL DIPHOSPHATE SYNTHASE* (*SlGGPS2*) and *PHYTOENE SYNTHASE* (*SlPSY1*), detected in the transgenic lines explains, at least in part, these phenotypes and suggests a role of SlPIF4 in the regulation of fruit ripening and carotenogenesis (Figure 2E).

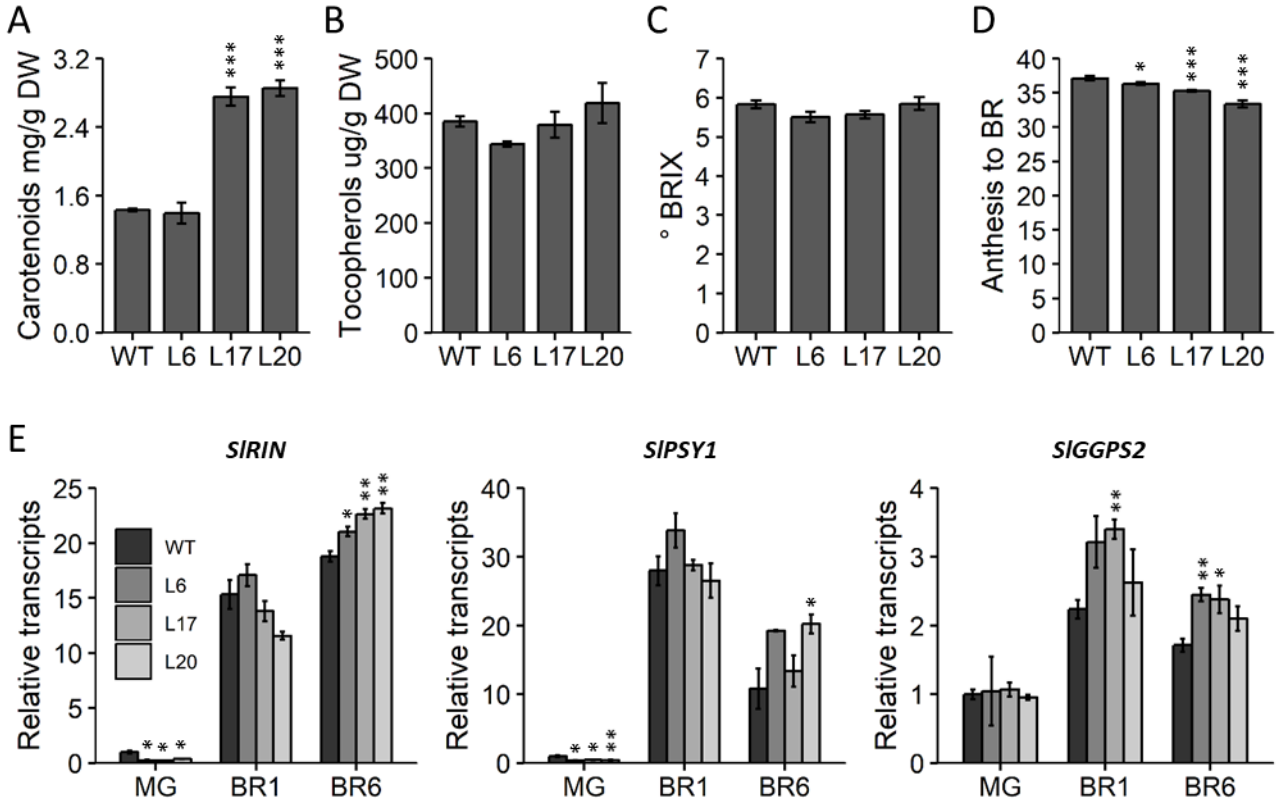

Figure 2. *SlPIF4* silencing affects tomato fruit quality. A, B, C, Carotenoid, tocopherol and ° BRIX in ripe fruits (12 days post breaker, BR, stage). D, Ripening time from anthesis to BR stage. E, mRNA abundance relative to wild type MG of differentially expressed genes involved in carotenogenesis. Values represent means ± SE of at least three biological replicates, each composed of at least 4 fruits (A B, E), 10 individual fruits (C) and 90 individual fruits (D). Significant differences with wild type control are denoted by asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: MG, mature green; BR1-12, 1-12 days after BR stage*; RIN*, *RIPENING INHIBITOR*; *PSY1*, *PHYTOENE SYNTHASE; GGPPS2*, *GERANYL GERANYL DIPHOSPHATE SYNTHASE*; WT, wild type; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20.

SlPIF4 silencing impacts flowering and fruit production

Flowering control by AtPIF4 has been extensively reported in *A. thaliana* (Brock et al., 2010; Kumar et al., 2012; Thines et al., 2014; Galvão et al., 2015; Seaton et al., 2015; Fernández et al., 2016). In this species, AtPIF4 induces the florigen *FLOWERING LOCUS T* (*AtFT*) directly

by binding to its promoter and indirectly by repressing *microRNA156* (*AtmiR156*) expression. Flowering in tomato is regulated similarly; *SlmiR156* represses the expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* and *15* (*SlSPB3* and *SlSBP15*) in both apex and leaf. In turn, these proteins induce the expression of *SINGLE FLOWER TRUSS* (*Sl*SFT), *AtFT* ortholog, in leaves and *FALSIFLORA* (*SlFA*) in shoot apices (Silva et al., 2019). SlSFT protein is translocated to the apex and together with FA induces flowering (Molinero-Rosales et al., 2004). However, the role of SlPIF4 in this regulatory network has not been addressed yet. Thus, we tested whether flowering was also affected by *SlPIF4* deficiency in tomato. In silenced lines, a significant reduction in flower number was observed, which reflected in reduced fruit production in 18-weeks old plants (Figure 3A-B). Interestingly, no changes in flowering time were observed between the studied genotypes, when either the number of leaves until the first truss or the number of days until the anthesis of the first flower per plant were scored (Supplemental Figure S2). In order to understand the molecular mechanism underneath this phenotype, the miR156-SPB-SFT/FA module that regulates flowering in tomato (Silva et al., 2019) was profiled in leaves and shoot apices harvested from of WT and L20 30-day-old young plants (Figure 3C-H). *SlPIF4* showed to be under-expressed in apex in comparison to leaves, and silencing was confirmed in both organs. Downregulation of *SlSFT* and *SlFA* florigens was observed in leaves and apices from SlPIF4-silenced plants, respectively. Moreover, the abundance of miR156 increased in the apex of transgenic plants, which negatively correlated with its targets *SlSBP3* and *SlSBP15* in the same organ. These data demonstrated that SlPIF4 regulates flowering in tomato reinforcing the hypothesis that PIF4 clade plays a conserved role in Angiosperms.

SlPIF4 silencing impacts vegetative growth and fruit size

To better understand at which extent constitutive silencing affected fruit yield, we compared different growth and production parameters in WT and L20 homozygous plants. At an early age (5-week-old), silenced plants were visually smaller than WT (Figure 4A). Differences in size were accentuated during the life cycle, and 18-week-old plants showed clear differences in size and fruit production (Figure 4B), displaying a reduction of 15% in vegetative and 23% in fruit weight was observed, accounting for a total reduction of 21% in plant aerial mass (Supplemental Table S2). Interestingly, fruit production was affected beyond number, as individual red fruits were smaller in mass and diameter compared to WT (Figure 4B and Supplemental Table S2). These

developmental differences could not be attributed to altered carbon assimilation rates, since no alterations in photosynthesis were detected (Supplemental Table S3). Instead, the observed phenotype is most likely caused by reduction of auxin levels (Figure 4C) and aggravated by the reduction in overall carbon assimilation due to lowered leaf area (Figure 4A) and number (Supplemental Table S2).

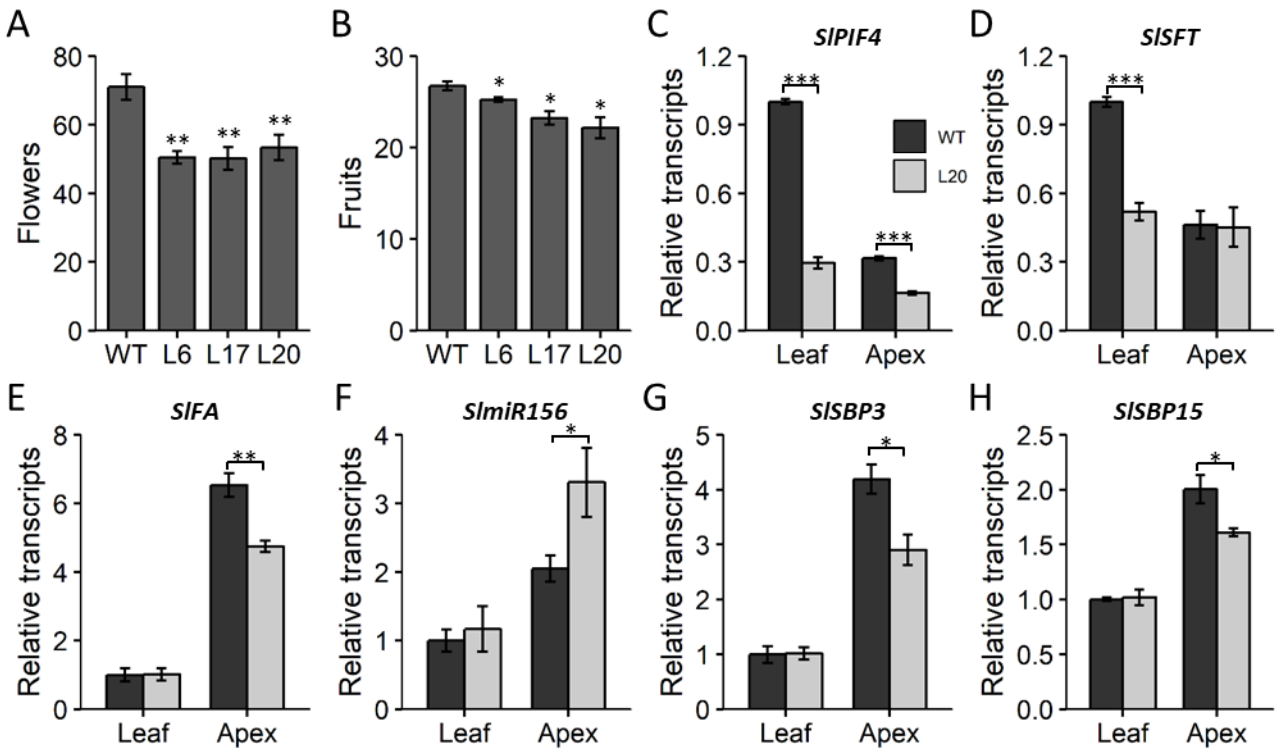

Figure 3. *SlPIF4* silencing affects plant development and fruit yield. A, B, Total flower and fruit number produced by T2 18-week-old plants. C-H, Transcript profile of flowering genes in 30-day-old T4 plants. Values represent means ± SE of at least 6 different plants (A,B) or 3 biological replicates, composed of 2 leaves or apices (C-H). Significant differences with WT control are denoted by asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: WT, wild type; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20; *PIF4*, *PHYTOCHROME INTERACTING FACTOR 4*; *SFT*, *SINGLE FLOWER TRUSS*; *FA*, *FALSIFLORA*; *SBP3* and *15*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* and *15*; *miR156*, *microRNA 156*.

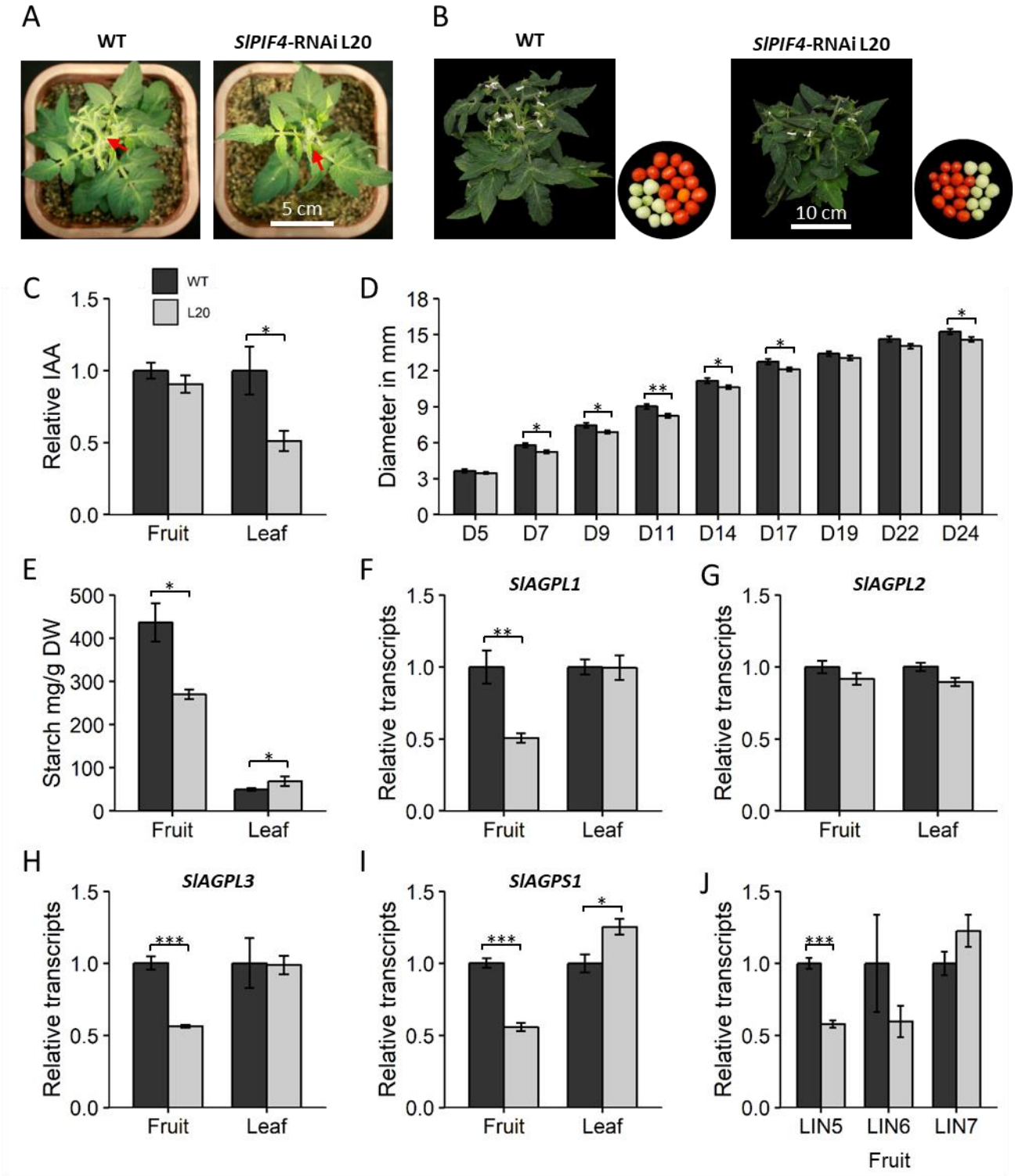
A
WT
SlPIF4-RNAi L20
5 cm
B
WT
SlPIF4-RNAi L20
10 cm
C
WT
L20
Relative IAA
Fruit
Leaf
D
Diameter in mm
D5
D7
D9
D11
D14
D17
D19
D22
D24
E
Starch mg/g DW
Fruit
Leaf
F
SlAGPL1
Relative transcripts
Fruit
Leaf
G
SlAGPL2
Relative transcripts
Fruit
Leaf
H
SlAGPL3
Relative transcripts
Fruit
Leaf
I
SlAGPS1
Relative transcripts
Fruit
Leaf
J
Relative transcripts
LIN5
LIN6
LIN7
Fruit

Figure 4. *SlPIF4* silencing affects growth and source-sink relationship. (A-B) Representative 5- (A) and 15- (B) week-old plants . A, Developmental delay in *SlPIF4*-silenced line; red arrow indicates the first inflorescence. B, Differences in size and fruit production. Relative auxin (C), starch (E) and transcript profile of starch biosynthetic and cell wall invertase genes (F-J) in immature green fruits and source leaves. D, Size differences between WT and silenced fruits of 5-24 days post anthesis (D5 to D24). Values represent means ± SE of at least 3 biological replicates composed of 2 leaves or 4 fruits (C,E-J), or 20 individual fruits (D). Significant differences with wild type control are denoted by asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: WT, wild type; L20, homozygous T4 35S::SlPIF4-RNAi L20; *AGPL1-3*, *ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1-3; AGPS1, ADP-GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1; LIN5-7, LYCOPERSICUM INVERTASE 5-7*

On the other hand, although differences in size appeared early in fruit development (Figure 4D), impaired fruit growth in *SlPIF4*-silenced plants could not be directly explained by the reduction of auxin levels, since no differences were detected in immature fruits (Figure 4C). Interestingly, carbohydrate profiling revealed a shift in sugar partitioning in the silenced lines. While no changes in soluble sugars were observed (Supplemental Table S4), starch was accumulated at higher levels in leaves and reduced in fruits from the L20 homozygous transgenic plants (Figure 4E). These observations were in accordance with the expression profile of ADP-GLUCOSE PYROPHOSPHORYLASE (AGPase) large and small subunit encoding genes (*SlAGPL1*, *SlAGPL2*, *SlAGPL3* and *SlAGPS1*), involved in starch biosynthesis, in both organs (Figure 4F-I). Additionally, expression of the flower- and fruit-specific invertase encoding gene *LYCOPERSICUM INVERTASE 5* (*SlLIN5*) (Fridman et al., 2004) was reduced in transgenic fruits (Figure 4J), which could be indicative of reduced sink strength caused by *SlPIF4* silencing. Thus, these further support the functional conservation of PIF4 in regulating plant growth and auxin biosynthesis but also illustrate a new role for SlPIF4 protein in fruit yield.

SlPIF4 participates in thermomorphogenesis

Beyond light, temperature is a key factor regulating plant growth and development (Kami et al., 2010; Quint et al., 2016), and many studies performed in *A. thaliana* placed AtPIF4 as an integrator of light and temperature responses (Franklin et al., 2011; Sun et al., 2012; Gangappa

and Kumar, 2017). To address whether tomato SlPIF4 also participates in temperature perception, hypocotyl elongation was analyzed in seedlings maintained for 3 days in either ambient (25 °C) or high temperature (30 °C) under day-neutral photoperiod. Only WT seedlings responded to the treatment and showed longer hypocotyls at 30 °C, while the hypocotyl length of *SlPIF4*-silenced seedlings remained unchanged (Figure 5A-B). Expression analysis revealed the up-regulation of *YUCCA FLAVIN MONOOXYGENASES*, *SlYUC8A* and *SlYUC8C*, in WT seedlings in high temperature compared to *SlPIF4* silenced ones (Figure 5C), suggesting that the observed high temperature-associated elongation is the consequence of auxin biosynthesis enhancement, demonstrating SlPIF4 involvement in temperature responsiveness.

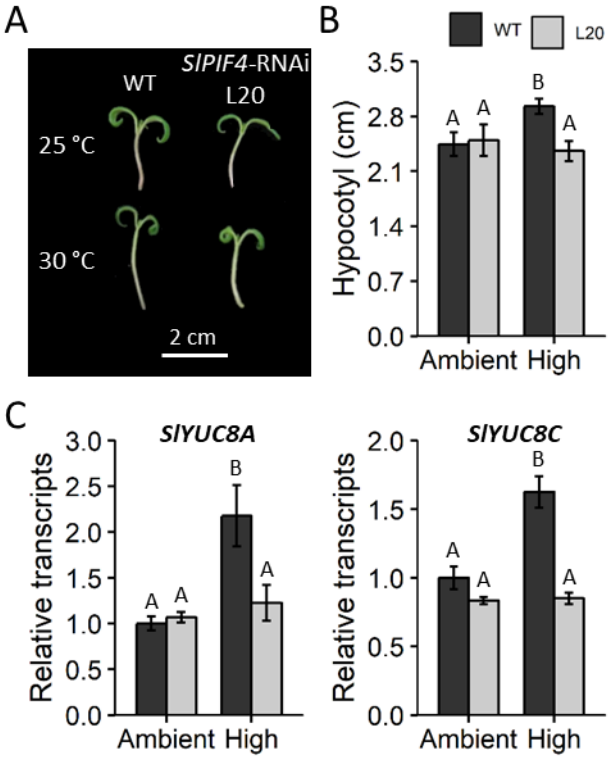

Figure 5. SlPIF4 participates in temperature response. A, Representative seedlings depicting differences in size in response to temperature. B Hypocotyl length. C, Relative transcript profile of auxin biosynthetic genes. Data shown are mean ± SE of at least 14 seedlings (B) or 3 (C) biological replicates, composed of 5 seedlings each. Significant differences with WT control are denoted by letters (ANOVA followed by Fisher's LSD test). Abbreviations denote the following: WT, wild type; L20, T4 homozygous 35S::SlPIF4-RNAi L20; *YUC8A-C, YUCCA FLAVIN MONOOXYGENASE 8A-C.*

Tomato PIF4 promotes age-induced leaf senescence

In *A. thaliana*, AtPIF4 and AtPIF5 promote both age and dark-induced senescence by activating *ORESARA 1* (*ORE1*) transcription factors, genes involved in chlorophyll breakdown, such as *STAYGREEN* (*SGR*), and repressing chloroplast maintainer *GOLDEN2-LIKE 1* (*GLK1*) (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). Although the same downstream effectors are involved in leaf senescence in tomato (Lira et al., 2017), the role of SlPIF4 in this signaling pathway has not been addressed so far. To investigate its involvement, leaves without any signs of senescence (non-senescent, NS), with initial yellowing (early-senescent, ES) and advanced yellowing (late-senescent, LS) were harvested from WT plants. Leaves from corresponding phytomeres from *SlPIF4*-silenced plants were also collected. Visually, silenced leaves remained greener than control (Figure 6A), suggesting that senescence was delayed in these plants. Lowered expression of senescence marker *SENESCENCE ASSOCIATED GENE 12* (*SlSAG12*) confirmed this hypothesis. Also, higher expression of chloroplast maintainer *SlGLK1*, as well as reduced levels of senescence-associated transcription factors *SlORE1S23* and *SlORE1S26*, as well as *SlSGR1* possibly contributed to the observed staygreen phenotype (Figure 6B). Thus, similarly to that described in *A. thaliana*, SlPIF4 participates of the senescence-inducing pathway.

DISCUSSION

Although *PIF* genes have been extensively studied in *A. thaliana* for over twenty years (Ni et al., 1998), only recently they have been identified in tomato (Rosado et al., 2016). The only *SlPIFs* studied so far were *SlPIF1a* and *SlPIF3*, which have been demonstrated to regulate fruit nutraceutical value (Llorente et al., 2016; Gramegna et al., 2019). Aiming at expanding our knowledge on the potential biotechnological use of PIF protein family in crop species; here we comprehensively characterized *S. lycopersicum PIF4*-silenced plants taking into account the role of PIF4 in flowering time and fruit setting in A. thaliana (Brock et al., 2010) and; more recently, in biomass production in switchgrass (Yan et al., 2018).

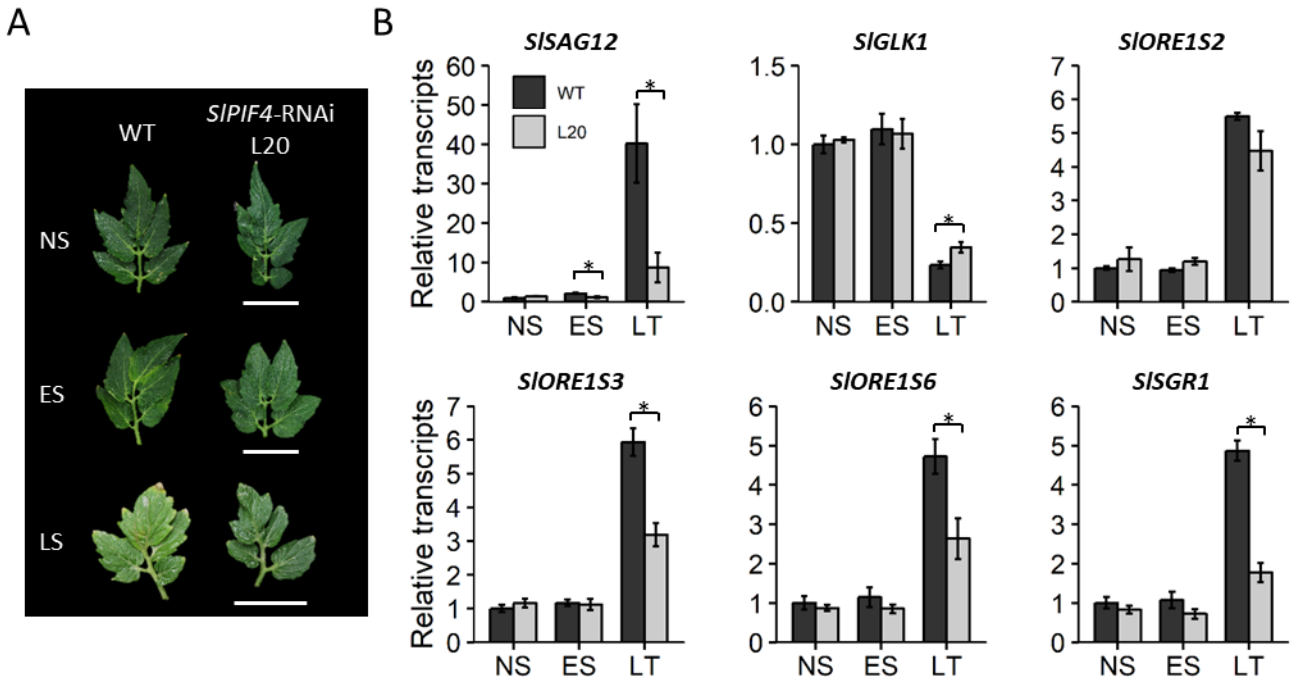

Figure 6. *SlPIF4* silencing delays age-induced leaf senescence. A, Representative non-senescent (NS, 6th phytomer), early-senescent (ES, 4th phytomer) and late-senescent (LS, 1st and 2nd phytomers) leaves from WT and *SlPIF4*-silenced 18-week-old plants. White bars represent 5 cm scales. B, Transcript profile of senescence related genes. Data shown are mean ± SE of at least 3 individual leaves. Significant differences with WT in two-tailed *t*-test : P<0.05 *. Abbreviations indicate the following: WT, wild type; L20, 35S::SlPIF4-RNAi L20; *SAG12, SENESCENCE ASSOCIATED GENE 12; GLK1, GOLDEN-2 LIKE 1; ORE1S02-6, ORESARA 1 LIKE 2-6; SGR1, STAY-GREEN 1*.

Generated *SlPIF4*-silenced plants showed downregulation of *SlPIF1a* and *SlPIF3* exclusively in red fruits and leaves, respectively (Figure 1C). However, this downregulation cannot be attributed directly to transgene expression since it was not observed in other stages. Additionally, since the fragment used for the silencing construct was on the 3'UTR (Figure 1B) and an off-target analysis was carefully performed, it is more likely that differential expression of *SlPIF1a* and *SlPIF3* was a side effect of *SlPIF4* silencing rather than co-silencing. This is in accordance with the regulatory network proposed for *A. thaliana*, in which PIFs regulate each other at the transcriptional level (Leivar and Monte, 2014).

Interestingly, although *SlPIF4* is poorly expressed in WT ripening fruits (Figure 1A), the silencing of this gene had a considerable effect on ripening process (Figure 2, Supplemental Figure S1). The late increase in expression of the master ripening regulator *SlRIN* observed in BR6 transgenic fruits cannot explain the ripening advance, but may contribute to carotenoid accumulation and induction of *SlPSY1* in ripe fruits (Fujisawa et al., 2011) (Figure 2). We, thus, propose that the faster ripening observed in *SlPIF4*-downregulated fruits is associated to temporal advancement of *SlRIN* expression, rather than the increase in absolute transcription of this gene, and uncover a novel function for tomato PIFs in regulating ripening time. The upregulation of *SlPSY1* and *SlGGPS2* expression, whose encoded enzymes act upstream in carotenogenesis, explains the accumulation of all carotenoid forms in ripe fruits (Supplemental Table S1). It is unlikely, though, that *SlPIF4* directly regulates carotenogenesis, because no differential carotenoid accumulation occurred in MG fruits, when *SlPIF4* is highly expressed (Supplemental Table S1, Figure 1A). Interestingly, expression of *SlPIF1a* and *SlPSY1/SlGGPS2* was inversely correlated in BR6 fruits (Figure 1C, Figure 2E). Considering the role of SlPIF1a as repressor of *SlPSY1* in tomato(Llorente et al., 2016), it is possible that the downregulation of *SlPIF1a* in BR6 *SlPIF4*-silenced fruits contributed to carotenoid accumulation (Figure 1C, Figure 2A and 2E). No changes in tocopherols were detected in *SlPIF4*-silenced fruits (Figure 2B), suggesting a unique role for SlPIF3. Nevertheless, the presented data demonstrate a functional convergence of tomato PIFs in regulating ripening and fruit nutritional quality (Llorente et al., 2016; Bianchetti et al., 2018; Gramegna et al., 2019).

Manipulation of flowering related traits is a key-strategy to improve fruit yield in tomato (Krieger et al., 2010). Indeed, *SlPIF4* silencing had an impact on fruit production derived from lowered flower number (Figure 3, Supplemental Table S2), which agreed with a conserved function of PIF4 Angiosperm homologs, as previously observed in *A. thaliana*, rice and maize (Kumar et al., 2012; Kudo et al., 2017; Shi et al., 2018). Although both, tomato and Arabidopsis PIF4, induce flowering via miR156-SPB-florigen (SlSFT or AtFT) module (Figure 3,(Xie et al., 2017), *SlPIF4* silencing only affected flower number (Figure 3), but not flowering time (Supplemental Figure S2), in opposition to findings in *A. thaliana pif4* mutant (Brock et al., 2010; Thines et al., 2014; Galvão et al., 2015). This result was expected since domesticated tomato is a day-neutral species (Soyk et al., 2017), while *A. thaliana* is a long day plant (Cho et al., 2017). Wild tomato species flower earlier under short days. Loss of photoperiod sensitivity in

domesticated varieties has been associated with mutations at *SELF PRUNING 5G* (*SP5G*) *locus*, an anti-florigen. Such mutations reduce the expression of this gene under long-day conditions, therefore attenuating the photoperiodic response (Cao et al., 2016; Soyk et al., 2017; Zhang et al., 2018). Interestingly, since SlPHYB1 regulates *SP5G* expression (Cao et al., 2018), SlPIF4 likely participates in flowering time regulation in wild species. Additionally, in *A. thaliana*, DELLA proteins, which are flowering repressors involved in gibberellin signaling, inhibit the activity of AtPIF4 (De Lucas et al., 2008; Xu et al., 2016). A recent work in tomato has shown that tomato DELLA homolog, PROCERA, actually induces flowering via the miR156-SPB-SFT module (Silva et al., 2019). In this sense, investigation of tomato PROCERA-PIF4 interaction could reveal new layers of species-specific regulation of flowering.

In silenced plants, lowered fruit number was accompanied by a reduction in ripe fruit size (Figure 2, Supplemental Table S2), revealing a critical function of SlPIF4 in determining tomato yield. This phenotype was attributed to both impaired vegetative growth and altered source-sink relationship (Figure 4). These observations are in accordance with a previous study in tomato, showing the importance of fruit-localized PHY for sugar partitioning and sink-strength (Bianchetti et al., 2018). Fruit-specific *SlPHYA* and *SlPHYB2* silencing causes over-accumulation of starch in immature fruits, which correlates with upregulation of genes involved in starch biosynthesis and cell-wall invertases, such as *SlLIN5*. Since silencing of *SlPIF4* had the opposite effect on starch synthesis and sink-strength (Figure 4), we propose that SlPHYA and SlPHYB2 regulate these processes via SlPIF4. A link between sugars and PIFs has been previously reported in *A. thaliana*. In this species, sugars induce the expression of *AtPIF4* and *AtPIF5*, coupling growth to carbon availability (Lilley et al., 2012; Sairanen et al., 2012). Here we show that SlPIF4 controls sugar partioning, regulating photoassimilate exportation from source leaves towards sink organs.

The results showed here for adult plants (Figure 4) and seedlings (Figure 5) suggest that SlPIF4 regulates growth by inducing auxin biosynthesis, which is in agreement with observations in *A. thaliana* (Nozue et al., 2007; Niwa et al., 2009; Kunihiro et al., 2011; Nieto et al., 2015), rice (Todaka et al., 2012) and maize (Shi et al., 2018) reporting PIF4 as growth regulator. Additionally, loss of temperature responsiveness in *SlPIF4*-silenced seedlings reveals another conserved feature for PIF4 clade in Angiosperms (Figure 5) (Koini et al., 2009). Recent works have placed PHYTOCHROME B (PHYB) as an integrator of light and temperature perception and PIF4 as a key protein in mediating the responses to both signals in *A. thaliana* (Jung et al., 2016; Legris et

al., 2016). In this context, future studies investigating interactions between SlPIF4, SlPHYB1 and SlPHYB2 will add invaluable information to understanding photo- and thermomorphogenesis in tomato.

Finally, *SlPIF4*-silenced plants displayed a delay in age-induced leaf senescence (Figure 6) explained by the downregulation of *SlORE1* transcription factor encoding genes. These senescence-associated proteins negatively regulate chloroplast maintainer SlGLK1 and upregulate the expression of chlorophyll degradation enzymes, such as *SlSGR* (Lira et al., 2017); thus, resulting in the staygreen phenotype observed in the old transgenic leaves (Figure 6). It has been demonstrated that, in *A. thaliana*, AtPIF4 regulates *SGR*, *ORE* and *GLK* by directly binding to specific motifs in their promoter regions (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). Moreover, the overexpression of maize ZmPIF4 and ZmPIF5 accelerates leaf senescence in *A. thaliana* (Shi et al., 2018). Then, these data strongly support the previously suggested functional conservation of PIF4 among Angiosperms (Rosado et al., 2016).

Taken together, our results present new and conserved roles for tomato PIF4, as previously suggested by our group (Rosado et al., 2016), highlighting the importance of evolutionary studies to uncover regulatory mechanisms in plant physiology. Overall, the pleiotropic effects observed in *SlPIF4*-silenced plants not only highlight the importance of PIFs for plant development, but also suggest that manipulation of light signaling is an efficient strategy to improve tomato yield and quality.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Sampling

SlPIF4 silenced lines were generated by constitutively expressing an intron-spliced hairpin RNA construct containing a 180 bp fragment of the 3'UTR region of *SlPIF4 locus* (Solyc07g043580). To avoid off-target effects, the construct was designed to have minimal complementarity with other genes, especially other *SlPIFs*, and then the sense/antisense fragment was used as query for a BLAST search against the Sol Genomics Network database (www.solgenomics.net). The fragment was amplified from cDNA with primers listed in Supplemental Table S5, cloned into pK7GWIWG2(I) (Karimi et al., 2002) and introduced into tomato (*Solanum lycopersicum* L.) cv. Micro-Tom (MT) harboring wild-type *SlGLK2* allele

(Carvalho et al., 2011) via *Agrobacterium*-mediated transformation according to Pino et al. (2010), with modifications described in Bianchetti et al. (2018). The presence of the transgene was confirmed by PCR using the primers 35S forward and RNAi-specific reverse (Supplemental Table S5). After silencing verification by RT-qPCR, three transgenic lines with a reduction of approximately 60% in *SlPIF4* mRNA level were selected for further analyses: 35S::SlPIF4-RNAi L6, L17 and L20. Two different generations of silenced plants were used in this work: L6, L17 and L20 segregating lines in T2 and; L20 homozygous line in T4.

Plants were grown in 6L pots containing 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, Brazil) and vermiculite, supplemented with 1 g L^{-1} of NPK 10:10:10, 4 g L^{-1} of dolomite limestone and 2 g L^{-1} Yoorin Master® (Yoorin Fertilizantes, Brazil). Cultivation was carried in growth chamber with controlled light and temperature conditions (250 µmol m^{-2} s^{-1}, 12h/12h photoperiod, 25±2 °C) and manual irrigation. For senescence analysis, T4 L20 plants were grown in greenhouse (25±2 °C) with natural light conditions. Two T2 experiments were set: one for non-destructive total flower and fruit number, and another one for fruit harvesting. Third leaves completely expanded from 90-day-old plants were collected. Fruit pericarp were sampled at mature green (MG), breaker 1 (BR1, 1 day after breaker), 6 days after breaker (BR6) and 12 days after BR (BR12). All further experiments were performed with T4 homozygous L20 plants. For colorimetric parameter measurement, fruits at MG stage were harvested and kept into a 0.5 L sealed transparent vessel and continuously flushed with ethylene-free humidified air (approximately 1 L min-1) at 12h/12h photoperiod conditions, 25±2 °C and air relative humidity at 80 ± 5%. Colorimetric parameters were scored at MG, BR, BR1, BR2, BR3, BR6 and BR12. For flowering experiments, the third leaf and shoot apex of 30-day-old plants were harvested. For growth and source-sink relationship analyses, the sixth leaves and immature green fruits from 12-week-old plants were collected. Yield was scored in 15-week-old plants. Ripe tomato size parameters were determined using Tomato Analyzer software (Rodríguez et al., 2010). Immature fruit diameter was measured with digital calipers. Hypocotyl lengths were obtained from images analyzed in ImageJ software (https://imagej.nih.gov/ij/). Temperature experiments were performed *in vitro*. For that, seeds were sown in MS growth media (Murashige and Skoog, 1962) and kept in the dark for 5 days at 25±2 °C, seedlings were subsequently transferred to 12h/12h photoperiod conditions, under either 25±2 °C or 30±2 °C for 3 days. All samples were harvested

around 4-6 h after light turned on, immediately frozen in liquid nitrogen and stored at –80 °C until use.

Quantitative RT-qPCR

RNA extraction, cDNA synthesis and qPCR were performed as described by Quadrana et al. (2013) following MIQE guidelines (Bustin et al., 2009). Stem loop pulse reverse transcription was performed as described previously by Varkonyi-Gasic et al. (2007). qPCR was carried out in QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) using 2X Power SYBR Green Master Mix (Life Technologies) in a 10 µL final volume. Quantitation cycle values and PCR efficiencies were obtained from absolute fluorescence data analyzed in LinRegPCR software package (Ruijter et al., 2009). Expression values were normalized with TIP41 and EXPRESSED reference genes (Expósito-Rodríguez et al., 2008). All primers and accession numbers can be found in Supplemental Table S5.

Fruit color, carotenoid, tocopherol and BRIX determination

Fruit color and intensity (Hue angle and Chroma) were determined using a Konica Minolta CR-400 colorimeter as described in Su et al. (2015).Carotenoid extraction was carried out as described in Bianchetti et al. (2018) with modifications. Briefly, 20 mg of freeze-dried fruit pericarps were homogenized sequentially with 100 µl of saturated NaCl, 200 µl of dichloromethane, and 1 ml of hexane:diethyl ether (1:1, v/v). Supernantant was collected after centrifugation and pellets were re-extracted additional 3 times with 500 µl hexane:diethyl ether mixture. Supernatant fractions were combined, vacuum-dried, suspended in 200 µl of acetonitrile and filtered through 0.45 µm membrane. Tocopherol extraction was perfomed as described by Lira et al. (2016).Briefly, 25 mg of freeze-dried fruit pericarps were homogenized sequentially in 1.5 mL methanol, 1.5 mL chloroform and 2.5 mL TrisNaCl (Tris 50 mM pH 7.5, NaCl 1M) solution. Following centrifugation, organic fraction was collected and samples were re-extracted in 2 mL chloroform. Fractions were combined, 3.mL of it were vacuum dried, suspended in 200 µl of hexane: tert-butyl methyl ether (90:10, v/v) and filtered through 0.45 µm membrane. Carotenoids and tocopherol levels were determined by high-performance liquid chromatography (HPLC) in an Agilent 1100 as described in Lira et al. (2017). Total soluble sugars measured as ° BRIX were

determined in ripe (BR12) fruits as follows. Fresh pericarp tissue was homogenized with metallic beads and briefly span. ° BRIX of resulting juice was measured in a portable digital refractometer NR151 (J.P. Selecta).

Hormone analysis

3-Indoleacetic acid (IAA) was extracted and quantified as in Silveira et al. (2004). Briefly, 1 g of powdered tissue was homogenized in a buffer containing 80% ethanol, 1% polyvinylpyrrolidone-40 and [^{3}H]IAA, used as an internal standard. Samples were incubated and subsequently centrifuged. The supernant was collected and concentrated in speedvac. Volume was adjusted to 3 mL with water, and the pH adjusted to 2.5. The organic fraction, obtained following double extraction with ethyl ether, was completely vacuum-dried, redissolved in 150 µL methanol and filtered through 0.45 µm membrane. Auxin levels were determined by high-performance liquid chromatography (HPLC) in 5 lm C18 column (Shimadzu Shin-pack CLC ODS), with a fluorescence detector (excitation at 280 nm, emission at 350 nm). Fractions containing IAA were collected and analyzed in the scintillation counter (Packard Tri-Carb) to estimate losses during the procedure.

Leaf Gas-Exchange and Fluorescence Measurements

Gas-exchange and chlorophyll fluorescence parameters were measured in the third leaf completely expanded from 90-day-old plants, as described in Lira et al. (2017), using a portable open gas-exchange system (LI-6400XT system; LI-COR) equipped with an integrated modulated chlorophyll fluorometer (LI-6400-40; LI-COR). Photosynthesis parameters were calculated as in Maxwell and Johnson (2000).

Starch and soluble sugar quantification

Starch and soluble sugars were extracted and determined as described in Bianchetti et al. (2017) and Bianchetti et al. (2018), respectively.

Statistical analyses

Statistical analyses were performed using the Rstudio (https://www.rstudio.com/) and Infostat software (Di Rienzo, 2009). Appropriate test and number of biological replicates used in each experiment are indicated in figure and table descriptions.

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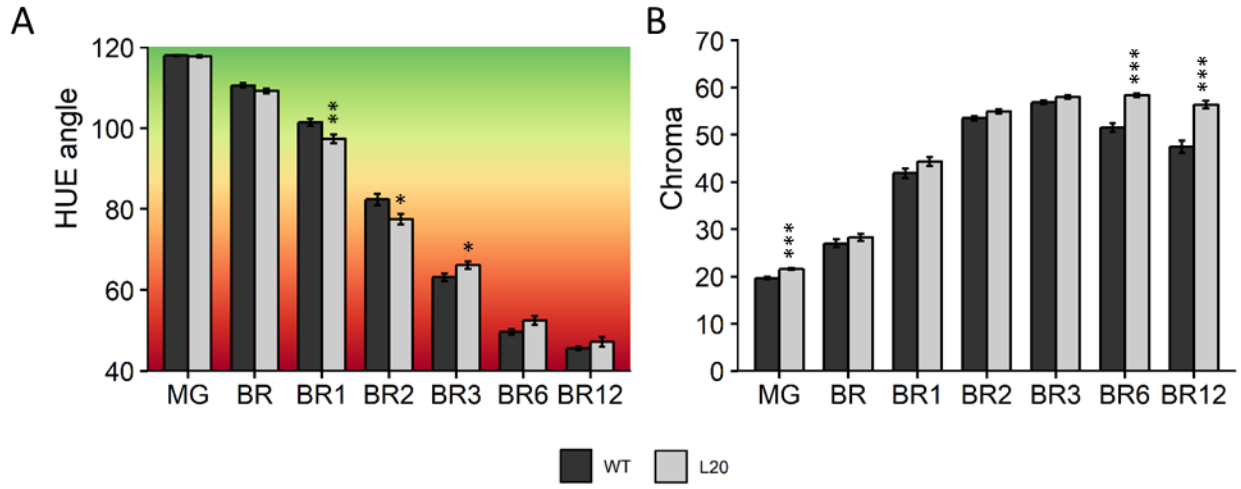
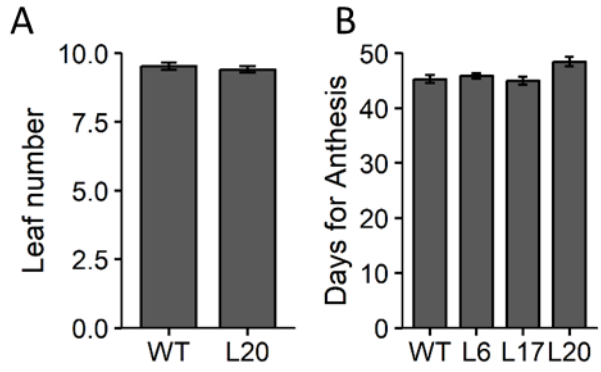
SUPPLEMENTAL DATA



Supplemental Figure S1. Off-vine ripening is affected in *SlPIF4*-silenced fruits. Fruit color estimated by Hue angle (A) and Chroma (B). Values represent means ± SE of at least 20 individual fruits. Significant differences with WT control are denoted by asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: MG, mature-green; BR, breaker stage; BR1-12, 1-12 days after BR; WT, wild type; L20, 35S::SlPIF4-RNAi L20.



Supplemental Figure S2. *SlPIF4* silencing doesn't affect flowering time. A, number of leaves before the first flower truss in T4 plants. B, Days post sowing until opening of the first flower. In T2 plants Means represent values of at least 18 plants (A) and 9 plants (B). Abbreviations indicate the following: WT, wild type; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20.

Supplemental Table S1. Carotenoid content in fruits.

	Mature green			
	WT	PIF4-RNAi_L6	PIF4-RNAi_L17	PIF4-RNAi_L20
Lycopene	nd	nd	nd	nd
Lutein	39.12 ± 2.58	35.61 ± 1.74	38.1 ± 1.35	36.54 ± 1.71
β-carotene	22.42 ± 0.88	22.43 ± 1.13	23.66 ± 0.1	21.6 ± 0.48
Phytoene	nd	nd	nd	nd
Phytofluene	nd	nd	nd	nd
Total carotenoids	64.25 ± 1.66	56.43 ± 0.8	60.89 ± 0.98	59.53 ± 1.33

	Ripe			
	WT	L6	L17	L20
Lycopene	1172.78 ± 18.34	1142.35 ± 78.57	**2196.86 ± 31.58**	**2438.06 ± 81.41**
Lutein	17.77 ± 1.17	16.83 ± 1.08	**30.49 ± 2.44**	**22.29 ± 0.55**
β-carotene	19.97 ± 0.41	19.46 ± 0.83	**26.65 ± 0.69**	**24.72 ± 0.24**
Phytoene	77.78 ± 4.37	71.32 ± 6.32	**126.55 ± 5.71**	**136.37 ± 2.72**
Phytofluene	38.88 ± 2.71	39.08 ± 3.36	**65.64 ± 2.97**	**75.85 ± 1.98**
Total carotenoids	1431.29 ± 13.18	1393.69 ± 100.94	**2754.44 ± 81.97**	**2855.5 ± 79.56**

Carotenoid content (ug per g of dry weight) in mature green and ripe fruits (12 days post breaker stage). Values represent means ± SE of at least three biological replicates, each composed of at least 4 fruits. Significant differences with wild type control are denoted in bold (two-tailed t-test; $P<0.05$). Abbreviations indicate the following: WT, wildtype; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20 .

Supplemental Table S2. Yield parameters in wild type and *SlPIF4*-silenced plants.

	WT	L20
Total aerial mass (g)	77.03 ± 2.32	**60.73 ± 0.49**
Vegetative mass (g)	19.51 ± 0.72	**16.58 ± 0.14**
Total fruit mass (g)	57.46 ± 1.68	**44.36 ± 0.45**
Ripe fruit mass (g)	37.19 ± 1.44	**30.36 ± 0.62**
Dry vegetative mass (g)	2.62 ± 0.08	**2.18 ± 0.04**
Water content (vegetative)	87%	86%
Dry fruit mass (g)	3.89 ± 0.17	**3.31 ± 0.03**
Fruit water content	85%	86%
Harvest index	0.75	**0.73**
Flower number	30.43 ± 0.83	**25.71 ± 0.74**
Fruit number	21.5 ± 0.71	**18.67 ± 0.94**
Green fruits	7.63 ± 0.66	6.13 ± 0.4
Red fruits	12 ± 0.44	11.78 ± 0.53
Total leaf number	17 ± 0.62	**14.13 ± 0.67**
Individual red fruit mass (g)	2.94±0.06	**2.64±0.07**
Individual red fruit slice area (mm^2)	249.76±4.03	**224.22±6.14**
Individual red fruit maximum width (mm)	17.73±0.16	**16.93±0.26**
Individual red fruit slice pericarp area (mm^2)	112.06±1.79	**100.71±2.72**
Individual red fruit pericarp thickness (mm)	2.06±0.02	**1.95±0.03**

Growth and yield parameters in 15-week old plants. Significant differences with wild type control are denoted in bold (two-tailed t-test; $P<0.05$). Abbreviations indicate the following: WT, wildtype; 35S::SlPIF4-RNAi L20 .

Supplemental Table S3. Photosynthesis parameters.

	Photosynthetic carbon assimilation [1]	Stomatal conductance[2]	Electron transport rate[3]	Leaf transpiration4
WT	11.71±0.24	0.34±0.01	112.23±3.96	4.06±0.04
L6	10.86±0.73	0.34±0.04	105.33±2.79	3.41±0.37
L17	12.2±0.91	0.43±0.07	107.7±1.5	3.99±0.41
L20	13.81±0.77	0.46±0.05	111.65±1.47	4.65±0.43

Photosynthetic parameters determined in source leaves of 2 month-old plants. Values represent means ± SE of at least five leaves from different plants. Significant differences with wild type control are denoted in bold (two-tailed t-test; $P<0.05$). Abbreviations indicate the following: WT, wildtype; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20; PSII, photosystem II. 1, µmol CO_2 m^{-2} s^{-1}. 2, µmol H_2O m^{-2} s^{-1}. 3, µmol m^{-2} s^{-1}. 4, mmol H_2O m^{-2} s^{-1}.

Supplemental Table S4. Soluble sugars.

	Immature fruit		Leaf	
	WT	L20	WT	L20
Glucose	9.06±0.47	8.01±0.25	1.85±0.2	1.96±0.26
Frutose	9.36±0.39	9.85±0.26	4.15±0.05	4.24±0.36
Sucrose	2.74±0.22	2.24±0.1	3.8±0.17	4.9±0.33

Soluble sugars (ug per g of fresh weight) in immature green fruits and source leaves. Values represent means ± SE of at least three biological replicates, each composed of at least 4 fruits or 2 leaves from different plants. Significant differences with wild type control are denoted in bold (two-tailed t-test; $P<0.05$). Abbreviations indicate the following: WT, wildtype; L20, 35S::SlPIF4-RNAi L20.

Supplemental Table S5. Primers used in this work.

Gene/Amplicon	Locus	Sequences F/R
SlPIF4_RNAi	Solyc07g043580	CACCGTACTAGACTCTAGCAATCACTACTGG / CATATGCCAACTACTAATGTGCTCC
35S	-	CTATCCTTCGCAAGACCC / -
SlExpressed	Solyc07g025390	GCTAAGAACGCTGGACCTAATG / TGGGTGTGCCTTTCTGAATG
SlTIP41	Solyc10g049850	ATGGAGTTTTTGAGTCTTCTGC / GCTGCGTTTCTGGCTTAGG
SlPIF1a	Solyc09g063010	AACTTCTTGCTTTGCTCTCTG / GCTCCGCCCATAAATCA
SlPIF1b	Solyc06g008030	TAGTATGGCAAAATGGTGGAG / CGGCGTCACAACTCGGTG
SlPIF3	Solyc01g102300	AAGGCTTCCCAATAATGC / CCATCAGACCAAACTTCCC
SlPIF4	Solyc07g043580	GGCTTAGGTTCACATACAG / TGATGGTGTCGTTGTCTC
SlRIN	Solyc05g012020	TCAAACATCATGGCATTGTGGTG / TGCATTTTCGGGTTGTACATTATCG
SlPSY1	Solyc03g031860	CGATGGTGCTTTGTCCGATAC / CTCATCAACCCAACCGTACC
SlGGPS2	Solyc04g079960	GTTGATTCATGGGGTCAAGC / CAAATCGCCTTTTCAGCTACG
SlSFT	Solyc03g063100	GTTGTTGGTCGTGTGGTAGG / ACTTCAACCCTTGGCTGGTT
SlFA	Solyc03g118160	AGGGGAAGAGGATGAGGAAA / GATGCTCCCTTTGTCTCTCG
miR156 / reverse universal	MIMAT0000167	CCTGAGTGACAGAAGAGAGTG / GTGCAGGGTCCGAGG
SlSBP3	Solyc10g009080	CAAGTTGAACGGGCACCTAC / TGGCAAATGACAGAAGAGAGAG
SlSBP15	Solyc10g078700	GGTTCAGCTACCAGGACCAG / TGTGAACTTGGCTGTTGACC
SlAGPL1	Solyc01g109790	AGCAGACTACTACCAAACAG / ATTCCAATCGGTACTTTCC
SlAGPL2	Solyc07g019440	CTGAAATTATCCCTTCTGCTG / ACTTCACTGTTCCAATATCCTC
SlAGPL3	Solyc01g079790	CGCGCTACTTCGTAATAACC / CCATCAATTCTCCATTGCA
SlAGPS1	Solyc07g056140	TGTAAGATTCACCATTCCGT / TCTTCTATAATTGCTCCCTCTG
SlLIN5	Solyc09g010080	TTGGAAGGGATTGAGAATCG / AATTCCAGCCCATCCTTTCT
SlLIN6	Solyc10g083290	AACCCGCTATCTACCCGTCT / GGGCTTGATCCACTTACGAA
SlLIN7	Solyc09g010090	TCTTGACTTTGGCTGGGTTC / TTCACGACGCACTGAGTTTC
SlYUC8A	Solyc06g008050	ACATCTTCCACCCTCTCTTTACT / TGAAAGCAGAACACGGGC
SlYUC8C	Solyc09g064160	TTGCTACTGGGGAGAATGCC / ACCAACGACCACCACTTTCT
SlSAG12	Solyc02g076910	ATGTCCTCCTCAAAGCCAAA / TTTCAGTTGGTGTAGCCCTT
SlGLK1	Solyc07g053630	GCTGTAGAGCAACTAGGTGTAGATAAGG / CAACTCGCTGCCTCCACTTC
SlORE1S2	Solyc02g088180	ACAACAGCGAGAAGTAGTGG / GCATCAATCCAGAATCTCCATAC
SlORE1S3	Solyc03g115850	ACATTTCAGGGCTTGTGAGA / AGGTGAATTGTTGAAGGAATTGAT
SlORE1S6	Solyc06g069710	GATTCTGCTACTGCTACTGCTT / GGATCTTGAACCCCAAATGAAG
SlSGR1	Solyc08g080090	GCAAAGAACTCCCTGTGGTT / CCCACCAGAAGAAGATGAGG

CONSIDERAÇÕES FINAIS

"A story must be told or there'll be no story, yet it is the untold stories that are most moving."

J. R. R. Tolkien

Considerando-se a importância da luz para o desenvolvimento vegetal, a manipulação da percepção e sinalização luminosa é uma estratégia eficiente para a engenharia de diversos caracteres simultaneamente. No entanto, a falta de conhecimento sobre os fatores de transcrição PIF, componentes centrais da rede regulatória controlada por luz, limita a aplicação dessa estratégia em tomateiro. Neste contexto, este trabalho teve por objetivo a caracterização dos genes *PIF* em *Solanum lycopersicum*. Para isto, foram estabelecidos três objetivos específicos.

O primeiro visou a identificação dos genes *SlPIF*. A partir de uma extensa análise filogenética, foram descobertos seis genes em tomateiro e foi estabelecida a relação de ortologia com os genes correspondentes da espécie modelo *A. thaliana*. Ainda, por meio da datação dos eventos de divergência entre genes parálogos, foi possível compreender a importância dos eventos de duplicação genômica ao longo da história evolutiva de Viridiplantae na diversificação dessa família gênica em Solanaceae. Diferentes pressões evolutivas atuaram sob os genes duplicados, culminando na sub- e neofuncionalização de diversos deles, atestada pelo perfil transcricional dos *SlPIF* sob diferentes condições luminosas e contextos fisiológicos. Por fim, foi possível propor a forte conservação funcional dentro do clado PIF4, comparando-se o comportamento dos transcritos de *SlPIF4* ao dos seus ortólogos *AtPIF4* e *AtPIF5* sob semelhantes condições. Estes conhecimentos foram aplicados nos capítulos seguintes.

O segundo objetivo teve por fim investigar o papel de SlPIFs na determinação da qualidade nutricional em tomateiro, especificamente na regulação da síntese de VTE. Para isto, primeiramente foi necessário comprovar a influência da luz no metabolismo de VTE. Em frutos destacados, a luz induz o acúmulo de VTE por meio da ativação de genes dessa rota biossintética, de maneira PHY-dependente. Em seguida, viu-se que esta regulação PHY-dependente é mediada pela proteína SlPIF3, que se liga diretamente ao promotor do principal gene que regula o acumulo de VTE, *GGDR*, inibindo-o. Desta forma, descobriu-se mais uma rota plastidial regulada por luz e o primeiro fator de transcrição a regular diretamente a biossíntese de VTE.

O terceiro objetivo propôs a caracterização de linhagens silenciadas constitutivamente para o gene *SlPIF4*, escolhido como alvo dada a sugerida semelhança funcional aos ortólogos em *A. thaliana*. Nestas plantas, foram observados efeitos pleiotrópicos durante o desenvolvimento vegetativo, floração, frutificação e desenvolvimento dos frutos. O silenciamento teve como principais consequências a diminuição da produtividade, causada pelo crescimento reduzido,

alterado particionamento de carbono e reduzida produção de flores; bem como o aumento do conteúdo de carotenoides nos frutos. Estes resultados comprovam concretamente a conservação funcional do clado PIF4, comparando-se os efeitos sobre a produtividade em diferentes espécies (*e.g. A. thaliana*, *O. sativa*, *Z. mays*). Sugerem, ainda, que as diferentes SlPIFs até o momento estudadas (SlPIF1a, SlPIF3 e SlPIF4) convergem na determinação do conteúdo nutricional de tomateiro.

Concluindo, este trabalho trouxe contribuições valiosas à área, conectando-se a estudos anteriores do grupo sobre determinação do conteúdo de VTE, regulação da senescência foliar e papel dos fitocromos na fisiologia dos frutos. Porém, deixa diversas questões abertas e sugere novos caminhos a serem explorados. Por exemplo, não se sabe se diversificação transcricional dos genes parálogos *SlPIF1a* e *SlPIF1b* acarreta em diferenças funcionais significativas entre as proteínas por eles codificadas; e também se a manipulação dos níveis de SlPIF3 afeta a produção de VTE sob condições normais de cultivo. Tampouco foi possível encontrar uma explicação robusta para os efeitos do silenciamento de *SlPIF4* sobre o tempo de amadurecimento e o aumento do conteúdo de carotenoides em frutos, sugerindo a possível participação de algum fator ainda desconhecido. Por fim, a observada perda de responsividade à temperatura em plantas silenciadas para *SlPIF4*, abre portas para o estudo aprofundado do efeito desse fator ambiental na fisiologia do tomateiro. Trabalhos atuais do grupo mostram um envolvimento dos PHYs na percepção de temperatura em folhas e frutos. Neste contexto, o estudo das *SlPIF4*-silenciadas sob diferentes temperaturas poderia iluminar os mecanismos regulatórios da termo-responsividade em tomateiro e auxiliar a obtenção de cultivares resistentes a condições ambientais extremas.