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Dedicatória

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“Na vida, não existe nada a temer, mas a entender.”

- Marie Curie

“Todas as vitórias ocultam uma abdicação”

-Simone de Beauvoir

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Resumo

A importância da luz para o desenvolvimento das plantas vai muito além da fotossíntese. Este estímulo ambiental controla desde a germinação até a senescência. Desde a sua percepção, por meio de fotorreceptores, até o desencadeamento da resposta fisiológica, diversos fatores participam da transdução do sinal luminoso. Dentre eles, recentemente, foram identificadas as proteínas portadoras do domínio B-BOX (BBXs). As proteínas BBXs, são fatores de transcrição do tipo dedos de zinco, quase exclusivamente estudadas em *A. thaliana*, que pertencem a uma família que está subdividida em cinco grupos de acordo com o número de domínios B-BOX e à presença de domínio CCT (CONSTANS, CONSTANS-LIKE e TIMING OF CAB1). As proteínas BBX que foram funcionalmente caracterizadas até o momento mostraram ser reguladores de diversos processos tais como fotomorfogênese, regulação fotoperiódica da floração, evitação de sombra e respostas a estresses bióticos e abióticos. Neste contexto, o presente trabalho teve como objetivo geral, caracterizar os genes que codificam as proteínas que contém domínio BBX presentes no genoma de *Solanum lycopersicum*. Para isso, foi realizado um detalhado perfil transcricional dos genes *SlBBXs* dos grupos IV e V em folhas e frutos em diferentes estágios de desenvolvimento e em hipocótilos crescidos sob diferentes condições de luminosidade. Os resultados mostraram que os genes *SlBBXs* são modulados transcricionalmente pela luz e pelo estado de desenvolvimento dos plastídios (cloroplastos e/ou transição de cloroplasto a cromoplastos). Para aprofundar a compreensão do papel dessas proteínas na fisiologia da planta, foi realizada a caracterização fenotípica do gene *SlBBX28*. O silenciamento constitutivo de *SlBBX28* utilizando a estratégia de RNAi, resultou em plantas de menor porte e com menos flores por inflorescência. Além disso, a deficiência de SlBBX28 reduziu o alongamento de hipocótilos crescidos no escuro. Utilizando diversas abordagens experimentais, foi possível demostrar que SlBBX28 participa do controle do crescimento, floração e estiolamento por meio da regulação do metabolismo e sinalização de auxinas.

Abstract

The importance of light for plant development goes far beyond photosynthesis. This environmental stimulus controls from germination to senescence. From photoreceptors-mediated towards the triggering of the physiological response, several factors participate in the transduction of the light signal. Among them, proteins carrying the B-BOX domain (BBXs) were recently identified. The members of the BBX protein family are zinc finger transcription factors, almost exclusively studied in A. thaliana, that cluster into five groups according to the number of B-BOX domains and the presence of a CCT domain (CONSTANS, CONSTANS-LIKE and TIMING OF CAB1). The BBX proteins that have been functionally characterized so far have shown to be regulators of several processes such as photomorphogenesis, photoperiodic regulation of flowering, shade avoidance and responses to biotic and abiotic stresses. In this context, the present work aimed to characterize the genes that encode BBX domain containing proteins present in *Solanum lycopersicum* genome. For this, a detailed transcriptional profile of the *SlBBX* genes of groups IV and V was carried out in leaves and fruits at different stages of development and in hypocotyls grown under different light conditions. The results showed that the *SlBBX* genes are transcriptionally modulated by light and by the developmental state of plastids (chloroplasts and/or chloroplast-to-chromoplast transition). To deepen the understanding of the role of these proteins in plant physiology, we performed the functional characterization of *SlBBX28* gene. The RNAi-mediated constitutive silencing of *SlBBX28* resulted in smaller plants with fewer flowers per inflorescence. Furthermore, SlBBX28 deficiency reduced dark-grown hypocotyl elongation. By using several experimental approaches, it was possible to demonstrate that SlBBX28 participates in the control of growth, flowering and etiolation by regulating auxin metabolism and signaling.

1. Introdução

1.1. Sinalização luminosa

A luz é um fator essencial para o crescimento, desenvolvimento e reprodução vegetal, controlando diversos processos tais como a germinação, fotossíntese, fototropismos, estabelecimento e manutenção de ritmos circadianos e floração (Quail 2002; Jiao *et al.* 2007). A percepção do sinal luminoso ocorre por meio de fotorreceptores, tais como fitocromos, UVR8, fototropinas e criptocromos (Gyula *et al*. 2013). Após a percepção, desencadeia-se uma complexa cascata de sinalização, a qual culmina na expressão diferencial de genes e, por fim, na indução de uma dada resposta fisiológica (Demotes-Mainard *et al.* 2016). Os primeiros fotorreceptores identificados foram os fitocromos (PHYs) (Mohr 1972), sendo atualmente os mais extensivamente caracterizados (Butler *et al*. 1959; Clack *et al.* 1994). Os PHYs são compostos por uma apoproteína ligada a um cromóforo (Hahn *et al.* 2006) e atuam como homodímeros, possuindo duas formas de acordo com a luz incidente. A luz vermelha (665 nm) converte os PHYs da forma Pr para Pfr, que é o estado biologicamente ativo. Essa alteração é reversível, pois a luz vermelho-distante (730 nm) pode reverter a forma Pfr à Pr (Inoue *et al.* 2017). A conversão entre as formas se dá devido à isomerização do cromóforo de acordo com o comprimento de onda absorvido que, por sua vez, acarreta em alterações conformacionais na apoproteína. Esta mudança, altera a exposição do domínio de localização nuclear, o qual somente encontra-se exposto na forma Pfr, a qual é, consequentemente, a única forma capaz de ser translocada para o núcleo das células (Quail 2002; Nagatani 2004; Chen *et al.* 2005; Fankhauser e Chen 2008).

No núcleo, o Pfr é capaz de regular diversas proteínas através de sua atividade de quinase (Chen e Chory 2011; Shin *et al.* 2016). Um exemplo é a inativação do complexo proteico formado por CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), DETIOLATED1 (DET1), DAMAGE DNA BINDING1 (DDB1) e CULLIN4 (CUL4) (Chory *et al.* 1989). Este complexo, na ausência de luz, induz a degradação proteossômica de diversos fatores promotores da fotomorfogênese, como é o caso do fator de transcrição ELONGATED HYPOCOTYL 5 (HY5) (Lau e Deng 2012; Hoecker 2017). Já na presença de luz, o Pfr inibe a COP1, a qual migra para o citoplasma deixando livre o regulador positivo do sinal luminoso HY5 (Hoang *et al.* 2019). Desta

forma, com a inativação do complexo COP1:DET1:DDB1:CUL4, HY5 se liga a motivos regulatórios específicos no DNA, induzindo a expressão de diversos genes evolvidos em processos como alongamento e proliferação celular, desenvolvimento plastidial, acúmulo de pigmentos e assimilação de nutrientes (Gangappa e Botto 2016). Outros reguladores negativos do sinal luminoso são os fatores de transcrição PHYTOCHROME INTERACTING FACTOR (PIFs). As proteínas PIFs são degradadas na presença de PHYs ativos e se acumulam na ausência de luz regulando negativamente a transcrição de diversos genes envolvidos na fotomorfogênese (Leivar e Quail 2011).

1.2. O efeito da manipulação da sinalização luminosa sobre a produtividade e qualidade nutricional de frutos de tomateiro

O tomateiro, *Solanum lycopersicum* L., pertence à família Solanaceae, seção Lycopersicon, a mesma de diversas outras espécies de importância agronômica tais 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. Particularmente, pela importância para a nutrição humana e a biologia dos frutos, *S. lycopersicum* se consolidou como modelo para o estudo da fisiologia de frutos carnosos (Carrari & Fernie 2006). 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 e do acúmulo de diversos compostos nutracêuticos antioxidantes, tais como vitamina C (ácido ascórbico), vitamina E (VTE, tocoferóis) e carotenoides. Dentre estes últimos, predomina o licopeno, pigmento que determina a característica cor vermelha dos frutos maduros; e a pró- vitamina A, o β-caroteno (Klee & Giovannoni 2011; Egea *et al.* 2010). Desta forma, a importância nutricional do fruto está intimamente ligada à atividade plastidial que, por sua vez, está fortemente regulada pela luz a qual tem papel fundamental na divisão, desenvolvimento e metabolismo dos cloroplastos (Azari *et al.* 2010; Powell *et al.* 2012).

Como mencionado acima, as proteínas SlDET1 (ou HP2) e SlDDB1 (ou HP1) são reguladores negativos da resposta à luz. A perda de função ou a redução da expressão destes fatores resulta no aumento do número e do volume dos cloroplastos,

assim como de maiores teores de clorofilas em frutos verdes, resultando em maiores quantidades de carotenoides, tocoferóis, flavonoides e fenilpropanoides nos frutos maduros (Davuluri *et al.* 2004; Enfissi *et al.* 2010). De forma semelhante, a repressão dos transcritos de outros dois reguladores negativos da transdução do sinal luminoso, o SlCUL4 e o SlCOP1, resulta no maior acúmulo de carotenoides em frutos maduros de tomateiro (Wang *et al.* 2008; Liu *et al.* 2004).

A modulação da expressão das proteínas SlPIF também afeta a qualidade dos frutos. Foi demonstrado que SlPIF1a e SlPIF4 inibem a expressão de genes envolvidos na carotenogênese reduzindo o acúmulo de carotenoides nos frutos (Llorente *et al.* 2016; Rosado *et al.* 2019). Já a proteína SlPIF3 modula o acúmulo de tocoferóis durante o amadurecimento por meio da inibição transcricional do gene *GERANILGERANIL DIFOSFATO REDUTASE (GGDR)* (Gramegna *et al*. 2018).

Não somente reguladores negativos do sinal luminoso afetam a qualidade nutricional dos frutos de tomateiro, mas também os positivos e fotorreceptores. Foi reportado que os PHYs são essenciais nesse cenário, regulando tanto o acúmulo de açucares e carotenoides (Bianchetti *et al*. 2018), quanto de vários outros antioxidantes promotores da saúde tais como, tocoferóis e flavonoides (Alves *et al.* 2020). O fator de transcrição GOLDEN2-LIKE 2 (SlGLK2), o qual é essencial para a correta diferenciação e manutenção da atividade plastidial, mostrou exercer um efeito positivo sobre o acúmulo de carotenoides e tocoferóis em frutos (Powell *et al.* 2012; Nguyen *et al*. 2014; Lupi *et al*. 2019). Por fim, o silenciamento constitutivo por RNAi de *SlHY5* reduz os teores de clorofilas e a formação de tilacoides nos cloroplastos de frutos imaturos, bem como diminuiu o conteúdo de carotenoides totais em frutos maduros (Liu *et al.* 2004). O perfil de expressão gênica de plantas mutantes para *SlHY5*, demonstrou que esse regulador controla o amadurecimento e qualidade nutricional por meio da regulação transcricional e traducional de seus genes alvo (Wang *et al.* 2021).

A manipulação do sinal luminoso também tem sido proposta como uma estratégia para aumentar a produtividade em algumas culturas (Xu *et al.* 2020). Alguns exemplos foram reportados para arroz e batata (Tan *et al.* 2016; Sheng *et al.* 2016; Abelenda *et al.* 2016). Particularmente em tomateiro, foi descrito que o silenciamento de *SlPIF4* compromete a produção de frutos devido a sua participação no controle do florescimento (Rosado *et al.* 2019).

No entanto, a maioria destas abordagens manipulam fatores que atuam no início da cascata de percepção e transdução do sinal luminoso, o qual acarreta diversos efeitos

pleiotrópicos. Assim, a intervenção em etapas mais a jusante da sinalização luminosa pode permitir resultados mais precisos. Nesse sentido, as proteínas pertencentes à família de fatores de transcrição BBX (B-BOX), as quais foram caracterizados como reguladores da sinalização luminosa, se apresentam como interessantes alvos para o estudo e eventual manipulação com fins biotecnológicos (Talar e Kielbowicz-Matuk 2021).

Em *Arabidopsis thaliana*, diversas BBXs foram descritas como reguladores do fator de transcrição HY5 ou reguladas por ele. Particularmente interessante é o caso da AtBBX28, um regulador negativo da sinalização luminosa induzido pela luz. Na ausência de luz, AtCOP1 ubiquitina AtHY5 e AtBBX28, resultando na degradação de ambas proteínas pelo proteossoma 26S. Na presença de luz, AtBBX28 interage fisicamente com AtHY5, reduzindo sua capacidade promotora da fotomorfogênese. Desta forma, AtBBX28 atua atenuando a transdução do sinal luminoso dependente de AtHY5 (Lin *et al.* 2018).

Embora haja um crescente acúmulo de informações acerca do papel das BBXs como parte da transdução de sinal luminoso, a maioria está restrita aos processos de estiolamento e desestiolamento de plântulas em *A. thaliana*, sendo escassas as informações em outros processos, e em outras espécies, tema que pretende abordar o presente trabalho.

1.3. Proteínas BBXs

As proteínas BBXs são fatores de transcrição do tipo dedos de zinco que atuam em diversos processos fisiológicos como a fotomorfogênese, regulação fotoperiódica da floração, evitação de sombra e respostas a estresses bióticos e abióticos (Lin *et al.* 2018; Min *et al.* 2015; Crocco *et al.* 2010; Luo *et al*. 2018; Liu *et al*. 2020; Xu *et al*. 2022). Estas proteínas são caracterizadas pela presença de um ou dois domínios B-BOX e pela presença ou ausência do domínio CCT (CONSTANS, CONSTANS-like, TIMING OF CAB1) no extremo C terminal (Khanna *et al.* 2009). A topologia de domínios é o critério utilizado para a subdivisão desta família de fatores de transcrição em cinco grupos estruturais (Grupos I-V). Os membros do grupo I e do grupo II são caracterizados pela presença de dois domínios B-BOX e um domínio CCT. O Grupo III contém um único domínio B-BOX e um CCT. O grupo IV é caracterizado pela presença de dois domínios B-BOX, mas sem domínio CCT. Por fim, o grupo V é composto por

proteínas com apenas um domínio B-BOX (Khanna *et al.* 2009; Gangappa e Botto 2014).

Funcionalmente, tanto o domínio B-BOX quanto o CCT já foram caracterizados como domínios importantes para a atividade de regulação da transcrição gênica das BBXs. Além disto, o B-BOX também é responsável por mediar interações proteína-proteína (Gangappa e Botto 2014), inclusive com COP1 (Song *et al.* 2020), além do sinal de localização nuclear já ter sido identificado neste domínio (Min *et al.* 2015). Já o domínio CCT é importante para o transporte para o núcleo das proteínas de grupo I, II e III (Gendron *et al.* 2012). O motivo VP (VALINA-PROLINA) presente em algumas proteínas BBXs, foi descrito como mediador da interação física com COP1(Holm *et al*. 2001; Datta *et al*. 2006).

Inicialmente descritas em *A. thaliana,* as proteínas BBXs desta espécie são as mais extensamente caracterizadas, com informação funcional para 26 das 32 AtBBXs identificadas no genoma. Interessantemente, enquanto os membros dos grupos I e II estão maiormente envolvidos no controle da floração, os membros dos grupos IV e V estão mais associados à regulação da fotomorfogênese (Gangappa e Botto 2014; Yadukrishnan *et al.* 2018). O grupo IV inclui 8 membros (AtBBX18-AtBBX25). AtBBX20 (Fan *et al.* 2012; Wei *et al.* 2016), AtBBX21 (Datta *et al.* 2007; Holtan *et al.* 2011), AtBBX22 (Datta *et al.* 2008) e AtBBX23 (Chang *et al.* 2008; 2011) são reguladores positivos da resposta à luz; enquanto AtBBX18 (Wang *et al.* 2013; Zhang X e Lin R 2017), AtBBX19 (Wang *et al.* 2014; 2015), AtBBX24 (Yan *et al.* 2011) e AtBBX25 (Gangappa *et al.* 2013) são reguladores negativos da fotomorfogênese. Essa função antagônica está associada com a capacidade de interagir com HY5 e o efeito que dita interação produz sobre a atividade de HY5 (Gangappa e Botto 2016). Por exemplo, mutações nos domínios BBX de AtBBX21, AtBBX22, AtBBX24 e AtBBX25 impedem a interação com AtHY5. A interação de AtBBX21 e AtBBX22 aumenta a capacidade dos complexos de induzir a expressão dos genes alvo (Datta *et al*. 2007; 2008; Bursch *et al.* 2020). Pelo contrário, a interação de AtBBX24 e AtBBX25 com HY5 gera heterodímeros inativos reduzindo a capacidade dos mesmos de regular a transcrição (Gangappa e Botto 2013). Análises de epístase entre proteínas BBX e COP1 mostraram que AtBBX20, AtBBX21 e AtBBX22 reprimem a ação de COP1; enquanto que AtBBX24 e AtBBX25 a estimulam (Datta *et al*. 2007; 2008; Fan *et al*. 2012; Gangappa *et al.* 2013). O grupo V abrange cinco membros (AtBBX28-AtBBX32), todos reguladores negativos da fotomorfogênese (Song *et al.* 2020; Holtan *et al.* 2011).

AtBBX28 e AtBBX29 interagem fisicamente com HY5 reduzindo sua atividade de indutor transcricional. Por sua vez, HY5 reprime a expressão de *AtBBX30* e *AtBBX31* os quais induzem a expressão de *AtBBX28* e *AtBBX29* (Song *et al.* 2020). Ainda, AtBBX32 interage com o regulador positivo AtBBX21, inativando-o e, reduzindo assim, a capacidade de HY5 de regular a expressão de seus genes alvo (Holtan *et al.* 2011).

Nos últimos anos, o papel das proteínas BBX em outras espécies tem sido investigado. Buscas em escala genômica permitiram a identificação e análise da família BBX em espécie como videira (Wei *et al.* 2020), banana (Chaurasia *et al.* 2016), amendoim (Jin *et al.* 2020), soja (Wu *et al.* 2014), orquídeas (Cao *et al.* 2019), batata (Talar *et al.* 2017), beterraba açucareira (Chia *et al.* 2008), algodão (Cai *et al.* 2017), cinco espécie de Poaceae (*i.e.* milho, arroz, sorgo, *Brachypodium distachyon* e painço; Shalmani *et al.* 2019), e sete espécies de Rosaceae (*i.e.* rosa, pêssego, maça, pera, morango, cereja e framboesa; Shalmani *et al.* 2018). Dados de transcriptoma e RT-qPCR em videira indicaram que as proteínas VvBBX desempenham um importante papel no desenvolvimento e amadurecimento dos frutos modulando diversos sinais hormonais (Wei *et al.* 2020). Recentemente, a expressão de genes codificantes para proteínas BBXs regulados pela luz foi associada ao acúmulo de carotenoides em *Cucumis sativus* (Obel *et al.* 2022).

Em *S. lycopersicum*, inicialmente foram identificados 29 genes *SlBBXs*, dos quais alguns foram descritos como responsivos a estresse abiótico e fitohormônios (Chu *et al*. 2016). Em 2019, a proteína SlBBX codificada pelo *locus* Solyc01g110180, chamada de SlBBX20, foi caracterizada funcionalmente. A sobre-expressão do gene resultou em frutos verdes com cloroplastos mais desenvolvidos e consequentemente, em frutos maduros com maiores quantidades de carotenoides. Experimentos de transativação, um híbrido e retardo em gel demostraram que SlBBX20 induz diretamente a transcrição do gene *PHYTOENE SYNTASE1* (*SlPSY1*), o qual codifica a primeira enzima envolvida na carotenogênese (Xiong *et al*. 2019). A SlBBX20 também mostrou promover o acúmulo de antocianinas em diversos tecidos e órgãos por meio da regulação transcricional do gene codificante da DIHIDROFLAVONOL REDUTASE (Luo *et al.* 2021). Finalmente, o produto do *locus* Solyc01g110180, mostrou também ser responsável pela indução do gene HY5 em resposta à UV-B (Yang *et al*. 2022). Por outro lado, foi descrito que a proteína SlBBX19, codificada pelo *locus* Solyc01g110370, regula o acúmulo de açúcares em frutos uma vez que foi demostrado

que a mesma interage com o promotor da *ADP-GLUCOSE PYROPHOSPHOSPHORYLASE LARGE SUBUNIT 1*, enzima limitante para a síntese de amido (Veltkamp 2021). Recentemente, a SlBBX17 codificada pelo *locus* Solyc07g052620, foi caracterizada como um regulador negativo do crescimento vegetativo e que aumenta a tolerância ao calor em tomateiro (Xu *et al.* 2022).

Um estudo recente do nosso grupo realizou uma nova busca por proteínas BBXs codificadas no genoma de tomateiro, identificando 31 genes *SlBBXs* (Lira *et al.* 2020). Uma análise filogenética com sequências de *S. lycopersicum* e *A. thaliana* revelou a distribuição dos genes em cinco grupos, de forma semelhante ao reportado para as BBXs de *A. thaliana*. Interessantemente, foi identificado no grupo II um sub-grupo, nomeado grupo VI, que divergiu perdendo o domínio CCT, embora vestígios do mesmo possam ainda ser identificados (Figura 1). Através de uma análise filogenética mais ampla com sequencias de *S. lycopersicum, A. thaliana,* Chlorophytas (*Volvox carteri, Volvox carteri f. nagariensis, Ostreococcus tauri, Ostreococcus lucimarinus CCE9901, Micromonas pusilla CCMP1545, Micromonas commoda, Coccomyxa subellipsoidea C-169, Chlorella variabilis, Chlamydomonas reinhardtii*) e proteínas de *Homo sapiens* com domínio B-BOX como grupo externo, foi sugerido um modelo para explicar a história evolutiva das proteínas BBX (Figura 2). Segundo este, o ancestral dos organismos fotossintetizantes possuiria um gene com somente um domínio B-BOX. Antes da divergência de Chlorophyta, houve uma duplicação deste gene ancestral, adquirindo um segundo domínio no clado que originou as sequencias de estrutura IV e adquirindo o domínio CCT no clado ancestral dos outros quatro grupos. Após a divergência de Chlorophyta, um evento de duplicação gênica com ganho de um segundo domínio B-BOX originou o grupo estrutural I. Posteriormente, o grupo estrutural III divergiu sem aquisição de novos domínios, enquanto que um segundo domínio-BOX surgiu independentemente no ancestral dos grupos II e V, sendo que neste último, tanto o domínio CCT quanto o segundo B-BOX foram perdidos após divergência do grupo II (Lira *et al.* 2020).

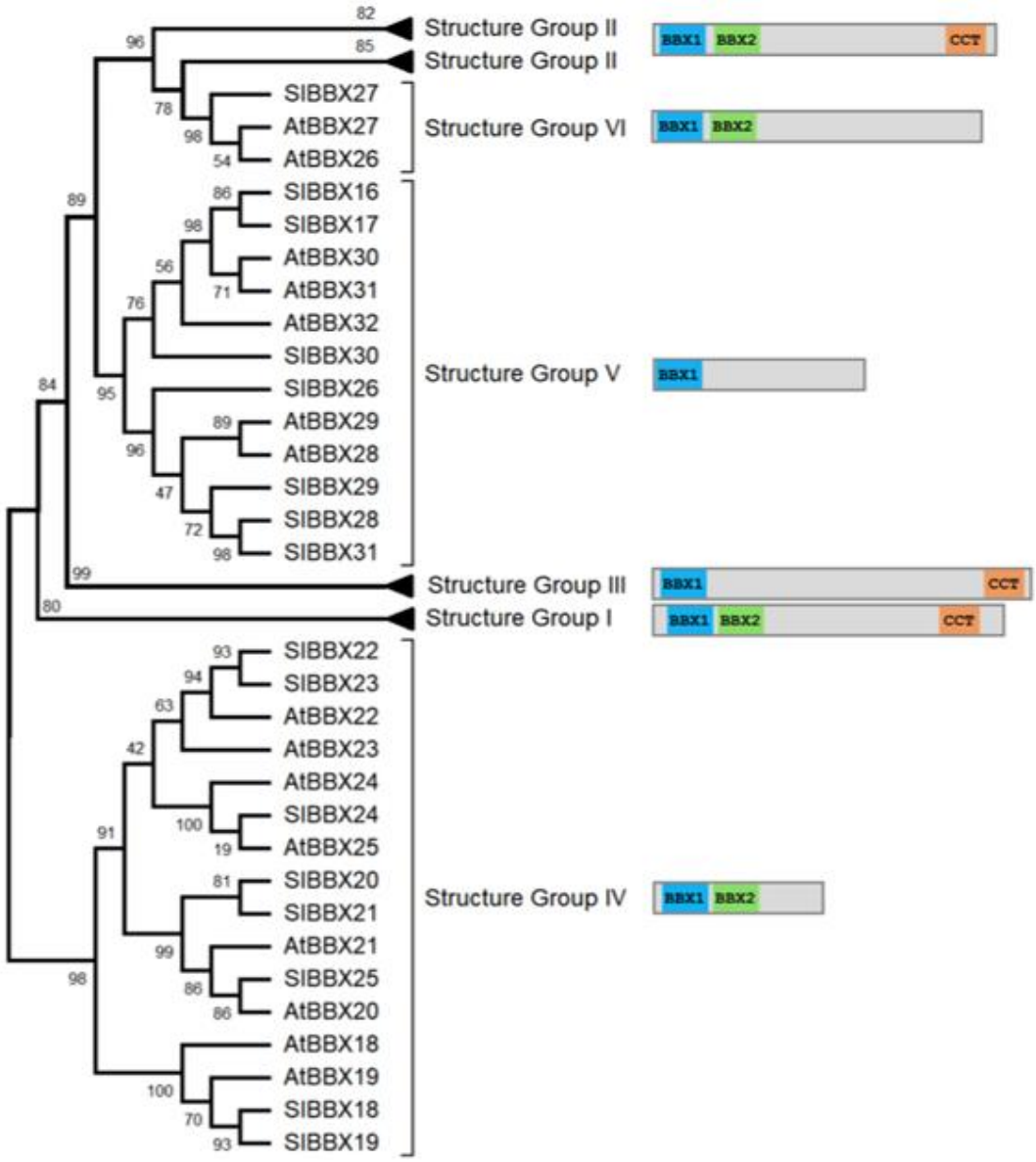

Figura 1: Análise filogenética das proteínas BBX de *A. thaliana* e *S. lycopersicum*. Reconstrução filogenética obtida a partir do alinhamento das proteínas BBX de *A. thaliana* e *S. lycopersicum*. Os grupos foram nomeados de acordo com os grupos de estrutura descritas em *A. thaliana* e a distribuição dos domínios de cada clado foi determinada usando a sequência consenso. Estão detalhados os grupos IV e V que possuem o maior número de proteínas BBXs descritas como reguladoras do sinal luminoso. Retirado de Lira *et at.* (2020).

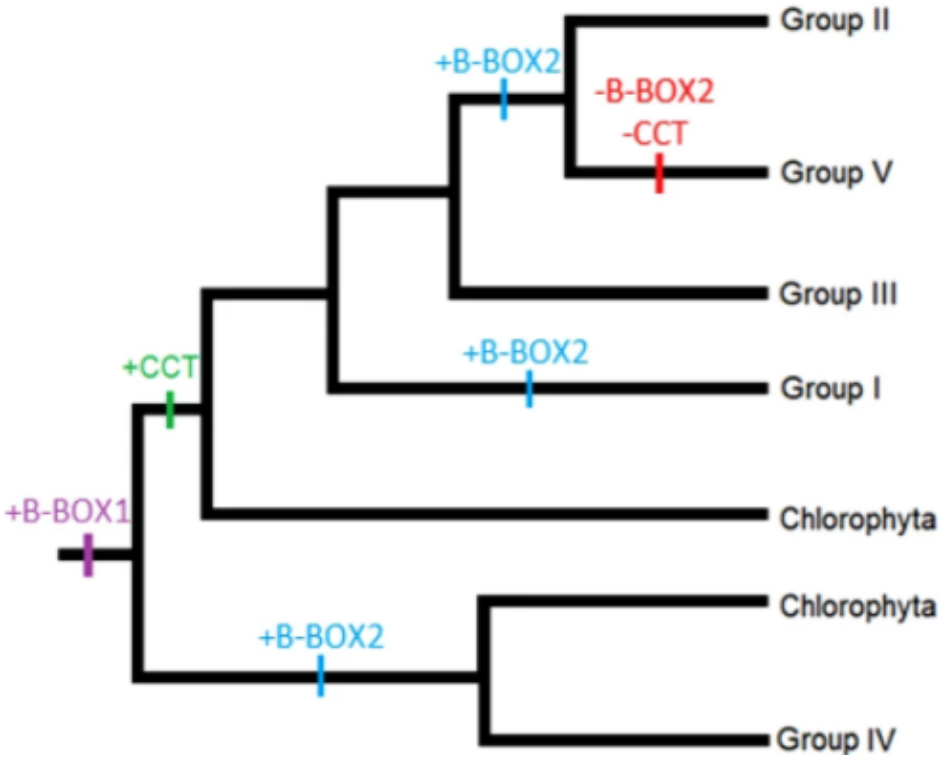

Figura 2: Modelo proposto para explicar a história evolutiva da família de proteínas BBX. Os domínios B-BOX1 e CCT mostram terem origens únicas, enquanto que o domínio B-BOX2 tem origens independentes em três diferentes clados, sendo perdido juntamente com o domínio CCT no grupo estrutural V. Retirado de Lira *et at.* (2020).

A partir dos dados da árvore filogenética (Figura 1), foi possível identificar os genes *SlBBXs* pertencentes aos grupos IV e V, os quais agrupam a maior parte das BBXs caracterizadas como integrantes da transdução do sinal luminoso, bem como possíveis ortólogos para a proteína AtBBX28, genes estudados no presente trabalho. Assim, considerando a importância da transdução do sinal luminoso para a determinação da qualidade nutricional e produtividade em tomateiro, e das proteínas BBXs nesta rota de sinalização, o aprofundamento da caracterização destas proteínas nesta espécie é um campo promissor de estudos capazes de fornecer subsídios para futuras estratégias biotecnológicas.

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Light and ripening-regulated BBX protein-encoding genes in *Solanum lycopersicum*

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Light controls several aspects of plant development through a complex signalling cascade. Several B-box domain containing proteins (BBX) were identified as regulators of *Arabidopsis thaliana* seedling photomorphogenesis. However, the knowledge about the role of this protein family in other physiological processes and species remains scarce. To fill this gap, here BBX protein encoding genes in tomato genome were characterised. The robust phylogeny obtained revealed how the domain diversity in this protein family evolved in Viridiplantae and allowed the precise identification of 31 tomato SlBBX proteins. The mRNA profiling in different organs revealed that *SlBBX* genes are regulated by light and their transcripts accumulation is directly affected by the chloroplast maturation status in both vegetative and fruit tissues. As tomato fruits develops, three *SlBBXs* were found to be upregulated in the early stages, controlled by the proper chloroplast differentiation and by the PHYTOCHROME (PHY)-dependent light perception. Upon ripening, other three *SlBBXs* were transcriptionally induced by RIPENING INHIBITOR master transcriptional factor, as well as by PHY-mediated signalling and proper plastid biogenesis. Altogether, the results obtained revealed a conserved role of *SlBBX* gene family in the light signalling cascade and identified putative members affecting tomato fruit development and ripening.

Zinc finger transcription factors (TFs) comprise one of the most important families of transcriptional regulators in plants and play a central role in plant growth and development regulation, as well as in biotic and abiotic stress responses[1,2]. Among these TFs, B-box domain containing proteins (BBX) belong to a subclass characterised by the presence of one or two zinc finger B-box domains, which are predicted to be involved in protein–protein interactions[3]. BBX proteins were classified into five structure groups, according to the number of B-box and CCT (CONSTANS, CONSTANS-like and TIMING OF CAB1) domains and VP (valine-proline) motifs. Members of group I are characterised by the presence of two B-box domains in tandem, one CCT domain and one VP motif. Group II is similar to group I, also presenting two B-box domains and one CCT domain, but no VP motif. Group III contains a single B-box domain and a CCT. Group IV is characterised by the presence of two B-box domains but without CCT domain. Finally, group V is composed by proteins with just one B-box domain[3,4]. Although the VP is mentioned as a group I exclusive motif, it has already been identified in several proteins belonging to group III, IV and V; thus, the presence of the VP motif differs members from structure group I from II, but evidences show that it is not exclusive to the first[5].

Out of the 32 BBX proteins identified in *Arabidopsis thaliana*, 21 have already been functionally characterised, being described as regulators of various processes such as seedling photomorphogenesis[6,7], photoperiodic flowering regulation[8], shade avoidance[9], and responses to biotic and abiotic stresses[10]. Interestingly, 14 BBX proteins were also found to be components of the light signalling transduction pathway[4,6,11,12], with 12 of them belonging to groups IV (8 proteins) and V (4 proteins). Four of the light-signalling group IV proteins act as positive regulators—AtBBX20[13], AtBBX21[14], AtBBX22[15] and AtBBX23[16]—and the other four play a negative role—AtBBX18[17], AtBBX19[18], AtBBX24[19] and AtBBX25[20,21]. In the case of group V, only repressors of light signal transduction were reported, AtBBX28[6], AtBBX30[7], AtBBX31[7] and AtBBX32[22].

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BBX proteins act by the direct or indirect interaction with central components of the light signal transduction network, including the transcription factors ELONGATED HYPOCOTYL 5 (HY5), HOMOLOG OF HY5 (HYH) and PHYTOCHROME INTERACTING FACTORs (PIFs), and the protein-ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)[4,23]. For instance, AtBBX21 and AtBBX22 promote HY5 transcript accumulation and can be tagged for proteasomal degradation via COP1-mediated ubiquitination[14,24,25]. In contrast, AtBBX24 and AtBBX25 downregulate light signalling by the physical interaction with HYH and HY5[20,26]. Interestingly, AtBBX28 was characterised as a light-induced light repressor, as it physically represses HY5 transcriptional regulatory activity and is marked for degradation in darkness by COP1[6]. Yet, it was demonstrated that PIF3 and PIF1 transcription factors signalling cascade regulates *AtBBX23* transcription, whose product physically interacts with HY5 inducing photomorphogenesis in *A. thaliana* seedlings[12].

The above-described links between BBXs and light signalling have been almost exclusively explored in seedling photomorphogenesis, and their role in other light-controlled physiological processes, such as plastid development and maintenance, plant architecture and fruit development, which are important determinants of crop yield and nutritional quality[27], remains elusive. In this context, although the effect of light perception and signalling in tomato (*Solanum lycopersicum* L.) fruit productivity and nutraceutical composition has been increasingly demonstrated[28–35], the association of the BBX protein family with light in this species is still elusive. In tomato, 29 BBX domain encoding genes were identified and reported to be modulated by abiotic stress and phytohormones[36]. Additionally, the Solyc01g110180 locus encodes the only deeply characterised tomato BBX, which is a positive regulator of fruit carotenogenesis[37].

Here, a comprehensive genome survey allowed the identification of 31 BBX protein-encoding loci in tomato genome. A robust phylogenetic reconstruction corroborated the monophyletic nature of the five previously identified structure groups and allowed the proposition of a new interpretation of the evolutionary history of this protein family. Further, we focused on the transcriptional profile of the 15 genes belonging to groups IV and V, revealing their association with organ greening and light signalling. Additionally, six genes were either up- or downregulated from immature fruit stages towards ripening. Finally, it was addressed whether the mRNA accumulation of these six genes is regulated by PHYTOCHROME (PHY)-mediated light perception and/or plastid development and differentiation.

Materials and methods

Plant material, growth conditions and sampling. Different tomato (*Solanum lycopersicum* L.) cv. Micro-Tom genotypes were used for *SlBBXs* transcriptional analysis: control genotype harbouring the wild-type *GOLDEN-2 LIKE 2 (SlGLK2)* allele (WT)[38]; uniform ripening *Slglk2* mutant, which is deficient in SlGLK2, the master transcription factor controlling fruit chloroplast differentiation and maintenance[33] and; fruit-specific transgenic lines silenced for *SlPHYA* (*SlphyA*) and *SlPHYB2* (*SlphyB2*)[30]. Although Micro-Tom cultivar is deficient in brassinosteroid biosynthesis due to the weak mutation *dwarf (d)*, it has been extensively demonstrated that represents a convenient and adequate model system to study fruit biology[39]. In this work we used Micro-Tom variety because we have all the germplasm collection in this background, including *Slglk2* mutant and the fruit-specific *SlPHY*-silenced transgenic lines.

For the experiments with seedlings, seeds were in vitro germinated in the darkness as described in[40]. After 2 days, seedlings were either kept in the darkness or transferred to the light (12 h photoperiod) for another 7 days, when hypocotyls and cotyledons were sampled.

Leaves and fruits were harvested from plants cultivated in 2L rectangular plastic pots containing a 1:1 mixture of substrate and vermiculite supplemented with NPK 10:10:10, dolomite limestone ($MgCO_3 + CaCO_3$) and magnesium thermophosphate (Yoorin), under controlled temperature (between 23 °C and 27 °C), daily automatically irrigation by capillarity, and under natural light conditions (13 h photoperiod and 250–350 $\mu molm^{-2}$ s^{-1} of incident photo-irradiance) in a biosafety level 1 greenhouse.

Source and sink leaves were harvested from 4 and 8th phytomer closest to the base of the plant, respectively, of plants with 40-day-old plants[34]. Fruit pericarp, without placenta and locule walls, was collected from fruits at different stages: (i) immature green 3 (IG3, approximately 8 days post-anthesis); (ii) immature green 5 (IG5, approximately 15 days post-anthesis); (iii) mature green (MG, when the placenta displays a gelatinous aspect, approximately 26 days post-anthesis); (iv) breaker (Br, beginning of ripening process when the fruit begins to present a yellowish coloration, approximately 32 days post-anthesis); (v) Br3 (three days after breaker stage, the fruits presents orange coloration); (vi) Br5 (5 days after breaker stage). Fruits were sectioned in three parts: (i) pedicellar, also known as the green shoulder, where developed chloroplast are predominately located, (ii) stylar region, which lacks developed chloroplasts), and (iii) the middle region that was discarded. For all the experiments, at least four pools of fruits (biological replicates) were harvested from at least five plants. Samples were frozen in liquid nitrogen and stored at − 80 °C freezer until processing. Mature green fruits were used for chromatin immunoprecipitation assay.

Phylogenetic analysis. For phylogenetic analysis BBX proteins from plant species representing angiosperms and Chlorophyta, as well as from Homo sapiens (as outgroup) were used. The loci encoding BBX proteins were retrieved from: Phytozome 12.1 (https://phytozome.jgi.doe.gov) database for *Arabidopsis thaliana, Chlamydomonas reinhardtii, Solanum lycopersicum* and *Volvox carteri* and, from NCBI ref-seq database (https://www.ncbi.nlm.nih.gov/refseq/) for *Chlorella variabilis, Coccomyxa subellipsoidea C-169, Homo sapiens, Micromonas commode, Micromonas pusilla CCMP1545, Ostreococcus lucimarinus CCE9901*, *Ostreococcus tauri* and *Volvox carteri f. nagariensis* (Supplementary Table S1).

Sequences from *A. thaliana*[3] and tomato[36] were named as previously reported. Amino acid sequences were aligned with Expresso T-COFFEE[41] and the phylogeny was reconstructed as described in[42]. Briefly, the protein

alignment was subjected to maximum likelihood phylogenetic reconstruction (PHYML 3.0) by JTT model with the proportion of invariable sites and gamma shape parameter estimated from the data sample. The obtained tree was optimized by tree topology and branch length, improved by subtree pruning and regrafting, and the branch support was calculated by the approximate likelihood-ratio test Shimodaira-Hasegawa-like (aLTR SH-like).

Reverse transcriptase quantitative PCR analysis (RT-qPCR). RNA extraction, complementary DNA (cDNA) synthesis, primer design and RT-qPCR assays were performed as described by[43]. Primer sequences used are detailed in Supplementary Table S2. qPCR reactions were carried out in a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) using 2X Power SYBR Green Master Mix reagent (Life Technologies) in a 10 µL final volume. Absolute fluorescence data were analysed using the LinRegPCR software package[44] in order to obtain quantitation cycle (Cq) values and calculate PCR efficiency. Expression values were normalised against the geometric mean of two reference genes, *TIP41* and *EXPRESSED*, according to[43]. A permutation test lacking sample distribution assumptions[45] was applied to detect statistical differences ($P<0.05$) in expression ratios using the algorithms in the fgStatistics software package version 17/05/2012[46].

Chromatin immunoprecipitation assay (ChIP). Full-length cDNA encoding RIPENING INHIBITOR transcription factor (*SlRIN*, Solyc05g012020) without the stop codon was amplified with the primers listed in Supplementary Table S2. The fragment was cloned into pENTR/DTOPO using Gateway technology (Invitrogen). The entry plasmids were recombined into pK7FWG2[47] using LR Clonase (Invitrogen) to produce 35S::SlRIN-GFP fusion protein. The construct obtained was introduced into *Agrobacterium tumefaciens* (GV3101) for further infiltration. ChIP assay followed by qPCR was performed as described in[34]. Briefly, MG fruits were agroinfiltrated with *35S::SlRIN-GFP* construct, kept for 3 days under 16 h/8 h photoperiod, and fixed with formaldehyde to promote the cross-linking 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 TF binding motif for each promoter region and the coding region of *SlACTIN4* gene[48] as control non-binding region (Supplementary Table S2) were used.

Data analyses. Differences in parameters were analysed using Infostat software version 17/06/2015[49]. When the data set showed homoscedasticity, Student's *t*-test ($P<0.05$) was performed to compare transgenic lines against the control genotype. In the absence of homoscedasticity, a non-parametric comparison was performed by applying the Mann–Whitney test ($P<0.05$). All values represent the mean of at least three biological replicates.

Transcription factor binding motifs were identified on the 3000 bp upstream of the transcription initiation site using PlantPAN 2.0[50].

Results

***Solanum lycopersicum* harbours similar diversity of BBX protein-encoding genes than *A. thaliana*.** The BBX TF family has been extensively studied in *A. thaliana*, whose proteins were classified into five groups accordingly to the domain structure[3,5]. Similar classification was reported for other species such as tomato[36], potato[51], rice[52] and grapevine[53]. However, not all provided a phylogeny with high branch support for the groupings and the lack of outgroup led the evolutionary history of the protein family ambiguous.

To provide robust phylogenetic information, BBX domain-containing protein sequences from tomato and *A. thaliana* were retrieved from Phytozome database (https://phytozome.jgi.doe.gov) (Supplementary Table S1). This survey led to the identification of two additional loci encoding BBX proteins in the tomato genome, that were named *SlBBX30* and *SlBBX31*, following the previously nomenclature published for this species[36]. *A. thaliana* sequences were named according to the nomenclature adopted by[3] (Supplementary Table S1).

The phylogenetic reconstruction (Fig. 1a) grouped the sequences according to their domain structure as previously reported in *A thaliana*[4], confirming the monophyletic nature of the five structure groups. Regarding the tree topology, structure group IV appeared isolated from the other four groups, while groups II and V clustered together. Interestingly, AtBBX26 and AtBBX27 were previously classified in the structure group V[4], while SlBBX27 was found clustered with group III proteins[36]; the three were described as a single B-box domain containing protein. Here, it was found that these three proteins contain indeed two BBX domains and grouped together as a subclade of structure group II without CCT domain, being referred as structure group VI (Fig. 1a). When the structure group VI sequences (i.e. AtBBX26, AtBBX27 and SlBBX27) and three representative sequences of structure group II (i.e. AtBBX10, AtBBX11 and AtBBX12) were aligned, the CCT motif could be clearly identified in the latter and some conserved residues could also be found in structure group VI sequences (Fig. 1b). Thus, this result indicates that the structure group VI diverged from structure group II sequences that lost the CCT motif.

The above described topology is in agreement with the one obtained for grapevine[53], but is not with two other well supported phylogenies[5,54]. The approach applied here differed from the previously reported in two methodological aspects: human (*H. sapiens*) B-box domain-containing proteins were obtained from NCBI refseq database (https://www.ncbi.nlm.nih.gov/refseq/) (Supplementary Table S1) and used as an outgroup in the analysis; and the structure-based multiple sequence alignment whose accuracy surpass sequence-based only packages was applied[41].

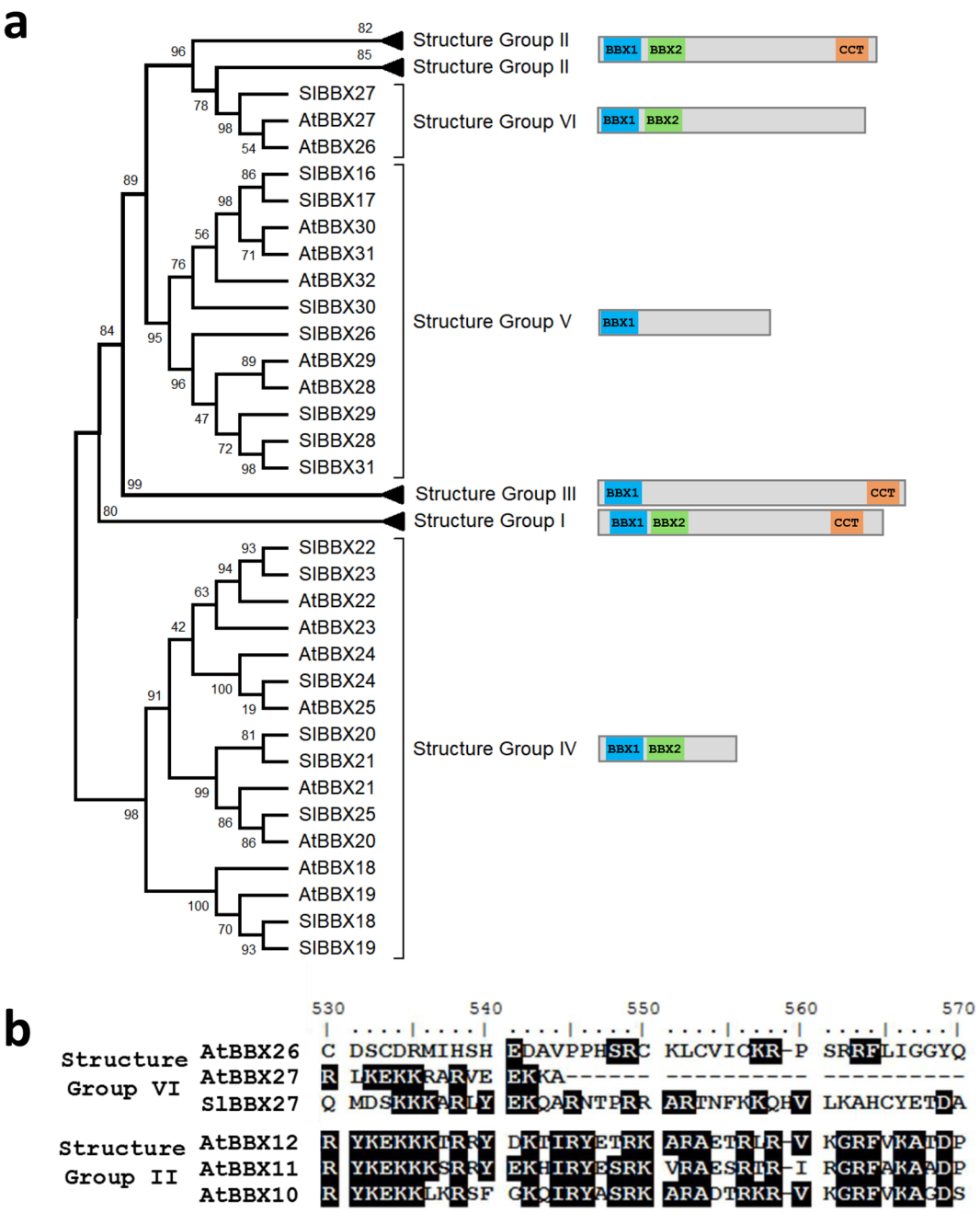

Figure 1. Phylogenetic presentation of *A. thaliana* and tomato BBX proteins. (**a**) Phylogenetic reconstruction obtained from the alignment of *A. thaliana* and tomato BBX proteins. The clusters were named accordingly to the structure groups described for A. thaliana and the domain architecture of each clade was determined using the consensus sequence. (**b**) A highlight of CCT motif alignment of structure group II representatives and the corresponding region of structure group VI sequences. Shading threshold = 60%.

Thus, to further confirm the obtained topology and bring information about the evolutionary history of this protein family, another phylogenetic analysis was performed including sequences from Chlorophyta species (Supplementary Table S1). The same above described topology for only tomato and *A. thaliana* was obtained. As the structure group VI was identified as a subclade of group II, group VI was collapsed with group II sequences to simplify the visualization (Fig. 2a). Moreover, two Chlorophyta clusters were observed, one grouping with the structure group IV and other with the clade composed of structure groups I/II/III/V. This indicated that the Viridiplantae ancestral, as means before the divergence of Chlorophyta and land plants, had two BBX-coding genes, one of which was subjected to three duplication events along land plants evolution.

The consensus sequence for the B-box and CCT domains was identified for each group (Fig. 2b). The CCT domain appear to have one single origin in the ancestral sequence of the structure groups I/II/III/V, before the divergence of Chlorophyta and land plants. It is not clear whether the ancestral proteins had one or two BBX

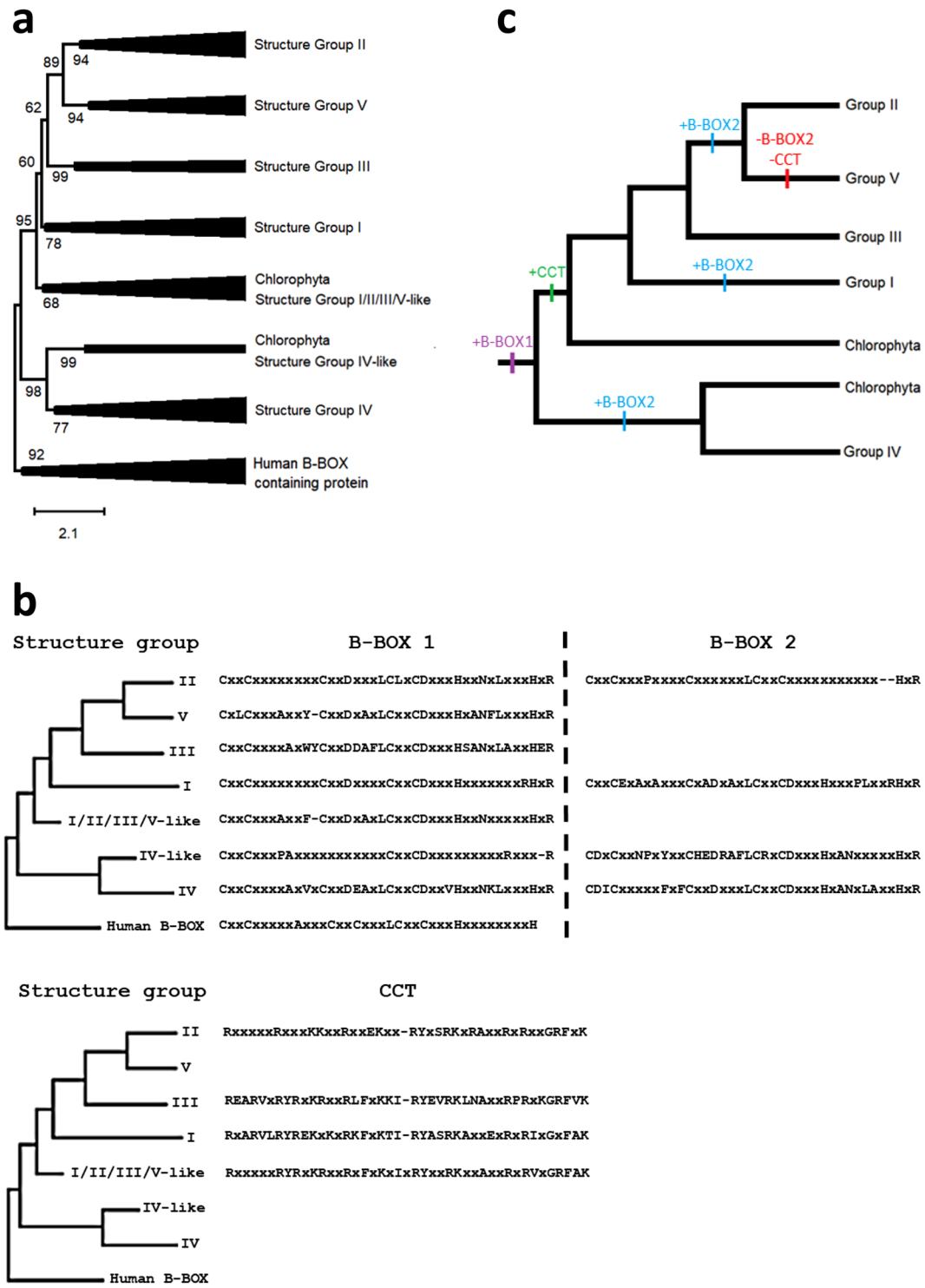

Figure 2. Evolution of BBX proteins. (**a**) Phylogenetic reconstruction obtained from the alignment of *A. thaliana,* tomato, chlorophyta and human B-box domain containing proteins. The clusters were named accordingly to the structure groups described for *A. thaliana*. The sequences information is available in Supplementary Table S1. (**b**) Consensus sequence for B-box and CCT domains (identity ≥ 60%). (**c**) Proposed hypothesis for domain evolution in the BBX protein family. While the B-box1 and CCT domains appear to have single origins along the evolution of these proteins, the B-box2 domain evolved independently three times.

domains. Based on the domain consensus, B-box1 seems to have a single origin, while B-box2 may have arisen several times independently, i.e. in the ancestral of the structure group IV clade, in structure group I group and

in the ancestral of the structure groups II/V. Regarding the latter, the alignment of the sequences of both groups revealed that some B-box2 domain conserved residues could be still identified in structure group V members, however none could be identified in structure group III (Supplementary Fig. S1). Thus, this indicates that B-box2 appeared in the ancestral of structure group II and V after the divergence from group III. The occurrence of only B-box1 domain in structure group V is the consequence of the divergence of B-box2 and a deletion in the ancestral sequence that resulted in the loss of the CCT domain.

Concluding, these results bring evidences that the ancestral Viridiplanteae harboured two B-box containing proteins; the ancestral of group IV with two B-box domains and the ancestral of group I/II/III/V-like clade with a single B-box domain. This later, after the divergence of land plants and Chlorophyta, diverged into four structure groups in which B-box2 domain arose two times independently (Fig. 2c).

The expression pattern of groups IV and V *SlBBX* genes is influenced by the stage of plastid development in both vegetative and fruit tissues. To gain insight into the link between BBX proteins and light signalling in tomato, we explored the transcription pattern of *SlBBX* genes that belong to the structure groups IV and V in organs bearing chloroplast at distinct light-regulated developmental stages, such as source and sink leaves, etiolated and de-etiolated seedlings and, fruits from immature to ripe stages[29,34,40].

As shown in Fig. 3a, *SlBBX* genes were significantly more expressed in source leaves than in sink counterparts, excepting *SlBBX25* and *SlBBX30* whose mRNA remained invariable. *SlBBX20* was the gene that showed the most expressive induction, approximately six times (Supplementary Table S3).

Transcript abundance of these *SlBBX* genes was also analysed under etiolation (skotomorphogenesis) and de-etiolation (photomorphogenesis) conditions in hypocotyls and cotyledons (Fig. 3b, Supplementary Table S3). Interestingly, most of the *SlBBX* genes showed higher levels of mRNA in cotyledons compared to hypocotyls, both in dark-grown (*SlBBX18*, *SlBBX19*, *SlBBX20*, *SlBBX22*, *SlBBX23*, *SlBBX24*, *SlBBX25*, *SlBBX26*, *SlBBX28* and *SlBBX30*) and light-grown (*SlBBX18*, *SlBBX21*, *SlBBX23*, *SlBBX24*, *SlBBX25*, *SlBBX26*, *SlBBX28* and *SlBBX29*) seedlings. Light exposure upregulated five (*SlBBX18*, *SlBBX24*, *SlBBX17*, *SlBBX28* and *SlBBX30*) and eight (*SlBBX18*, *SlBBX21*, *SlBBX16*, *SlBBX17*, *SlBBX26*, *SlBBX28*, *SlBBX29* and *SlBBX31*) genes in hypocotyls and cotyledons, respectively.

Finally, the transcript pattern of *SlBBXs* belonging to structure groups IV and V was profiled throughout fruit development and ripening. Since there is a chloroplast development gradient along the longitudinal axis in wild type (WT) tomato fruits[55], they were sectioned in pedicellar (with more and more developed chloroplasts) and stylar (with less and poorly developed chloroplasts) portions. As the profiles from both sections were mostly similar (Supplementary Fig. S2), we focused the analysis on the pedicellar portion (Fig. 3c, Supplementary Table S4). Most *SlBBX* genes exhibited substantial variations in the mRNA accumulation within the analysed stages. Interestingly, six genes showed clear association with either early development or ripening of fruits: *SlBBX19* (Solyc01g110370), *SlBBX20* (Solyc12g089240) and *SlBBX26* (Solyc10g006750) were strongly upregulated upon ripening triggering, as means from MG to Br stage; while, the amount of *SlBBX16* (Solyc12g005750), *SlBBX28* (Solyc12g005660) and *SlBBX29* (Solyc02g079430) mRNA was higher at green stages of fruit development gradually declining afterwards. The most expressive fold changes were observed for *SlBBX20* and *SlBBX16*, which were eight times more and ten times less expressed from IG3 towards fully ripe Br5 fruits, respectively.

The comparison of the relative mRNA accumulation levels of groups IV and V *SlBBX* genes among all the four organs analysed displayed no evident organ or structural specificity; however, except for *SlBBX20* and *SlBBX22*, they showed the highest expression either in source leaves or cotyledons (Supplementary Fig. S3). To sum up, the results showed that the plastid type and developmental stage (i.e. proplastid, chloroplast or chromoplast) seem to affect the transcript accumulation pattern of these 15 *SlBBX* genes in leaves, hypocotyls, cotyledons and fruits.

***SlBBX* genes associated with fruit early development or ripening are regulated by SlPHY and/or SlGLK2.** The identification of *SlBBXs* whose transcript profile is associate with fruit development and the importance of plastidial metabolism for determining nutraceutical content of tomato fruit, led to the investigation whether SlGLK2, a transcription factor essential for fruit chloroplast differentiation and activity maintenance[33,55], and PHY-mediated light perception[29] participate in the transcriptional regulation of the six above highlighted *SlBBX* genes (i.e. *SlBBX16*, *SlBBX19*, *SlBBX20*, *SlBBX26*, *SlBBX28* and *SlBBX29*). The hypothesis that SlGLK2- and/or PHYs regulate these genes was reinforced by the finding, in their promoter regions, of at least one HY5 (key inductor of PHY-mediated photomorphogenesis[56,57]), PHYTOCHROME INTERACTING FACTORs (PIF; key repressor of PHY-mediated photomorphogenesis[58]), or GLK binding motifs[59] (Supplementary Fig. S4). *Slglk2* mutant, which encodes a truncated and inactive version of the protein[55], and two fruit-specific *SlPHY*-silenced transgenic genotypes were used for the mRNA profiling. Out of the five tomato PHYs[60], fruit-specific functional characterization highlighted two as major contributors to fruit physiology: SlPHYA, a positive regulator of tomato plastid division machinery; SlPHYB2, a negative regulator of chlorophyll accumulation[30] and; both, inductors of fruit carotenogenesis.

Among the *SlBBX* genes downregulated during fruit development, *SlBBX28*, regardless punctual fluctuations, did not show clear pattern of SlPHY- and SlGLK2-dependent regulation (Fig. 4). In the case of *SlBBX29*, while the lack of SlGLK2 led to a reduced transcript amount at IG3; SlPHYs have opposite effects at MG stage. Yet, *SlBBX16* regulation appears to be more complex, at the peak of expression (i.e. IG3 stage) SlPHYA- and SlPHYB2-deficiency enhanced mRNA accumulation level. On the contrary, SlGLK2 seemed to have an inductive effect at green stages of fruit development (Fig. 4, Supplementary Table S5). The biological significance of the transcript level differences in the tested genotypes from Br to Br5 is questionable due to the extremely low amount of mRNA detected in ripening stages of WT genotype (i.e. the mRNA level of *SlBBX16* at Br stage is only 3% of the IG3 value, Supplementary Table S4).

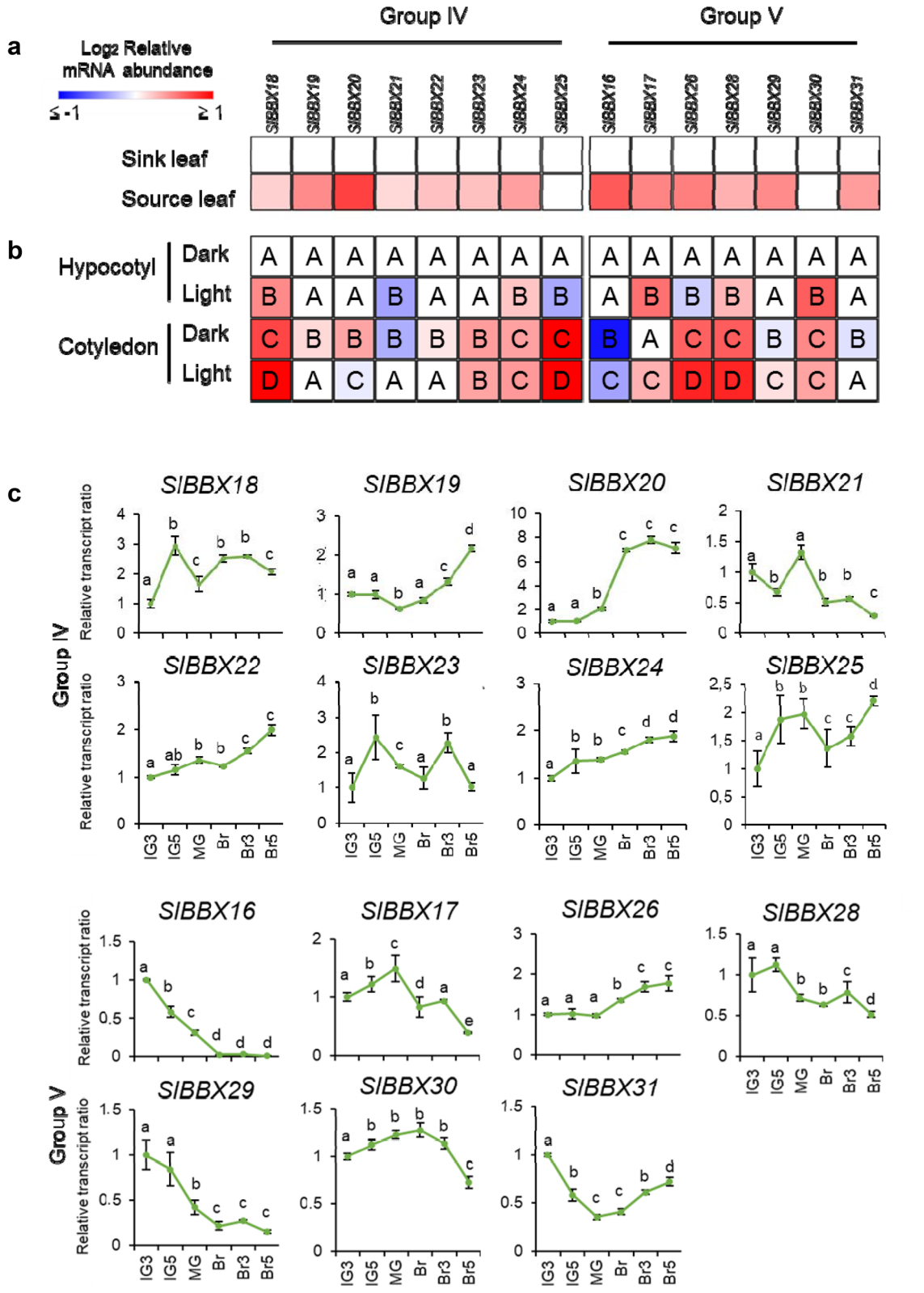

Figure 3. Transcript profile of structure group IV and V *SlBBX* genes. (**a**) Heatmap representation of the relative transcript ratio of *SlBBXs* in sink and source leaves from the 8th and the 4th phytomers of 40-day-old plants, respectively. Values are means of at least three biological replicates. Colored squares represent statistically significant differences in relation to the sink leaf sample ($P < 0.05$). Relative transcript values are detailed in Supplementary Table S3. (**b**) Heatmap representation of the relative transcript ratio of SlBBXs in etiolated and de-etiolated hypocotyls and cotyledons. Values are means of at least three biological replicates. Different letters represent statistically significant differences among the samples within each gene ($P < 0.05$). Relative transcript values are detailed in Supplementary Table S3. (**c**) Relative transcript ratio of SlBBXs in the pedicellar (top) portion throughout fruit development and ripening. Data were normalised against the IG3 sample. Values are means ± SE of at least three biological replicates. Different letters indicate statistically significant differences between fruit stages ($P < 0.05$). IG3: immature green 3; IG5: immature green 5; MG: mature green; Br: breaker; Br3: 3 days after Br; Br5: 5 days after Br.

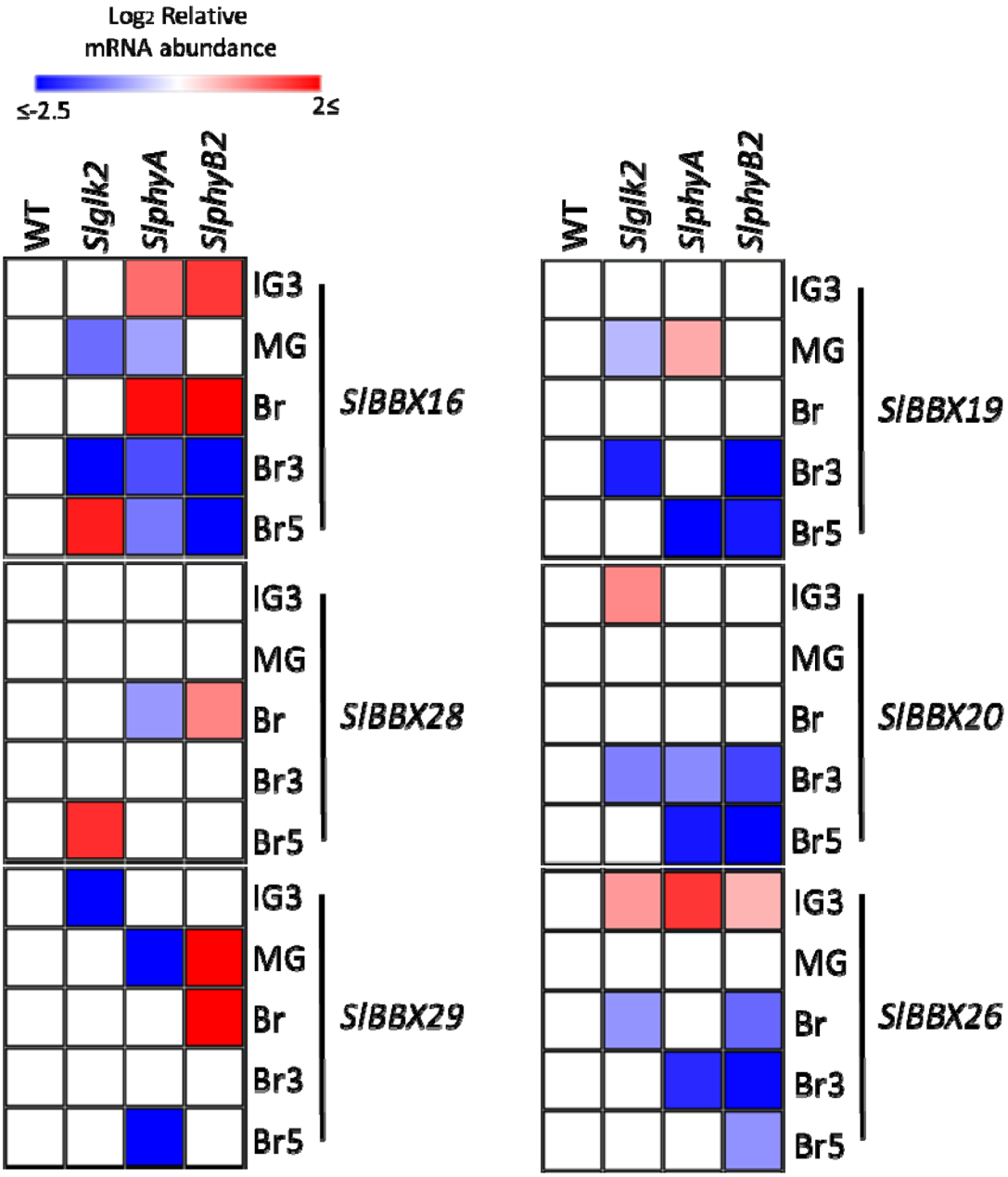

Figure 4. Transcriptional profile of *SlBBXs* in developing fruits of tomato lines impaired in light perception or chloroplast differentiation. The relative mRNA abundance of the six *SlBBXs* modulated by ripening was addressed in fruits of wild type plants (WT), SlGLK2-deficient mutant (*Slglk2*, Lupi et al. 2019), and fruit-specific *SlPHYA*- and *SlPHYB2*-silenced (*SlphyA* and *SlphyB2*) lines[30]. Values were normalised against the respective WT sample and are means of at least three biological replicates. The relative transcript values are detailed in Supplemental Table S5. Statistically significant differences relative to WT samples are colored ($P < 0.05$). IG: immature green 3; MG: mature green; Br: breaker; Br3: 3 days after Br; Br5: 5 days after Br.

The ripening induction observed in *SlBBX19*, *SlBBX20* and *SlBBX26* was attenuated in *SlPHYA*- and *SlPHYB2*-silenced fruits as well as in the SlGLK2-deficient genotype. This is clearly shown by the downregulation of their expression from Br towards Br5, suggesting that SlGLK2- and SlPHY-mediated signalling cascade stimulate the expression of these genes.

RIPENING INHIBITOR (SlRIN) regulates ripening-dependent expression of *SlBBXs*. SlRIN is a master TF controlling tomato fruit ripening[61] whose binding motif $C(CT)(AT)_6(AG)G$ was identified after a genome wide ChIP-Seq experiment[62,63]. On the promoter region (3000 bp upstream the transcription initiation site) of the three ripening-induced *SlBBX* genes (i.e. *SlBBX19*, *SlBBX20* and *SlBBX26*), putative RIN binding motifs were identified (Fig. 5a). To address whether SlRIN directly interacts with the promoter of the aforementioned genes, a *35S::SlRIN-GFP* construct was transiently expressed in WT mature green tomato fruits followed

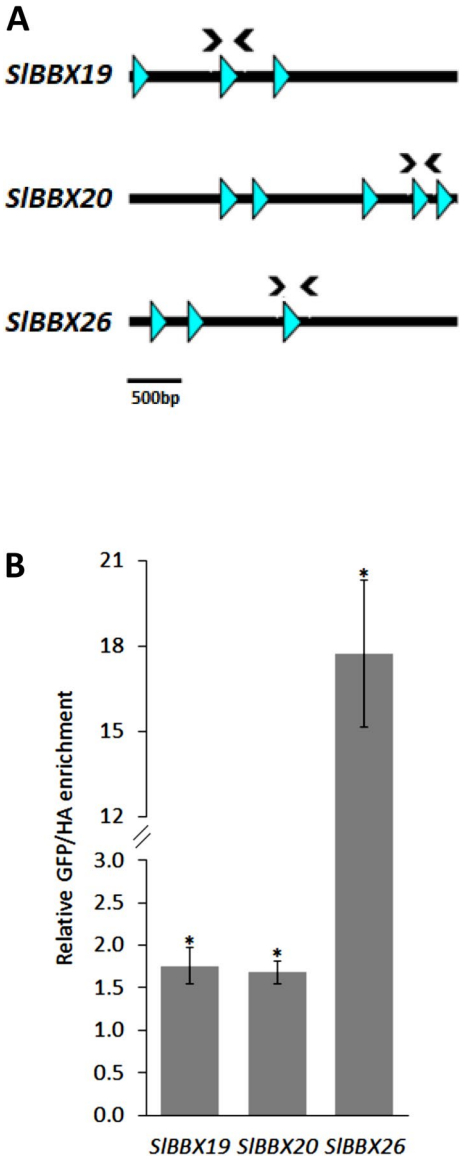

Figure 5. SlRIN binds to the ripening-induced *SlBBXs* promoter. (**A**) SlRIN binding motifs (C(CT)(AT)6(AG)G) blue triangles) in the promoter region (3000 bp upstream of the + 1 base) of the three ripening-induced *SlBBX* genes. Arrows indicate the positions of the primers used for ChIP-qPCR assay. (**B**) ChIP-qPCR experiment performed in tomato fruits transiently expressing *35S::SlRIN-GFP* using anti-GFP and anti-HA (as negative control) antibodies. Asterisks denote statistically significant differences ($P < 0.05$) to the respective anti-HA sample.

by a ChIP-qPCR assay with anti-GFP or negative control anti-HA antibodies. The anti-GFP immunoprecipitated chromatin showed to be enriched for all *SlBBX* promoters tested (Fig. 5b), demonstrating that SlRIN physically binds the regulatory region of *SlBBX19*, *SlBBX20* and *SlBBX26*, explaining the above-mentioned ripening-associated upregulation.

Discussion

Over the past years, BBX protein family was surveyed in several species such as apple[64], *A. thaliana*[4], grapevine[53], orchids[65], pear[54], rice[52], potato[51], *Arachis duranensis*[66] and tomato[36], being classified in five groups accordingly to the domain composition of the proteins. The comprehensive phylogenetic analysis performed in this work (Fig. 2a) provided evolutionary validation of this classification by revealing that the structure groups corresponded to well sustained monophyletic clusters. A foundational work[3] performed a phylogenetic analysis of *A. thaliana* BBX protein family that was further revised by[5], which proposed a model for BBXs evolutionary trajectory in green plants. Although the phylogeny topology obtained here does not reflect the evolutionary model proposed by[5], two pieces of evidences showed by the phylogenetic analysis of B-box domains reported by these authors support the clustering observed here: (i) B-box2 domain from groups IV and I are more closely related

than group II B-box2 and; (ii) B-box1 domain from groups II and V are the most closely related. Moreover, some methodological differences might have increased the accuracy of the topology obtained here: i) the incorporation of an outgroup; (ii) the multiple sequence alignment carried out with structure-based information[41] and; (iii) the algorithm used for the multiple sequence alignment is consistency-based, whose accuracy is increased in comparison to matrix-based ones such as ClustalW[67].

Our analysis showed that some *A. thaliana* and tomato proteins, previously reported as members of the structure group V[3,4] and II[36], respectively, are actually members of a new structure group, VI, which is diverging from group II after the loss of the CCT domain. As also observed for punctual examples belonging to groups II and V[4], these results suggest that some BBX proteins lost a domain in a recent evolutionary event, but conserve other common characteristics of their structure group.

Concluding, based on phylogenetic and domain structure analyses, we propose that the ancestral Viridiplanteae harboured two B-box domain containing proteins that originated structure group IV-like and structure group I/II/III/V-like clades, respectively. Moreover, while B-box1 and CCT domains seem to single origins in the evolutionary history of this protein family, B-box2 arose three time, independently (Fig. 2c).

Functional studies regarding B-box domain encoding genes were performed almost exclusively in *A. thaliana* seedlings and, interestingly, especially members of structure group IV and V, were characterised as components of the light signalling cascade[13,14,16,18–21,24]. By employing different photoreceptors, plants can track light intensity, quality, periodicity and direction. Among photoreceptors, PHYs are codified by a small gene family, with members playing different roles gathering information for adjusting plant development and metabolism to the changing environment[68]. Once activated by light, PHYs phosphorylate several nuclear proteins controlling their function[69]. Among them, E3 ubiquitin ligase COP1 activity and stability is negatively modulated by PHYs[70]. Free of COP1 repression, the transcription factor HY5 is able to induce and repress the expression of photomorphogenesis- and skotomorphogenesis-related genes, respectively[57]. Several reports have pinpointed the major contribution of the above described light signal transduction pathway for determining tomato fruit yield and nutritional quality[30–35,71,72]. However, regarding *SlBBX* genes, only the locus Solyc01g110180, here named as *SlBBX25*, has been functionally characterised up to date, being described as a COP1-repressed positive regulator of chloroplast biogenesis, whose constitutive overexpression leads to dwarf plants bearing ripe fruits with increased carotenoid content[37]. Thus, it remains to be explored in a broader manner the role of BBX proteins in light-regulated physiological processes in tomato.

Here, in structure group IV and V, which encompasses most of the light-regulated BBX proteins described in *A. thaliana,* 15 tomato sequences were identified (Fig. 1). Then, they were transcriptionally profiled in source and sink leaves, seedling de-etiolation, and along fruit development and ripening (Fig. 3). The comparison of the mRNA accumulation level among the different profiled organs revealed that *SlBBX* transcripts accumulate most in source leaves or cotyledons (Supplementary Fig. S3), which is mostly in line with the profile previously reported in tomato[36]. The vast majority of *SlBBXs* displayed higher amounts of mRNA in source than in sink leaves hinting a correlation with chloroplast number and activity (Fig. 3a). The pattern of mRNA accumulation during seedlings skoto- and photomorphogenesis showed that out of the 15 analysed genes, 8 showed to be induced by light (*SlBBX16*, *SlBBX17*, *SlBBX18*, *SlBBX24*, *SlBBX28*, *SlBBX29*, *SlBBX30* and *SlBBX31*); while only four showed to be light-downregulated (*SlBBX19*, *SlBBX20*, *SlBBX22* and *SlBBX25*) in at least hypocotyl or cotyledon. Two genes showed inversed pattern in response to light in both organs (*SlBBX21* and *SlBBX26*) and one was invariable (*SlBBX23*). These results indicate that tomato BBX genes that belong to structure group IV and V are light responsive, like observed in *A. thaliana*[4], and most are light-induced. The expression pattern of BBX encoding genes in *Solanum tuberosum* during de-etiolation was also addressed and the expression of most of the genes belonging to structural groups IV and V was modulated upon illumination of etiolated leaves[51]. This profile provides further evidences about a link between mRNA levels of BBX proteins from structure groups IV and V and plastid biogenesis and differentiation, revealing that they are affected, to some extent, by the light signalling cascade.

Regarding fruit development and ripening (Fig. 3c), six genes stood out as their transcripts were gradually reduced from green stages towards ripening (*SlBBX16, SlBBX28* and *SlBBX29*) or sharply induced upon this process triggering (*SlBBX19, SlBBX20* and *SlBBX26*), indicating that their expression is also modulated by the plastid developmental stage, i.e. chloroplast to chromoplast transition. Interestingly, with the exception of *SlBBX19* and *SlBBX26*, the mRNA accumulation profile observed here was in agreement with that reported by[36].

Led by the particular pattern found in fruits for *SlBBX16*, *SlBBX19*, *SlBBX20*, *SlBBX26*, *SlBBX28* and *SlBBX29*, together with the occurrence in their promoter regions of binding motifs for TFs involved in the light signalling cascade (i.e. PIF, HY5 and GLK, Supplementary Fig. S4), their transcripts were profiled in genotypes with altered fruit light perception or without proper fruit chloroplast differentiation (Fig. 4). The three *SlBBX* genes downregulated from immature towards ripe stages showed induction by chloroplast maturation and light (Fig. 3a,b) and, except for *SlBBX28* that did not show alterations of its transcript abundance, *SlBBX16* and *SlBBX29* were induced in a SlGLK2- and SlPHY-dependent manner at green stages. SlGLK2, directly and/or indirectly, *i.e.* inducing chloroplasts biogenesis and maintenance[33,55], promoted the mRNA accumulation of *SlBBX16* and *SlBBX29* at green stages of fruit development (Fig. 4). Interestingly, it was shown that SlPHYB2 represses *SlGLK2* mRNA accumulation[30] thus, explaining the inducible effect of SlPHYB2 deficiency on the expression of these genes at green stages (Fig. 4). Finally, *SlPHYA*-silenced fruits displayed reduced number of chloroplasts with limited differentiation of its intermembranous structure[30], which may be associated with the *SlBBX16* and *SlBBX29* downregulation detected in this genotype at MG stage.

The disruption of PHY-mediated light signalling or chloroplast differentiation by the lack of active SlGLK2 attenuated the ripening-associated transcript accumulation of *SlBBX19, SlBBX20* and *SlBBX26.* The minor effects observed in early stages indicate that these genes are rather induced along ripening than repressed during green stages of tomato fruit development. Since the mRNA amount of *SlGLK2* is almost undetectable from breaker

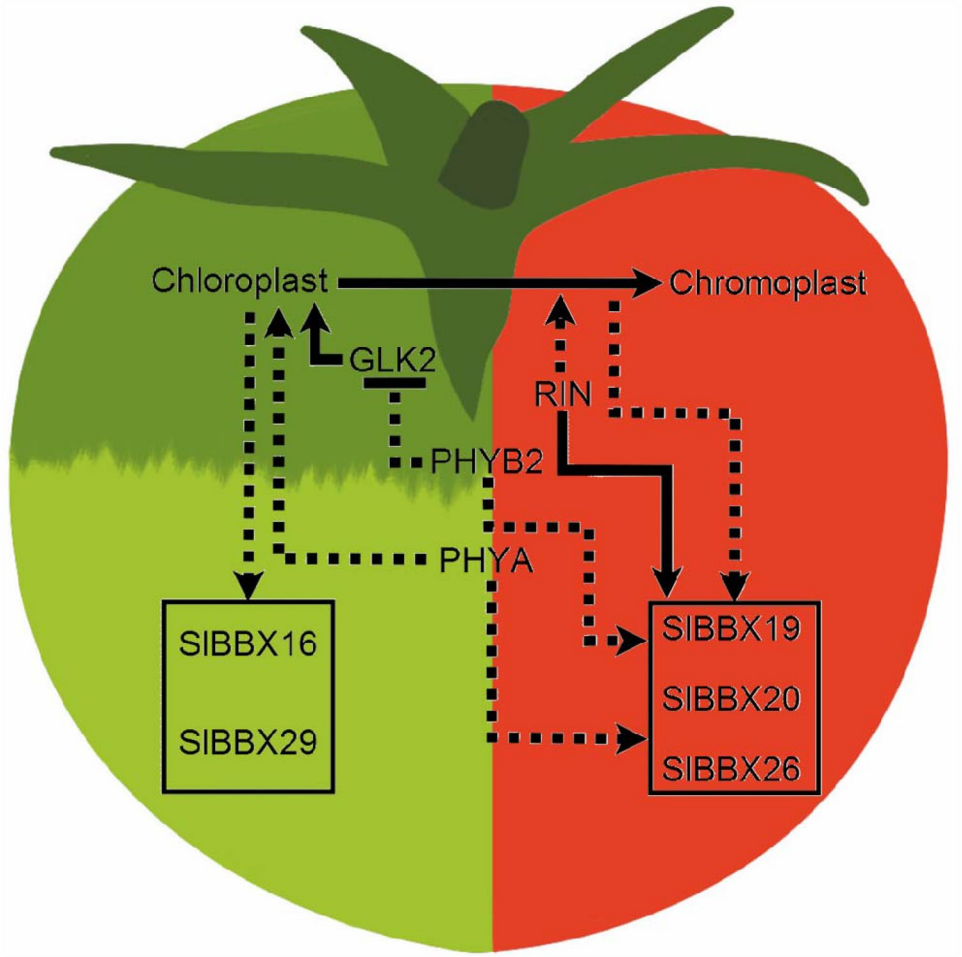

Figure 6. Proposed regulatory network for the control of fruit development- and ripening-associated *SlBBX* genes. During early tomato fruit development, SlGLK2 induces the expression of several genes leading to chloroplast differentiation. SlPHYs have an inverse effect over plastidial development at green stages. While SlPHYB2 inhibits SlGLK2 transcript accumulation, SlPHYA positively controls chloroplast division regulators[30]. Chloroplast biogenesis and maturation positively influence *SlBBX16* and *SlBBX29* transcript accumulation. As the fruit matures, the transcript abundance of both these *SlBBX* genes decreases. Once ripening initiates, the conversion of chloroplast to chromoplast begins and SlRIN accumulates, activating the expression of several ripening associated genes, including *SlBBX19*, *SlBBX20* and *SlBBX26*. During ripening, these three *SlBBX* genes are also positively regulated by SlPHYs, probably, through the repression of several light signalling negative regulators, such as COP1 and PIFs. The absence of properly differentiated chloroplast due to SlGLK2 deficiency attenuates the upregulation of *SlBBX19*, *SlBBX20* and *SlBBX26* during ripening. Continuous lines indicate direct effect; dotted lines indicate that the effects may not be due to direct interaction. Arrow-ended lines indicate induction; bar-ended lines indicate repression.

towards fully ripe stage[33,55], the observed reduction in mRNA level in *Slglk2* mutant for these three genes at ripening stages might be an indirect effect of the fewer and not fully differentiated chloroplasts in this genotype[33,55], which are further converted into chromoplasts as ripening proceeds[73]. In a similar way, *SlPHYA*-silenced fruits also displayed poorly developed chloroplasts in the green stages[30] that, as aforementioned, might lead to the observed reduction in the transcription of the three *SlBBX* genes. Interestingly, the observed downregulation of *SlBBX19* in the lack of PHYA or PHYB2 was also reported for its *A. thaliana* ortholog, *AtBBX19*, in *AtphyA* and *AtphyB* mutant seedlings[12]. As chlorophyll degrades, the chlorophyll self-shading effect is reduced allowing the pass of sunlight through the flesh of green fruit. Light shifts the photoequilibrium of PHYs to the active form promoting the inactivation of their downstream negative effectors SlPIFs and leading to the upregulation of light-dependent ripening associated genes[31,72]. As PIF-binding motifs were identified in *SlBBX19*, *SlBBX20* and *SlBBX26* promoters (Supplementary Fig. S4), these TFs that are altered in *SlphyA* and *SlphyB2*[30] might downregulate the accumulation of these *BBX* transcripts in the PHY deficient lines.

Moreover, the ripening-associated mRNA accumulation of *SlBBX19, SlBBX20* and *SlBBX26* raised the hypothesis of the involvement of the master regulator of tomato fruit ripening SlRIN[61] in the regulation of these genes. Indeed, in the promoter region of all three genes, RIN-binding motifs were found (Fig. 5a) and, by ChIP-qPCR, the direct binding of SlRIN was confirmed (Fig. 5b). This is in line with the previously reported ChIP-Seq results

that showed the direct interaction between SlRIN and *SlBBX20* promoter[63], and also with the reduced mRNA amount of this gene in *SlRIN*-silenced fruits[74]. Altogether, these results indicate that *SlBBX19, SlBBX20* and *SlBBX26* are light- and SlRIN-regulated, playing a role in tomato fruit ripening.

Collectively, data obtained here provided a robust phylogenetic analysis of BBX proteins, giving a new perspective of the events that led to the diversification of these proteins in six structure groups. A comprehensive transcriptional profile of 15 *SlBBX*s revealed a correlation of mRNA amounts with the state of chloroplast development, as well as their regulation by the light signalling cascade. Additionally, a more detailed profiling in fruits led to the identification of three putative SlRIN-regulated ripening-associated *SlBBX* genes and other three loci associated with the early fruit development (Fig. 6). These results give insights on putative roles of SlBBX proteins in other light-regulated physiological process aside seedling photomorphogenesis and allow the identification of putative candidates for further characterization that may affect tomato fruit development and/or ripening.

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Author contributions

B.S.L. designed and performed most of the experiments, analysed the data and wrote the article with contributions of all the authors; M.J.O., L.S., R.T.A.W., A.C.D.L. and D.R. performed the experiments. L.F. designed the experiments, contributed to data analysis and complemented the writing; M.R. designed the experiments, contributed to data analysis and wrote the article with contributions of all the authors.

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Competing interests

The authors declare no competing interests.

Additional information

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7. ANEXO 2

Iniciadores utilizados

NOME	SEQUÊNCIA	*LOCUS*
Construção dos vetores e genotipagem		
SlEXPRESSED	F- TGGGTGTGCCTTTCTGAATG	Solyc07g025390
	R- GCTAAGAACGCTGGACCTAATG	
M13	F- GTAAAACGACGGCCAG	
	R- GGAAACAGCTATGAC	
NPTII	F- GAACAAGATGGATTGCACGC	
	R- GAAGAACTCGTCAAGAAGGC	
35S-Promotor	CCCACTATCCTTCGCAAG	
35S-Terminador	CCAAAATCCAGTGACCTGCA	
SlBBX28-RNAi	F- GGAGGAAATTGATTTGGAAGATATTCAAG	Solyc12g005660
	R- GATTCTATCGGGTCGGGTTG	
RT-qPCR		
SlTIP41	F- GCTGCGTTTCTGGCTTAGG	Solyc10g049850
	R- ATGGAGTTTTTGAGTCTTCTGC	
SlEXPRESSED	F- TGGGTGTGCCTTTCTGAATG	Solyc07g025390
	R- GCTAAGAACGCTGGACCTAATG	
SlBBX28-RT-qPCR	F- GACAGAGAAGAGGAAGAGAAGG	Solyc12g005660
	R- CCACCGTCGCTGAACATC	
SlYUC8A	F- ACATCTTCCACCCTCTCTTTACT	Solyc06g008050
	R- TGAAAGCAGAACACGGGC	
SlYUC8C	F- TTGCTACTGGGGAGAATGCC	Solyc09g064160
	R- ACCAACGACCACCACTTTCT	
SlSFT	F- GTTGTTGGTCGTGTGGTAG	Solyc03g063100
	R- ACTTCAACCCTTGGCTGGTT	
SlBBX29	F- GGTCCCACTGTTTCTGTTTG	Solyc02g079430
	R- CATCATCTTCTTCTTCTTCCG	
SlBBX31	F- GTTTGTGTTGGGACTGTGATG	Solyc07g053140
	R- GGTGGAGGCGTCGTATTTGAC	
SlFUL2	F- ATCTCTGTGCTTTGCGATGC	Solyc03g114830
	R- GCTCTTTCATACTCAATGTGTC	

F: Senso, R: Ante senso *LOCUS*: ID do gene de acordo com o banco de dados do Sol Genomics Network (https://solgenomics.net)