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**Manipulação da senescência foliar e da degradação de
clorofila visando o melhoramento de frutos**

**Manipulation of leaf senescence and chlorophyll degradation
aiming fruit improvement**

Tese apresentada ao Instituto de Biociências da
Universidade de São Paulo, para a obtenção de Título de
Doutor em Ciências, na Área de Botânica.

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Laboratório de Genética Molecular de Plantas

São Paulo

2017

Ficha Catalográfica

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Manipulação da senescência foliar e da degradação da clorofila visando o melhoramento de frutos.

Número de páginas: 161

Tese (Doutorado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Botânica.

1. Tomate 2. *Solanum lycopersicum* 3. Senescência 4. Tocoferol 5. Vitamina E 6. Produtividade 7. Clorofila

Universidade de São Paulo. Instituto de Biociências. Departamento de Botânica.

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To the ones I care the most,

I dedicate

The creation of a single world comes from a huge number of fragments and chaos.

Hayao Miyazaki

THANKFULNESS

Once you meet someone, you never really forget them. in Spirited Away
Life is suffering. It is hard. The world is cursed. But still you find reasons to keep on living. in Princess Mononoke
We each need to find our own inspiration. Sometimes it's not easy. in Kiki's Delivery Service
The smallest act of kindness is worth more than the grandest intention. Oscar Wilde

There are many people I am thankful, my family, friends and colleagues. They all, in different ways, helped me to carry on and finish everything that is part of this thesis. Each everyday act defines how much each person is important to me and how I cherish their support, so I don't find the urge to name everyone, especially as it would be woeful if I miss a name.

I must also thank the Botany Department of Biosciences Institute of University of Sao Paulo for the academic and structural support, and FAPESP for the grant and financial support.

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RESUMO

As folhas, para a maioria das espécies vegetais, são o principal órgão responsável pela fixação de carbono. Durante o desenvolvimento foliar, o potencial fotossintético aumenta até a folha atingir a sua maturidade. Consequentemente, no momento que o programa de senescência se inicia, a folha apresenta a maior taxa de fotossíntese, a qual passa então a declinar conforme o cloroplasto se desorganiza e a maquinaria fotossintética é degradada. Apesar da redução na fixação de carbono, o catabolismo de macromoléculas possibilita a remobilização de nutrientes para os órgãos dreno em desenvolvimento. Neste contexto, atrasar a senescência destaca-se como uma promissora estratégia para aumento da produtividade, uma vez que estende o período de máxima fixação de carbono das folhas. Outra estratégia que tem recebido atenção por, potencialmente, regular a capacidade fotossintética e afetar a qualidade nutricional dos órgãos coletáveis é a manipulação da degradação da clorofila. Durante o catabolismo deste pigmento, o fitol liberado é reciclado podendo ser armazenado (*i.e.* na forma de ésteres de fitil com ácidos graxos), ser utilizado na síntese de novas moléculas de clorofila ou ser incorporado na rota biossintética de tocoferóis. Estes últimos compostos, por seu potencial antioxidante, possuem alto valor nutracêutico. No entanto, a maior parte dos estudos sobre senescência e degradação de clorofila foi realizada na planta modelo *Arabidopsis thaliana* ou em gramíneas, tornando escassas as informações relativas a plantas com frutos carnosos de interesse para a dieta humana. Nesse âmbito, o tomateiro, *Solanum lycopersicum*, é um excelente modelo de estudo não apenas pela disponibilidade de recursos genético e genômicos, mas também pela importância agrônoma e nutricional desta espécie. Assim, este trabalho pretende expandir o conhecimento acerca dos efeitos da manipulação da degradação de clorofila e da senescência sobre o metabolismo e produtividade do tomateiro, bem como sobre a qualidade nutricional dos frutos. De modo a se avaliar as consequências de alterações na degradação de clorofila, iniciou-se por identificar e caracterizar em tomateiro as enzimas clorofilase e feofitinase, as quais catalisam a defitilação da molécula de clorofila. Uma vasta análise filogenética, evolutiva e transcricional permitiu a identificação de dois grupos de clorofilases, um dos quais estaria envolvido na plasticidade de respostas a estímulos e o outro na homeostase dos níveis de clorofila. Já para feofitinase, somente um grupo foi identificado, o qual está relacionado a processos fisiologicamente programados que levam à degradação de clorofila (*i.e.* senescência foliar e amadurecimento de frutos). Dado o panorama obtido, a feofitinase foi escolhida para ser constitutivamente silenciada de modo a se avaliar as consequências para o metabolismo de folhas e frutos. Como consequência do silenciamento, as linhagens transgênicas mostraram-se incapazes de degradar clorofila durante a senescência, mas, embora com um atraso nas etapas iniciais, a degradação ao longo do amadurecimento de frutos não

foi comprometida. Diversos parâmetros fotossintéticos e bioquímicos foram indicativos de fotoinibição, possivelmente em virtude de uma deficiência na reciclagem da clorofila em folhas. Isto acarretou em um aumento na exportação de açúcares para frutos, incrementando a concentração de açúcares solúveis nos frutos maduros, que, em contrapartida, resultou na queda nos teores de carotenoides. A queda nestes compostos antioxidantes foi, ao menos parcialmente, compensada por um aumento nos níveis de tocoferóis. Os resultados indicaram que a feofitinase possui um papel além da degradação de clorofila associada à senescência, e que sua manipulação leva ao desenvolvimento de frutos com maior teor de açúcares solúveis e de tocoferóis ao custo da redução no de carotenoides. Desta forma, estas evidências suportam a manipulação da clorofila como estratégia para o melhoramento de frutos carnosos. Para investigar o efeito da senescência sobre a produtividade e qualidade de frutos foi escolhido o fator de transcrição ORESARA1, o qual está amplamente caracterizado em *A. thaliana* e é considerado um regulador chave no desencadeamento deste processo. A partir de uma extensa análise filogenética e da caracterização de sua regulação, um putativo ortólogo foi selecionado como alvo para silenciamento. Como consequência desta manipulação, folhas apresentaram os níveis de clorofila incrementados. Além disto, taxas fotossintéticas maiores que as do genótipo controle foram mantidas por maior tempo indicando que a iniciação da senescência foi retardada. Assim, estas plantas produziram um maior número de frutos, consequentemente, aumentando o índice de colheita dessas linhagens. Os frutos maduros apresentaram maiores teores de açúcares solúveis e de tocoferóis. Os resultados demonstraram que o retardo do início da senescência é uma estratégia efetiva para aumento da produtividade de tomateiro. Coletivamente, os resultados obtidos aprofundam o conhecimento acerca dos impactos da degradação de clorofila e senescência sobre o metabolismo de plantas com frutos carnosos, além de prover estratégias para se incrementar a produtividade e a qualidade nutricional de frutos.

ABSTRACT

Leaves are responsible for the majority of the fixed carbon in most plant species. Along leaf development, the photosynthetic capacity increases until the organ reaches maturity. Consequently, at the onset of senescence the leaves have the highest photosynthetic activity, then, as the chloroplasts are dismantled and the photosynthetic machinery is degraded, leaves gradually lose the rate of carbon assimilation. Although the capacity to fix carbon declines as senescence progresses, nutrient remobilization from macromolecule degradation nourishes the developing sink organs. In this regard, delaying leaf senescence stands out as a promising strategy to increase plant yield as extends the window of time with maximum carbon fixation rate. Another approach that is receiving much attention is the manipulation of chlorophyll degradation once it potentially regulates photosynthetic capacity and affects the nutritional quality of harvestable organs. As chlorophyll is degraded, the released phytol is recycled and can be either stored (*i.e.* as fatty acid phytol esters), used for chlorophyll synthesis or be incorporated in tocopherol biosynthesis. Tocopherols have high nutraceutical value due to their antioxidant properties. However, the majority of the studies regarding senescence and chlorophyll degradation were carried out in the model plant *Arabidopsis thaliana* or grasses, creating a knowledge gap about these processes in fleshy fruit-bearing plants of human diet interest. In this regard, the tomato, *Solanum lycopersicum*, is an excellent model not only for the genetic and genomic resources, but also for its agronomic and nutritional importance. Thus, this project aims to extend what is known about the effects of chlorophyll degradation and senescence manipulation over the metabolism and yield of tomato plants, as well as fruit nutritional quality. In order to evaluate the consequences of alteration in chlorophyll degradation, first the enzymes chlorophyllase and pheophytinase, both capable of dephytylating the chlorophyll molecule, were identified and characterised. An extensive phylogenetic, evolutive and transcriptional analysis allowed the identification of two groups of chlorophyllases, one putatively involved in the response to different stimuli, while the other may act in chlorophyll homeostasis. As for pheophytinase, only one group was identified, being related to physiologically programmed processes that trigger chlorophyll degradation (*i.e.* leaf senescence and fruit ripening). Given this scenario, pheophytinase was chosen to be constitutively knocked-down in order to evaluate the effects over the metabolism of leaves and fruits. As consequence of this manipulation, transgenic plants were impaired in the leaf senescence-associated chlorophyll breakdown, but, although with an initial delay, fruit ripening-associated degreening was not compromised. Several photosynthetic and biochemical parameters were signs of photoinhibition, possibly due to a deficiency in chlorophyll recycling in leaves. This led to an increase in sugar exportation towards fruits, ultimately increasing soluble sugar content of

ripe fruits. However, as a consequence, carotenoid levels were reduced, what, at least partially, it was compensated by an increase in tocopherol content. The results indicated that pheophytinase plays a role beyond senescence-associated chlorophyll degradation and its manipulation led to the development of fruit with increased soluble sugars and tocopherols at the cost of lowering carotenoid levels. Thus, these evidences support the manipulation of chlorophyll breakdown as a strategy for improvement of fleshy fruit plants. In order to address the effects of senescence over yield and fruit quality, the transcription factor ORESARA1, which has been widely characterised in *A. thaliana* and is considered a key regulator of senescence initiation, was targeted. After a comprehensive phylogenetic analysis and the characterization of the regulatory mechanisms, one putative ortholog was selected to be silenced. As consequence of this manipulation, leaves displayed increased chlorophyll content. Moreover, as senescence was delayed, the extent of photosynthetic activity of the leaves was also expanded. As the number of fruits was increased in the knockdown lines, this reflected in an increment in the harvest index. Ripe fruits accumulated more soluble sugars and tocopherols. Collectively, the results support the manipulation of leaf senescence as a strategy for tomato yield improvement. Altogether, data obtained enhance the knowledge about the impacts of chlorophyll degradation and leaf senescence over the metabolism of fleshy-fruit plants, providing strategies to increase yield and nutritional quality of fruits.

GENERAL INTRODUCTION

1. Senescence: regulation and manipulation for crop improvement

Plants, as sessile organisms, must cope with environmental changes by physiological adjustments. The capability to do so is an essential evolutionary trait as it affects directly the fitness in a specific ecological setting (Fenner, 1998). These adjustments may have different magnitudes accordingly to the stimulus, varying from minor metabolic shifts to changes in the developmental program (Sultan, 2000). In this context, leaves play a major role. As these organs are responsible for most of the light-harvested energy, leaves are involved in light perception, which is a major abiotic factor that regulates plant development (Gyula *et al.*, 2003).

Light-driven adjustments are particularly important in the regulation of carbon fixation. By controlling the phyllotaxies, chloroplast differentiation and positioning and, leaf shape, light absorption is optimized (Buschmann *et al.*, 1978; Smith, 2000; Weston *et al.*, 2000; Inoue *et al.*, 2008, Tsukaya, 2013). During leaf development, the rate of carbon fixation and, consequently, the availability of photoassimilates to the developing parts of the plant gradually increases until it reaches the maximum. Then, at a certain point, the interplay of environmental and endogenous signals triggers the leaf senescence and the rate of photosynthesis starts to decrease (Fig. 1).

Figure 1. Leaf lifespan dynamics. Representation of the chlorophyll content (green) and net carbon assimilation (blue) during leaf lifespan. Both parameters increase gradually as the leaf grows and develop. At the onset of senescence occurs, the net carbon assimilation is at its most, but declines afterwards as the chloroplast structure is disassembled as senescence progresses. Adapted from Thomas and Howarth (2000).

Senescence is an age-dependent process that can occur at different levels (*i.e.* cellular, tissue, organ or organism level) and leads to a massive catabolism program that enables nutrient remobilization and, ultimately, death or end of the organ lifespan (Noodén, 1988). Yet, not only age, but also many internal and external factors, such as fruit ripening, seed setting, temperature, light,

drought, nutrient availability and pathogen infection, can trigger the senescence program (Noodén and Guiamet, 1989; Hensel *et al.*, 1993; Nam, 1997).

The capacity to initiate a coordinated catabolism program in response for both, endogenous and exogenous factors, is critical for improving the fitness (Lim *et al.*, 2007). As chloroplasts are the first organelles to be degraded (Dodge, 1970), the recycling of chloroplasts lipids and proteins is an important source of nutrients for sink organs. For example, nitrogen salvaging can account for up to 80% of the final content in grains and the fatty acid content can decrease up to 80% in senescent leaves (Ischebeck *et al.*, 2006; Padham *et al.*, 2007; Yang and Ohlrogge, 2009; Watanabe *et al.*, 2013; Girondé *et al.*, 2015). Since, during their lifespan, chloroplasts are the sources of carbon and are major sources of nitrogen while being degraded, it is expected a fine tuning of leaf senescence to guarantee the proper carbon and nitrogen partitioning to developing organs.

Differently to chloroplasts, the nucleus and mitochondria are only degraded in later senescence stage (Thomson and Platt-Aloia, 1987; Noodén, 1988), as, along the process, the cell remains transcriptionally and metabolically active. Thus, in the early stages of senescence, chloroplasts are disrupted and carbon assimilation ceases while the nucleus remains active, shifting the genetic programme that, ultimately, leads to cell death (Yen and Yang, 1998; Quirino *et al.*, 2000; Simeonova *et al.*, 2000; Cao *et al.*, 2003).

For all the above mentioned, the onset of senescence stands on the point of equilibrium between vegetative growth, by means carbon fixation, and chloroplast dismantling and nutrient remobilisation. As the delay or impairment of leaf senescence has been proposed as a strategy to improve plant yield (Rossi *et al.*, 2015), it must be evaluated to which extend it is possible to retard senescence initiation without compromising nutrient remobilisation.

Plants deficient in distinct aspects of senescence process were characterized and named as *stay green* genotypes (Thomas and Howarth, 2000; Thomas and Ougham, 2014). This classification includes a great diversity of mutations that affects from chlorophyll catabolism to key regulators of the senescence initiation or progression (Thomas and Ougham, 2014). For most of these mutants, the genetic bases that underneath the stay green phenotype remain unknown. Nonetheless, several genes that are upregulated during senescence, named senescence-associated genes (SAGs), have been identified (Buchanan-Wollaston, 1997; Nam, 1997; Gepstein *et al.*, 2003; Guo *et al.*, 2004; Lin *et al.*, 2004; Buchanan-Wollaston *et al.*, 2005). The ones firstly characterised has backed strategies for yield improvement (Guo and Gan, 2014;), specially *SAG12* that encodes a senescence-specific vacuolar cysteine proteinase which was first identified in *Arabidopsis thaliana* (Lohman *et al.*, 1994).

Due to the high specificity of *SAG12* promoter, it has been widely used to drive the expression of *ISOPENTENYL TRANSFERASE* (*IPT*) aiming to delay leaf senescence (Gan and Amasino, 1995; Guo and Gan, 2014). This approach relies upon the inhibitory effects of cytokinins over senescence (D'Agostino and Kieber, 1999), as the first-committed and rate limiting step of this hormone synthesis is catalysed by *IPT* (Barry *et al.*, 1984). Increasing the levels of this enzyme at an early stage of senescence promotes a cytokinin-driven blockage of this process progression and a reversion to the cell vegetative program (Gan and Amasino, 1995).

The effectiveness of *P_{SAG}:IPT* expression was first addressed in *Nicotiana tabacum* rendering plants with delayed senescence, increased biomass and no morphogenetic abnormalities as consequence of the ectopic cytokinin synthesis (Gan and Amasino, 1995). However, if grown under limited nitrogen supply, developing organs were penalized, showing nutritional deficiency phenotypes, such as low chlorophyll content, due to the shortage of this nutrient (Jordi *et al.*, 2000). The success of this approach led to the usage of promoters of other senescence-associated genes to drive *IPT* expression, such as the one from the *SENESCENCE ASSOCIATED RECEPTOR PROTEIN KINASE* (*SARK*) (Rivero *et al.*, 2009; Peleg *et al.*, 2011, Kuppu *et al.*, 2013), *Solanum lycopersicum* *AGPase S1* (Luo *et al.*, 2005), *Gossypium hirsutum* *CYSTEINE PROTEINASE* (*Ghcysp*) (Liu *et al.*, 2012), *A. thaliana* *SAG13* (Swartzberg *et al.*, 2006) and *Oryza sativa* *SAG39* (Liu *et al.*, 2010). Besides *N. tabacum*, increase in biomass and/or yield was also reported in other plants, such as *A. thaliana* (Zhang *et al.*, 2000), *Brassica napus* (Luo *et al.*, 2002), *G. hirsutum* (Liu *et al.*, 2012), *Arachis hypogaea* (Qin *et al.*, 2011) and *O. sativa* (Peleg *et al.*, 2011).

Although the *P_{SAG}:IPT* strategy has proven to be successful to delay senescence and increase yield, the activation of *SAG* transcription occurs after the signal that triggers the onset of senescence. Thus, another interesting approach to manipulate leaf senescence targets the transcription factors (TFs) involved in the initiation of the process (Breeze *et al.*, 2011; Li *et al.*, 2012; Guo, 2013; Woo *et al.*, 2013). In this regard, three families of TFs have been characterised as major senescence regulators, the MYB (Warner *et al.*, 2007; Guo and Gan, 2011; Zhang *et al.*, 2011), the WRKY (Zentgraf *et al.*, 2010) and the NAC (Guo and Gan, 2006; Kim *et al.*, 2009; Lee *et al.*, 2012; Hickman *et al.*, 2013).

The NAM/ATAF/CUC (NAC) family includes a great number of TF that are involved in many processes along plant development (Mitsuda *et al.*, 2005; Olsen *et al.*, 2005; Puranik *et al.*, 2009, Zhu *et al.*, 2012; Maugarny-Cales *et al.*, 2016) and has received special attention in the past few years by

the characterisation of NAC-domain containing proteins as key regulators of senescence (Thomas and Ougham, 2014; Kim *et al.*, 2016), particularly ORESARA1 (ORE1).

The delayed leaf senescence phenotype allowed the identification of the *A. thaliana ore1* mutant (Oh *et al.*, 1997), which was later characterised as defective in a NAC-domain containing protein. The functional characterisation of *AtORE1* demonstrated that the encoded protein is a key TF involved in senescence triggering (Kim *et al.*, 2009; Balazadeh *et al.*, 2010a; 2010b; Du *et al.*, 2013; Matallana-Ramirez *et al.*, 2013; Rauf *et al.*, 2013; Kim *et al.*, 2014; Garapati *et al.*, 2015; Qiu *et al.*, 2015; Zhang *et al.*, 2015). *AtORE1* transcript accumulation is induced by several senescence-promoting stimuli, such as ethylene, abscisic acid and darkness, while repressed by light and *miR164* (Kim *et al.*, 2009). Once translated, the protein induces the expression of *SAGs* while repressing chloroplast maintenance-related genes by physically interacting and inactivating GOLDEN 2-LIKE (GLK) TF (Rauf *et al.*, 2013) (Fig. 2).

Figure 2. ORE1 regulatory network. ORE1 promotes senescence by directly activating senescence-associated genes while represses the chloroplast maintenance signal by inactivating GOLDEN 2-LIKE transcription factors. Diverse senescence inducing stimuli, such as hydrogen peroxide (H₂O₂), nitric oxide (NO), age, abscisic acid (ABA), ethylene and darkness induces ORE1 transcription while it is repressed by light signal and the transcripts are regulated by the microRNA miR164. Blue lines, positive regulation; red lines, negative regulation; grey lines, physical interaction; dashed line, presumed regulation without direct evidence; yellow squares, stimuli; orange squares, regulators involved in the signal transduction; purple circles, transcription factors; PHYB, PHYTOCHROME B (red/far red light receptor); PIF, PHYTOCHROME

INTERACTING FACTOR (transcription factor negatively regulated by light); EIN, ETHYLENE INSENSITIVE (ethylene signal transduction factor); ABI, ABA INSENSITIVE (ABA signal transduction factor); ATAF1, ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR 1 (NAC domain-containing protein involved in ABA signalling).

Although the knowledge regarding senescence keeps increasing, this process has been mostly addressed in the model plant *A. thaliana* or in grasses that undergo whole plant senescence after the seed filling phase, thus, there is a gap of knowledge about the regulation and the impacts of its manipulation in other species. This is particularly interesting in freshly fruit bearing plants that do not undergo whole plant senescence during fruit/seed maturation. Moreover, many events that occur during senescence such as the degradation of chlorophyll are still not fully elucidated, but are critical for the proper nutrient remobilisation.

2. Chlorophyll degradation

Probably the most notorious aspect of leaf senescence is the loss of greenness turning into a yellowish tone, this symptom is even an established senescence marker (Lim *et al.*, 2007). However, there is more involved in this phenomenon than just a cosmetic change in leaf colour.

The chlorophyll, as most tetrapyrrole molecules, is a compound that can lead to oxidative damage once, if not properly regulated, excitation by light can generate reactive oxygen species (open Camp *et al.*, 2003). Thus, it is paramount a fine regulation over its metabolism. As senescence takes place, the cell program is shifted from chloroplast maintenance to catabolism and the expression of several proteases and chlorophyll-degrading enzymes is enhanced, leading to the disassemble of chlorophyll-protein complexes and increase in the nutrients available for remobilisation to sink organs (Ougham *et al.*, 2008; Havé *et al.*, 2016).

As chlorophyll is a complex molecule, composed by a tetrapyrrole head with a central magnesium ion condensed to a phytol-derived side chain (Tanaka and Tanaka, 2007), its degradation requires several steps. In fact, chlorophyll breakdown can be divided in two steps. The first occurs within the chloroplasts and comprehends the removal of the magnesium ion, dephytylation and catabolite inactivation. The second step takes place in the cytosol and vacuole and involves the modifications of the linear tetrapyrrole molecule until its final storage in senescing vacuoles (Christ and Hörtensteiner, 2014). These steps are part of the so called “PHEOPHORBIDE A

OXYGENASE/phyllobilin (PAO/PB) pathway” and is considered the canonical chlorophyll degradation pathway (Hörtensteiner, 2013).

In order to be accessible for the enzymes, first the chlorophyll must be released from the protein complexes that are associated with this pigment. The STAY-GREEN PROTEIN (SGR) not only disrupt the chlorophyll-protein interaction, but also, by physically interacting with them, it brings together the chlorophyll-degrading enzymes, creating a multiprotein complex that channel all the reactions until the catabolite is inactivated (*i.e.* is no longer a potential source of reactive oxygen species) (Sakuraba *et al.*, 2012; 2013) (Fig. 3).

The chlorophyll-degrading multiprotein complex only catabolites chlorophyll *a* due to the PAO affinity (Pružinská *et al.*, 2003, Hörtensteiner and Kräutler, 2011). So, chlorophyll *b* molecules must be reduced by the subsequent action of CHLOROPHYLL-B REDUCTASE (CBR) (Kusaba *et al.*, 2007) and 7-HYDROXYMETHYL CHLOROPHYLL A REDUCTASE (HCAR) (Meguro *et al.*, 2011) to chlorophyll *a* (Fig. 3). Further, the magnesium ion and phytol chain are released from the chlorophyll molecule, then the subsequent reactions modify just the tetrapyrrole head.

Until recently, it was thought that chlorophyll was first dephytylated by the action of CHLOROPHYLLASE (CLH) (Tsuchiya *et al.*, 1999), releasing phytol and chlorophyllide, the latter was then dechelated, producing the cyclic tetrapyrrole, pheophorbide *a* (Fig. 3). However, evidences from genotypes defective for CLH questioned the role of this enzyme in senescence-associated degreening (Schenk *et al.*, 2007). This led to the identification of another dephytylase, the PHEOPHYTINASE (PPH), that removes the phytol chain from pheophytin *a*, the magnesium-free chlorophyll catabolite, generating pheophorbide *a* (Schelbert *et al.*, 2009) (Fig. 3). As *pph* mutant is impaired in senescence-associated chlorophyll degradation (Schelbert *et al.*, 2009) and PPH, but not CLH, is able to physically interact with SGR and other chlorophyll degrading enzymes (Sakuraba *et al.*, 2012; 2013), it is now considered to be the senescence-associated dephytylating enzyme (Hörtensteiner, 2013; Christ and Hörtensteiner, 2014).

Regarding the removal of the magnesium ion, it has for long been speculated whether a metal chelating substance or an enzyme would be response for this reaction (Hörtensteiner, 2013). Recently it was elucidated that SGR protein is responsible for the dechelating activity of chlorophyll *a* rendering pheophytin *a* (Shimoda *et al.*, 2016) (Fig. 3).

Thus, after dephytylation, either by CLH or by PPH, pheophorbide *a* is linearized by the action of PAO (Pružinská *et al.*, 2003) rendering the red chlorophyll catabolite, which is inactivated by the

by CHLOROPHYLASE (CLH), releasing the phytol side chain and producing chlorophyllide *a* (Chlide *a*), which is chelated by a still unknown substance, rendering Pheide *a*. Either way, Pheide *a* is linearized into the red chlorophyll catabolite (RCC) by the action of PHEOPHORBIDE A OXYGENASE (PAO), then, this catabolite is inactivated by the reaction catalysed by the RED CHLOROPHYLL CATABOLITE REDUCTASE (RCCR). Finally, the primary fluorescent chlorophyll catabolite (pFCC) is produced and will be translocated to the cytosol where it will be modified and stored in the vacuole. Enzyme are positioned accordingly to the physical interaction between each of them. Red arrowheads indicate the order of reactions when the compound produced is not shown. 7-HMChl, 7- hydroxymethyl chlorophyll *a*; FCC, fluorescent chlorophyll catabolite; NCC, non-fluorescent chlorophyll catabolite. The square colours indicate the fluorescence spectrum of the compound. Adapted from Sakuraba *et al.* (2013).

3. Plastid isoprenoid-derived compounds: chlorophyll, tocopherols and carotenoids

The plastidial MEP pathway produces geranylgeranyl diphosphate (GGDP), which is a branching point that diverts the metabolic flux towards the biosynthesis of carotenoids. Further, the geranylgeranyl diphosphate reductase (GGDR) converts GGDP to phytyl diphosphate (PDP), a limiting precursor for the synthesis of chlorophyll and tocopherols (Lichtenthaler, 1999; DellaPenna and Pogson, 2006; Quadrana *et al.*, 2013; Almeida *et al.*, 2015) (Fig. 4). As mentioned above, chlorophyll degradation releases phytol, which can be recycled producing PDP. Besides the importance for chlorophyll homeostasis, phytol salvage is the main source of PDP for tocopherol biosynthesis (Ischebeck *et al.*, 2006; vom Dorp *et al.*, 2015; Almeida *et al.*, 2016).

Figure 4. Biosynthetic pathway of carotenoids, chlorophylls and tocopherols. The metabolism of chlorophylls (green), carotenoids (orange) and tocopherols (blue) is tightly linked by geranylgeranyl pyrophosphate (GGDP). This isoprenoid is produced by the plastidial methylerythritol 4-phosphate (MEP) pathway is a precursor for carotenoids synthesis and, once reduced to phytyl-diphosphate (PDP), for chlorophylls and tocopherols. From chlorophyll degradation, the phytol produced can be phosphorylated into phytyl-phosphate (PP) that can be further phosphorylated into PDP that can feed *de novo* chlorophyll synthesis or the tocopherol pathway. Dotted lines indicate that intermediate steps are omitted. Adapted from Almeida *et al.*, 2015.

Both, carotenoids and tocopherols, are essential for the protection of plant cells against oxidative damage, thus, preserving the integrity of the reaction centres and, consequently, photosynthetic activity (Krieger-Liszkay *et al.*, 2008; Esteban *et al.*, 2009). As consequence of chlorophyll photo-excitability, the reactions centres of photosystem I and II become major generating sites of reactive oxygen species (Asada, 2006; Krieger-Liszkay, 2005; Triantaphylidès and Havaux, 2009).

Tocopherols scavenge oxygen singlets and inhibit the propagation of lipid peroxidation that can disrupt the membranous structure of the cell (Munné-Bosch and Alegre, 2002; Havaux *et al.*, 2005; Kruk *et al.*, 2005; Krieger-Liszkay *et al.*, 2008).

In the case of carotenoids, each type of this major pigment family acts in a different way to protect the cellular function (Britton, 1998; Hirschberg, 2001). For instance, β -carotene is component of the photosystem II (Loll *et al.*, 2005) and not only quench singlet oxygen, but also acts in the cyclic electron flow (Krasnovsky, 1997; Miyake *et al.*, 2002; Telfer *et al.*, 2003; Frank and Brudvig, 2004; Krieger-Liszkay *et al.*, 2008; Telfer, 2014). The xanthophylls, specially violaxanthin and zeaxanthin that are part of the so-called xanthophyll or violaxanthin cycle, have a major role as component of the non-photochemical quenching. Their capability to dissipate the excess of energy absorbed by the chlorophylls relies on the enzymatic de-epoxidation of violaxanthin into zeaxanthin, afterwards, the pool of violaxanthin is restored by epoxidation of zeaxanthin (Demmig-Adams, 1990; Young *et al.*, 1997; Dreuw *et al.*, 2003; Demmig-Adams and Adams, 2006; Latowski *et al.*, 2011; Jahns and Holzwarth, 2012). Another xanthophyll cycle, which is also a component of the non-photochemical quenching, is the lutein epoxide cycle, in which lutein epoxide is reversibly converted into lutein (Bungard *et al.*, 1999; García-Plazaola *et al.*, 2007).

These antioxidants compounds are not only important for the maintenance of plant cellular functions, but also for human health (Hunter and Cahoon, 2007). As humans are incapable of *de novo* synthesis of vitamin A, it must be supplemented by the diet, to which, especially in developing countries, plant carotenoids account for most of the daily intake (West and Darnton-Hill, 2008). Since they are converted to vitamin A by the intestinal cells, carotenoids are classified as provitamin A compounds, however, only of them are known to have provitamin A activity: α -carotene, β -carotene, γ -carotene and β -cryptoxanthin (Yonekura and Nagao, 2007; Grune *et al.*, 2010). From those, β -carotene is the one with highest activity and conversion to vitamin A (Yeum and Russel, 2002; Harrison, 2005; Grune *et al.*, 2010; Haskell, 2012). This carotenoid is particularly of interest since vitamin A deficiency is a public health problem, especially in developing countries such those for Africa and Asia, that can lead to childhood blindness, reduced immunity and increased mortality risk from gastrointestinal diseases and measles (Beaton *et al.*, 1993; Olson, 1994; West and Darnton-Hill, 2008; World Health Organization, 2009; Haskell, 2012). Indeed, to suppress the nutritional deficiency, one of the most well-known approach was the development of the provitamin A-rich golden rice (Paine *et al.*, 2005), yet it is still to be approved for consumption.

β -carotene is not the only carotenoid important for human health, lutein and zeaxanthin accounts together, approximately, 54% of the carotenoid composition of the macular pigment (Bone *et al.*, 1993; Landrum and Bone, 2001) and have also been detected into primate brains (Craft *et al.*, 2004; Vishwanathan *et al.*, 2013). Lutein and zeaxanthin, not only can prevent macular degeneration

that can cause blurred or even loss of the vision (Krinsky *et al.*, 2003; Abdel-Aal *et al.*, 2013), but also recent evidences unravelled a correlation between the concentration of these pigments and the cognitive function of elderly people and people with cognitive-impairing diseases, such as Alzheimer's disease (Johnson *et al.*, 2013; Nolan *et al.*, 2014; Renzi *et al.*, 2014; Vishwanathan *et al.*, 2014).

The effects of tocopherol on human health are less known. These lipid-soluble antioxidants are component of the cell membranes maintaining their integrity and protecting them against oxidative stress. Immune cells are particularly enriched in tocopherols and, it has been recently reported that vitamin E boosts the immune system effectiveness, especially with ageing (Pae *et al.*, 2012; Mocchegiani *et al.*, 2014). Moreover, by scavenging free radicals and, consequently, controlling the oxidative state of the cells, it has been suggested that tocopherols play a role in preserving the genome stability, thus protecting against some types of cancer (Claycombe and Meydani, 2001; Virtamo *et al.*, 2014; Peh *et al.*, 2016), atherosclerosis (Terasawa *et al.*, 2000) and loss of neuron integrity (Ulatowski *et al.*, 2014).

For all the above mentioned, it becomes evident that the metabolism of chlorophyll, carotenoids and tocopherols are entwined and is not only important for plant development and physiology, but also have implications on the nutritional quality of harvestable organs. In this regard, the tomato (*Solanum lycopersicum*) fruit is an interesting model to study the interconnections of these pathways as the fruit ripening couples chlorophyll degradation, tocopherol synthesis and active MEP pathway for carotenoid accumulation (Seymour *et al.*, 2013; Almeida *et al.*, 2015).

4. The tomato, *Solanum lycopersicum*

Tomato is one of the most important crop in the world economy, occupying the eleventh position in the rank of tonnes harvested in 2014, and is the most consumed fruit (*Food and Agriculture Organization of the United Nations*, FAO, <http://fao.org/faostat>, 2016). Also, it is a model plant for the study of fleshy fruit development and metabolism, whose genome has been fully sequenced (Giovannoni, 2004; 2007; Tomato Genome Consortium, 2012).

As briefly mentioned above, during tomato fruit ripening, which is triggered by the ethylene and the respiration burst, a great diversity of metabolic changes occurs that results in the changes in flavour, savour, firmness and colour (Giovannoni, 2004; Carrari and Fernie, 2006; Giovannoni, 2007; Barsan *et al.*, 2010; Bian *et al.*, 2011; Egea *et al.*, 2011; Barsan *et al.*, 2012). Interestingly, the

conversion of chloroplasts into chromoplasts, couples chlorophyll degradation and the enhancement of MEP pathway for carotenoid accumulation (Seymour *et al.*, 2013), both sources of the prenyl precursor for tocopherol biosynthesis (Almeida *et al.*, 2015). Thus, this species stands out as an interesting model to explore the link between chlorophyll, tocopherols and carotenoid. Additionally, tomato is an important source of these compounds as it is highly and widely consumed worldwide (García-Closas *et al.*, 2004; Canene-Adams *et al.*, 2005; Reboul *et al.*, 2006; Jesús Periago *et al.*, 2009; Perveen *et al.*, 2013).

The resulting chemical composition directly affects the commercial value of tomato fruit. Beyond the nutraceutical content (*i.e.* carotenoids and tocopherols), the most important chemical trait for industry is the total soluble solids (*i.e.* *brix*) as it determines the viscosity of the fruit juice. Definitively, fruit number and size are also main traits as they define yield. Thus, several studies attempt to unravel the mechanisms and the genetic determinants that underneath those traits (Baxter *et al.*, 2005; Schauer *et al.*, 2006; Bermúdez *et al.*, 2008; Fernie and Schauer, 2009; Kamenetzky *et al.*, 2010; Klee and Tieman, 2013; Tieman *et al.*, 2017). Indeed, different strategies increased tomato yield (Krieger *et al.*, 2010; Bermúdez *et al.*, 2014; Heldens *et al.*, 2015; Soyk *et al.*, 2017) and nutraceutical content (Römer *et al.*, 2000; Fraser *et al.*, 2001; Davuluri *et al.*, 2005; Enfissi *et al.*, 2005; Fraser *et al.*, 2007; Apel and Bock, 2009), however, it remains to be addressed the impact of alterations in chlorophyll degradation and leaf senescence on plant physiology and fruit production.

5. References

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OBJECTIVES

This work aimed to improve the knowledge regarding the dynamics of chlorophyll dephytylation and the consequence of its manipulation for fruit development and metabolism as well as to elucidate if alterations in leaf senescence is a potential strategy to improve tomato yield. Thus, this thesis had the following objectives:

- I. Identify and characterise the chlorophyll dephytylating enzymes in tomato genome.
- II. Evaluate how perturbation in chlorophyll dephytylation affects tomato plant development and fruit metabolism and nutritional content.
- III. Identify putative leaf senescence regulator and address the physiological consequences of delaying leaf senescence over tomato fruit yield and nutritional value.

CHAPTER I. CHLOROPHYLL DEPHYTYLATING ENZYMES DYNAMICS IN *SOLANUM LYCOPERSICUM*

The results, methodology and discussion of this chapter are presented as a paper published in *Gene*. Supplementary material corresponds to the attachment I.

Lira, B. S., de Setta, N., Rosado, D., Almeida, J., Freschi, L., & Rossi, M. (2014). Plant degreening: evolution and expression of tomato (*Solanum lycopersicum*) dephytylation enzymes. *Gene*, 546(2), 359-366.

Abstract

Chlorophyll is the most abundant pigment on earth and due its high photo-excitability necessitates a tight regulation of its degradation pathway. Chlorophyll breakdown sequentially involves a dephytylation step, tetrapyrrole ring opening and catabolite inactivation to avoid phototoxicity of degradation products, resulting in a linear tetrapyrrole molecule that is stored in the vacuole. There are two possible ways by which the phytol chain can be hydrolysed: directly from the chlorophyll *a* molecule by the action of CHLOROPHYLASE (CLH); or from pheophytin, the magnesium-free chlorophyll catabolite, by the reaction catalysed by PHEOPHYTYNASE (PPH). Although CLH was first identified, cumulative evidences questioned its role in the senescence-associated chlorophyll degradation, leading to the identification of PPH in *Arabidopsis thaliana*. While the plastidial AtPPH is responsible for the senescence-associated chlorophyll degradation, the subcellular localization of AtCLH is arguable and its absence does not impact chlorophyll degradation. In order to address the dynamics of these dephytylating enzymes in tomato leaf senescence and fruit ripening, the genome of *Solanum lycopersicum* was surveyed for genes encoding putative orthologs of these enzymes. While a single copy of PPH-encoding gene was found four copies of CLH-encoding genes were identified. A comprehensive phylogenetic analysis using species with fully sequenced and assembled genomes revealed that CLH group into two clades. An evolutionary analysis of the coding sequences revealed that PPH and Group II of CLH evolved under purifying selection, while Group I of CLH exhibited a relaxed selection, containing codons evolving by both purifying selection and neutral evolution. Moreover, a comprehensive transcription profile upon senescence-inducing treatments and along fruit ripening brought extra insights about the function of these genes. The expression of the CLH belonging to Group II showed correlation with the chlorophyll levels; while the mRNA accumulation pattern of Group I was variable in response to

hormonal treatments and ripening. *SlPPH* transcript levels were upregulated along senescence and ripening. Collectively, the data obtained allowed the proposition of a model in which: *PPH* evolves under a high selective constraint and is associated to chlorophyll degradation in physiologically programmed processes, such as senescence and fruit ripening; Group I *CLH*, which is under a relaxed selective constraint, are responsive to environmental and hormonal stimuli, playing a role in plant adaptation plasticity and; Group II *CLH* that is evolving under high selective constraint acts in chlorophyll recycling.

Plant degreening: evolution and expression of tomato (*Solanum lycopersicum*) dephytylation enzymes

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ARTICLE INFO

Article history:

Received 5 November 2013

Received in revised form 21 May 2014

Accepted 22 May 2014

Available online 24 May 2014

Keywords:

Chlorophyllase

Pheophytinase

Senescence

Ripening

Tomato

ABSTRACT

Chlorophyll is the most abundant pigment on earth and even though it is known that its high photo-excitability necessitates a tight regulation of its degradation pathway, to date there are still several steps in chlorophyll breakdown that remain obscure. In order to better understand the 'degreening' processes that accompany leaf senescence and fruit ripening, we characterized the enzyme-encoding genes involved in dephytylation from tomato (*Solanum lycopersicum*). A single pheophytinase (PPH) gene and four chlorophyllase (CLH) genes were identified in the tomato genome. A phenetic analysis revealed two groups of CLHs in eudicot species and further evolutionary analysis indicated that these enzymes are under diverse selection pressures. A comprehensive expression profile analysis also suggested functional specificity for these dephytylating enzymes. The integrated analysis allows us to propose three general roles for chlorophyll dephytylation: i) PPH, which is under high selective constraint, is responsible for chlorophyll degradation during developmentally programmed physiological processes; ii) Group I CLHs, which are under relaxed selection constraint, respond to environmental and hormonal stimuli and play a role in plant adaptation plasticity; and iii) Group II CLHs, which are also under high selective constraint, are mostly involved in chlorophyll recycling.

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1. Introduction

Chlorophyll (Chl) is responsible for light absorption during photosynthesis, a process upon which life on Earth depends. Chl is composed of a tetrapyrrole ring with a central magnesium ion and a phytol chain and its metabolism can be divided into three phases: biosynthesis, the chlorophyll cycle, and degradation. However, in part due to its complex biochemical structure, various aspects of Chl metabolism are still poorly understood, and this is especially true for those associated with its degradation (Hörtensteiner, 2013). In general, the Chl degradation pathway resembles bacterial detoxification processes, and for many

years it was considered to be a simple Chl detoxification phenomenon (Hörtensteiner, 2006). However, recent studies indicate that the process is more complicated since more Chl derived catabolites are being discovered, although few of these have been assigned a biological function (Hörtensteiner, 2013; Hörtensteiner and Kräutler, 2011).

Chl breakdown sequentially involves a dephytylation step, tetrapyrrole ring opening and catabolite inactivation to avoid phototoxicity of Chl degradation products, resulting in a linear tetrapyrrole molecule that is stored in the vacuole. For many years, it was accepted that dephytylation was catalyzed by chlorophyllase (CLH), an enzyme that hydrolyzes Chl *a* to generate phytol and chlorophyllide (Chlide *a*), a compound that is further converted to pheophorbide *a* (Pheide *a*) by a still unknown metal dechelataase. The enzyme pheophorbide *a* oxygenase (PAO) then opens the tetrapyrrolic backbone structure, producing the red chlorophyll catabolite (RCC) that follows the "PAO pathway" of Chl breakdown until catabolite inactivation (Hörtensteiner, 2013). However, data obtained from experiments with *Arabidopsis thaliana* double mutants demonstrated that CLHs are not required for senescence-related Chl breakdown in vivo (Schenk et al., 2007). Furthermore, subcellular localization analyses have resulted in contradicting observations since *Arabidopsis* CLHs have been reported to be cytoplasmic proteins (Schenk et al., 2007), while it was demonstrated that a *Citrus* CLH resides in the plastid and is post-transcriptionally

Abbreviations: ABA, abscisic acid; Chl, chlorophyll; Chlide, chlorophyllide; CLH, chlorophyllase; dN, non-synonymous distance; dS, synonymous distance; gf, green flesh mutant; LTR, likelihood ratio test; MS, Murashige and Skoog media; Nc, number of codons; PAO, pheophorbide *a* oxygenase; PAR, photosynthetically active radiation; pFCC, primary fluorescent chlorophyll catabolite; Pheide, pheophorbide; Pheo, pheophytin; PPH, pheophytinase; RCC, red chlorophyll catabolite; SAG12, senescence associated gene 12; SE, standard error; SGR, stay green protein; UTR, untranslated regions.

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<http://dx.doi.org/10.1016/j.gene.2014.05.051>

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regulated by cleavage (Azoulay-Shemer et al., 2008, 2011; Harpaz-Saad et al., 2007). This discrepancy might reflect a difference in experimental approaches, but this issue remains to be resolved. In 2009, Schelbert et al. identified an *A. thaliana* plastidial protein that dephytylates the Mg-free Chl *a*, pheophytin (Pheo *a*), yielding phytol and Pheide *a*. This new dephytylating enzyme was named pheophytinase (PPH) and shown to be associated with the thylakoid membrane as part of a Chl breakdown complex recruited by the STAY-GREEN protein (SGR). Interestingly, this degradation complex involves all the enzymes that catalyze the stepwise degradation of Chl to the compound known as primary fluorescent chlorophyll catabolite (pFCC), allowing metabolic channeling of phototoxic Chl breakdown intermediates. Subsequently, pFCC is exported from the plastid to the vacuole (Sakuraba et al., 2013). Interestingly, CLH is not part of this enzymatic complex (Sakuraba et al., 2012), suggesting different and/or complementary roles for the two dephytylation enzymes.

Chl degradation has been extensively investigated and most studies to date have used leaf senescence as the associated experimental system. Leaf senescence is a highly regulated process that involves several changes in cell structure, metabolism, and gene expression. The earliest and most evident cell structure change is the breakdown of the chloroplast, accompanied by catabolism of chlorophyll and macromolecules (e.g. proteins, lipids, and RNA). Regarding gene expression, leaf senescence is characterized by the decrease of genes related to photosynthesis and protein synthesis and by increased expression of senescence-associated genes (SAGs) (Lim et al., 2007). Senescence is an integrated response of plants to endogenous developmental and external environmental stimuli, and plant hormones play an important role in signaling. Our current understanding of the relationship between environmental responses and leaf senescence comes from the study of senescence response to the phytohormone abscisic acid (ABA), involved in response to abiotic stresses such as drought, high salt condition, and low temperature and; jasmonic acid (JA) and salicylic acid (SA) that participate in biotic stress regulation. Meanwhile, ethylene has extensively been described as a major hormone in hastening age-dependent leaf senescence (Khan et al., 2013). However, relatively little is known about other examples when degreening occurs, such as the mechanisms of Chl degradation that accompany fruit ripening (Hörtensteiner, 2013). Fleshy fruit ripening involves major biochemical and physiological alterations that influence texture, flavor and aroma as well as color (Seymour et al., 2013). This complex metabolic process is mainly controlled by ABA and ethylene. In the climacteric fruits such as tomato, there is an increase in ABA preceding the increase in ethylene. Exogenous application of ABA induces ethylene through the transcriptional activation of the biosynthesis genes, while the suppression of ABA leads to a delay in fruit ripening (McAtee et al., 2013). Additionally, this hormone has also been associated with fruit growth in tomato. ABA accumulates at the end of the expansion phase, and ABA-deficient mutants have reduced fruit size (Nitsch et al., 2012). In this framework, the cultivated tomato, *Solanum lycopersicum*, whose fruit exhibits clear color changes during maturation, is a potentially valuable model for comparative studies of degreening during leaf senescence and fruit ripening in relationship with their hormonal control. To our knowledge, the only report describing Chl degradation-associated genes in *S. lycopersicum* (Efrati et al., 2005) involved the mapping of three CLH genes and a PAO gene as part of a search for candidates for the *gf* (green flesh) mutant phenotype, which was later demonstrated to be SGR (Barry et al., 2008).

In this current study, we performed a genomic and evolutionary characterization of tomato CLH and PPH genes, in order to gain a more comprehensive understanding of their role in Chl dephytylation. Additionally, the expression patterns of these genes in leaves submitted to different hormonal treatments and during fruit development and ripening were analyzed. Our data demonstrate that the diversity of dephytylating enzymes is associated with different selective constraints and expression profiles, suggesting functional specialization.

2. Materials and methods

2.1. Phenetic and evolutionary analyses

Tomato CLH and PPH gene sequences were identified by using the coding sequence of both *A. thaliana* CLH genes (AT1G19670 and AT5G43860) and the PPH gene (AT5G13800) to interrogate the tomato genome sequence, available at the Sol Genomics Network (<http://solgenomics.net/>). The chromosomal locations were determined by the position of the closest marker, based on the Tomato-EXPEN-2000 map (<http://solgenomics.net/>). In silico prediction of the protein subcellular localization was performed using TargetP (Emanuelsson et al., 2007), Cello v2.5 (Yu et al., 2006), SherLoc2 (Briesemeister et al., 2009), MultiLoc2 (Blum et al., 2009), BaCellO (Pierleoni et al., 2006), Plant-mPloc (Chou and Shen, 2010), ChloroP (Emanuelsson et al., 2007) and iPSORT (Bannai et al., 2002). Other plant sequences that are homologous to the *A. thaliana* CLHs and PPH genes were identified by searching the Phytozome 9.0 database (<http://www.phytozome.net/>), NCBI (<http://www.ncbi.nlm.nih.gov>) and Sol Genomics Network, using the tBLASTx program (Altschul et al., 1990). The sequences were aligned using the MUSCLE package available in the MEGA 5.2 software with default parameters (Tamura et al., 2007) in a codon-based manner and inspected manually. The alignment was analyzed using the Neighbor-Joining method, the distances were calculated according to the best model pointed by MEGA 5.2 software and the tree topology was evaluated with 5000 bootstrap replications. Detailed information of all sequences used for the analyses is shown in Table S1.

The evolutionary analysis was performed as previously described by Almeida et al. (2011). Non-synonymous (*dN*) and synonymous (*dS*) distances, as well as their standard error (SE) values were estimated by the Nei–Gojobori method (p-distance) using the MEGA 5.2 software. In order to preserve the reading frames, the alignment gaps were deleted. Alignments are presented in Figs. S1 to S10. Codon bias was determined by the effective number of codons (Nc) value computed by the CodonW program (mobyli.pasteur.fr/cgi-bin/portal.py?form¼codonw). The Nc value varies from 21, when only one codon is used per amino acid, to 61, when synonymous codons for each amino acid are used at similar frequencies. One-way ANOVA with Tukey's posthoc test was performed using the InfoStat software (www.infostat.com, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) to evaluate significant differences in codon usage. In order to compare codon evolution models to determine selective constraint, three models were fitted using the CODEML program of the PAML suite (Yang, 2007). The first model, M0, assumes that all codons across the sequences have the same *dN/dS* ratio (ω). The value for ω provides an indication of the selection at the protein level: $0 < \omega < 1$ indicates purifying selection; $\omega = 1$ is neutral evolution; and $\omega > 1$ points to the presence of positive selection. Model M1a suggests the existence of two classes of codons, a proportion with $0 < \omega < 1$ and the remainder of codons with $\omega = 1$. Model M2a indicates three types of codon evolution: purifying selection, neutral evolution, and positive selection. The fit of models M0 versus M1a, and M1a versus M2a was evaluated by a likelihood ratio test (LRT), comparing the difference in log likelihoods with a χ^2 distribution two times (Yang, 2007).

2.2. Plant material

S. lycopersicum L. (cv. MicroTom) seeds were obtained from the Laboratory of Hormonal Control of Plant Development (ESALQ, Universidade de São Paulo). The plants were grown in 1 L pots in a greenhouse under automatic irrigation (four times a day) at an average mean temperature of 25 °C, 11.5 h/13 h (winter/summer) photoperiod and 250–350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of incident photo-irradiance. Fruit pericarp material (without placenta and locule walls) at the green (G, ~1.5 cm in diameter), mature green (MG, jelly placenta and ~2.5 cm in diameter),

yellow (Y, ~2.5 cm in diameter) and ripe (R, ~2.5 cm in diameter) stages was harvested at 15, 25, 28 and 45 days after anthesis. For phytohormone-regulated senescence assay, tomato seeds were surface-sterilized according to Pino et al. (2010) and germinated on half strength MS salts (Murashige and Skoog, 1962), 30 g L⁻¹ sucrose and 2 g L⁻¹ Phytagel (Sigma, St. Louis, MO, USA). The growth chamber conditions were 400 $\mu\text{mol PAR m}^{-2} \text{s}^{-1}$ with a 12 h photoperiod and air temperature of $25 \pm 1^\circ \text{C}$. Four-week-old in vitro plants were treated with 100 μM abscisic acid (ABA), 100 μM salicylic acid (SA) or 5 mL L⁻¹ ethylene (ET). Treatment with 500 $\mu\text{L L}^{-1}$ 1-methylcyclopropene (MCP), an inhibitor of ethylene perception (Blankenship and Dole, 2003), started three weeks before harvest time and was renewed weekly. Proper volumes of the gaseous ET and MCP solutions were added in the headspace of the sealed flasks containing the plants to achieve the final concentrations mentioned above. The preparation of MCP followed the manufacturer's instructions (SmartFresh® powder 0.14%). Source leaf tissue from the second node from the bottom of the plant was collected after two and four days of hormonal treatment. MCP-treated plants were harvested at the exact same age of those exposed to

hormonal treatments. All samples were frozen in liquid nitrogen, powdered and stored at -80°C .

2.3. Chlorophyll measurement

For chlorophyll *a* and *b* quantification, 50 mg of homogenized tissue was incubated with *N,N*-dimethylformamide at room temperature in darkness for 1 h, the supernatant was collected and the extraction was repeated one more time. The absorption spectrum of the combined supernatants at 647 and 664 was measured and the pigment content was calculated as described by Lichtenthaler (1987). Chlorophyll data were analyzed by ANOVA followed by a Tukey test ($p < 0.05$) using the Infostat software (Di Rienzo et al., 2011).

2.4. qPCR

RNA extraction and qPCR reactions were performed as described by Quadrona et al. (2013). The PCR primers used are listed in Table S2. The *SLPAO* (Soly11g066440) and *SISAG12* (Soly02g076910) genes were

Fig. 1. Genomic characterization of *S. lycopersicum* chlorophyllase and pheophytinase genes. (A) Chromosomal positioning of SLCLH1 (Soly06g053980.2), SLCLH2 (Soly09g065620.2), SLCLH3 (Soly09g082600.1), SLCLH4 (Soly12g005300.1) and SLPPH (Soly01g088090.1) according to the closest marker mapped onto the Tomato-EXPEN-2000 map (<http://solgenomics.net/>). T0834 (32 cM) for SLCLH1, C2_At4g02680 (56.3 cM) for SLCLH2, cLET-42-02 (72 cM) for SLCLH3, TG180 (9 cM) for SLCLH4 and T1677 (46 cM) for SLPPH. (B) Schematic representation of gene structures. Black boxes and lines indicate exons and introns, respectively. Untranslated regions (UTRs) are represented as white boxes for those genes with available information. The gray arrows represent the primers used for the qPCR experiments. Scale bar indicates 200 bp. The representation was made with Exon-Intron Graphic Maker (<http://wormweb.org/exonintron>).

used as controls for chlorophyll degradation and senescence related gene expression, respectively. 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). Data were analyzed with LinRegPCR software (Ruijter et al., 2009) to obtain Ct values and to calculate primer efficiency. Expression values were normalized to the mean of two constitutively expressed genes, TIP41 and Expressed (Quadrana et al., 2013). A permutation test, which lacks sample distribution assumptions (Pfaffl et al., 2002), was used to detect statistical ($p < 0.05$) differences in expression levels between samples and developmental stages using the algorithms in the fgStatistics software (Di Rienzo, 2009). The normalized expression pattern was presented by a heat map constructed with GENE-E program (<http://www.broadinstitute.org/cancer/software/GENE-E/>).

3. Results

3.1. Genomic and evolutionary characterization of PPH and CLHs

In order to evaluate the diversity of dephytylation enzymes, the tomato genome sequence (The Tomato Genome Consortium, 2012) was searched using *A. thaliana* CLHs and PPH coding sequences as queries. Four *CLH*-like and one *PPH*-like genes were identified, distributed on chromosomes 1, 6, 9 and 12 (Fig. 1A). These genes were named and will be referred to hereafter as *SICLH1*, *SICLH2*, *SICLH3*, *SICLH4*, and *SIPPH*. *SIPPH* contains seven exons and there is variation in the *SICLH* gene structures, with two or three exons (Fig. 1B). In order to predict the subcellular location of tomato proteins, eight different programs were tested using the protein sequence of AtPPH as control, which is experimentally demonstrated to localize in chloroplasts (Schelbert et al., 2009). Interestingly, all tested programs accurately predicted AtPPH to be located in plastid. However, for tomato proteins the predictions were ambiguous. Most of the programs indicated that SIPPH is a plastidial protein whereas for SICLHs suggested chloroplastic, cytosolic and even, plasma membrane localization (Table S3).

The topology of the PPH tree was for the most part in agreement with the phylogenetic distribution of the species and among the plant species considered, PPH was found to be mostly encoded by a single copy gene. When paralogs were found, as was the case for *Glycine max* and *Brassica rapa*, the sequences clustered together, indicating species-specific gene duplications (Fig. 2A).

The *CLH* phenetic analysis revealed one distinct clade containing all the Eudicot sequences, while those of the monocotyledonous species, conifers and *Eucalyptus grandis* grouped together in a not well supported clade (Fig. 2B). In agreement with that reported by Gupta et al. (2012), within the Eudicot clade, two groups could be distinguished, which are highlighted as Group I and Group II. All the analyzed species were represented by at least one *CLH* copy in each group, suggesting ancient gene duplication in the flowering plant lineage.

An integrated analysis of the phenogram, combined with the limited functional data available further revealed some interesting features. AtCLH1 (Benedetti et al., 1998; Liao et al., 2007; Tsuchiya et al., 1999), BoCLH1 (Büchert et al., 2011) and CsCLH3 (Jacob-Wilk et al., 1999), which belong to Group I, have been shown to be hormone or environmental stimuli-responsive enzymes; however, their paralogs are constitutively expressed, or suggested to be non-responsive to hormone treatments (Büchert et al., 2011; Tang et al., 2004).

The existence of two types of dephytylation enzymes in land plants, namely two *CLH*-encoding genes and a single *PPH*, is intriguing in terms of their putative physiological roles as well as the evolutionary history of their functional differentiation. We therefore explored the patterns of sequence diversification by applying a likelihood ratio test (LRT) to evaluate the selective constraints in which these sequences are evolving. Three different models were tested. The first, M0, assumes that all codons have the same dN/dS (ω). The second, M1a, proposes

Fig. 2. Phenogram of PPHs and CLHs. Sequence diversity for PPHs (A) and CLHs (B). Details of sequence data are listed in Table S1. Only genes from species with fully sequenced genomes were included in the analysis. Within the Eudicots, two groups of CLHs, Groups I and II, could be distinguished. *S. lycopersicum* sequences are highlighted with open circles. Filled and open rhombuses indicate CLH sequences that have been functionally characterized as hormone-responsive or non-responsive, respectively. Bootstrap values are shown on the branches. *Arabidopsis thaliana* (At), *Brassica oleracea* (Bo), *Brassica rapa* (Br), *Citrus clementina* (Cc), *Citrus sinensis* (Cs), *Eucalyptus grandis* (Eg), *Glycine max* (Gm), *Nicotiana tabacum* (Nt), *Oryza sativa* (Os), *Picea sitchensis* (Ps), *Populus trichocarpa* (Pt), *Ricinus communis* (Rc), *Solanum lycopersicum* (Sl), *Solanum tuberosum* (St), *Sorghum bicolor* (Sb), *Vitis vinifera* (Vv), and *Zea mays* (Zm).

that a subset of codons is under purifying selection while the others exhibit neutral evolution. The third model, M2a, is able to categorize codons in three classes: those with purifying selection, those with a neutral evolution pattern, and the remainder that are under positive selection. In order to avoid data misinterpretation, the Nc was estimated and the comparison between species did not show statistically significant differences, indicating that there is no codon usage bias. No positive selection was detected for any of the groups of sequences tested (data not shown). When phylogenetically distant sequences were compared (all PPHs, all CLHs, Group I-CLHs or Group II-CLHs, Table 1), PPH and CLHs showed evidence of evolving in accordance with the M1a model, with both neutral and constrained evolving codons. However, looking exclusively at Solanaceae or Brassicaceae sequences, a different scenario was uncovered. While, PPH and Group II-CLHs have codons constrained exclusively by purifying selection, Group I-CLHs exhibited a relaxed selection, containing codons evolving by both purifying selection and neutral evolution (Table 1).

3.2. Expression profiles of tomato PPH and CLH genes upon hormone treatments and along fruit development and ripening

To generate additional evidence for an association between the roles of the different dephytylating enzymes with distinct physiological processes, we performed a comprehensive expression analysis in leaves submitted to different hormonal treatments and during fruit development and ripening (green (G), mature green (MG), yellow (Y) and ripe (R) stages). Chl *a* and Chl *b* levels decreased upon ET, ABA and SA treatments while the leaves grown in the presence of 1-methylcyclopropene (MCP), an inhibitor of ethylene perception, displayed higher levels of Chls (Fig. 3). As expected, Chl content also declined during ripening (Fig. 4). ET, ABA and SA treatments triggered leaf senescence as demonstrated by the increase of the mRNA levels of the *SISAG12* gene, which is a senescence marker (Weaver et al., 1998). In MCP treated leaves, *SISAG12* showed reduced amount of transcripts compared with control. *PAO* has shown to be ubiquitously expressed in green tissues to avoid the phototoxicity of Pheide *a* (Hörtensteiner, 2013). Thus, we might speculate that the increment in Chl content observed upon MCP treatment would result in increased levels of *PAO* mRNA as observed in Fig. 5 although, to our knowledge, this correlation is still unexplored. In agreement with previous report, *PAO* expression correlated positively with Chl degradation induced by ET, ABA and SA (Pružinska et al., 2003) (Fig. 5, Tables S4, S5).

Transcripts corresponding to the dephytylating enzymes *SICLH1*, *SICLH4* and *SIPPH* were detected in all samples tested. *SICLH2* showed expression in leaves, but only very low amounts of mRNA were detected in fruits and exclusively at the G stage. None of the analyzed samples

Fig. 3. Chlorophyll content upon hormonal treatment. Chlorophyll *a* and chlorophyll *b* content in leaves collected after 0, 2 and 4 days of hormonal treatment. MCP refers to 1-methylcyclopropene. Asterisks indicate statistically significant values compared with non-treated T0 ($p < 0.05$).

showed detectable levels of *SICLH3* expression. In leaves *SICLH2* and *SICLH4* showed low and similar expression levels, which represent 4 and 8-fold less than *SIPPH* and *SICLH1*, respectively. This difference in the magnitude of expression enlarges in fruits where *SIPPH* and *SICLH1* displayed more than 50-fold mRNA than *SICLH2* and *SICLH4* (Table S6). A detailed analysis of the temporal and spatial expression patterns suggested that the four enzymes have distinct functional roles (Fig. 5, Table S4). *SICLH1* expression correlated with Chl content showing a mild decrease following the three treatments that induced leaf senescence. In the presence of MCP, leaves maintained *SICLH1* transcript level displaying a little reduction after 4 days. *SICLH2* expression peaked after two days of MCP, ET and ABA treatment, while the presence of SA inhibited *SICLH2* mRNA accumulation compared against non-treated samples. *SICLH4* expression exhibited variable responses upon growth regulator application. Leaves of plants grown in the presence of MCP accumulate less, yet constant, amount of *SICLH4* mRNA than controls. Upon ET application, *SICLH4* expression decreased

Table 1
Evolutionary analysis of CLH and PPH sequences.

	Number of sequences	Number of codons	Mean dN ± SE	Mean dS ± SE	Mean Nc ± SD ^a	M0			M1a			2Δl (M1a–M0) ^g		
						ω ^b	p0 ^c	lnl ^d	ω0	p0	ω1 ^e		p1 ^f	lnl
All CLHs	38	234	0.330 ± 0.014	0.723 ± 0.010	51.14 ± 8.64	0.23984	1.00000	-16042.717743	0.17541	0.69088	1.00000	0.30912	-15781.792075	521.85*
Group I	13	281	0.186 ± 0.011	0.613 ± 0.015	50.08 ± 3.04	0.15071	1.00000	-5917.189525	0.09879	0.80370	1.00000	0.19630	-5845.329127	143.72*
Group II	16	269	0.325 ± 0.013	0.637 ± 0.013	55.97 ± 4.21	0.28053	1.00000	-8068.742702	0.14568	0.60939	1.00000	0.39061	-7912.028941	313.43*
Solanaceae Group I	4	278	0.140 ± 0.011	0.355 ± 0.023	53.46 ± 1.96	0.28452	1.00000	-2231.814979	0.18233	0.83215	1.00000	0.16785	-2225.767086	12.09*
Brassicaceae Group I	3	324	0.066 ± 0.050	0.323 ± 0.239	60.82 ± 0.31	0.21138	1.00000	-1936.781773	0.02253	0.73739	1.00000	0.26261	-1930.121126	13.32*
Solanaceae Group II	4	288	0.090 ± 0.009	0.348 ± 0.026	47.38 ± 2.17	0.16221	1.00000	-2084.000701	0.14081	0.96064	1.00000	0.03936	-2082.982486	2.04
Brassicaceae Group II	3	317	0.053 ± 0.041	0.325 ± 0.270	55.57 ± 0.52	0.17562	1.00000	-1797.414114	0.09724	0.88243	1.00000	0.11757	-1794.771181	5.29
All PPHs	18	326	0.143 ± 0.009	0.635 ± 0.013	53.92 ± 2.42	0.12057	1.00000	-8204.934624	0.07911	0.86475	1.00000	0.13525	-8069.057688	271.75*
Solanaceae PPHs	3	478	0.054 ± 0.006	0.197 ± 0.017	50.72 ± 1.71	0.23623	1.00000	-2787.421353	0.17129	0.90511	1.00000	0.09489	-2786.327631	2.18
Brassicaceae PPHs	3	476	0.071 ± 0.019	0.313 ± 0.038	54.33 ± 1.15	0.23429	1.00000	-3047.214210	0.19376	0.93393	1.00000	0.06607	-3046.592305	1.24

^a Nc: effective number of codons and the corresponding standard deviation (SD). No significant differences in codon usage were identified ($p < 0.01$). ^b ω0: ω estimates for the codons under purifying selection. ^c p0: estimated proportion of codons under purifying selection. ^d lnl: log likelihood of model. ^e ω1: ω estimates for the codons under neutral evolution. ^f p1: estimated proportion of codons under neutral evolution. ^g 2Δl (M1a–M0): the likelihood ratio statistics (2Δl) is approximated by the χ^2 distribution (degree of freedom = 1). Null hypotheses (M0) rejected is highlighted in dark gray and indicated with an asterisk in the 2Δl (M1a–M0) ($p < 0.01$). Null hypotheses (M0) accepted are highlighted in light gray.

Fig. 4. Chlorophyll content during fruit development and ripening. (A) Phenotypic characteristics of the fruits. (B) Chlorophyll *a* and chlorophyll *b* content in fruits. Fruit pericarp material was sampled during natural development and ripening at four different stages: green (G), mature green (MG), yellow (Y) and ripe (R). Different letters indicate statistically significant values compared with the corresponding G stage ($p < 0.05$).

after 2 days of treatment and rapidly returned to basal levels. ABA and SA application inhibited *SICLH4* mRNA accumulation, which decreased 76% and 90% after 4 days, respectively. Finally, *SIPPH* showed an

expression profile that mirrored the Chl *a* content and *SICLH1* expression pattern, suggesting its involvement in senescence-induced chlorophyll degradation.

Fig. 5. Expression of the dephytylating enzyme-encoding genes in leaves and fruits. Schematic view of the Chl degradation pathway showing the relative expression profile of *SICLH1*, *SICLH2*, *SICLH4* and *SIPPH* assayed by qPCR. Leaves were analyzed after 0, 2 or 4 days of treatment with 1-methylcyclopropene (MCP), ethylene (ET), abscisic acid (ABA) or salicylic acid (SA). Fruit transcript profiles were performed at green (G), mature green (MG), yellow (Y) and ripe (R) stages. *SISAG12* and *SIPAO* were included as controls. Values represent mean relative expression calculated from the means of two technical replicates and normalized against non-treated samples (day 0) or green fruits (G). Leaf expression data were expressed as the ratio between the treatment value and the corresponding untreated control. The expression values and the corresponding statistically significant differences ($p < 0.05$) between samples are detailed in Tables S4 and S5. Gray boxes denote not detected. Chl *a*, chlorophyll *a*; Pheo *a*, pheophytin *a*; Chlide *a*, chlorophyllide *a*; Pheide *a*, pheophorbide *a*; RCC, red chlorophyll catabolite; CLH, chlorophyllase; PPH, pheophytinase; PAO, pheophorbide *a* oxygenase; SAG12, senescence-induced marker gene.

Once tomato fruits are fully expanded at the MG stage, one of the most obvious visible changes is a modification of color resulting from the degradation of chlorophyll and the accumulation of carotenoid pigments. Indicative of the early onset of chlorophyll degradation, the *SIPPH* and *SICLH1* showed a peak of expression at the MG stage, which decreased towards the R stage. In contrast, *SICLH4* displayed a fluctuating expression profile with higher mRNA levels in the G and Y stages and lower amount of transcripts in the MG and R stages (Fig. 5, Table S5).

4. Discussion

Although the cytotoxic effects of Chl metabolism intermediates are well known, there are still many poorly understood steps in the Chl degradation pathway. Moreover, since most studies have focused on Chl breakdown during leaf senescence, knowledge of Chl degradation during fruit ripening is very limited (Hörtensteiner, 2013). We therefore performed a comparative study of both processes in *S. lycopersicum* focusing on the gene encoding dephytylating enzymes. A survey of the tomato genome (The Tomato Genome Consortium, 2012) revealed four *CLH*-like and a single *PPH*-like genes.

In silico prediction of subcellular localization suggested that, as described for the *A. thaliana* ortholog, *SIPPH* is located in the chloroplast (Schelbert et al., 2009). In agreement with the divergent data obtained from different experimental approaches (Azoulay-Shemer et al., 2008, 2011; Harpaz-Saad et al., 2007), for *SICLHs* the protein sequence analyses were not conclusive with respect to organelle targeting. The phenetic analysis showed that, with the exception of *E. grandis* and *Oryza sativa*, the analyzed species had two or three copies of *CLH* genes. Interestingly, among the fourteen species examined, *Solanum tuberosum* and *S. lycopersicum* were the only examples with four *CLHs*, suggesting a lineage-specific duplication. In contrast, *PPH* was apparently present as a single copy gene. The existence of two groups of *CLHs* suggests functional diversification, and a selective constraint study revealed that different dephytylating-encoding genes are likely subjected to distinct selective pressures. Moreover, the differing expression profiles discussed below further suggests the existence of functional specificity. However, it is important to note that in *Citrus*, chlorophyllase is subjected to N- and C-terminal processing, so post-translational regulatory mechanisms may also participate in the regulation of enzyme activity (Azoulay-Shemer et al., 2011).

Our expression data indicate that *SIPPH* is the principal dephytylating enzyme acting during leaf senescence, as has been demonstrated for the ortholog in *A. thaliana* (Schelbert et al., 2009), and its expression coincides with Chl *a* catabolism. The levels of *SICLH1* mRNA gradually decreased correlating with Chl amount during hormone-induced leaf senescence. The same behavior was observed for the *CLH* of *Ginkgo biloba* during autumnal yellowing (Tang et al., 2004) and *AtCLH2* along dark-induced senescence (Liao et al., 2007), which belong to the same group of *SICLH1*. These results are indicative of Group II *CLH* involvement in the homeostasis of Chls in leaves, as was previously suggested by Tang et al. (2004). In contrast, *SICLH2* and *SICLH4* displayed variable expression patterns upon the tested stimuli. This is in agreement with all functional studies performed with Group I *CLHs* that have indicated responsiveness to external and hormonal stimuli, such as abscisic acid (ABA) (Gupta et al., 2012), methyl-jasmonate (Kariola et al., 2005; Schenk et al., 2007; Tsuchiya et al., 1999), wounding (Benedetti and Arruda, 2002; Kariola et al., 2005), pathogen elicitors (Kariola et al., 2005) and salicylic acid (Kariola et al., 2005). It is worth to remark the drastic reduction of *SICLH2* and *SICLH4* expression in response to SA, which after 2 days of treatment the mRNA levels dropped to 2% and 19%, respectively. The corresponding ortholog gene from *A. thaliana*, *AtCLH1*, has also shown to modulate SA signaling. During an SA-dependent defense response, cell damage causes Chl leaking. As a result of the phototoxic nature of this pigment, the delay of free Chl degradation by the *AtCLH1* transcriptional inhibition enhanced reactive oxygen

species accumulation, which boosts defense response (Kariola et al., 2005).

Regarding *SICLH3*, the absence of detectable levels of mRNA described here is in accordance with RNAseq public data available at Tomato Functional Genomic Database at Sol Genomics Network (<http://solgenomics.net/>) for *S. lycopersicum* cv. Heinz and *Solanum pimpinellifolium*. However, a comprehensive analysis of the gene sequence did not indicate any feature that could suggest that *SICLH3* locus was a pseudogene.

The expression of the gene encoding the dephytylating enzymes during fruit degreening showed similar behavior from what was observed during leaf senescence. At the MG stage, when fruits have reached their maximum size and Chl content before the triggering of ET biosynthesis (Giovannoni, 2004), *SICLH1* exhibited the highest levels of mRNA accumulation, which then decreased during ripening together with both Chl *a* and *b* levels. From G to MG stage, *SIPPH* expression increased over 40-fold. This expression pattern indicates that *SIPPH* and *SICLH1* transcriptional regulation is ET-independent in fruits. The correlation of *SICLH1* expression patterns with Chl content again indicates its involvement in pigment homeostasis. Additionally, *SIPPH* up-regulation demonstrates that Chl degradation during tomato degreening mostly depends on this enzyme. However, the transcriptional induction of *SICLH1* observed in MG stage (2.5-fold) suggests a synergistic action of *SIPPH* and *SICLH1* on fruit Chl catabolism during chloroplast to chromoplast transition. Additionally, as observed in leaves, the expression of *SICLH4* showed to be responsive to ABA displaying reduction of mRNA levels following ABA accumulation at late expansion (G) and early ripening (Y) phases (McAtee et al., 2013; Seymour et al., 2013), further underlining the responsiveness of Group I *CLHs* to phytohormones. However, in light of the extremely low expression level of *SICLH4*, the role of Group I *CLHs* in tomato ripening is questionable.

The data presented here, together with previous studies, indicate three main physiological roles for the dephytylating enzymes. *PPH* acts as part of a multi-enzymatic complex during developmentally programed physiological processes such as leaf senescence and fruit ripening, and so experiences a high degree of selective constraint (Sakuraba et al., 2012, 2013). *CLHs* belonging to Group I respond to external and hormonal stimuli. Since this group of enzymes is under relaxed purifying selection, a certain degree of sequence variability may have helped the development of tailored defense response pathways according to the varying environment and pathogen pressures during the evolutionary history. Finally, *CLHs* belonging to Group II are involved in Chl homeostasis and phytol recycling in leaves and fruits, and are subjected to higher levels of purifying selection (Almeida et al., 2011; Ischebeck et al., 2006; Quadrana et al., 2013; Tang et al., 2004; Vavilin and Vermaas, 2007). In summary, an integrated analysis of the results obtained from the genomic, evolutionary and expression studies of tomato *PPH* and *CLH*-encoding genes, demonstrated that the different Chl dephytylation enzymes play specific roles in plant physiology and plant-environment interactions.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2014.05.051>.

Conflict of interest

There are no conflict of interest.

Acknowledgments

BSL and JA were recipients of FAPESP fellowships and MR was funded by a fellowship from CNPq. This work was supported by FAPESP 2012-12531-8 (Brazil). The authors thank Rohm and Haas Company for generous donation of the 1-methylcyclopropene (SmartFresh®) used for the experiments.

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CHAPTER II. EFFECT OF PERTURBATION IN CHLOROPHYLL DEPHYTYLATION OVER PLANT DEVELOPMENT AND METABOLISM

The results, methodology and discussion of this chapter are presented as a paper published in *Plant & Cell Physiology*. Supplementary material corresponds to the attachment II.

Lira, B. S., Rosado, D., Almeida, J., de Souza, A. P., Buckeridge, M. S., Purgatto, E., Guyer, L., Hörtensteiner, S., Freschi, L. & Rossi, M. (2016). Pheophytinase knockdown impacts carbon metabolism and nutraceutical content under normal growth conditions in tomato. *Plant and Cell Physiology*, 57(3), 642-53.

Abstract

The characterisation of the dephytylating enzymes (Chapter I) gave evidences that PHEOPHYTINASE (PPH) plays a major role in the senescence- and fruit ripening-associated chlorophyll degradation in tomato. Thus *SIPPH*-knockdown tomato transgenic lines were generated. In a first paper, the biochemistry of chlorophyll degradation was extensively explored. The silenced plants exhibited impairment in senescence-associated chlorophyll degradation, but, except for a slight initial delay, the same was not observed along fruit ripening (Guyer *et al.*, 2014 – attachment III). Further, to address the impact of *SIPPH* knocked down on plant physiology and, in particular on fruit metabolism, a detailed phenotypic characterisation was performed. Interestingly, *SIPPH*-knockdown plants displayed symptoms of photoinhibition, such as reduced carbon assimilation, electron transport rate and photosystem II operating efficiency and increased non-photochemical quenching, suggesting that transgenic plants were limited in the photochemical dissipation of the absorbed energy. This is in agreement with the observed increment in carotenoids, such as xanthophylls and β -carotene, for dealing with this excess of energy, avoiding the accumulation of reactive oxygen species that could prejudice cellular functions. Carbon metabolism was also altered in leaves of *SIPPH*-knockdown plants. The reduction in starch and the accumulation of soluble sugars like glucose and sucrose hint the enhancement of carbon exportation from sources towards sinks organs. Supporting this hypothesis, fruit sugar content was increased ultimately leading to the development of ripe fruits with increased soluble sugar content. In turn, the increased sugar content downregulated carotenoid biosynthesis, what was, at least partially, compensated by an increased in the tocopherol content. Thus, it appears that *SIPPH* plays a role beyond leaf senescence-associated degreening. By participating in chlorophyll recycling, reduction in this enzyme negatively affected carbon

assimilation without penalising the yield. Moreover, SLPPH deficiency disturbed isoprenoid metabolism resulting in fruits with altered nutraceutical composition.

Pheophytinase Knockdown Impacts Carbon Metabolism and Nutraceutical Content Under Normal Growth Conditions in Tomato

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(Received October 5, 2015; Accepted January 20, 2016)

Although chlorophyll (Chl) degradation is an essential biochemical pathway for plant physiology, our knowledge regarding this process still has unfilled gaps. Pheophytinase (PPH) was shown to be essential for Chl breakdown in dark-induced senescent leaves. However, the catalyzing enzymes involved in pigment turnover and fruit ripening-associated degreening are still controversial. Chl metabolism is closely linked to the biosynthesis of other isoprenoid-derived compounds, such as carotenoids and tocopherols, which are also components of the photosynthetic machinery. Chls, carotenoids and tocopherols share a common precursor, geranylgeranyl diphosphate, produced by the plastidial methylerythritol 4-phosphate (MEP) pathway. Additionally, the Chl degradation-derived phytol can be incorporated into tocopherol biosynthesis. In this context, tomato turns out to be an interesting model to address isoprenoid-metabolic cross-talk since fruit ripening combines degreening and an intensely active MEP leading to carotenoid accumulation. Here, we investigate the impact of PPH deficiency beyond senescence by the comprehensive phenotyping of *SIPPH*-knockdown tomato plants. In leaves, photosynthetic parameters indicate altered energy usage of excited Chl. As a mitigatory effect, photosynthesis-associated carotenoids increased while tocopherol content remained constant. Additionally, starch and soluble sugar profiles revealed a distinct pattern of carbon allocation in leaves that suggests enhanced sucrose exportation. The higher levels of carbohydrates in sink organs down-regulated carotenoid biosynthesis. Additionally, the reduction in Chl-derived phytol recycling resulted in decreased tocopherol content in transgenic ripe fruits. Summing up, tocopherol and carotenoid metabolism, together with the antioxidant capacity of the hydrophilic and hydrophobic fractions, were differentially affected in leaves and fruits of the transgenic plants. Thus, in tomato, PPH plays a role beyond senescence-associated Chl degradation that, when compromised, affects isoprenoid and carbon metabolism which ultimately alters the fruit's nutraceutical content.

Keywords: Carotenoids • Chlorophyll degradation • Pheophytinase • *Solanum lycopersicum* • Tocopherol • Tomato.

Abbreviations: DXS, 1-deoxy-D-xylulose-5-P synthase; ETR, electron transport rate; GGDR, geranylgeranyl diphosphate reductase; GGPP, geranylgeranyl diphosphate; MEP, methylerythritol 4-phosphate; NPQ, non-photochemical quenching; PDP, phytyl diphosphate; PPH, pheophytinase; SGR, STAY GREEN 1; TEAC, Trolox equivalent antioxidant capacity; Chl, Chlorophyll; PSII, Photosystem II; PSY, phytoene synthase; VTE, vitamin E; SK, shikimate; VTE5, phytol kinase; VTE6, phytyl-phosphate kinase; PAO, pheophorbide a oxygenase; PDS, phytoene synthase; LCY, lycopene β -cyclase chloroplast-specific; CYC, lycopene β -cyclase chromoplast-specific; R_d , respiration; E, transpiration; g_s , conductance; Fv/Fm, dark-adapted PSII maximum quantum efficiency; A, CO₂ assimilation; qP, open PSII centers; Φ PSII, PSII operating efficiency; PPHL, PPH-like; O₂, singlet oxygen.

Introduction

Photosynthesis, the process upon which life relies, converts light energy by coupling chlorophyll (Chl) with an electron transport chain. The extension of photosynthetic activity for longer periods, by delaying leaf senescence, might lead to improvement in carbon assimilation. Thus, this phenotype, called stay green, has been considered as a strategy for breeding for yield (Rossi et al. 2015) and has been classified as cosmetic or functional (Thomas and Howarth 2000). Genotypes with deficiency in senescence-associated Chl degradation are considered cosmetic, since the non-visible components of senescence are mostly unaffected (Thomas and Ougham 2014). This seems to be the case in the *Arabidopsis thaliana* pheophytinase (*AtPPH*) mutant (*pph-1*). Pheophytinase (PPH) catalyzes the dephytylation of pheophytin *a*, releasing phytol, the Chl lipophilic chain. *pph-1* is unable to degrade Chl and the functionality of photosynthesis is strongly affected upon induction of senescence

(Schelbert et al. 2009). Similar results have been obtained for the *Solanum lycopersicum* ortholog gene, *SIPPH* (Guyer et al. 2014), as Chl breakdown is impaired in dark-induced senescent leaves of *SIPPH*-knockdown plants. The fruits of transgenic lines showed a transient delay of Chl degradation that was accompanied by a transitory accumulation of pheophytin *a*, but, ultimately, they degraded Chl and pheophytin *a* like the wild-type genotype. These results demonstrate the involvement of *SIPPH* in ripening-associated degreening; however, they also indicate that other dephytylating activities may compensate for the reduced levels of PPH in transgenic fruits.

Another elusive mechanism regarding Chl metabolism is Chl turnover. It has been demonstrated that chlorophyll synthase re-esterifies chlorophyllide *a*; nevertheless the enzyme responsible for the hydrolysis of the ester bond yielding chlorophyllide *a* and free phytol is still unknown (Lin et al. 2014, Zhang et al. 2015). Pheophytin *a* and Chl *a* differ only in one magnesium ion; thus, it seems likely that the de-esterification is catalyzed by a PPH-like enzyme. Interestingly, pheophytin *a* recycling might also be needed for proper photosystem turnover as it is known to be the primary electron acceptor in Photosystem II (PSII) (Vass and Cser 2009). Furthermore, *PPH*-like genes are found from cyanobacterial to angiosperm genomes and have been shown to be localized in plastids (Schelbert et al. 2009, Guyer et al. 2014).

Chl metabolism is closely linked to biosynthesis of carotenoid and tocopherol, which are the major lipid-soluble antioxidants of the thylakoid membrane and consequently, important players in maintenance of photosynthetic activity (Havaux et al. 2005). These isoprenoid-derived compounds are synthesized from geranylgeranyl diphosphate (GGPP) produced by the plastidial methylerythritol 4-phosphate (MEP) pathway (DellaPenna and Pogson 2006) (Fig. 1). While GGPP is the substrate for the first enzyme of carotenoid biosynthesis, phytoene synthase (PSY), the Chl and tocopherol prenyl side chain derives from phytyl diphosphate (PDP), which in turn is produced by geranylgeranyl reductase (GGDR) (Almeida et al. 2015). Tocopherols, which belong to the vitamin E (VTE) family of compounds, are further synthesized from the condensation of PDP and homogentisate from the plastidial shikimate (SK) pathway (Almeida et al. 2011). Additionally, PDP for tocopherol biosynthesis can also originate from the activity of phytol kinase (VTE5) and phytyl-phosphate kinase (VTE6), which recycle Chl degradation-derived phytol (Ischebeck et al. 2006, Valentin et al. 2006, vom Dorp et al. 2015, Almeida et al. 2016).

Although Chl, tocopherols and carotenoids share a common precursor, few studies have provided comprehensive insight into the regulation of their metabolic network (Quadrana et al. 2013, Zhang et al. 2014, Almeida et al. 2015, Zhang et al. 2015). It has been demonstrated that STAY GREEN 1 (SISGR), a Chl degradation regulatory protein, controls lycopene and β -carotene accumulation by interacting directly with SIPSY1 during tomato ripening (Luo et al. 2013). Recent evidence indicates that PDP for tocopherol biosynthesis is mostly supplied by Chl degradation (Zhang et al. 2015). In seeds of *A. thaliana*, neither PPH nor chlorophyllases, the other known Chl-dephytylating enzyme, seems to be responsible for producing Chl-derived phytol for tocopherol biosynthesis, although

AtPPH-overexpressing lines displayed increased levels of tocopherol (Zhang et al. 2014). Taken together, these data suggest the existence of an unknown source of phytol for tocopherol biosynthesis as well as an as yet unknown complex regulatory network of isoprenoid-derived compounds.

Tomato (*Solanum lycopersicum*) turns out to be an interesting model to address the above-described isoprenoid-metabolic cross-talk since, during fruit ripening, chloroplast to chromoplast transition combines degreening and an intensely active MEP, leading to carotenoid accumulation (Egea et al. 2011, Barsan et al. 2012, Seymour et al. 2013), while the total tocopherol content remains constant (Quadrana et al. 2013). Moreover, tomato is one of the most widely consumed fruits worldwide and is a major source of these nutraceutical compounds for the human diet (Canene-Adams et al. 2005, Chun et al. 2006).

In this work, we further characterized *SIPPH*-knockdown plants to address the role of PPH in Chl, carotenoid and tocopherol homeostasis and the physiological impact of PPH deficiency beyond senescence. The transgenic plants showed altered photosynthetic parameters that were reflected in modifications in carbon partitioning. Carotenoid and tocopherol content and composition were affected differently in leaves and fruits of *SIPPH*-knockdown plants, which could be explained by the transcriptional profile of the respective biosynthetic genes as well as by the influence of sugars over carotenoid metabolism. In addition, antioxidant activities in both organs were altered in accordance with carotenoid and tocopherol contents. Finally, a comprehensive survey of the tomato genome revealed the presence of three other *PPH*-like genes. The transcriptional profiles suggested that they all might play a role in leaf Chl metabolism, while *SIPPHL3* could, in addition, be involved in ripening-associated degreening. The integrated data analysis showed that *SIPPH* deficiency affects isoprenoid metabolism in an organ-specific manner, modifying the nutritional content of the edible fruit.

Results

Isoprenoid-derived compounds and Trolox equivalent antioxidant capacity (TEAC) profiles are altered in *SIPPH*-knockdown plants

Aiming to investigate the effect of PPH deficiency on the metabolism of carotenoids and tocopherol, three independent *SIPPH*-knockdown lines, with at least 85% reduction of the *SIPPH* mRNA level (Supplementary Fig. S1), were characterized.

Linear (lycopene) and cyclized (β -carotene, lutein, violaxanthin and neoxanthin) carotenoids were measured (Table 1). In leaves, all detected carotenoids were increased in a statistically significant manner, except for lutein that remained unaltered. On the other hand, a contrasting scenario was found in fruits. While no significant changes were found at the mature green stage, ripe fruits of *SIPPH*-knockdown plants showed reduced carotenoid levels compared with the control genotype. Again, lutein was the only measured compound that showed similar levels to wild-type fruits.

Fig. 1 Chl, tocopherol and carotenoid metabolism. Schematic representation of the interconnection between Chl synthesis and degradation (green), carotenogenesis (orange) and tocopherol biosynthetic (blue) pathways. Dotted lines denote that intermediate steps were omitted. Enzymes whose genes have been transcriptionally profiled in this work are indicated. Enzymes and compounds are named according to the following abbreviations: DXS, 1-deoxy-D-xylulose-5-P synthase; GGDR, geranylgeranyl diphosphate reductase; PSY, phytoene synthase; PDS, phytoene desaturase; LCY β , chloroplast-specific β -lycopene cyclase; CYC β , chromoplast-specific β -lycopene cyclase; GGPP, geranylgeranyl diphosphate; PDP, phytyl diphosphate; PP, phytyl phosphate; PPH, pheophytinase; PAO, pheophorbide *a* oxygenase; pFCC, primary fluorescent Chl catabolite; VTE5, phytol kinase; VTE6, phytyl phosphate kinase; MPBQ, 2-methyl-6-geranylgeranylbenzoquinol; VTE1, tocopherol cyclase; VTE2, homogentisate phytyl transferase; VTE3, 2,3-dimethyl-5-phytylquinol methyltransferase; VTE4, tocopherol C-methyl transferase; MEP, methylerythritol 4-phosphate. Adapted from Almeida et al. (2016).

Similar to that described for carotenoids, distinct tocopherol profiles were observed between organs. The levels of α -, γ - and total tocopherol in leaves of *SIPPH*-knockdown plants were similar to those observed in the wild type; however, the transgenic lines also presented detectable amounts of δ -tocopherol. Mature green fruits of the transgenic plants accumulated higher levels of α - and γ -tocopherol, which resulted in about a 12% increase in total tocopherol content. At the ripe stage, the amount of total tocopherol decreased in *SIPPH*-knockdown lines as a consequence of the reduction of the minor tocopherol forms, γ - and δ -, whose extent was not compensated by the increment in α -tocopherol (Table 2).

As *SIPPH* knockdown affected the profile of tocopherols and carotenoids, the antioxidant capacity in leaf and fruit extracts was estimated by the TEAC assay. The hydrophilic fraction of leaves and ripe fruits showed increased TEAC in transgenic plants, while reduced levels were detected at the mature

green stage (Fig. 2A). In terms of antioxidant capacity of the lipophilic fraction, leaves of *SIPPH*-knockdown plants did not differ significantly from the wild-type genotype. Interestingly, the fruit TEAC profile in the non-polar extract of transgenic lines displayed the opposite pattern to that observed in the hydrophilic fraction (Fig. 2B).

These data indicate that the metabolism of isoprenoid-derived compounds as well as the antioxidant capacity is differentially adjusted in response to *SIPPH* deficiency in leaves and fruits.

SIPPH deficiency alters the transcript profile of tocopherol and carotenoid biosynthetic enzyme-encoding genes

To address whether the alterations in tocopherol and carotenoid contents observed in transgenic plants are explainable by a

Table 1 Carotenoid contents in *SIPPH* knockdown lines

	WT	L17	L24	L27
<i>Leaf</i>				
Neoxanthin	41.82 ± 7.63	52.39 ± 6.92	65.56 ± 8.8	56.11 ± 6.97
Violaxanthin	36.57 ± 10.36	66.23 ± 10.17	71.36 ± 12.65	70.87 ± 14.32
Lutein	52.75 ± 7.05	53.03 ± 7.38	61.25 ± 6.37	59.94 ± 4.69
Lycopene	ND	ND	ND	ND
β-Carotene	39.16 ± 6.08	60.81 ± 5.66	64.32 ± 6.4	69.7 ± 10.5
<i>Mature green fruit</i>				
Neoxanthin	2.00 ± 0.39	1.81 ± 0.24	2.28 ± 0.18	2.22 ± 0.6
Violaxanthin	2.16 ± 0.32	1.80 ± 0.12	2.38 ± 0.23	2.33 ± 0.5
Lutein	6.16 ± 0.99	5.74 ± 0.69	6.22 ± 0.52	5.76 ± 1.32
Lycopene	ND	ND	ND	ND
β-Carotene	1.74 ± 0.10	1.77 ± 0.16	2.07 ± 0.15	1.89 ± 0.07
<i>Ripe fruit</i>				
Neoxanthin	0.19 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.02
Violaxanthin	0.50 ± 0.05	0.38 ± 0.02	0.34 ± 0.06	0.38 ± 0.04
Lutein	2.40 ± 0.22	2.20 ± 0.11	2.24 ± 0.13	2.22 ± 0.16
Lycopene	411.04 ± 11.51	326.42 ± 34.76	271.3 ± 16.62	283.32 ± 10.53
β-Carotene	10.13 ± 0.72	9.02 ± 0.32	7.25 ± 0.78	7.88 ± 1.28

Statistically significant differences between the wild-type (WT) control and transgenic lines are indicated in bold (*t*-test, *P* < 0.05). Values represent means ± SD from at least three biological replicates. Values are expressed in μg g⁻¹ FW. ND, not detected.

Table 2 Tocopherol content in *SIPPH*-knockdown lines

	WT	L17	L24	L27
<i>Leaf</i>				
α-Tocopherol	326.49 ± 3.59	322.41 ± 12.32	301.85 ± 13.54	308.35 ± 15.61
β-Tocopherol	ND	ND	ND	ND
γ-Tocopherol	5.01 ± 0.94	6.11 ± 0.88	6.32 ± 1.04	4.36 ± 0.48
δ-Tocopherol	ND	2.05 ± 0.33	2.31 ± 0.19	1.81 ± 0.20
Total tocopherol	331.50 ± 6.07	330.57 ± 22.65	310.48 ± 14.42	314.51 ± 20.09
<i>Mature green fruit</i>				
α-Tocopherol	134.88 ± 1.97	149.08 ± 8.38	141.04 ± 2.81	146.74 ± 4.90
β-Tocopherol	2.78 ± 0.18	2.38 ± 0.22	2.60 ± 0.36	2.70 ± 0.22
γ-Tocopherol	6.28 ± 0.79	11.86 ± 0.50	13.96 ± 2.01	14.33 ± 1.53
δ-Tocopherol	ND	ND	ND	ND
Total tocopherol	143.94 ± 2.43	163.32 ± 4.32	157.60 ± 3.58	163.77 ± 2.77
<i>Ripe fruit</i>				
α-tocopherol	145.54 ± 17.48	167.40 ± 13.76	165.67 ± 22.62	170.72 ± 30.55
β-tocopherol	3.24 ± 0.18	2.85 ± 0.27	3.22 ± 0.13	3.00 ± 0.21
γ-tocopherol	93.07 ± 8.23	27.47 ± 6.11	36.49 ± 3.42	50.21 ± 4.39
δ-tocopherol	2.10 ± 0.15	1.28 ± 0.08	1.35 ± 0.10	1.31 ± 0.11
Total-tocopherol	243.94 ± 25.88	198.99 ± 25.41	206.72 ± 11.56	225.23 ± 17.69

Statistically significant differences between the wild-type (WT) control and transgenic lines are indicated in bold (*t*-test, *P* < 0.05). Values represent the means ± SD from at least three biological replicates. Values are expressed in μg g⁻¹ DW. ND, not detected.

Fig. 2 Trolox equivalent antioxidant capacity (TEAC) in *SIPPH*-knockdown lines. The TEAC assay was carried out in hydrophilic (A) and lipophilic (B) extracts of leaves and fruits at mature green and ripe stages. Values represent the mean $\pm$ SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) between the control genotype and transgenic lines are marked with an asterisk.

transcriptional regulation of the respective biosynthetic genes, the mRNA levels of isoprenoid metabolism-related genes (Fig. 1) were profiled in leaves and fruits (Fig. 3; Supplementary Table S1).

Due to the role of SGR protein in recruitment of Chl catabolic enzymes (Sakuraba et al. 2012) and regulation of carotenoid biosynthesis (Luo et al. 2013), the transcript profile of *SISGR* was also evaluated. Interestingly, while no effect was found in leaves and mature green fruits, significantly lower amounts of *SISGR* mRNA were detected at breaker and ripe stages of *SIPPH*-knockdown fruits. Pheophorbide *a* oxygenase (PAO) is another member of the Chl catabolic enzyme complex (Sakuraba et al. 2012), and *SIPAO* also showed reduced levels of mRNA in leaves and fruits of *SIPPH*-knockdown plants, indicating that these plants are, to some extent, also impaired in tetrapyrrole ring breakdown. In *A. thaliana* the overexpression and silencing of *AtSGR1* resulted in higher and lower amounts, respectively, of *AtPPH* mRNA (Sakuraba et al. 2014). Reciprocally, the results presented here showed that the reduced expression of *SIPPH* leads to the down-regulation of *SISGR* and *SIPAO*, suggesting the existence of transcriptional co-regulation of the members of the Chl catabolic enzyme complex.

During tomato fruit ripening, the expression of the 1-deoxy-D-xylulose-5-P synthase-encoding gene (*SIDXS*) increases while the amount of transcripts of *SIGGDR* diminishes. These changes in the transcriptional profile enhance the availability of isoprenoid intermediates for fruit carotenoid accumulation (Quadrana et al. 2013, Almeida et al. 2015). Interestingly, the fruits of the transgenic plants showed a reversed pattern of expression, displaying higher and lower levels of transcripts for *SIGGDR* and *SIDXS*, respectively. Conversely, the leaves of the *SIPPH*-knockdown plants showed increased amounts of mRNA for *SIDXS*.

Carotenoid metabolism was addressed by profiling the transcript amount of *PHYTOENE SYNTHASE* (*SIPSY1* and *SIPSY2*), *PHYTOENE DESATURASE* (*SIPDS*) and *LYCOPENE β -CYCLASE* (the chloroplast-specific *SILCY β* and the chromoplast-specific *SICYC β*) genes. It is worth mentioning

Fig. 3 Transcript profile of Chl degradation, carotenogenesis and tocopherol biosynthetic genes in leaves and during fruit ripening of *SIPPH*-knockdown lines. Heatmap representation of the statistically significant ($P < 0.05$) relative transcript amount compared with the corresponding wild-type sample. The expression values are detailed in Supplementary Table S1. Abbreviations: *SISGR*, stay-green; *SIPAO*, pheophorbide *a* oxygenase; *SIDXS*, 1-deoxy-D-xylulose-5-P synthase; *SIGGDR*, geranylgeranyl diphosphate reductase; *SIVTE1*, tocopherol cyclase; *SIVTE2*, homogentisate phytyl transferase; *SIVTE3(1)*, 2,3-dimethyl-5-phytylquinol methyltransferase; *SIVTE3(2)*, tocopherol C-methyl transferase; *SIVTE4*, phytyl kinase; *SIVTE5*, phytyl phosphate kinase; *SIPSY1*, phytoene synthase; *SIPSY2*, phytoene desaturase; *SIPDS*, chloroplast-specific β -lycopene cyclase; *SILCY β* , chromoplast-specific β -lycopene cyclase; LF, leaf; MG, mature green fruit pericarp; BR, breaker fruit pericarp; RR, ripe fruit pericarp. Values represent means from at least three biological replicates. *SIVTE3(2)* was not evaluated in fruits (gray boxes) because it is leaf specific (Quadrana et al. 2013).

that in chloroplasts of wild-type plants, both *SIPSY* genes and *SILCY β* are highly expressed, contributing to the biosynthesis of photosynthesis-associated carotenoids. In fruits, *SIPSY2* expression is insignificant, while *SICYC β* is the most abundant *LYCOPENE β -CYCLASE*, being 7-fold more expressed than *SILCY β* (Supplementary Table S2). As an overall pattern, *SIPPH* silencing led to the down-regulation of the analyzed genes, with the exception of *SILCY β* at breaker and ripe stages.

The mRNA levels of tocopherol biosynthetic [*SIVTE1*, *SIVTE2*, *SIVTE3(1)*, *SIVTE3(2)* and *SIVTE4*] and phytol recycling (*SIVTE5* and *SIVTE6*) enzyme-encoding genes were also evaluated. In comparison with the control genotype, leaves of transgenic plants displayed a reduction in *SIVTE4* and *SIVTE3(2)* expression, whereas in breaker fruits *SIVTE2*, *SIVTE4* and *SIVTE5* showed lower amounts of transcript. The latter was also reduced at the mature green stage in *SIPPH*-deficient

plants. Moreover, reduced amounts of mRNAs for *SIVTE6* were also observed in breaker and ripe fruits of *SIPPH*-knockdown plants compared with controls.

These data showed that *SIPPH* silencing alters the expression of several isoprenoid metabolism-related genes in leaves and fruits, reinforcing the tight link between Chl, carotenoid and tocopherol metabolisms.

SIPPH knockdown affects photosynthesis and primary carbon metabolism

Given that *SIPPH* deficiency led to significant changes in carotenoid and tocopherol content, the effect on plant growth and photosynthesis was also evaluated.

The height and node number data were monitored during 13 weeks of growth and, although some transitory changes in plant height were observed up to the ninth week, shoot growth in *SIPPH*-knockdown lines was largely similar to that of wild-type plants (Supplementary Fig. S2).

Regarding Chl content, no significant alterations were identified in source leaves, mature green and ripe fruits. However, age-induced senescent leaves and petioles had impaired Chl degradation in transgenic lines (Supplementary Fig. S3).

Photosynthetic parameters were evaluated in source leaves of 7-week-old plants (Table 3). *SIPPH* silencing did not affect respiration (R_d), transpiration (E), conductance (g_s) and dark-adapted PSII maximum quantum efficiency (F_v/F_m). However, reduction in CO_2 assimilation (A), proportion of open PSII centers (qP), PSII operating efficiency (Φ_{PSII}) and electron transport rate (ETR), as well as an increase in non-photochemical quenching (NPQ) were detected in comparison with the wild type. These results implied that reduced transcript levels of *SIPPH* alter the energy usage of excited Chl.

Starch and soluble sugar composition was further evaluated in *SIPPH*-knockdown plants (Fig. 4). In leaves, the allocation of fixed carbon as starch diminished, whereas sucrose and glucose accumulated at higher levels. In fruits, sucrose was remarkably increased in transgenic plants in all three stages analyzed. Additionally, breaker fruits of *SIPPH*-knockdown plants showed higher levels of starch and glucose, whereas higher glucose and fructose amounts were detected in ripe fruits of most transgenic lines. The starch and soluble sugar profile revealed an altered pattern of carbon distribution in the analyzed organs of transgenic lines that suggest enhanced sucrose export from leaves towards sink organs.

The tomato genome harbors four PPH-like genes

SIPPH knockdown led to diverse effects of the plant's physiology by affecting leaf senescence-associated degreening (Guyer et al. 2014), metabolism of isoprenoid-derived compounds, antioxidant capacity and carbon allocation. Nevertheless, Chl is still degraded during fruit ripening. Aiming at the identification of the missing dephytylating enzymes, a comprehensive survey for putative α/β -hydrolases homologous to *SIPPH* resulted in the identification of four clades in plant genomes (Fig. 5; Supplementary Table S4). One, named PPH, included the previously functionally characterized enzymes, AtPPH (Schelbert et al. 2009) and *SIPPH* (Guyer et al. 2014). The other three

Table 3 Photosynthetic parameters in *SIPPH*-knockdown lines

	WT	L17	L24	L27
A	13.35 ± 0.86	12.16 ± 0.89	11.92 ± 0.93	11.85 ± 0.94
R_d	2.46 ± 0.17	2.31 ± 0.14	2.37 ± 0.20	2.42 ± 0.17
E	4.05 ± 0.17	3.92 ± 0.28	3.90 ± 0.14	4.03 ± 0.14
g_s	0.34 ± 0.02	0.30 ± 0.04	0.31 ± 0.03	0.31 ± 0.04
F_v/F_m	0.61 ± 0.02	0.62 ± 0.02	0.63 ± 0.02	0.60 ± 0.01
qP	0.38 ± 0.01	0.33 ± 0.03	0.34 ± 0.03	0.32 ± 0.03
NPQ	0.97 ± 0.04	1.01 ± 0.05	1.18 ± 0.12	1.15 ± 0.07
Φ_{PSII}	0.22 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
ETR	98.45 ± 3.11	84.98 ± 2.99	86.51 ± 7.49	85.27 ± 8.71

Photosynthetic parameters were measured in the sixth fully expanded leaf from the base of 7-week-old plants. Statistically significant differences between the wild-type (WT) control and transgenic lines are indicated in bold (t -test, $P < 0.05$). Values represent the means ± SD from at least three biological replicates. Carbon assimilation rate (A) and leaf dark respiration (R_d) are expressed in $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Transpiration (E) and leaf stomatal conductance (g_s) are expressed in $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$. F_v/F_m , dark-adapted PSII maximum quantum efficiency; qP, proportion of open PSII centers; Φ_{PSII} , PSII operating efficiency; NPQ, non-photochemical quenching; ETR, electron transport rate.

contained uncharacterized α/β -hydrolase-like genes and were named PPH-LIKE (PPHL) 1–3.

The tomato genome harbors a single copy gene in the PPH, PPHL1 and PPHL2 clade; while two paralogs were identified for the PPHL3 clade. All of the predicted protein sequences contained the PPH motif with a high degree of similarity and the conserved serine active residue (Schelbert et al 2009), suggesting that these proteins share the catalytic domain described for *SIPPH* (Supplementary Fig. S4). As expression was not detected for PPHL3.1 (Solyc02g085070) in the public RNA-seq database (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi), this gene was not further analyzed.

The abundance of mRNA of the identified *SIPPHL* genes was profiled in leaves and during fruit ripening in wild-type plants (Fig. 6; Supplementary Table S1). The three genes showed >5-fold more transcripts in leaves than in fruits. The amount of *SIPPHL1* mRNA decreased during ripening while *SIPPHL2* maintained constant levels from the mature green to the ripe stage. Similarly to *SIPPH*, the amount of *SIPPHL3* mRNA increased at the breaker stage. It is worth highlighting that the mRNA levels of *SIPPHL* genes were up to 6-, 80-, 300- and 1,000-fold less than *SIPPH* in leaves, mature green, breaker and ripe fruits, respectively, *SIPPHL3* being the gene with the highest expression after *SIPPH* (Supplementary Table S2). To evaluate co-silencing in *SIPPH*-knockdown plants, the *SIPPHL* transcripts were further profiled (Fig. 7). Interestingly, a considerable level of co-silencing in leaves and fruits was verified up to 80% and 60% for *SIPPHL1* and *SIPPHL3*, respectively. *SIPPHL2* was only silenced in breaker fruits of the *SIPPH*-knockdown plants. Although the fragment of 400 bp of the hairpin RNA (RNAi) construct used for *SIPPH* silencing does not contain the conserved PPH motif, it spans a highly conserved region shared by the four genes (Supplementary Fig. S5), explaining the observed off-target silencing. The expression levels suggest that *SIPPHL* genes are more likely to play a role in leaf Chl metabolism than in fruits. However, taking into account the mRNA abundance and profile fluctuation, *SIPPHL3* might also

Fig. 4 Starch and soluble sugar content in *SIPPH*-knockdown lines. The content of starch and soluble sugars (glucose, fructose and sucrose) was measured in leaves and fruits at the mature green and ripe stages. Values represent the mean $\pm$ SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) between the control genotype and transgenic lines are marked with an asterisk.

be involved in degreening during ripening, even though further experiments are needed to address the role of these *SIPPH*L genes precisely.

Discussion

Our knowledge of Chl metabolism has significantly increased in recent years, especially regarding tetrapyrrole biosynthesis (Tanaka and Tanaka 2007), the Chl cycle (Tanaka and Tanaka 2011) and senescence-associated degradation (Hörtensteiner 2013). However, a few critical processes, such as Chl turnover (Lin et al. 2014) and ripening-induced degreening (Guyer et al. 2014), remain elusive. Interestingly, although induced upon senescence and onset of ripening, *AtPPH* and *SIPPH* are transcribed in non-senescent source leaves and in developing green fruits (Schelbert et al. 2009, Lira et al. 2014). Beyond the impairment in leaf Chl degradation associated with dark-induced senescence and the delay in fruit degreening described in tomato (Guyer et al. 2014), this work investigated the effects that PPH deficiency has on the content of close metabolically linked compounds, photosynthetic performance and primary carbon metabolism in an integrated manner under normal growth conditions.

Although without apparent effect on vegetative growth, *SIPPH* deficiency led to a slight reduction in carbon assimilation. This might be a consequence of photoinhibition as evidenced by a reduced Φ_{PSII} and ETR, and by the increment in the NPQ (Table 3). The xanthophyll cycle is one of the main components of NPQ that dissipate excessively absorbed photons via harmless heat (Wilhem and Selmar 2011). In this sense, the increase in violaxanthin content observed in leaves of the *SIPPH*-knockdown plants (Table 1) is indicative of an enhancement of the xanthophyll cycle. Additionally, neoxanthin and β -carotene were also increased in leaves of transgenic plants, and their role as PSII-derived singlet oxygen (1O_2) quenchers has been extensively described (Pospíšil 2012). Interestingly, *SIDXS*, the first MEP enzyme-encoding gene, showed up-regulation, whereas a reduced level of expression of *SILCYB* was observed in the transgenic leaves (Fig. 3). Since no increment in Chl was verified (Supplementary Fig. S3), the alterations in the expression profile described might have resulted in higher precursor availability for carotenogenesis, thus explaining the increment in photosynthesis-associated carotenoids in *SIPPH*-knockdown plants. Interestingly, a similar transcript pattern for carotenogenesis-related genes was found in the *sgr* tomato mutant (Almeida et al. 2015). Regarding tocopherol, the reduction of the amount of *SIVTE4* mRNA (Fig. 3) accounts for the slight decrease in α -tocopherol and the increase of δ -tocopherol towards detectable levels; however, these fluctuations did not affect the total tocopherol content in transgenic plants (Table 2). These data indicate that the leaves of *SIPPH*-knockdown plants accumulate carotenoids as a mechanism to attenuate photoinhibition (Fanciullino et al. 2014). Additionally, other compensatory protective mechanisms, such as those that contribute to the observed increment of the hydrophilic TEAC in leaves (Fig. 2A) (e.g. the ascorbic acid–glutathione cycle; Foyer and Shigeoka 2011), are likely to guarantee a constant F_v/F_m in *SIPPH*-knockdown plants. This is in agreement with the observation in the tocopherol-deficient *vte1* Arabidopsis mutant, in which the enhancement of NPQ is accompanied by a compensatory increment in hydrophilic antioxidants (Kanwischer et al. 2005). Additionally, accumulation of xanthophylls was observed in response to limited PSII photoinhibition in this mutant. Furthermore, the xanthophyll- and tocopherol-deficient *vte1 npq1* double mutant displayed severe oxidative symptoms, pointing to the overlapping antioxidant function of both compounds (Havaux et al. 2005). Interestingly, the allocation of fixed carbon as starch diminished, whereas sucrose accumulated at higher levels in leaves of the transgenic plants (Fig. 4). This shift in partitioning, away from starch and towards soluble sugars, has been described as being associated with photoinhibition, due to the role of sucrose as a stress signal and antioxidant protective agent (Bolouri-Moghaddam et al. 2010). Tomato plants showed enhanced sucrose accumulation in leaves upon low temperature treatment, together with reduction in *gP*, Φ_{PSII} and ETR, as well as an increase in NPQ (Liu et al. 2012), in agreement with that observed in *SIPPH*-knockdown plants. Considering that in tomato carbon is mostly translocated in the form of sucrose (Barker et al. 2000), this carbohydrate profile hints at enhanced carbon export from the leaves towards sink organs.

Fig. 5 Phenetic analysis of PPH homologous protein sequences. PPH homologs were identified in the Phytozome database ([Supplementary Table S4](#)). Tree topology reveals four clades: the already functionally characterized PPH clade and three as yet uncharacterized clades that were named PPH-LIKE1–3.

Accordingly, fruits of *SIPPH*-knockdown plants accumulated higher amounts of starch and soluble sugars ([Fig. 4](#)). It has already been demonstrated that the increment in sugar availability in sink organs led to the reduction of carotenoid accumulation by repressing the expression of enzyme-encoding genes of the carotenoid biosynthetic pathway in tomato (Mortain-Bertrand et al. 2008). In agreement with this, *SIDXS* expression is highly reduced in fruits of the *SIPPH*-deficient plants, limiting the input of precursors towards GGPP. The genes encoding the first two committed steps in carotenogenesis also showed reduced levels of expression: *SIPSY1*—which is the most highly expressed paralog in fruits (Tomato Genome Consortium 2012) and its essential role in carotenogenesis during ripening, has already been demonstrated (Fantini et al. 2013)—and *SIPDS*. Thus, it is reasonable to propose that the massive reduction in carotenoid content observed in the fruits of transgenic plants ([Table 1](#)) is linked to the increased sugar availability. Additionally, the relatively higher reduction of lycopene may also be the consequence of a failure of *SILCYβ* transcriptional repression during ripening ([Fig. 3](#)). The perturbation in Chl metabolism in *SIPPH*-knockdown plants resulted in an altered tocopherol profile in fruits as well ([Table 2](#)). Taking into

account that tomato mature green fruits harbor an active photosynthetic ETR (Piechulla et al. 1987), the slight increment in total tocopherol suggests a tuning of protective antioxidant compounds to deal with oxidative stress. Chl degradation-derived phytol is the main source of PDP for tocopherol synthesis in tomato, as demonstrated by the tocopherol depletion described in *SIVTE5*-silenced plants (Almeida et al. 2016). Although *SIVTE5* and *SIVTE6* are down-regulated in *SIPPH*-knockdown fruits, such a severe phenotype is not verified because of the failure of transcriptional repression of *SIGGDR* during ripening, which deflects GGPP from carotenogenesis towards PDP biosynthesis ([Fig. 3](#)) (Quadrona et al. 2013, Almeida et al. 2015). However, the de novo PDP supply is not enough to maintain wild-type levels of tocopherol in ripe fruits. The transgenic fruits showed TEAC inverted patterns in hydrophilic and hydrophobic fractions ([Fig. 2](#)), which were negatively and positively correlated, respectively, to the variation in lipophilic antioxidant content, especially total tocopherol. These results provided evidence for the existence of a compensatory regulation between antioxidant mechanisms and reinforced the role of tocopherol as an $^1\text{O}_2$ scavenger in PSII (Kaiser et al. 1990, Fukuzawa et al. 1997).

Fig. 6 Transcript profile of *SIPPH* and *SIPPHL* genes in leaves and during fruit ripening of the wild-type genotype. Data represent the mean $\pm$ SD from at least three biological replicates and are expressed as relative transcript amount compared with the corresponding mature green stage. Statistically significant differences ($P < 0.05$) compared with the mature green stage are marked with an asterisk.

Based on the collected data and the state of knowledge, it seems reasonable to propose that PPH, beyond the already demonstrated function in senescence degreening, plays a role in photosystem-associated pigment recycling. The identification of three *SIPPH-like* genes brings new players to the Chl metabolic network for further investigation (Fig. 5). In this sense, Arabidopsis orthologs of *SIPPH-like2* and *SIPPH-like3* were previously proposed to be associated with steady-state Chl turnover (Lin et al. 2014). According to the expression pattern, *SIPPH-like3* appears to be a candidate responsible for the delayed ripening-associated degreening observed in *SIPPH*-knockdown fruits (Fig. 6). It is worth highlighting that co-silencing of the *SIPPHL* genes (Fig. 7) has two implications: they could partially compensate the deficiency of *SIPPH* in fruit degreening, and also the above-described phenotypes might be a consequence not only of the *SIPPH* knockdown but also of the slight reduction in the expression of any of the *SIPPHL* genes.

In summary, *SIPPH* silencing in tomato affects photosynthesis, carbon partitioning and, in an organ-specific manner, isoprenoid metabolism, which, ultimately, results in fruits with reduced levels of nutraceutical compounds but enriched levels of soluble sugars.

Materials and Methods

Plant material, growth conditions and sampling

SIPPH-silenced transgenic lines (L17, L24 and L27) were generated in a previous study (Guyer et al. 2014) by constitutively expressing an intron-spliced RNAi

construct targeted at *SIPPH* (Solyc01g088090) in *S. lycopersicum* (cv Ailsa Craig). T_2 plants were grown in a greenhouse under automatic irrigation (four times a day) at an average mean temperature of 25°C, 11.5/13 (winter/summer) light hours and 250–350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of incident photoirradiance. Plant height and leaf number were measured weekly. The sixth fully expanded leaves from the base of 7-week-old plants were harvested in the middle of the light period. Pericarp samples from fruits at mature green, breaker and ripe stages were harvested at approximately 39 (displaying jelly placenta), 42 and 52 d after anthesis, respectively. All samples were frozen in liquid N_2 , powdered and stored at -80°C . For tocopherol content determination, leaf and pericarp samples were dried by lyophilization before extraction.

Quantitative PCR analysis

RNA extraction, cDNA synthesis, primer design and qPCR assays were performed as described by Quadrona et al. (2013). Primer sequences used are detailed in [Supplementary Table S3](#). qPCRs were carried out in a 7500 real-time PCR system (Applied Biosystems) using 2 $\times$ SYBR Green Master Mix reagent (Life Technologies) in a 20 μl final volume. Absolute fluorescence data were analyzed using the LinRegPCR software package (Ruijter et al. 2009) in order to obtain quantitation cycle (Cq) values and calculate PCR efficiency. Expression values were normalized against the geometric mean of two reference genes, TIP41 and EXPRESSED, according to Quadrona et al. (2013). A permutation test lacking sample distribution assumptions (Pfaffl et al. 2002) was applied to detect statistical differences ($P < 0.05$) in expression ratios using the algorithms in the fgStatistics software package version 17/05/2012 (Di Rienzo 2009).

Tocopherol and pigment quantification

Tocopherols were extracted from approximately 25 mg DW as described in Almeida et al. (2015). The samples were adjusted to 4 ml final volume. Aliquots of 3 ml were dried under N_2 and dissolved in 200 μl of mobile phase composed of hexane/*tert*-butyl methyl ether (90:10). Chromatography was carried out on a Hewlett-Packard series 1100 HPLC system coupled with a fluorescence

Fig. 7 Transcript profile of *SIPPHL* genes in *SIPPH*-knockdown plants in leaves and during fruit ripening. Data represent the mean $\pm$ SD from at least three biological replicates and are expressed as relative transcript amount compared with the corresponding wild-type sample. Statistically significant differences ($P < 0.05$) compared with the mature green stage are marked with an asterisk.

detector (Agilent Technologies series 1200) on a normal-phase column (LiChrosphere® 100 Diol Si; 250 mm $\times$ 4.0 mm, 5 μ m; Agilent Technologies) at room temperature, with the mobile phase running isocratically at 1 ml min⁻¹. Eluted compounds were detected by excitation at 295 nm and fluorescence was quantified at 330 nm.

Chl extraction was carried out as described in Porra et al. (1989). A 1 ml aliquot of dimethylformamide (DMF) was added to 100 or 300 mg FW for leaf or fruit samples, respectively. After sonication for 5 min and further centrifugation at 9,000 $\times$ g for 10 min, the supernatant was collected. The procedure was repeated until total removal of tissue green color. Spectrophotometer measurements were performed at 664 and 647 nm.

Carotenoid extraction was modified from Srino et al. (2009). Aliquots of 200 mg FW were sequentially extracted with a solution of saturated NaCl, dichloromethane and hexane: diethyl ether (1 : 1). After centrifugation, the supernatant was collected and the last step was repeated three more times. Samples were dried by vacuum and dissolved in 200 μ l of acetonitrile. Chromatography was carried out on an Agilent Technologies series 1100 HPLC system on a normal-phase Phenomenex column (Luna C18; 250 $\times$ 4.6 mm, 5 μ m particle diameter) at room temperature with a flow rate of 1 ml min⁻¹. The mobile phase was a gradient of ethyl acetate (A) and acetonitrile: water 9 : 1 (B): 0–4 min with 20% A/80% B; 4–30 min with 65% A/35% B; 30–35 min with 65% A/35% B; 35–40 min with 20% A/80% B. Eluted compounds were detected between 340 and 700 nm and quantified at 450 nm.

Trolox equivalent antioxidant capacity (TEAC)

Leaf and pericarp samples of 100 and 500 mg FW, respectively, were ground in liquid nitrogen and subsequently homogenized in 1 ml of 100 mM sodium acetate buffer pH 7.0. The homogenate was incubated at 4C for 30 min and

then centrifuged for 10 min at 4C at 5,000 $\times$ g. The supernatant was immediately analyzed for hydrophilic antioxidant activity and the pellet was resuspended in 1.5 ml of hexane, incubated at 4C for 30 min and then centrifuged for 10 min at 4C at 5,000 $\times$ g. This procedure was performed three times and the organic extract was reduced to 500 μ l under vacuum and immediately analyzed for lipophilic antioxidant activity. Both hydrophilic and lipophilic antioxidant activities were spectrophotometrically determined as the deactivation of an activated 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS⁺)) solution prepared as described in Re et al. (1999) and compared with a standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Absorbance was read at 734 nm after 2 h of incubation at 25C and the results were expressed as TEAC.

Leaf gas exchange and fluorescence measurements

Gas exchange and Chl fluorescence parameters were measured in the sixth fully expanded leaf from the base of 7-week-old plants between 10 and 12 h using a portable open gas-exchange system incorporating infra-red CO₂ and water vapor analyzers (LI-6400XT system; LI-COR) equipped with an integrated modulated Chl fluorometer (LI-6400-40; LI-COR). The reference CO₂ concentration was held at 400 μ mol mol⁻¹ and leaf temperature at 28C for all measurements. Air humidity inside the leaf chamber was equivalent to values measured inside the greenhouse (approximately 75%). Carbon assimilation rate (*A*), leaf stomatal conductance (*g*_s), leaf dark respiration (*R*_d), transpiration (*E*) and fluorescence parameters were measured at 800 μ mol PPFD m⁻² s⁻¹. For the values of minimal (*F*_o) and maximal (*F*_m) fluorescence and leaf dark respiration, leaves were dark-adapted for 30 min before a saturating pulse of light. The parameters derived from Chl fluorescence, including dark-adapted PSII maximum quantum efficiency (*F*_v/*F*_m), proportion of open PSII centers (photochemical quenching, *qP*), PSII operating efficiency (Φ _{PSII}), NPQ and ETR were calculated according to Maxwell and Johnson (2000).

Quantification of starch and soluble sugars

Extraction and quantification of soluble sugars and starch were performed as described in Freschi et al. (2010) with some modifications. Briefly, 100 and 300 mg FW of leaf and pericarp samples, respectively, were extracted in 1 ml of 80% ethanol for 15 min at 80C and the supernatants were recovered by centrifugation (5,000 $\times$ g, 15 min). Pellets were re-extracted five times and all supernatants were combined and reduced to dryness under vacuum. Starch content was determined from dried pellets as described in Suguiyama et al. (2014). The supernatant residue was resuspended in 50 μ l of pyridine and derivatized for 40 min at 60C with 20 μ l of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. Sample volumes of 1 μ l of the trimethylsilylated extracts were analyzed on a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) and total ion current spectra were recorded in the mass range of 50–700 atomic mass units in scanning mode. The chromatograph was equipped with a fused-silica capillary column (30 m, ID 0.25 mm, 0.5 μ m thick internal film) DB-5 MS stationary phase using helium as the carrier gas at a flow rate of 4.5 ml min⁻¹. The initial running condition was 100C for 5 min, followed by a gradient up to 320C at 8C min⁻¹. The injector temperature was 250C and the following mass spectrometry (MS) operating parameters were used: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 230C; interface temperature, 260C. The endogenous metabolite concentration was obtained by comparing the peak areas of the chromatograms with commercial standards.

Phenetic analysis

For the phenetic analysis, the Phytozome v10.1 database (Goodstein et al. 2012) was surveyed for AtPPH homologous sequences (Supplementary Table S4). The retrieved protein sequences were aligned by Clustal in MEGA 6.0 software (Tamura et al. 2013) using default parameters. The tree reconstruction from the obtained alignment was performed using the PHYML 3.0 algorithm (Guindon et al. 2010) hosted at <http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html> with the following parameters: LG substitution model, four substitutions rate categories; tree optimization by topology and branch length; and improvement by subtree pruning and regrafting. From the data set, the proportion of invariable sites, equilibrium of frequencies

and gamma shape parameter were estimated. Branches were supported by SH-like support.

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 transgenic lines against the wild-type control genotype. 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. A parameter was considered to be affected by *SIPPH* silencing if at least two out of the three transgenic lines differed significantly from the wild-type genotype.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Brazil) [fellowships to B.S.L., D.R., J.A. and A.P.S.; grant to M.R.]; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brazil) [fellowships to M.R. and M.S.B.]; Universidade de São Paulo (USP; Brazil); the Swiss National Science Foundation [grants to S.H.].

Disclosures

The authors have no conflicts of interest to declare.

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CHAPTER III. MANIPULATION OF LEAF SENESCENCE AS A STRATEGY TO IMPROVE TOMATO YIELD

The results, methodology and discussion of this chapter are presented as a manuscript accepted for publication in *Plant Physiology*. Supplementary material corresponds to the attachment IV.

Lira, B.S., Gramegna G., Trench, B., Alves, F.R.R., Silva, E.M., Silva, G.F.F., Thirumalaikumar, V.P., Lupi, A.C.D., Demarco, D., Purgatto, E., Nogueira, F.T.S., Balazadeh, S., Freschi, L. & Rossi, M. Manipulation of a senescence-associated gene improves fleshy fruit yield in *Solanum lycopersicum* (submitted manuscript).

Abstract

Leaves are the major sources of photoassimilates in most plant species. When the senescence program begins, carbon assimilation is the highest, thus, as consequence of its progression, the photosynthetic rate gradually decreases, while the intense macromolecular catabolism turns the senescing leaf in a source of nutrient for other organs. In this scenario, delaying leaf senescence has been proposed as a strategy to increase crop yield, although the evidences were mostly recruited from grasses species, which undergoes whole plant senescence after the filling phase. Thus, there is scarce knowledge regarding the application of such approach to improve the yield of a fleshy fruit species like the tomato *Solanum lycopersicum*. To address this question, the key regulator of senescence initiation ORESARA1 (ORE1) was chosen to be knocked-down in order to delay leaf senescence initiation and, this way, increase the photosynthetically active period of leaves. First, tomato genome was surveyed and three putative *AtORE1* orthologs were identified. These candidates were characterised with regards to their transcriptional profile upon senescence-inducing treatments, regulation by the *SlmiR164*, capability to physically interact with SIGLKs, subcellular localization and capacity to revert the *stay green* phenotype of *ore1 A. thaliana* mutant. All three putative orthologs were induced by the hormonal treatments, directly or indirectly regulated by *SlmiR164*, physically interacted with both SIGLK1 and SIGLK2, are localised in the nucleus and partially rescued *Atore1* phenotype. Thus, the one more closely related to AtORE1 in the phylogenetic reconstruction, namely *SLOREIS02*, was chosen. Leaves of *SLOREIS02*-knockdown plants showed increased chlorophyll content and were able to maintained higher photosynthetic rate for longer periods, displaying delay in dark-induced senescence. *SLOREIS02*-silenced plants produced more fruits without affecting the vegetative development, which resulted in a considerable enhancement in

harvest index. Higher assimilation together with increased yield hint increased carbon exportation from leaves towards fruits, which indeed accumulated more sugars, leading to ripe fruits with higher soluble solid content. Collectively, data obtained support the manipulation of the onset of senescence as an effective strategy to improve tomato yield.

Running title: Senescence manipulation enhances tomato yield

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Article title: Manipulation of a senescence-associated gene improves fleshy fruit yield in *Solanum lycopersicum*

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One sentence summary: Senescence-associated gene knockdown increases carbon exportation towards sink organs increasing plant yield.

List of 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; G.G. designed and performed the confocal and protein-protein interaction experiments and wrote the article with contributions of all the authors; B.T. performed some experiments; F.R.R.A. performed the gas exchange and chlorophyll fluorescence analysis; A.C.D.L. performed some experiments; D.D assisted the preparation of TEM samples and obtained the micrographs; E.P. performed soluble sugar quantification; E.M.S. and G.F.F.S. produced the transgenic plants *OEmiR164a*; F.T.S.N. supervised E.M.S. and G.F.F.S.; V.P.T. generated the transgenic *Atore1* lines overexpressing each *S/ORE1* putative ortholog under the supervision of S.B.; 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.

Funding information:

B.S.L., G.G., F.R.R.A. and B.T. were recipients of FAPESP fellowships, M.R., F.T.S.N. and D.D. were funded by a fellowship from CNPq. A.C.D.L. was recipient of CAPES fellowship. This work was partially supported by grants from FAPESP (Brazil), CNPq (Brazil), CAPES (Brazil) and USP (Brazil).

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Abstract

Senescence is the process that marks the end of leaves lifespan. As it progresses, the massive macromolecular catabolism dismantles the chloroplasts and, consequently, decreases the photosynthetic capacity of these organs. Thus, senescence manipulation is a strategy to improve plant yield by extending the leaf photosynthetically active window of time. However, it remains to be addressed if this approach can improve fleshy fruit production and nutritional quality. One way to delay senescence initiation is by regulating key transcription factors (TFs) involved in triggering this process, such as the NAC TF ORESARA1 (ORE1). Here, three senescence-related NAC TFs from *Solanum lycopersicum* were identified, namely SLORE1S02, SLORE1S03 and SLORE1S06. All three genes showed to be responsive to senescence-inducing stimuli and post-transcriptionally regulated by the microRNA *miR164*. Moreover, the encoded proteins physically interacted with the chloroplast maintenance related TF SIGLKs. This characterization led to the selection of a putative tomato *ORE1* as target gene for RNAi knockdown. Transgenic lines showed delayed senescence and enhanced carbon assimilation that, ultimately, increased the number of fruits and their total soluble solid content. Additionally, the fruit nutraceutical composition was enhanced. In conclusion, the data provide robust evidence that the manipulation of leaf senescence is an effective strategy for yield improvement in fleshy fruit-bearing species.

Keywords: *Solanum lycopersicum*, tomato, *ORESARA1*, *GOLDEN2-LIKE*, senescence, yield.

Introduction

The ever-growing demand for food and biofuels has been a significant driving force behind studies focussed on crop yield manipulation. As a complex trait, many strategies can lead to yield improvement, such as manipulation of morphogenetic patterns, source activity, source to sink communication or sink activity (Rossi et al., 2015). Delaying senescence to extend leaf photosynthetic activity is considered one mechanism to strengthen source activity aiming to increase the net carbon availability for sink organs (Thomas & Ougham, 2014; Rossi et al., 2015). The expression of *ISOPENTENYL TRANSFERASE (IPT)*, which encodes the enzyme responsible for the rate-limiting step in cytokinin biosynthesis, under the control of the *SENESCENCE ASSOCIATED GENE 12 (SAG12)* promoter has been extensively used for delaying leaf senescence (Gegersen et al., 2013). Once the expression is activated during senescence by the *SAG12* promoter, the increment in cytokinin levels blocks senescence progression thereby retaining photosynthetically active leaves. This approach has proven to successfully block or attenuate both age- and stress-related leaf senescence in different crop species like rice (Liu et al., 2010), cassava (Zhang et al., 2010), wheat (Sykorova et al., 2008) and tomato (Swartzberg et al., 2006). With the increase in the knowledge about the genetic control of senescence, transcription factors (TFs), such as those belonging to the WRKY, MYB and NAM/ATF1/CUC2 (NAC) families, stand out as regulators of this process (Balazadeh et al., 2008). The extensive NAC family regulates a diversity of processes along plant development (Mitsuda et al., 2005, Olsen et al., 2005, Puranik et al., 2012). Particularly, the *Arabidopsis thaliana ORESARA1* gene (*AtORE1*) has received special attention for encoding a master regulator of senescence initiation (Oh et al., 1997, He et al., 2005, Kim et al., 2009, Balazadeh et al., 2010, Kim et al., 2011, Rauf et al., 2013), as *Atore1* mutant exhibits a delayed senescence phenotype (Oh et al., 1997, Kim et al., 2009). Besides being induced by ethylene, salt, darkness and abscisic acid (Kim et al., 2009, Balazadeh et al., 2010, Kim et al., 2011, Sakuraba et al., 2014; Song et al., 2014,) *AtORE1* is also post-

transcriptionally regulated by microRNA *miR164* (Kim et al., 2009). *AtORE1* not only induces senescence-related genes, but also physically interacts with and inactivates the chloroplast maintenance-related TF GOLDEN2-LIKE (GLK), shifting the cell from a photosynthetically active program towards senescence (Rauf et al., 2013, Garapati et al., 2015). GLKs have been shown to control chloroplast development in several species, in which usually a pair of paralogs with functional redundancy occurs in the genome of several species (Fitter et al., 2002, Waters et al., 2008, 2009). In tomato, *Solanum lycopersicum*, the paralogs exhibit organ specificity; while *SIGLK1* is mostly expressed in vegetative organs, *SIGLK2* is exclusively transcribed in the stylar portion of the fruits (Powell et al, 2012, Nguyen et al., 2014). Interestingly, *Slgk2* mutation, which was fixed in many cultivated varieties, negatively impacts fruit chloroplast differentiation; on the other hand, *SIGLK2* overexpression increases fruit starch, soluble sugar and carotenoid content (Powell et al., 2012).

Although being a model plant for fleshy fruit physiology, the senescence regulatory network and its impacts on fruit development and quality in tomato remains elusive. Here, putative *AtORE1* orthologs, namely *SIORE1S02*, *SIORE1S03* and *SIORE1S06*, were identified in tomato genome. The genes were functionally characterised regarding their responsiveness to senescence-inducing treatments, regulation by *SlmiR164* and capability to physically interact with SIGLKs and to complement *Atore1* mutant phenotype. Moreover, *SIORE1S02*-knockdown plants displayed delayed senescence, enhanced carbon assimilation and altered source-sink sugar partitioning leading to the development of more fruits with increased soluble sugar content. The results obtained provide robust evidences that the manipulation of leaf senescence is a promising strategy for yield improvement in the fleshy fruit-bearing species.

Results

***Solanum lycopersicum* genome harbours three putative senescence-related *AtORE1* orthologs**

118 To identify putative *S. lycopersicum* *AtORE1* orthologs, the protein sequences of all NAC
 119 transcriptions factors of *S. lycopersicum* and *A. thaliana*, retrieved from the Plant Transcription
 120 Factors Database (<http://planttfdb.cbi.pku.edu.cn/>), were aligned (Supplemental Table 1). The
 121 phylogenetic reconstruction (Supplemental Fig. S1) allowed the selection of three putative NAC
 122 domain-containing proteins (Supplemental Fig. S2), named *SIORE1S02*, *SIORE1S03* and
 123 *SIORE1S06* accordingly to the chromosome localization (Fig. 1). Then, their responsiveness to
 124 senescence-inducing treatments was addressed.

Figure 1. Phylogenetic representation of the NAC transcription factor subfamily that includes *AtORE1* and *SIORE1s*. Detail of the phylogenetic reconstruction obtained from the alignment of the protein sequence of all NAC transcription factors of *Arabidopsis thaliana* (green) and *Solanum lycopersicum* (red) obtained from the Plant Transcription Factor Database (Supplemental Table 1). Three putative *AtORE1* orthologs (in bold), named *SIORE1S02*, *SIORE1S03* and *SIORE1S06* according to their chromosome position, were identified.

125 The transcript profile of *SIORE1S02*, *SIORE1S03* and *SIORE1S06* was evaluated upon different *in*
 126 *vitro* treatments for senescence induction, *i.e.* treatment with ethylene, darkness, jasmonic acid,
 127 salicylic acid or salt (Fig. 2). Confirming the induction and progression of senescence, all

treatments triggered significant reduction in leaf chlorophyll content as well as down- and up-regulation of *S/CLK1* (the chloroplast maintenance-related TF) and *S/SAG12* (the senescence-associated gene) genes, respectively (Supplemental Fig. S3). The mRNA profile of the three *SIORE1s* was modulated by the various treatments. While *SIORE1S03* was induced in all cases, the level of *SIORE1S02* mRNA increased upon the application of ethylene, salicylic acid and salt solution, and *SIORE1S06* was upregulated by ethylene and darkness. Thus, although with differences, all three genes were transcriptionally activated by senescence-inducing stimuli.

Figure 2. Transcript profile of *SIORE1s* in response to senescence-inducing treatments. Transcript profile of *SIORE1s* in leaves of 4-week-old *in vitro*-grown plants after senescence-inducing treatments. Values represent the mean $\pm$ SE from at least three biological replicates normalized against the zero hour sample. Each treatment relative transcript ratio is expressed as the ratio between treatment value and the corresponding untreated control (*). Statistically significant differences relative to the zero hour treatment are represented with closed symbols ($P < 0.05$).

The transcriptional profile of *SIORE1s* during fruit development and ripening was also assessed (Fig. 3). For all three *SIORE1s*, the highest expression levels were identified at early immature stages declining towards the ripe stage. Thus, although in leaves these genes were induced by ethylene, mRNA accumulation did not coincide with the ripening-associated climacteric peak of ethylene production typically observed at the breaker (BR) stage. This result indicates that, although a role in early fruit development cannot be ruled out, a direct function of *SIORE1s* along fruit ripening seems unlikely, as revealed by the expression pattern in this organ. Moreover, the data suggest an organ-specific ethylene-mediated regulation of *SIORE1* genes.

Figure 3. Transcript profile of *SIORE1s* along fruit development and ripening. IG: immature green stage; MG: mature green stage; BR: breaker stage; BRX: X days after breaker stage. Values represent the mean $\pm$ SE from at least three biological replicates normalized to the corresponding IG3 sample. Statistically significant differences are represented with letters ($P < 0.05$).

***SIORE1s* are regulated by *SlmiR164* and their encoded proteins physically interact with SIGLKs and partially recover the *Atore1* phenotype**

In silico analysis of the coding sequences revealed putative binding sites for *SlmiR164* in all three *SIORE1s*. However, this site is disrupted by an insertion of 29 bp in *SIORE1S02* (Fig. 4A). To ascertain if this insertion affects *SlmiR164* recognition, a modified 5' RACE was performed to detect cleaved transcripts, using RNA extracted from non-senescent leaves. This allowed the identification of *SlmiR164*-guided cleavage transcripts of *SIORE1S03* and *SIORE1S06*, but not of *SIORE1S02* (Fig. 4A).

To gain further knowledge about the role of *SlmiR164* for *SIORE1s* regulation, the mRNA of the three genes was profiled in transgenic lines harbouring a 35S:*AtMIR164a* construct (abbreviated as *OEmiR164a*) in non-senescent, early senescent and late senescent leaves (Fig. 4B). The mRNA pattern of *SIGLK1* and *SISAG12* was also assessed as senescence markers. In wild-type plants

(WT), leaf senescence was accompanied by a progressive downregulation in *SlmiR164* and *SIGLK1* and upregulation of *SISAG12*, *SIORE1S02*, *SIORE1S03* and *SIORE1S06*. As expected, *OEmiR164a* lines displayed high levels of *SlmiR164* in both, non-senescent and senescent leaves stages. As a result, a lag in *SIGLK1* decrease and *SISAG12* accumulation, together with the increased chlorophyll levels, were indicatives of delayed senescence initiation (Fig. 4C). The marked reduction of *SIORE1S03* and *SIORE1S06* mRNA levels observed in *OEmiR164a* lines is in agreement to both being directly regulated by *SlmiR164*. Interestingly, the levels of *SIORE1S02* in non-senescent and senescing leaves were the same as those observed in non-senescent WT leaves, meaning that the maintenance of high levels of *SlmiR164* prevented the senescence-associated induction of this gene. Although *SlmiR164* is not capable of cleaving *SIORE1S02* transcripts, it regulates it by targeting a currently unknown factor that, in turn, drives the senescence-associated mRNA accumulation of this gene. Thus, *SlmiR164* directly controls the transcript levels of *SIORE1S03* and *SIORE1S06* and indirectly regulates the transcript abundance of *SIORE1S02*, what is in line with the known transcriptional regulation of *AtORE1* by *AtmiR164* (Kim et al., 2009).

A

SIORE1S02 800 AGCGAGAAGTAGTGGAGAGACATGTAATTCACATGTGACGTGTTTCTCCG 0/13
SIORE1S03 810 A-----GCCACGTGCACTGCTTCTCCA 9/9
SIORE1S06 814 A-----GCCACGTGCACTGCTTCTCCA 5/5
SlmiR164 5' T-----GCACGTGCCCTGCTTCTCCA

B

C

Figure 4. Regulation of *SIORE1s* by *SlmiR164*. **A**, Highlight of the alignment of *SIORE1s* and *SlmiR164* showing the putative binding site. The validation by a modified 5' RACE assay identified cleaved transcripts of *SIORE1S03* and *SIORE1S06*. The red arrowhead and numbers on the right indicate the inferred cleavage site and the fraction of positive cloned PCR products ending at the site, respectively. Shading threshold of alignment = 75%. **B**, Heatmap representing the transcript profile of non-senescent (NS), early senescing (ES) and late senescing (LS) leaves of wild type control (WT) and *OEmiR164a* (lines miR_2 and miR_3) genotype. Values represent mean of at least three biological replicates normalised against the NS WT sample. Statistically significant differences in comparison to NS WT are represented as coloured squares ($P < 0.05$). The relative transcript values are detailed in Supplemental Table S2. **C**, Chlorophyll content in NS, ES and LS leaves. Values represent the mean $\pm$ SE from at least three biological replicates. Asterisks denote statistically significant differences to WT control ($P < 0.05$.)

Moreover, AtORE1 is known to physically interacts with AtGLKs in the nucleus, preventing the activity of these chloroplasts maintenance TFs (Rauf *et al.*, 2013). Then, it was first addressed whether *SIORE1s* and *SIGLKs* are nucleus-targeted proteins. Confocal analysis of *Nicotiana tabacum* leaves transiently expressing transcription factor - GFP fusion proteins revealed that *SIORE1S02*, *SIORE1S03*, *SIORE1S06*, *SIGLK1* and *SIGLK2* were localized in the nucleus as the GFP fluorescence in all cases co-localized with the fluorescence of the DAPI marker (Supplemental Fig. S4). For the latter, the result confirmed that previously reported in tomato protoplasts by Tang *et al.* (2015). Next, bimolecular fluorescence complementation (BiFC) was used to test the physical interaction. The C-terminal of *SIORE1s* and *SIGLKs* were fused to the N- (VYNE) and C-terminal (VYCE) of the Venus YPF protein, respectively (Fig. 5). The interaction between AtORE1 and AtGLK2 was used as positive control. In all interactions tested, fluorescence was detected in

the nucleus, thus, all SIORE1s physically interact with both SIGLKs. Of note, a strong fluorescence signal was observed in the nucleolus in all SIORE1-SIGLK interactions, and also for AtORE1 and AtGLK2.

Figure 5. Analysis of SIGLKs and SIORE1s protein-protein interaction by Bimolecular Fluorescent Complementation (BiFC). VYNE and VYCE fusion proteins were transiently expressed in *N. tabacum* leaves by infiltration with *A. tumefaciens*. VYNE-Cnx6/VYCE-Cnx6 and AtORE1/AtGLK2 (Gehl et al., 2009) were used as technical and biological positive controls, respectively. Cnx6 and SIOREs/SIGLKs interaction were used as negative controls. SIOREs/SIGLKs interaction was confirmed as evidenced by the YFP fluorescence detected. YFP, DAPI nuclear marker, bright-field and merged signals are indicated above the panels. Bars = 20 μ m, except in VYNE-Cnx6/VYCE-Cnx6 images = 40 μ m.

It has been suggested that the ubiquitin proteasome system may be active in the nucleolus, where ubiquitin and proteasome were immunolocalized (Stępiński, 2012). In addition, this compartment provides a site for transient inactivation of enzymatic or regulatory proteins (Stępiński, 2014). As SIGLK2 was shown to be degraded by the ubiquitin-proteasome system (Tang et al., 2015), one may speculate that the ORE1-GLK complex is transferred to the nucleolus where GLKs are inactivated by ubiquitination and subsequently degraded in this compartment. These results show similarities between *SIORE1s* and *AtORE1* regulation. To address whether *SIORE1s* were able to recover the *Atore1* delayed-senescence phenotype, each *SIORE1s* was constitutively overexpressed in the Arabidopsis mutant background, *Atore1*-OE:*SIORE1S02*, *Atore1*-OE:*SIORE1S03* and *Atore1*-OE:*SIORE1S06*. In line with *AtORE1* overexpression in Col-0 background (Rauf et al., 2013), no visible phenotype was observed in young plants. Dark-induced senescence was assayed in detached leaves. After seven days, while *Atore1* leaves remained

green, the leaves overexpressing any of the *SIORE1s* acquired a yellowish tone indicative of ongoing senescence, but not to the same extent as the Col-0 genotype (Fig. 6). Thus, all three *SIORE1s* were able to partially recover the senescence delay in *Atore1* mutant.

Figure 6. *SIORE1s* partially recover *Atore1* senescence impairment. Detached leaves from three-month-old Col-0, *Atore1* mutant, *Atore1-OE:SIORE1S02*, *Atore1-OE:SIORE1S03* and *Atore1-OE:SIORE1S06* plants were kept in darkness for seven days to induce senescence. While *Atore1* displayed no signs of senescence, the yellowish colour of the genotypes overexpressing *SIORE1s* hints an ongoing senescence program, yet not to the extent observed in the Col-0 genotype. Bar = 1 cm.

***SIORE1S02*-knockdown in tomato increases photosynthesis and delays dark-induced senescence**

Among the three *SIORE1s*, *SIORE1S02* was chosen for stable RNA interference (RNAi) knockdown as this is the closest ortholog to *AtORE1* in the phylogenetic reconstruction, its transcript levels are highly modulated along senescence and indirectly regulated by *SlmiR164*, and the encoded protein physically interacts with SIGLKs and partially complement the *Atore1* mutant. Five

tomato transgenic lines were obtained and three lines displaying over 70% silencing (Supplemental Fig. S5), namely L1, L3 and L6, were chosen for further phenotyping. Interestingly, *SIORE1S03* and *SIORE1S06* were also reduced in leaves of *SIORE1S02*-knockdown lines, but not to the same extent as *SIORE1S02*. Since there is an extremely low degree of similarity among the RNAi target region of *SIORE1S02* and the coding sequences of *SIORE1S03* and *SIORE1S06*, the possibility of cross-silencing can be ruled-out. Instead, it is likely that *SIORE1S02* is an upstream positive regulator of *SIORE1S03* and *SIORE1S06* (Supplemental Fig. S6).

Gas exchange and chlorophyll fluorescence parameters were measured by using an infra-red gas analyser (IRGA) at two developing stages of the plant: (I) before the beginning of flowering stage, in the fifth leaf of 70-day-old plants (referred as young plant leaves - YPL), and (II) in fruit-bearing stage, in the sixth leaf of 120-day-old plants (referred as mature plant leaves - MPL). No differences were found in photosynthetic parameters in YPL. However, MPL from *SIORE1S02*-knockdown plants showed increased carbon assimilation (*A*) in comparison to the untransformed control genotype (MT), while all other measured parameters remained unaltered (Table 1).

Table 1. Photosynthetic parameters of *SIORE1S02*-knockdown lines.

Genotype	CO ₂ assimilation (<i>A</i>) ($\mu\text{mol CO}_2/\text{m}^2 \text{ s}$)	Electron transport rate (<i>ETR</i>) ($\mu\text{mol}/\text{m}^2 \text{ s}$)	Photochemical quenching (<i>qP</i>)	Non-photochemical quenching (<i>NPQ</i>)
MT YPL	4.31 $\pm$ 0.21	65.80 $\pm$ 3.16	0.28 $\pm$ 0.01	1.69 $\pm$ 0.21
MPL	3.92 $\pm$ 0.15	35.93 $\pm$ 3.90	0.18 $\pm$ 0.02	2.18 $\pm$ 0.21
L1 YPL	4.63 $\pm$ 0.64	75.51 $\pm$ 7.47	0.30 $\pm$ 0.03	1.57 $\pm$ 0.12
MPL	5.93 $\pm$ 0.84	49.91 $\pm$ 4.38	0.24 $\pm$ 0.02	2.13 $\pm$ 0.04
L3 YPL	8.55 $\pm$ 0.95	70.13 $\pm$ 6.71	0.31 $\pm$ 0.03	2.23 $\pm$ 0.34
MPL	5.28 $\pm$ 0.46	41.63 $\pm$ 1.34	0.21 $\pm$ 0.01	2.28 $\pm$ 0.19
L6 YPL	3.97 $\pm$ 0.46	66.31 $\pm$ 4.47	0.31 $\pm$ 0.02	1.87 $\pm$ 0.15
MPL	5.57 $\pm$ 0.08	38.68 $\pm$ 2.98	0.21 $\pm$ 0.01	1.76 $\pm$ 0.18

Values represent mean $\pm$ SD of at least three biological replicates. Statistically significant differences to MT control are in bold.

Chlorophyll levels in YPL and MPL were higher in transgenic plants than in equivalent leaves of the MT genotype (Fig. 7A). Whereas, at MPL stage, the density of chloroplasts per mesophyll cell was slightly higher in transgenic lines (Fig. 7B), the ultrastructure of this organelle was not

affected by the downregulation of *SIORE1S02* (Supplemental Fig. S7). Accordingly, *SIGLK1* mRNA levels were higher in both YPL and MPL of transgenic plants (Fig. 7C). Interestingly, while the senescence marker *SISAG12* was undetectable in YPL, lower levels were detected in transgenic MPL compared to the corresponding MT control (Fig. 7C). Together these results suggest a delay in senescence in *SIORE1S02*-knockdown plants. To further corroborate this hypothesis, the eighth leaf of 120-day-old plants was detached and kept in the dark for 10 days. While MT leaves turned yellowish, transgenic ones kept greenness, supporting the conclusion that *SIORE1S02* deficiency delays senescence (Fig. 7D).

Figure 7. *SIOR1S02*-knockdown affects leaf aging. **A**, Chlorophyll content in leaves. Values represent the mean $\pm$ SE from at least three biological replicates. Asterisks denote statistically significant differences to MT control ($P < 0.05$). YPL: young plant leaves; MPL: mature plant leaves. **B**, Number of chloroplasts per mesophyll cell of MPL. Values represent the mean $\pm$ SE from at least 45 cells. Asterisks denote statistically significant differences to MT control ($P < 0.05$). **C**, *SIGLK1* and *SISAG12* transcript ratio in YPL and MPL leaves. Values represent the mean $\pm$ SE from at least three biological replicates normalised against the respective sample from MT control. Asterisks denote statistically significant differences to MT control ($P < 0.05$). The relative transcript values are available in Supplemental Table S4. **D**, *SIOR1S02*-knockdown effect on dark-induced senescence. Detached leaflets from 120-day-old plants kept ten days in darkness retain greenness, while MT control leaflets turned yellow. Bar = 5 cm.

***SIORE1S02*-knockdown in tomato increases yield and brix**

As transgenic plants assimilate carbon for an extended period of time, the impact of *SIORE1S02*-knockdown over yield was evaluated. Transgenic plants produced more fruits than MT control plants, rendering a significant increase in harvest index (HI), as no difference was detected in aerial shoot mass (Fig. 8A). The transgenic fruits showed no alteration in the shape, weight and diameter (Supplemental Fig. S8). Besides, *SIORE1S02*-knockdown lines showed higher levels of

Figure 8. *SIORE1S02*-knockdown increases yield and *brix*. **A**, Harvest index, aerial part weight and ripe fruit number in MT and *SIORE1S02*-knockdown lines. Values represent the mean $\pm$ SE from at least six biological replicates. Asterisks denote statistically significant differences compared to MT control genotype ($P < 0.05$). **B**, Total soluble solids of ripe fruits measured in *brix* units. Values represent the mean $\pm$ SE from at least twelve biological replicates. Asterisks denote statistically significant differences compared to MT control genotype ($P < 0.05$).

soluble solids in ripe fruits as reflected by the increment in *brix* units (Fig. 8B), which is also an important trait in tomato, especially for industrial processing.

Enhanced HI and *brix* are typically due to increased levels of glucose, fructose and sucrose and are indicative of altered carbohydrate metabolism. Thus, the content of starch and soluble sugars in leaves and fruits was determined (Fig. 9). The fruits of transgenic plants showed an increased content of glucose in all analysed stages and of fructose in both ripening stages, which explains the increment in *brix* units. Moreover, starch content in mature green and breaker fruits of *SIORE1S02*-knockdown lines were also higher when compared to MT control. As expected, six days after the breaker stage, starch was totally degraded in all genotypes. In leaves, while YPL of transgenic plants accumulated higher starch and sucrose levels than MT control genotype; when fruits are developing, in the MPL, the allocation of fixed carbon as starch diminished, whereas sucrose still accumulated at higher levels. The upregulation in the leaf carbohydrate levels triggered by *SIORE1S02*-knockdown is consistent with the above-mentioned increase in carbon assimilation observed in the transgenic lines. Moreover, considering that in tomato carbon is mostly translocated as sucrose (Barker et al., 2000), this carbohydrate pattern suggests an enhanced carbon export from leaves towards sink organs in *SIORE102*-deficient plants.

Figure 9. *SIORE1S02*-knockdown alters sugar profile in leaves and fruits. Content of starch and soluble sugars in leaves and fruits of *SIORE1S02*-knockdown lines. Values represent the mean $\pm$ SE from at least three biological replicates. Asterisks denote statistically significant differences to MT control genotype ($P < 0.05$). ND: Not detected. YPL, young plant leaves; MPL, mature plant leaves; MG, mature green stage; BR, breaker; BR6: 6 days after BR, respectively.

258 **Isoprenoid metabolism is affected by *SIORE1S02*-knockdown tomato plants**

259 It was shown that lowering *SIORE102* transcript abundance affects chlorophyll accumulation and
260 carbon assimilation and partitioning. To better understand the origin of these metabolic
261 alterations and their impact of fruit chemical composition, isoprenoid-derived compounds (*i.e.*
262 chlorophyll, tocopherols and carotenoids, Supplemental Table S3) were quantified and the
263 mRNA levels of chlorophyll-, tocopherol- and carotenoid-metabolism associated genes profiled
264 (Supplemental Table S4) (Fig. 10). The analysed genes were those that showed to be
265 transcriptionally regulated key points of the biosynthetic pathways (Quadrana et al., 2013;
266 Almeida et al., 2015, 2016).

Figure 10. Isoprenoid metabolism. Schematic representation of the interconnection between Chl synthesis and degradation (green), carotenogenesis (orange) and tocopherol biosynthetic (blue) pathways. Dotted lines denote that intermediate steps were omitted. The heatmap represents statistically significant differences in relative transcript and metabolite amounts detected in *SIORE1S02*-knockdown lines compared to the corresponding organ of MT control genotype ($P < 0.05$). For simplicity, the mean of the three transgenic line values is represented when, at least, two were statistically significant different than MT control. The absolute metabolite and relative transcript values are detailed in Supplemental Table S3 and S4. Black and grey colours indicate that transcripts were not detected or not addressed, respectively. Enzymes and compounds are named according to the following abbreviations: GGDR, geranylgeranyl diphosphate reductase; PSY, phytoene synthase; PDS, phytoene desaturase; LCY β , chloroplast-specific β -lycopene cyclase; CYC β , chromoplast-specific β -lycopene cyclase; GGDP, geranylgeranyl diphosphate; PDP, phytol diphosphate; PP, phytol phosphate; CHLG, chlorophyll synthase; PPH, pheophytinase; PPHL1, pheophytinase-like 1; pFCC, primary fluorescent chlorophyll catabolite; VTE5, phytol kinase; VTE6, phytol phosphate kinase; MPBQ, 2-methyl-6-geranylgeranylbenzoquinol; VTE1, tocopherol cyclase; VTE2, homogentisate phytol transferase; VTE3, 2,3-dimethyl-5-phytylquinol methyltransferase; VTE4, tocopherol C-methyl transferase; MEP, methylerythritol 4-phosphate. Adapted from Almeida et al. (2016).

267 In MPL of *SIORE1S02*-knockdown plants, the chlorophyll recycling/biosynthesis was enhanced,
 268 as evidenced by the increase in *CHLOROPHYLL SYNTHASE (SICHLG)*, *PHEOPHYTINASE-LIKE 1*
 269 (*SIPPHL1*) and *PHYTOL KINASE (SIVTE5)* transcript abundance. Although the mRNA levels of
 270 *PHYTOENE SYNTHASE 1 (SIPSY1)* and *PHYTOENE DESATURASE (SIPDS)* were increased in YPL, no
 271 differences were found in leaf carotenoid contents, except for a slight reduction in lutein from

MPL. Regarding tocopherol synthesis, YPL showed similar levels of tocopherol in both genotypes, which is in line with the expression of the enzyme encoding genes that supply phytyl diphosphate (PDP) precursor, such as *GERANYLGERANYL DIPHOSPHATE REDUCTASE (SIGGDR)*, *SIVTE5* and *PHYTYL PHOSPHATE KINASE (SIVTE6)*. However, in *SIORE1S02*-knockdown MPL, due to chlorophyll biosynthesis enhancement, PDP availability for tocopherol synthesis is reduced compared to MT control resulting in reduced levels of this compound. It is worth mentioning that the most abundant form of vitamin E is α -tocopherol that perfectly correlates with the amount of total-tocopherol (Supplemental Table S3). Apparently, the maintenance of a highly active photosynthetic apparatus in MPL requires less amounts of antioxidant tocopherol compounds.

The impact of *SIORE102*-knockdown on fruit isoprenoid profile was distinct from those observed in leaves. There was no difference in fruit chlorophyll content between transgenic and control plants. Similarly, carotenoid metabolism remained almost unaffected, displaying punctual reductions in neurosporene and lutein content in MG and BR1 fruits, respectively. Tocopherols were more abundant in *SIORE1S02*-knockdown than in control fruits, which is in accordance to the transcript levels of *SIGGDR*, *SIVTE6*, *HOMOGENISATE PHYTYL TRANSFERASE (SIVTE2)*, *2,3-DIMETHYL-5-PHYTYLQUINOL METHYLTRANSFERASE (SIVTE3(1))*, *TOCOPHEROL CYCLASE (SIVTE1)* and *TOCOPHEROL C-METHYL TRANSFERASE (SIVTE4)*.

Therefore, *SIORE1S02*-knockdown plants showed organ-specific regulation of isoprenoid metabolism.

Discussion

The leaves are the major photosynthetic organs and the net carbon fixed during the photosynthetic period is critical for plants' fitness and sink organ development (Wu et al, 2012). Thus, source-sink relationship is tightly linked to leaf lifespan and, consequently, affected by the

fine control of senescence. In this regard, a straightforward approach to increase yield is to extend the photosynthetic function phase. This has been achieved in grasses by silencing NAC TF encoding genes. Although an increase in grain yield was observed due to extended grain filling time, seed quality was compromised by reduced nutrient remobilization (Waters et al., 2009; Liang et al., 2014). Yet, the impact of delayed senescence remains poorly explored in non-monocarpic species that do not undergo whole-plant senescence after the reproductive phase (Thomas, 2012).

To gain insight on how fleshy fruit development and yield can be affected by altering leaf senescence, the senescence master regulator *ORE1* was targeted in *S. lycopersicum*. Three putative orthologs were identified in the tomato genome, namely *SIORE1S02*, *SIORE1S03* and *SIORE1S06*. As all were induced by senescence-inducing treatments, regulated by *SlmiR164*, able to physically interact with SIGLKs and capable to rescue *Atore1* mutant, *SIORE1S02*, the phylogenetically closest one to *AtORE1*, was chosen for knockdown by RNA interference.

The reduction in the amount of *SIORE1S02* transcripts affected the metabolism of source leaves, both of young and mature plants. The increase in chlorophyll content and the expression of *SIGLK1* TF, together with the increment in carbon assimilation and the reduced *SISAG12* mRNA levels in leaves of mature plants, hint that senescence is delayed in transgenic plants. This was corroborated by the dark induction senescence assay on detached leaves. The aforementioned phenotype in leaves from the *SIORE1S02*-deficient plants might be explained, at least in part, because GLK TFs are known to coordinate the expression of genes related to chlorophyll synthesis (e.g. *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE*) and the photosynthetic machinery (e.g. light harvesting complex proteins) leading to proper chloroplast differentiation and maintenance (Waters et al., 2009; Nguyen et al., 2014). This is in agreement with the increased chloroplast number in mesophyll cells of mature leaves. Additionally, higher levels of *SICHLG* transcript, whose encoded protein is a key enzyme for chlorophyll biosynthesis and also

regulates the synthesis of chlorophyll-binding proteins (Shalygo et al., 2009), contributes to the extended photosynthetic active period in transgenic plants.

Tocopherol metabolism was also affected in leaves of *SIORE1S02*-knockdown plants. As chlorophyll, tocopherols are isoprenoid-derived compounds that play a main role in protecting photosynthetic machinery by either quenching oxygen singlet ($^1\text{O}_2$) or inhibiting the progression of lipid peroxidation (Havaux et al., 2005). It has been demonstrated that the recycling of chlorophyll degradation-derived phytol is the main source of tocopherol side chain in tomato (Almeida et al., 2015). In agreement with this, the delayed senescence progression proposed above for *SIORE1S02*-knockdown plants explains the reduction in tocopherol content measured in leaves from mature plants. In this sense, it has been suggested that increasing tocopherol content along senescence contributes to the proper dismantling and recycling of plastid thylakoid lipids (Chrost et al., 1999). Additionally, the boost in chlorophyll turnover, evidenced by the increment in mRNA abundance of *SIPPHL1* (Lin et al., 2016), *SIVTE5* (Almeida et al., 2016) and *SICHLG*, driving PDP towards chlorophyll synthesis, further support this statement.

The delayed senescence progression was also reflected in both leaf and fruit sugar composition. During the vegetative phase, before the onset of flower anthesis, while the fifth leaf from control young plants were the main source of assimilates for sink organs, in *SIORE1S02*-deficient plants, these leaves retained higher levels of fixed carbon mainly as starch, as older leaves retained high photosynthetic activity. As fruits began to develop, the starch was remobilised to sucrose and reallocated to these organs. The boosted carbon exportation in *SIORE1S02*-knockdown plants increased yield as the result of the increment in fruit number. Interestingly, the fruits developed slightly faster than the control genotype as the number of days to reach the breaker stage was reduced (Supplemental Fig. S9A). Additionally, BR1 and BR6, but not MG fruits were enriched in total soluble protein content, which might indicate that *SIORE1S02*-knockdown affect protein degradation during chloroplast-chromoplast transition (Supplemental Fig. S9B).

Regarding fruit nutritional quality, *SIGDDR* mRNA accumulated to higher levels in transgenic fruits during ripening increasing PDP availability for tocopherol synthesis. Moreover, in *SIORE1S02*-knockdown genotype, the tocopherol biosynthesis-related genes were upregulated along fruit ripening, contributing to the increment of this nutraceutical. Altogether, the metabolic alterations indicate that *SIORE1S02* deficiency delays ageing by means of extending chloroplast maintenance and carbon assimilation. Consequently, photoassimilate exportation towards fruits is enhanced, ultimately, improving harvest index and the content of tocopherol antioxidant and soluble sugars in ripe fruits. Thus, the data obtained suggests that the manipulation of leaf senescence is a promising strategy to improve fleshy fruit yield and metabolism.

Materials and Methods

Plant material, growth conditions and sampling

In vitro treatments for senescence induction were performed with wild type *Solanum lycopersicum* (cv Micro Tom, MT) harboring the wild-type allele *SIGLK2*. Tomato seeds were surface sterilized, sown and cultivated *in vitro* as described by Lira et al. (2014). Four-week-old plants had senescence induced either by application of ethylene (gaseous to a final concentration of 5 M.L⁻¹), jasmonic acid (gaseous to a final concentration of 30 µM), salicylic acid (liquid solution to a final concentration of 30 µM), salt (liquid NaCl solution to a final concentration of 300 mM) or kept in darkness. Leaf samples were collected 30 min, 1 h, 3 h, 6 h, 48 h-after treatment.

For *SIORE1s* fruit transcriptional profiling, tomato plants were grown in 1-L pots in a greenhouse under automatic irrigation in an average mean temperature of 25°C, 11.5/13 (winter/summer) light hours and 250-350 µmol m⁻² s⁻¹ of incident photo-irradiance. The pericarp from the top portion of the fruit was sampled at immature green 3 (IG3), immature green 5 (IG5), mature

374 green (MG), breaker (BR), breaker + 3 (BR3) and breaker + 5 (BR5) stages were harvested
375 approximately at 10, 22, 37 (displaying jelly placenta) and, approximately, 42 (when the first
376 signals of carotenoid accumulation are visualized), 45 and 48 days after anthesis, respectively.
377 All samples were frozen in liquid N₂, powdered and stored at -80°C.

378 The transgenic lines overexpressing *AtMIR164a* (miR_2 and miR_3) were generated via a 176 bp
379 fragment encompassing the *AtMIR164a* precursor (MIR164aAt2g47585) that was amplified
380 from genomic DNA of *Arabidopsis thaliana* ecotype Columbia (Col-0). The PCR product was sub-
381 cloned into TOPO TA (Invitrogen, <http://www.lifetechnologies.com>) and sequenced. The
382 confirmed *AtMIR164a* precursor was digested using the XbaI and Pst restriction enzymes, and
383 subsequently cloned into binary vector under the control of the CaMV 35S promoter. Once
384 cleaved, the mature *AtMIR164a* has the exact sequence of the mature *SlmiR164* (Accessions for
385 miRbase; <http://www.mirbase.org/>; *AtMIR164*: MI0000197; *SlmiR164*: MI0027570). The genetic
386 transformation was made in *Slglk2 Solanum lycopersicum* (cv Micro Tom) background according
387 to Pino et al. (2010). T2 plants were grown as above described and non-senescent (NS), early
388 senescing (ES) and late senescing (LS) leaves were collected. Leaves stages were set according
389 to the untransformed genotype yellowish phenotype.

390 For subcellular localization and BiFC assays, *Nicotiana tabacum* plants were cultivated for 4
391 weeks in 1-L pots as described above.

392 To evaluate the capability of *SIORE1S02*, *SIORE1S03* and *SIORE1S06* to recover *Atore1* mutant
393 phenotype, this mutant was independently transformed with the three tomato gene coding
394 sequences under the control of the constitutive CaMV 35S promoter. *A. thaliana* plants were
395 grown and transformed according to Balazadeh et al. (2010). Plants from three independent
396 lines of T2 generation were used for dark-induced senescence assay. A pool of 10 detached
397 leaves from three-month-old plants for each genotype were kept in darkness for seven days.

398 *SIORE1S02*-knockdown lines (L1, L3 and L6) were generated by constitutively expressing an
399 intron-spliced hairpin RNA (RNAi) construct of 186 bp targeted to *SIORE1S02* (Soly02g088180).

The fragment was cloned into pK7GWIWG2(I) (Karimi et al, 2002) and introduced in wild type *Solanum lycopersicum* (cv Micro Tom) background, which harbours the wild-type *SIGLK2* allele, following the protocol described in Pino et al. (2010). Gas exchange and chlorophyll fluorescence parameters were measured in the fifth leaf of 70-day-old plants (YPL) and in the sixth leaf of 120-day-old plants (MPL) between 10 and 12 h and were subsequently harvested for biochemical analyses and transcriptional profiling. T1 plants were grown as above described. Pericarp samples from fruits at MG, BR1 and BR6 stages were harvested. For tocopherol content determination, leaf and pericarp samples were dried by lyophilization before extraction. For yield evaluation, an independent set of plants without any destructive sampling was cultivated and all ripe fruits were sampled and weighted until the plants reach 160-day-old when destructive harvest took place. At harvest time, aerial part was weighted and harvest index calculated according to $HI = (\text{total ripe fruit mass}) * 100 / (\text{aerial part mass} + \text{total ripe fruit mass})$. Brix was measured in ripe fruits with a refractometer DR201-95 (Kruss).

Phylogenetic analysis

For the phylogenetic analysis, all NAC transcriptions factors of *S. lycopersicum* and *A. thaliana* (Supplemental Table S1) were retrieved from the Plant Transcription Factors Database (<http://planttfdb.cbi.pku.edu.cn/>). The retrieved protein sequences were aligned by Clustal in MEGA 6.0 software (Tamura et al. 2013) using default parameters. The tree reconstruction from the obtained alignment was performed using PHYML 3.0 algorithm (Guindon et al. 2010) hosted at <http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html> with the following parameters: LG substitution model, four substitutions rate categories, tree optimization by topology and branch length and, improvement by subtree pruning and regrafting. From the dataset, the proportion of invariable sites, equilibrium of frequencies and gamma shape parameter were estimated. Branches were supported by SH-like support.

qPCR and miRNA analysis

RNA extraction, complementary DNA (cDNA) synthesis, primer design and qPCR assays were performed as described by Quadrana et al. (2013). Primer sequences used are detailed in Supplemental Table S5. Stem loop pulse reverse transcriptase were performed as described previously in Varkonyi-Gasic et al., (2007) and modified 5'RACE were performed as described in Morea et al. (2016). 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 14 µL final volume. Absolute fluorescence data were analysed using the LinRegPCR software package (Ruijter et al. 2009) 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 Quadrana et al. (2013). A permutation test lacking sample distribution assumptions (Pfaffl et al. 2002) was applied to detect statistical differences ($P < 0.05$) in expression ratios using the algorithms in the fgStatistics software package version 17/05/2012 (Di Rienzo 2009).

Subcellular localization and BiFC assay

The full-length open reading frames encoding *AtORE1* (AT5G39610), *AtGLK2* (AT5G44190), SIGLKs and SIORE1s were amplified using specific primers listed in Supplemental Table S5 and cloned into pCR™ 8/GW/TOPO TA Cloning vector (Invitrogen). For the subcellular localization experiment, each recombination cassette was amplified using the universal M13 pair of primers and then recombined into the binary vector pK7FWG2 (Karimi et al., 2002) using LR clonase. For BiFC assay, each entry vector was recombined into the binary vector pDEST-VYCE(R)GW or pDEST-VYNE(R)GW (Gehl et al., 2009), which carry the C-terminal and N-terminal fragment of Venus YFP, respectively, using LR Clonase. All BiFC fusion proteins were tagged at the C-terminus. The binary vectors were introduced in *Agrobacterium tumefaciens* strain GV3101. For subcellular localization, cultures were resuspended in infiltration buffer (50 mM MES pH 5.6, 2

mM sodium phosphate buffer pH 7, 0.5% glucose and 200 μ M acetosyringone (Sigma-Aldrich)) to a final OD₆₀₀ of 0.5, incubated for 3 h in the dark at room temperature, and infiltrated into leaves of 4-week-old *Nicotiana tabacum* plants. For BiFC experiments, leaves were co-infiltrated with a mix of both cultures at 0.5 OD₆₀₀. To avoid SIGLKs degradation, 100 μ M of the proteasome inhibitor MG132 (Sigma-Aldrich #C2211) was added to the solution before infiltrating. After 48 or 72 h for localization and BiFC experiments, respectively, the transformed tissues were observed in a confocal laser microscope (Zeiss LSM 780-NLO). DAPI staining was performed by infiltrating 10 μ g/mL of water dissolved DAPI (Life Technologies #D1306) 20 min before the confocal microscope observation. GFP signal was captured over a 508-553 nm range after excitation at 488nm, while DAPI fluorescence was excited at 405 nm and captured over a 415-501 nm range.

The controls VYNE-Cnx6 and VYCE-Cnx6 constructs were kindly provided by Prof. Jörg Kudla, Institute of Plant Biology and Biotechnology, Münster, Germany. CNX6 is a protein that belongs to the complex of molybdopterin synthase involved in MoCo biosynthesis. As reported in Gehl et al. (2009), when VYNE-Cnx6 and VYCE-Cnx6 fusion proteins (approximately 40 KDa), are transiently co-expressed in *N. benthamiana* leaves by agroinfiltration, they localize not only in the cytoplasm but also in the nucleus. This is due to their small size that allows the diffusion through the nuclear pores, which exclusion size is 60 KDa. Thus, Cnx6 auto-dimerization was used as positive control, while VYNE-Cnx6/SIGLK2-VYCE and VYCE-Cnx6/SIORE1S02-VYNE interactions were used as negative controls.

Leaf gas exchange and fluorescence measurements

Gas exchange and chlorophyll fluorescence parameters were measured using a portable open gas-exchange system incorporating infra-red CO₂ and water vapor analysers (LI-6400XT system; LI-COR) equipped with an integrated modulated chlorophyll fluorometer (LI-6400-40; LI-COR). Reference CO₂ concentration was held at 400 μ mol mol⁻¹ and leaf temperature at 28°C for all

measurements. Air humidity inside the leaf chamber was equivalent to values measured inside the greenhouse (approximately 75%). Carbon assimilation rate (A), leaf stomatal conductance (g_s), transpiration (E) and fluorescence parameters were measured at $800 \mu\text{mol PPFD m}^{-2} \text{s}^{-1}$. For the values of minimal (F_o) and maximal (F_m) fluorescence and leaf dark respiration, leaves were dark-adapted for 30 min before a saturating pulse of light. The parameters derived from chlorophyll fluorescence, including dark-adapted PSII maximum quantum efficiency (F_v/F_m), proportion of open PSII centers (photochemical quenching, qP), PSII operating efficiency (Φ_{PSII}), non-photochemical quenching (NPQ) and electron transport rate (ETR) were calculated according to Maxwell and Johnson (2000).

Chloroplast number

Chloroplasts in leaf mesophyll cells were counted following the methodology described in Pyke (2011). Briefly, leaf pieces were fixed in 3.5% (v/v) glutaraldehyde solution for 1 h, then calcium ions were chelated by transferring the leaf samples to 0.1 M NaEDTA solution and keeping them for 4 h at 60°C and then overnight at 4°C . The pieces were transferred to a microscope slide and macerated with a blunt scalpel for tissue desintegration, and visualised by light microscopy.

Transmission electron microscopy

Leaf segments were fixed at 4°C in Karnovsky's solution [2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.2] for 24 h. After washing in buffer, the samples were post-fixed in buffered 1% (w/v) osmium tetroxide, washed, dehydrated in a graded series of acetone, and embedded in Spurr resin. The resin was polymerized at 60°C . Ultrathin sections were stained with saturated uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and observed using a Zeiss EM 900 transmission electron microscope.

Total soluble proteins, starch and soluble sugars quantification

Total soluble proteins extraction and quantification were performed as described in Jones et al. (1989). Starch and soluble sugars extraction were performed as described in Lira et al. (2014). Soluble sugar quantification was performed according to Mainardi et al. (2006). Briefly, 1 mL of the extract was evaporated under vacuum in a SpeedVac system. The residue was resuspended with 1 mL of ultrapure water and filtered through 0.22 µm membrane. Soluble sugars (i.e., glucose, fructose, sucrose) were analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex, Sunnyvale, CA, USA), using a Carbowax PA1 (250 x 4 mm, 5 µm particle size, Dionex) in an isocratic run with 18 mM NaOH as mobile phase. The content of each sugar was calculated with the standard curves made with pure glucose, fructose and sucrose (Sigma-Aldrich, Co).

Tocopherol and pigment quantification

Tocopherols were extracted from approximately 25 mg dry weight as described in Almeida et al. (2015). The samples were adjusted to 4 mL final volume. Aliquots of 3 mL were dried under N₂ and dissolved in 200 µL of mobile phase composed of hexane/*tert*-butyl methyl ether (90:10). Chromatography was carried out on a Hewlett–Packard series 1100 HPLC system coupled with a fluorescence detector (Agilent Technologies series 1200) on a normal-phase column (LiChrosphere® 100 Diol Si; 250 mm x 4.0 mm, 5 µm; Agilent Technologies, Germany) at room temperature, with the mobile phase running isocratically at 1 mL min⁻¹. Eluted compounds were detected by excitation at 295 nm and fluorescence was quantified at 330 nm.

Chlorophyll extraction was carried out as described in Porra et al. (1989). One mL of dimethylformamide (DMF) was added to 100 mg or 300 mg of fresh weight for leaf or fruit samples, respectively. After sonication for five min and further centrifugation of 9000 *g* for 10 min, the supernatant was collected. The procedure was repeated until total removal of tissue green colour. Spectrophotometer measurements were performed at 664 and 647 nm.

Carotenoid extraction was modified from Sérino et al. (2009). Aliquots of 200 mg of fresh weight were sequentially extracted with a solution of saturated NaCl, dichloromethane and hexane:diethyl ether (1:1). After centrifugation, the supernatant was collected and the last step was repeated three more times. Samples were dried by vacuum and dissolved in 200 µL of acetonitrile. Chromatography was carried out on Agilent Technologies series 1100 HPLC system on a normal-phase column Phenomenex (Luna C18; 250 x 4.6 mm; 5 µm particle diameter) at room temperature with a flow rate of 1 mL min⁻¹. The mobile phase was a gradient of ethyl acetate (A) and acetonitrile:water 9:1 (B): 0-4 min with 20% A/80% B; 4-30 min with 65% A/35% B; 30-35 min with 65% A/35% B; 35-40 min with 20% A/80% B. Eluted compounds were detected between 340-700 nm and quantified at 450 nm.

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, 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. A parameter was considered to be affected by *SIORE1S02* silencing if at least two out of the three transgenic lines differed significantly from the untransformed genotype.

Acknowledgments

The authors thank Prof. Dr. Javier Palatnik from IBR, CONICET, Argentina, for providing the p35S:AtMiR164a construct and Dr. Tania Misuzu Shiga from FCF, USP, Brazil, for the assistance in soluble sugar quantification.

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FINAL CONSIDERATIONS

This project objectives aimed to increase the knowledge regarding manipulation of chlorophyll degradation and of senescence over the metabolism and yield of a fleshy fruit species.

Although several advances were made in the past years regarding the regulation and processes involved in chlorophyll metabolism and senescence, most focus on the model plant *Arabidopsis thaliana* or on grasses. Thus, there is a lack of knowledge in other species and, specially, over the metabolism and development of fleshy fruit bearing species. In this sense, tomato (*Solanum lycopersicum*) comes out as an excellent model plant not only because its fruits undergo chloroplast-chromoplast conversion that involves chlorophyll degradation and accumulation of nutraceutical isoprenoid-derived compounds, but also for its economic value.

The first requirements for chlorophyll degradation is the removal of the phytol-derived chain and of the magnesium ion. Chlorophyll dephytylation can be catalysed either by chlorophyllase or by pheophytinase, here it was identified different evolutive constraints under which the coding genes of each enzyme evolved, reflecting physiological specificities for each of them. Thus, different enzymes play a role in chlorophyll dephytylation associated to physiologically programmed processes and in response to environmental stimuli.

When pheophytinase, the senescence-associated chlorophyll dephytylating enzyme, was knocked-down, tomato plants displayed signs of photoinhibition in leaves, yet, this enhanced fruit nutritional content by increasing the content of soluble sugars and tocopherols, however carotenoid accumulation was penalised.

Delaying senescence initiation have proved to be a promising strategy for yield improvement in tomato. The maintenance of photosynthetically active leaves for more time led to an increase in the harvest index as plants produced more fruits, which also accumulated more soluble sugars and tocopherols.

To conclude, as more information is becoming available in *A. thaliana* and other model crops, it is necessary to explore if the mechanisms are the same in fleshy fruit species and the possible applications for improving yield and/or nutritional values of crop species.

ARTICLES PUBLISHED

Directly related to this thesis

Lira, B. S., de Setta, N., Rosado, D., Almeida, J., Freschi, L., & Rossi, M. (2014). Plant degreening: evolution and expression of tomato (*Solanum lycopersicum*) dephytylation enzymes. *Gene*, 546(2), 359-366.

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With results obtained in the thesis, but are not part of it

Guyer, L., Hofstetter, S. S., Christ, B., **Lira, B. S.**, Rossi, M., & Hörtensteiner, S. (2014). Different mechanisms are responsible for chlorophyll dephytylation during fruit ripening and leaf senescence in tomato. *Plant physiology*, 166(1), 44-56.

Collaborations in others projects

de Godoy, F., Bermúdez, L., **Lira, B. S.**, de Souza, A. P., Elbl, P., Demarco, D., Alseekh, S., Insani, M., Buckeridge, M. S., Almeida, J., Grigioni, G., Fernie, A. R. & Rossi, M. (2013). Galacturonosyltransferase 4 silencing alters pectin composition and carbon partitioning in tomato. *Journal of experimental botany*, 64(8), 2449-2466.

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ATTACHMENT I

Table S1. Sequence information.

Name	Species	Accession number
<i>PsHCLH</i>	<i>Picea sitchensis</i>	BT070775 ^a
<i>PsHCLH2</i>	<i>Picea sitchensis</i>	EF677023 ^a
<i>PsHCLH3</i>	<i>Picea sitchensis</i>	EF087079 ^a
<i>AtCLH1</i>	<i>Arabidopsis thaliana</i>	NP_564094.1 ^a
<i>AtCLH2</i>	<i>Arabidopsis thaliana</i>	NP_199199.1 ^a
<i>BoCLH1</i>	<i>Brassica oleracea</i>	AF337544_1 ^a
<i>BoCLH2</i>	<i>Brassica oleracea</i>	AF337545_1 ^a
<i>BoCLH3</i>	<i>Brassica oleracea</i>	AF337546_1 ^a
<i>BrCLH1</i>	<i>Brassica rapa</i>	Bra036000 ^b
<i>BrCLH2</i>	<i>Brassica rapa</i>	Bra025756 ^b
<i>CcCLH1</i>	<i>Citrus clementina</i>	clementine0.9_016047m ^b
<i>CcCLH2</i>	<i>Citrus clementina</i>	clementine0.9_015518m ^b
<i>CsCLH1</i>	<i>Citrus sinensis</i>	orange1.1g020950m ^b
<i>CsCLH2</i>	<i>Citrus sinensis</i>	orange1.1g020188m ^b
<i>CsCLH3</i>	<i>Citrus sinensis</i>	orange1.1g020199m ^b
<i>EgCLH</i>	<i>Eucalyptus grandis</i>	Eucgr.B01023 ^b
<i>GmCLH1</i>	<i>Glycine max</i>	Glyma10g00570 ^b
<i>GmCLH2</i>	<i>Glycine max</i>	Glyma09g31250 ^b
<i>GmCLH3</i>	<i>Glycine max</i>	Glyma07g10840 ^b
<i>OsCLH1</i>	<i>Oryza sativa</i>	Os10g28370 ^b
<i>PtCLH1</i>	<i>Populus trichocarpa</i>	POPTR_0010s09240 ^b
<i>PtCLH2</i>	<i>Populus trichocarpa</i>	POPTR_0284s00200 ^b
<i>PtCLH3</i>	<i>Populus trichocarpa</i>	POPTR_0005s23590 ^b
<i>SbCLH1</i>	<i>Sorghum bicolor</i>	Sb02g012300 ^b
<i>SbCLH2</i>	<i>Sorghum bicolor</i>	Sb07g024090 ^b
<i>SbCLH3</i>	<i>Sorghum bicolor</i>	Sb01g021520 ^b
<i>SlCLH1</i>	<i>Solanum lycopersicum</i>	Solyc06g053980.2 ^c
<i>SlCLH2</i>	<i>Solanum lycopersicum</i>	Solyc09g065620.2 ^c
<i>SlCLH3</i>	<i>Solanum lycopersicum</i>	Solyc09g082600.1 ^c
<i>SlCLH4</i>	<i>Solanum lycopersicum</i>	Solyc12g005300.1 ^c
<i>StCLH1</i>	<i>Solanum tuberosum</i>	PGSC0003DMT400041941 ^c
<i>StCLH2</i>	<i>Solanum tuberosum</i>	PGSC0003DMT400016518 ^c
<i>StCLH3</i>	<i>Solanum tuberosum</i>	PGSC0003DMC400033622 ^c
<i>StCLH4</i>	<i>Solanum tuberosum</i>	PGSC0003DMC400037791 ^c
<i>ZmCLH1</i>	<i>Zea mays</i>	GRMZM2G103197_T01 ^b
<i>ZmCLH2</i>	<i>Zea mays</i>	GRMZM2G170734_T01 ^b
<i>AtPPH</i>	<i>Arabidopsis thaliana</i>	BAH19780 ^b
<i>BrPPH</i>	<i>Brassica rapa</i>	Bra023436 ^b
<i>BrPPH1</i>	<i>Brassica rapa</i>	Bra006215 ^b
<i>CsPPH</i>	<i>Citrus sinensis</i>	orange1.1g009834m ^b
<i>CcPPH</i>	<i>Citrus clementina</i>	clementine0.9_005955m ^b
<i>EgPPH</i>	<i>Eucalyptus grandis</i>	Eucgr.G03276.1 ^b
<i>GmPPH</i>	<i>Glycine max</i>	Glyma09g36010 ^b
<i>GmPPH1</i>	<i>Glycine max</i>	Glyma12g01320 ^b

<i>GmPPH2</i>	<i>Glycine max</i>	Glyma11g16070 ^b
<i>NtPPH</i>	<i>Nicotiana tabacum</i>	CAO99125.1 ^b
<i>OsPPH</i>	<i>Oryza sativa Japonica</i>	NP_001057593.1 ^b
<i>PtPPH</i>	<i>Populus trichocarpa</i>	POPTR_0009s05960 ^b
<i>RcPPH</i>	<i>Ricinus communis</i>	30221.m002221 ^b
<i>SbPPH</i>	<i>Sorghum bicolor</i>	Sb04g002990 ^b
<i>SlPPH</i>	<i>Solanum lycopersicum</i>	SGN-U572366 ^c
<i>StPPH</i>	<i>Solanum tuberosum</i>	PGSC0003DMT400017406 ^c
<i>VvPPH</i>	<i>Vitis vinifera</i>	CAO40741.1 ^b
<i>ZmPPH</i>	<i>Zea mays</i>	ACF87407.1 ^b

^a NCBI (<http://www.ncbi.nlm.nih.gov/>). ^b Phytozome (<http://www.phytozome.net/>). ^c SolGenomicsNetwork (<http://solgenomics.net/>).

Table S2. Primers used for qPCR.

Primer	Sequence 5' -3'				
RT-CLH1-F	CAT	GGC	TTC	ATT	GTT GCT CC
RT-CLH1-R	GCC	TGC	TAG	TCC	AAG TTT CGT C
RT-CLH2-F	GGC	TTC	ATG	CTC	CAA CCT AG
RT-CLH2-R	CGG	CAG	TAC	GGA	TTT GAG G
RT-CLH3-F	CTT	CTC	CTC	CAA	AGC CAC TC
RT-CLH3-R	GTA	ATC	CGT	CAG	GTA ACC AGT TT
RT-CLH4-F	ATT	CCG	CCT	CCA	ACC CGA CT
RT-CLH4-R	GCA	GCA	CAG	AGT	TGA GCT TCT TAG C
RT-PPH-F	TAT	GGA	GGG	AGC	AAG TAC GC
RT-PPH-R	TGG	AGG	GCA	GAG	GAA AAG TAC
RT-SAG12-F	ATG	TCC	TCC	TCA	AAG CCA AA
RT-SAG12-R	TTT	CAG	TTG	GTG	TAG CCC TT
RT-PAO-F	TCA	GAA	GTG	GGT	GAT ATG GA
RT-PAO-R	TAT	CCC	CGT	CAT	ACA CCT TA
RT-Expressed-F	GCT	AAG	AAC	GCT	GGA CCT AAT G
RT-Expressed-R	TGG	GTG	TGC	CTT	TCT GAA TG
RT-TIP41-F	ATG	GAG	TTT	TTG	AGT CTT CTG C
RT-TIP41-R	GCT	GCG	TTT	CTG	GCT TAG G

Table S3. Subcellular compartment prediction for *S. lycopersicum* PPH and CLH proteins.

Protein	Subcellular compartment					Chloroplast signal peptide	
	TargetP ^a	Cello v2.5 ^b	SherLoc2 ^c	MultiLoc2 ^d	BaCellLo ^e	Plant-PLoc ^f	iPSORT ^g
SLPPH	-	Cl	Cl	Ct	N	Cl	No Yes
SLCLH1	-	Cl/Ct	Cl	Ct	Cl	Ct	No Yes
SLCLH2	-	PM	Cl	Cl	Cl	Cl	No No
SLCLH3	-	PM	Cl	Ct	Cl	PM	Yes Yes
SLCLH4	-	Cl	Cl	Ct	Ct	Cl	No No
AtPPH	Cl	Cl	Cl	Cl	Cl	Cl	Yes Yes

AtPPH was used as a control of the prediction accuracy. Cl: chloroplast. N: nucleus. Ct: cytosol. PM: plasma membrane. -: compartment other than mitochondrion, chloroplast or secretory pathway.^a Emanuelsson et al. (2007).^b Yu et al. (2006).^c Briesemanster et al. (2009).^d Blum et al. (2009).^e Perleoni et al. (2006).^f Chou and Shen (2010).^g Bannai et al. (2002).

Table S4. Relative expression of genes encoding chlorophyll degradation-related enzymes in leaves submitted to different hormonal treatments.

Gene	Days after treatment	No treatment	Hormonal treatment			
			MCP	ET	ABA	SA
<i>SLC1H1</i>	0	1.00±0.18 ^{aA}	1.00±0.07 ^{aA}	1.00±0.07 ^{aA}	1.00±0.07 ^{aA}	1.00±0.07 ^{aA}
	2	0.83±0.08 ^{aA}	0.90±0.05 ^{aA}	0.63±0.05 ^{bB}	0.53±0.04 ^{bB}	0.30±0.03 ^{bC}
	4	0.90±0.08 ^{aA}	0.53±0.04 ^{bB}	0.39±0.02 ^{cC}	0.77±0.07 ^{cA}	0.46±0.02 ^{bB}
<i>SLC1H2</i>	0	1.00±0.09 ^{aA}	1.00±0.05 ^{aA}	1.00±0.05 ^{aA}	1.00±0.05 ^{aA}	1.00±0.05 ^{aA}
	2	0.10±0.01 ^{bA}	0.55±0.01 ^{bB}	0.20±0.02 ^{bC}	0.55±0.04 ^{bB}	0.02±0.00 ^{bD}
	4	0.13±0.02 ^{bA}	0.28±0.02 ^{cB}	0.12±0.01 ^{bA}	0.27±0.01 ^{cB}	0.05±0.01 ^{cC}
<i>SLC1H3</i>	0	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND
<i>SLC1H4</i>	0	1.00±0.17 ^{aA}	1.00±0.09 ^{aA}	1.00±0.09 ^{aA}	1.00±0.09 ^{aA}	1.00±0.09 ^{aA}
	2	0.96±0.14 ^{aA}	0.46±0.04 ^{bB}	0.33±0.03 ^{bC}	0.48±0.04 ^{bB}	0.19±0.02 ^{bD}
	4	0.84±0.14 ^{aA}	0.57±0.05 ^{bB}	0.90±0.06 ^{aA}	0.24±0.01 ^{bC}	0.10±0.01 ^{cD}
<i>SLPPH</i>	0	1.00±0.13 ^{aA}	1.00±0.07 ^{aA}	1.00±0.07 ^{aA}	1.00±0.07 ^{aA}	1.00±0.07 ^{aA}
	2	0.89±0.12 ^{aA}	0.60±0.03 ^{bB}	1.75±0.09 ^{bC}	1.74±0.20 ^{bC}	1.94±0.11 ^{bC}
	4	0.89±0.20 ^{aA}	0.85±0.06 ^{aA}	2.58±0.15 ^{cB}	1.49±0.08 ^{bC}	1.69±0.09 ^{cC}
<i>SLPAO</i>	0	1.00±0.15 ^{aA}	1.00±0.04 ^{aA}	1.00±0.04 ^{aA}	1.00±0.04 ^{aA}	1.00±0.04 ^{aA}
	2	1.17±0.19 ^{aA}	1.68±0.13 ^{bB}	2.50±0.15 ^{bC}	1.50±0.32 ^{abB}	3.12±0.21 ^{bD}
	4	0.93±0.12 ^{aA}	1.47±0.07 ^{bB}	2.72±0.18 ^{bC}	1.69±0.10 ^{bB}	1.73±0.10 ^{cB}
<i>SLsAG12</i>	0	1.00±0.18 ^{aA}	1.00±0.05 ^{aA}	1.00±0.05 ^{aA}	1.00±0.05 ^{aA}	1.00±0.05 ^{aA}
	2	0.79±0.14 ^{aA}	0.46±0.01 ^{bB}	9.38±1.89 ^{bC}	34.10±3.79 ^{bD}	9.21±1.19 ^{bC}
	4	0.89±0.16 ^{aA}	0.45±0.01 ^{bB}	20.12±4.70 ^{bC}	13.28±0.35 ^{cD}	20.90±0.87 ^{cC}

Values represent means ± SE from at least three biological replicates. The mean relative expression was calculated from means of two technical replicates and normalized against the samples from day 0. Different letters indicate statistically significant differences (p<0.05) in the time course (lower case) and among treatments (upper case) according to a permutation test, that lack assumptions of sample distribution (Pfaffl *et al.*, 2002). MCP: 1-methylcyclopropene; ET: ethylene; ABA: abscisic acid; SA: salicylic acid; ND: not detected.

Table S5. Relative expression of genes encoding chlorophyll degradation-related enzymes along fruit development and ripening.

Gene	Fruit stage			
	Green	Mature Green	Yellow	Ripe
<i>S1CLH1</i>	1.00±0.11 ^a	3.82±0.41 ^b	2.78±0.20 ^{ab}	1.14±0.23 ^a
<i>S1CLH2</i>	1.00±0.21	ND	ND	ND
<i>S1CLH3</i>	ND	ND	ND	ND
<i>S1CLH4</i>	1.00±0.10 ^a	0.14±0.03 ^b	0.51±0.03 ^a	0.02±0.01 ^c
<i>S1PPH</i>	1.00±0.08 ^a	42.03±3.51 ^b	21.96±1.85 ^c	6.50±1.40 ^d
<i>S1PAO</i>	1.00±0.18 ^a	2.39±0.27 ^b	5.04±0.35 ^c	5.93±1.00 ^c
<i>S1SAG12</i>	ND	ND	ND	ND

Values represent means ± SE from at least three biological replicates. The mean relative expression was calculated from means of two technical replicates and normalized against green fruits. Statistically significant differences ($p < 0.05$) between samples are indicated with different letters according to a permutation test, that lack assumptions of sample distribution (Pfaffl et al., 2002). ND: not detected.

Table S6. Relative expression among genes encoding chlorophyll degradation-related enzymes in the same tissue.

Gene	Non treated leaves (0 days)	Green fruits
<i>SICLH1</i>	4.51±0.50	112.42±4.20
<i>SICLH2</i>	1.00±0.10	1.00±0.12
<i>SICLH3</i>	ND	ND
<i>SICLH4</i>	0.75±0.09	2.09±0.31
<i>SIPPH</i>	8.12±0.84	110.88±2.39
<i>SIPAO</i>	13.21±0.85	485.69±9.11
<i>SISAG12</i>	0.92±0.11	ND

Values represent means ± SE from at least three biological replicates. The mean relative expression was calculated from means of two technical replicates and normalized against the corresponding *SICLH2* expression level. ND: not detected.

ATTACHMENT II

Supplementary Fig. S1 *SIPPH* transcript profile in *SIPPH*-knockdown lines. The extension of *SIPPH* silencing was addressed in leaves and fruits at mature green, breaker and ripe stages. Data represent the mean $\pm$ SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) between the wild type and transgenic genotype are marked with an asterisk.

Supplementary Fig. S2

Supplementary Fig. S2 Vegetative growth of *SIPPH*- knockdown lines. Plant height (A) and node number (B) were monitored for thirteen week. Values represent the mean $\pm$ SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) between the control genotype and transgenic lines are marked with an asterisk.

Supplementary Fig. S3

A

B

Supplementary Fig. S3 Chlorophyll content and impairment of age-induced senescence-associated degreening in *SIPPH*-knockdown lines. Chlorophyll was measured in leaves, mature green and ripe fruits. Data represent the mean $\pm$ SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) between the wild type and transgenic genotype are marked with an asterisk (A). Age-induced senescent leaves (above) and the corresponding petioles after leaf detachment (below) of 9-week-old plants (B).

Supplementary Fig. S4

Supplementary Fig. S4 Protein sequence alignment of AtPPH, SIPPH and SIPPHLs. The grey line localizes the PPH motif with the Ser active residue pointed by the arrow (Schelbert et al 2009). Residues were shaded with 75% identity threshold. Alignment representation was made in BioEdit (Hall et al. 1999).

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Supplementary Fig. S5

Supplementary Fig. S5 Alignment of SIPPHs along the fragment of the hairpin RNA (RNAi) construct used for *SIPPH*-knockdown plants (between arrows). Residues were shaded with 75% identity threshold. Alignment representation was made in BioEdit (Hall et al. 1999).

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Supplementary Table S1 Expression values of chlorophyll degradation, carotenogenesis and tocopherol biosynthetic genes in leaves and along fruit ripening of *SIPPH* - knockdown lines.

	<i>SISGR</i>	<i>SIPAO</i>	<i>SIDX5</i>	<i>SIGDDR</i>	<i>SIVTE1</i>	<i>SIVTE2</i>	<i>SIVTE3(1)</i>	<i>SIVTE3(2)</i>	<i>SIVTE4</i>	<i>SIVTE5</i>	<i>SIVTE6</i>	<i>SIPSV1</i>	<i>SIPSV2</i>	<i>SIPDS</i>	<i>SILCY β</i>	<i>SICYC β</i>
<i>Leaf</i>	WT	1.00 ± 0.22	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.18	1.00 ± 0.14	1.00 ± 0.17	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.15	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.07
	L17	0.78 ± 0.11	0.47 ± 0.04	2.07 ± 0.21	1.17 ± 0.07	0.85 ± 0.07	1.15 ± 0.21	0.95 ± 0.03	0.85 ± 0.06	0.57 ± 0.04	0.99 ± 0.14	0.86 ± 0.08	0.90 ± 0.07	0.56 ± 0.09	0.73 ± 0.04	0.94 ± 0.12
	L24	1.00 ± 0.24	0.56 ± 0.03	1.87 ± 0.17	1.11 ± 0.10	1.08 ± 0.15	1.21 ± 0.21	1.09 ± 0.23	0.95 ± 0.01	0.74 ± 0.02	1.09 ± 0.20	0.92 ± 0.08	0.94 ± 0.05	0.69 ± 0.13	0.89 ± 0.07	1.05 ± 0.09
	L27	0.90 ± 0.12	0.52 ± 0.04	1.94 ± 0.12	1.10 ± 0.12	1.43 ± 0.08	1.82 ± 0.19	0.85 ± 0.09	0.84 ± 0.05	0.70 ± 0.11	0.97 ± 0.06	0.99 ± 0.10	1.10 ± 0.09	0.81 ± 0.12	0.90 ± 0.11	1.07 ± 0.11
<i>Mature green fruit</i>	WT	1.00 ± 0.15	1.00 ± 0.11	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.15	1.00 ± 0.03	1.00 ± 0.15	ND	1.00 ± 0.04	1.00 ± 0.11	1.00 ± 0.05	1.00 ± 0.11	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.03
	L17	0.85 ± 0.10	0.50 ± 0.07	0.63 ± 0.06	1.27 ± 0.07	1.08 ± 0.14	1.04 ± 0.08	0.87 ± 0.14	ND	0.87 ± 0.09	0.85 ± 0.06	0.95 ± 0.07	1.15 ± 0.12	0.64 ± 0.03	0.83 ± 0.07	0.91 ± 0.10
	L24	1.12 ± 0.18	0.71 ± 0.12	0.73 ± 0.06	1.28 ± 0.09	0.92 ± 0.11	0.95 ± 0.14	1.11 ± 0.17	ND	0.91 ± 0.12	0.89 ± 0.04	0.96 ± 0.09	1.51 ± 0.18	0.47 ± 0.02	0.80 ± 0.08	0.85 ± 0.17
	L27	0.87 ± 0.17	0.63 ± 0.04	0.52 ± 0.08	1.43 ± 0.13	1.13 ± 0.16	1.08 ± 0.12	0.82 ± 0.08	ND	1.10 ± 0.16	0.70 ± 0.06	1.07 ± 0.07	1.13 ± 0.15	0.60 ± 0.15	0.87 ± 0.09	0.95 ± 0.08
<i>Breaker fruit</i>	WT	1.00 ± 0.04	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.13	ND	1.00 ± 0.08	1.00 ± 0.01	1.00 ± 0.12	1.00 ± 0.05	1.00 ± 0.09	1.00 ± 0.09	1.00 ± 0.05
	L17	0.77 ± 0.17	0.69 ± 0.07	0.95 ± 0.11	2.00 ± 0.17	0.89 ± 0.19	0.64 ± 0.01	0.95 ± 0.06	ND	0.65 ± 0.13	0.87 ± 0.03	0.66 ± 0.04	0.72 ± 0.13	1.22 ± 0.17	0.85 ± 0.08	0.51 ± 0.08
	L24	0.67 ± 0.24	0.60 ± 0.05	0.94 ± 0.07	1.43 ± 0.11	0.84 ± 0.17	0.49 ± 0.08	0.90 ± 0.06	ND	0.72 ± 0.09	0.79 ± 0.02	0.68 ± 0.08	0.67 ± 0.10	0.97 ± 0.11	0.67 ± 0.04	0.49 ± 0.03
	L27	0.71 ± 0.14	0.63 ± 0.05	1.04 ± 0.09	1.91 ± 0.15	0.82 ± 0.20	0.54 ± 0.12	0.89 ± 0.06	ND	0.67 ± 0.23	0.76 ± 0.06	0.63 ± 0.09	0.50 ± 0.15	0.72 ± 0.35	0.59 ± 0.04	0.44 ± 0.07
<i>Ripe fruit</i>	WT	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.08	1.00 ± 0.08	1.00 ± 0.10	1.00 ± 0.13	1.00 ± 0.24	ND	1.00 ± 0.14	1.00 ± 0.19	1.00 ± 0.04	1.00 ± 0.16	1.00 ± 0.2	1.00 ± 0.02	1.00 ± 0.08
	L17	0.32 ± 0.12	0.99 ± 0.11	0.55 ± 0.10	2.79 ± 0.21	0.91 ± 0.17	1.27 ± 0.22	0.89 ± 0.17	ND	0.86 ± 0.11	1.06 ± 0.09	0.59 ± 0.09	0.83 ± 0.18	0.92 ± 0.11	0.93 ± 0.09	1.10 ± 0.10
	L24	0.56 ± 0.17	0.99 ± 0.04	0.63 ± 0.11	2.45 ± 0.17	1.02 ± 0.20	1.14 ± 0.14	1.04 ± 0.09	ND	0.97 ± 0.08	1.10 ± 0.16	0.65 ± 0.05	0.89 ± 0.14	0.87 ± 0.15	1.02 ± 0.07	0.88 ± 0.13
	L27	0.21 ± 0.14	1.04 ± 0.08	0.76 ± 0.07	3.43 ± 0.20	0.96 ± 0.16	0.96 ± 0.10	0.86 ± 0.11	ND	1.14 ± 0.16	1.13 ± 0.10	0.57 ± 0.07	0.75 ± 0.16	0.84 ± 0.14	1.05 ± 0.09	0.93 ± 0.08

Statistically significant differences between the transcript ratio of transgenic and control plants are indicated in bold terms (t-test, $P < 0.05$). Values represent means from at least three biological replicates. ND: Not determined.

Supplementary Table S2 Relative transcript ratio.

	<i>Leaf</i>	<i>Mature green fruit</i>	<i>Breaker fruit</i>	<i>Ripe fruit</i>
<i>SIPPH</i>	1.00 ± 0.14	1.000 ± 0.160	1.000 ± 0.074	1.000 ± 0.4650
<i>SIPPHL1</i>	0.22 ± 0.02	0.040 ± 0.002	0.006 ± 0.001	0.004 ± 0.0020
<i>SIPPHL2</i>	0.16 ± 0.02	0.012 ± 0.001	0.003 ± 0.001	0.001 ± 0.0001
<i>SIPPHL3</i>	0.16 ± 0.01	0.040 ± 0.003	0.013 ± 0.001	0.007 ± 0.0020

	<i>Leaf</i>	<i>Mature green fruit</i>	<i>Breaker fruit</i>	<i>Ripe fruit</i>
<i>SIPSY1</i>	1.00 ± 0.16	1.00 ± 0.06	1.00 ± 0.13	1.00 ± 0.09
<i>SIPSY2</i>	0.60 ± 0.07	0.03 ± 0.01	0.0033 ± 0.0001	0.0042 ± 0.0008

	<i>Leaf</i>	<i>Mature green fruit</i>	<i>Breaker fruit</i>	<i>Ripe fruit</i>
<i>SILCYB</i> ¹	1.00 ± 0.09	1.00 ± 0.02	1.00 ± 0.16	1.00 ± 0.19
<i>SICYCB</i> ²	0.04 ± 0.008	3.13 ± 0.07	6.93 ± 0.52	3.54 ± 0.34

Statistically significant differences between the transcript ratio are indicated in bold terms (t-test, $P < 0.05$). Values represent means ± SD from at least three biological replicates and are expressed as relative transcript amount compared to *SIPPH*, *SIPSY1* and *SILCYB*, correspondingly. ¹Chloroplast-specific. ²Chromoplast-specific β-lycopene cyclase.

Supplementary Table S3 Primers used for qPCR.

Gene name	Tomato genome loci	Primer sequence (Foward/Reverse)
<i>SIVTE1</i>	Solyc08g068570	CGAACTCCTCATAGCGGGTATC CACGCCAGTAAACCGAGGC
<i>SIVTE2</i>	Solyc07g017770	CAATTCCAGTTCCTGCTGAG CCTCCAACATGCTCTTGCGTG
<i>SIVTE3(1)</i>	Solyc09g065730	CTTGACCAATCTCCTCATC GCACGCCTTTCCTCCAGG
<i>SIVTE3(2)</i>	Solyc03g005230	GCTAAGGCTAGGCAGAAGGAG CAGGCAACCCACCTATGG
<i>SIVTE4</i>	Solyc08g076360	CAGATCATCGTGCTGCTCAG CCTCTCTGCTTGACAGGAC
<i>SIVTE5</i>	Solyc03g071720	CGTATCAGGACGGGCTCGC TCACCACCACACATCATTGCTAATG
<i>SIVTE6</i>	Solyc07g062180	AGCACAAGCATCAGTGTCTG AAGAAAGCAGCCGCAATACC
<i>SIPPH</i>	Solyc01g088090	TATGGAGGGAGCAAGTACGC TGGAGGGCAGAGGAAAAGTAC
<i>SIPPHL1</i>	Solyc02g062610	GATTTGGTGCTTCTGCCTTTC GCTGTTTCTTCAGTTCCTTC
<i>SIPPHL2</i>	Solyc12g098660	AGGGCTATTCCATCCGATACC GTCTACCAAACCAAGGCTG
<i>SIPPHL3</i>	Solyc02g070490	GGGAACCTCTGTCGGAAGC GTGGACCTGTCACAATGGA
<i>SIPSY(1)</i>	Solyc03g031860	CGATGGTGCTTTGTCCGATAC CTCATCAACCCAACCGTACC
<i>SIPSY(2)</i>	Solyc02g081330	GCATCACACATAACTCCACAAGC CGCATTCTTCAACCATATCTCTG
<i>SILCYβ</i>	Solyc06g074240	GCACCCACATCAAAGCCAGAG GCCACATGGAGAGTGGTGAAG
<i>SICYCβ</i>	Solyc04g040190	TTGACTTAGAACCTCGTTATTGG AACAGTTCCTTTGTCATTATCT
<i>SIPDS</i>	Solyc03g123760	CGTTCCGTGCTTCTCCGC CTAGAACATCCCTTGCCTCCAG
<i>SIGGDR</i>	Solyc03g115980	CAGAGACGCTCGCTAAGG GCTTCAGAGTCTGTCCGATATC
<i>SIDXS</i>	Solyc01g067890	CAGGACTGGTGTGGTTTCAG GGGATAGTTCACAGTGTCC
<i>SIPAO</i>	Solyc11g066440	TCAGAAGTGGGTGATATGGA TATCCCCGTCATACACCTTA
<i>SISGR</i>	Solyc08g080090	GCAAAGAACTCCCTGTGGTT CCCACCAGAAGAAGATGAGG
<i>TIP</i>	Solyc10g049850	ATGGAGTTTTTGAGTCTTCTGC GCTGCGTTTCTGGCTTAGG
<i>EXPRESSED</i>	Solyc07g025390	GCTAAGAACGCTGGACCTAATG TGGGTGTGCCTTTCTGAATG

Supplementary Table S4 Sequences used for alignment.

Species	Phytozome accession	Clade
<i>Aquilegia coerulea</i>	Aquca_004_00646.1	PPH-LIKE2
<i>Aquilegia coerulea</i>	Aquca_010_00522.1	PPH-LIKE3
<i>Aquilegia coerulea</i>	Aquca_014_00014.1	PPH-LIKE1
<i>Aquilegia coerulea</i>	Aquca_014_00751.1	PPH
<i>Arabidopsis lyrata</i>	fgenes1_pgC_scaffold_7003305	PPH-LIKE1
<i>Arabidopsis lyrata</i>	fgenes2_kg6_1338_AT5G13800.2	PPH
<i>Arabidopsis lyrata</i>	fgenes2_kg6_2013_AT5G19850.1	PPH-LIKE2
<i>Arabidopsis lyrata</i>	fgenes2_kg7_417_AT4G36530.2	PPH-LIKE3
<i>Arabidopsis thaliana</i>	AT4G36530.2	PPH-LIKE3
<i>Arabidopsis thaliana</i>	AT5G13800.2	PPH
<i>Arabidopsis thaliana</i>	AT5G19850.1	PPH-LIKE2
<i>Arabidopsis thaliana</i>	AT5G38520.2	PPH-LIKE1
<i>Boechera stricta</i>	Bostr17584s0010.1	PPH-LIKE1
<i>Boechera stricta</i>	Bostr26527s0217.1	PPH-LIKE2
<i>Boechera stricta</i>	Bostr2902s0114.1	PPH
<i>Boechera stricta</i>	Bostr7867s1510.1	PPH-LIKE3
<i>Brachypodium distachyon</i>	Bradi1g23675.2	PPH-LIKE2
<i>Brachypodium distachyon</i>	Bradi1g35780.1	PPH
<i>Brachypodium distachyon</i>	Bradi2g25130.1	PPH-LIKE1
<i>Brachypodium distachyon</i>	Bradi3g52760.2	PPH-LIKE3
<i>Brassica rapa</i>	BraraA002261	PPH-LIKE3
<i>Brassica rapa</i>	BraraB005211	PPH
<i>Brassica rapa</i>	BraraC005831	PPH
<i>Brassica rapa</i>	BraraJ016091	PPH-LIKE2
<i>Carica papaya</i>	evmmmodelsupercontig_119	PPH-LIKE2
<i>Carica papaya</i>	evmmmodelsupercontig_197	PPH
<i>Carica papaya</i>	evmmmodelsupercontig_21	PPH-LIKE1
<i>Carica papaya</i>	evmmmodelsupercontig_6	PPH-LIKE3
<i>Chlamydomonas reinhardtii</i>	Cre10g456150	
<i>Chlamydomonas reinhardtii</i>	Cre12g514700	PPH
<i>Chlamydomonas reinhardtii</i>	Cre12g558550	PPH-LIKE2
<i>Chlamydomonas reinhardtii</i>	Cre16g664350	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10015550m	PPH-LIKE2
<i>Citrus clementina</i>	Ciclev10020543m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10020551m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10021411m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10021413m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10021643m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10021704m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10021706m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10023871m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10024042m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10024267m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10024539m	PPH-LIKE1
<i>Citrus clementina</i>	Ciclev10025882m	PPH-LIKE3
<i>Citrus clementina</i>	Ciclev10031731m	PPH
<i>Citrus sinensis</i>	orange1.1g009834m	PPH
<i>Citrus sinensis</i>	orange1.1g017361m	PPH-LIKE3
<i>Citrus sinensis</i>	orange1.1g021259m	PPH-LIKE2
<i>Coccomyxa subellipsoidea</i>	16804	

<i>Coccomyxa subellipsoidea</i>	19825	PPH
<i>Coccomyxa subellipsoidea</i>	38683	PPH-LIKE1
<i>Glycine max</i>	Glyma02G055200.1	PPH-LIKE3
<i>Glycine max</i>	Glyma09G226000.1	PPH
<i>Glycine max</i>	Glyma12G010600.1	PPH
<i>Glycine max</i>	Glyma16G138000.1	PPH-LIKE3
<i>Glycine max</i>	Glyma20G214000.1	PPH-LIKE1
<i>Glycine max</i>	GlymaU018300.1	PPH
<i>Gossypium raimondii</i>	Gorai003G013800.1	PPH-LIKE3
<i>Gossypium raimondii</i>	Gorai004G168500.1	PPH
<i>Gossypium raimondii</i>	Gorai008G112100.1	PPH
<i>Gossypium raimondii</i>	Gorai013G066300.1	PPH-LIKE1
<i>Gossypium raimondii</i>	Gorai013G244100.1	PPH-LIKE2
<i>Gossypium raimondii</i>	Gorai013G244300.1	PPH-LIKE2
<i>Linum usitatissimum</i>	Lus10003969	PPH-LIKE1
<i>Linum usitatissimum</i>	Lus10005320	PPH
<i>Linum usitatissimum</i>	Lus10016369	PPH
<i>Linum usitatissimum</i>	Lus10019755	PPH
<i>Linum usitatissimum</i>	Lus10028315	PPH-LIKE3
<i>Malus domestica</i>	MDP0000155922	PPH-LIKE1
<i>Malus domestica</i>	MDP0000212844	PPH-LIKE2
<i>Malus domestica</i>	MDP0000243945	PPH-LIKE2
<i>Malus domestica</i>	MDP0000255076	PPH-LIKE2
<i>Malus domestica</i>	MDP0000297656	PPH-LIKE2
<i>Malus domestica</i>	MDP0000299147	PPH-LIKE2
<i>Malus domestica</i>	MDP0000312878	PPH
<i>Malus domestica</i>	MDP0000469526	PPH-LIKE3
<i>Malus domestica</i>	MDP0000675548	PPH
<i>Manihot esculenta</i>	cassava4.1_005551m	PPH
<i>Manihot esculenta</i>	cassava4.1_009393m	PPH-LIKE2
<i>Manihot esculenta</i>	cassava4.1_010002m	PPH-LIKE3
<i>Manihot esculenta</i>	cassava4.1_010269m	PPH-LIKE1
<i>Medicago truncatula</i>	Medtr1g076920.1	PPH-LIKE1
<i>Medicago truncatula</i>	Medtr3g034670.1	PPH
<i>Medicago truncatula</i>	Medtr8g077185.1	PPH-LIKE3
<i>Micromonas pusilla</i>	108137	
<i>Micromonas pusilla</i>	108397	PPH-LIKE1
<i>Micromonas pusilla</i>	132413	PPH-LIKE2
<i>Micromonas pusilla</i>	159570	PPH-LIKE1
<i>Micromonas pusilla</i>	175092	PPH
<i>Micromonas pusilla</i>	57370	PPH
<i>Micromonas pusilla</i>	70909	
<i>Micromonas pusilla</i>	97456	PPH-LIKE2
<i>Mimulus guttatus</i>	MigutB01255.1	PPH
<i>Mimulus guttatus</i>	MigutC01319.1	PPH-LIKE1
<i>Mimulus guttatus</i>	MigutG01135.1	PPH-LIKE2
<i>Mimulus guttatus</i>	MigutH02536.1	PPH-LIKE3
<i>Mimulus guttatus</i>	MigutN01808.1	PPH
<i>Oryza sativa</i>	LOC_Os02g47620.1	PPH-LIKE3
<i>Oryza sativa</i>	LOC_Os05g34630.1	PPH-LIKE1
<i>Oryza sativa</i>	LOC_Os06g24730.1	PPH
<i>Oryza sativa</i>	LOC_Os07g38830.1	PPH-LIKE2

<i>Ostreococcus lucimarinus</i>	13371	PPH-LIKE2
<i>Ostreococcus lucimarinus</i>	37370	
<i>Ostreococcus lucimarinus</i>	3905	PPH
<i>Ostreococcus lucimarinus</i>	4818	PPH-LIKE1
<i>Physcomitrella patens</i>	Phpat009G000300.1	PPH-LIKE1
<i>Physcomitrella patens</i>	Phpat019G067300.1	PPH-LIKE2
<i>Physcomitrella patens</i>	Phpat025G034500.1	PPH
<i>Populus trichocarpa</i>	Potri003G219700.1	PPH-LIKE2
<i>Populus trichocarpa</i>	Potri005G121000.1	PPH-LIKE3
<i>Populus trichocarpa</i>	Potri009G054800.1	PPH
<i>Populus trichocarpa</i>	Potri019G063900.1	PPH-LIKE1
<i>Prunus persica</i>	ppa004221m	PPH
<i>Prunus persica</i>	ppa007170m	PPH-LIKE2
<i>Prunus persica</i>	ppa018218m	PPH-LIKE1
<i>Prunus persica</i>	ppa018291m	PPH-LIKE3
<i>Ricinus communis</i>	29634.m002117	PPH-LIKE3
<i>Ricinus communis</i>	29835.m000654	PPH-LIKE2
<i>Ricinus communis</i>	29955.m001167	PPH-LIKE1
<i>Ricinus communis</i>	30221.m002221	PPH
<i>Ricinus communis</i>	39680.m000014	PPH-LIKE2
<i>Selaginella moellendorffii</i>	e_gw173286	PPH-LIKE2
<i>Selaginella moellendorffii</i>	estExt_fgenes2_pgC_20078	PPH-LIKE1
<i>Selaginella moellendorffii</i>	estExt_Genewise1PlusC_20629	PPH-LIKE1
<i>Solanum lycopersicum</i>	Solyc01g088090.2	PPH
<i>Solanum lycopersicum</i>	Solyc02g062610.2	PPH-LIKE1
<i>Solanum lycopersicum</i>	Solyc02g070490.2	PPH-LIKE3
<i>Solanum lycopersicum</i>	Solyc02g085070.1	PPH-LIKE3
<i>Solanum lycopersicum</i>	Solyc12g098660.1	PPH-LIKE2
<i>Solanum tuberosum</i>	PGSC0003DMP400008100	PPH-LIKE2
<i>Solanum tuberosum</i>	PGSC0003DMP400011967	PPH
<i>Solanum tuberosum</i>	PGSC0003DMP400027144	PPH-LIKE3
<i>Solanum tuberosum</i>	PGSC0003DMP400036552	PPH-LIKE1
<i>Solanum tuberosum</i>	PGSC0003DMP400059242	PPH-LIKE3
<i>Sorghum bicolor</i>	Sobic002G351200.1	PPH-LIKE2
<i>Sorghum bicolor</i>	Sobic004G034600.1	PPH
<i>Sorghum bicolor</i>	Sobic004G267700.1	PPH-LIKE3
<i>Sorghum bicolor</i>	Sobic009G140600.1	PPH-LIKE1
<i>Theobroma cacao</i>	Thecc1EG000351t1	PPH-LIKE3
<i>Theobroma cacao</i>	Thecc1EG005808t1	PPH
<i>Theobroma cacao</i>	Thecc1EG007625t1	PPH-LIKE1
<i>Vitis vinifera</i>	GSVIVT01001445001	PPH
<i>Vitis vinifera</i>	GSVIVT01018934001	PPH-LIKE3
<i>Vitis vinifera</i>	GSVIVT01025450001	PPH-LIKE2
<i>Vitis vinifera</i>	GSVIVT01037084001	PPH-LIKE1
<i>Volvox carteri</i>	Vocar20009758m	PPH-LIKE2
<i>Volvox carteri</i>	Vocar20010293m	PPH-LIKE1
<i>Volvox carteri</i>	Vocar20014244m	PPH
<i>Volvox carteri</i>	Vocar20014805m	
<i>Zea mays</i>	GRMZM2G076631_P01	PPH-LIKE3
<i>Zea mays</i>	GRMZM2G109070_P01	PPH
<i>Zea mays</i>	GRMZM2G121516_P01	PPH-LIKE2
<i>Zea mays</i>	GRMZM5G815808_P01	PPH-LIKE1

ATTACHMENT III

Different Mechanisms Are Responsible for Chlorophyll Dephytylation during Fruit Ripening and Leaf Senescence in Tomato¹[W][OPEN]

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Chlorophyll breakdown occurs in different green plant tissues (e.g. during leaf senescence and in ripening fruits). For different plant species, the PHEOPHORBIDE A OXYGENASE (PAO)/phyllobilin pathway has been described to be the major chlorophyll catabolic pathway. In this pathway, pheophorbide (i.e. magnesium- and phytol-free chlorophyll) occurs as a core intermediate. Most of the enzymes involved in the PAO/phyllobilin pathway are known; however, the mechanism of dephytylation remains uncertain. During *Arabidopsis* (*Arabidopsis thaliana*) leaf senescence, phytol hydrolysis is catalyzed by PHEOPHYTINASE (PPH), which is specific for pheophytin (i.e. magnesium-free chlorophyll). By contrast, in fruits of different *Citrus* spp., chlorophyllase, hydrolyzing phytol from chlorophyll, was shown to be active. Here, we enlighten the process of chlorophyll breakdown in tomato (*Solanum lycopersicum*), both in leaves and fruits. We demonstrate the activity of the PAO/phyllobilin pathway and identify tomato PPH (SIPPH), which, like its *Arabidopsis* ortholog, was specifically active on pheophytin. SIPPH localized to chloroplasts and was transcriptionally up-regulated during leaf senescence and fruit ripening. SIPPH-silencing tomato lines were impaired in chlorophyll breakdown and accumulated pheophytin during leaf senescence. However, although pheophytin transiently accumulated in ripening fruits of SIPPH-silencing lines, ultimately these fruits were able to degrade chlorophyll like the wild type. We conclude that PPH is the core phytol-hydrolytic enzyme during leaf senescence in different plant species; however, fruit ripening involves other hydrolases, which are active in parallel to PPH or are the core hydrolases in fruits. These hydrolases remain unidentified, and we discuss the question of whether chlorophyllases might be involved.

Chlorophyll breakdown is an important physiological process in plants that occurs during different phases of plant development. Most obvious and eye-catching is the loss of green pigment color during autumnal leaf senescence in deciduous trees, but also the ripening phase of many fruits such as banana (*Musa acuminata*) and tomato (*Solanum lycopersicum*) includes massive degradation of chlorophyll.

For many years, chlorophyll degradation was considered a biological enigma (Hendry et al., 1987). Only the identification and structure determination of a first colorless nonfluorescent chlorophyll catabolite from senescing barley (*Hordeum vulgare*) as a (final) breakdown product (Kräutler et al., 1991) paved the way for the step-wise elucidation of a pathway of chlorophyll

degradation (for review, see Hörtensteiner and Kräutler, 2011; Kräutler and Hörtensteiner, 2013; Christ and Hörtensteiner, 2014). This pathway leads to the ultimate degradation of chlorophyll to a group of colorless, linear tetrapyrroles, termed phyllobilins (Kräutler and Hörtensteiner, 2013).

The pathway can be divided into two parts. Early reactions take place within senescing chloroplasts and result in the formation of a colorless primary fluorescent chlorophyll catabolite (*p*FCC; Fig. 1; Mühlecker et al., 1997). The reactions catalyzing the chlorophyll-to-*p*FCC conversion are commonly present in land plants (Hörtensteiner, 2013) and, therefore, represent the core part of the pathway. The second part of the chlorophyll degradation pathway is characterized by largely species-specific modifications at different peripheral positions within *p*FCC (indicated in Fig. 1 with R¹–R⁴) and ultimate conversion to respective nonfluorescent phyllobilins that represent the end products of chlorophyll breakdown in most species and are stored in the vacuole (Kräutler and Hörtensteiner, 2013).

To date, a total of four steps are known to be required for the conversion of chlorophyll *a* to *p*FCC. Except for the activity that is responsible for magnesium dechelation, genes encoding these catalytic activities have been identified in *Arabidopsis* (*Arabidopsis thaliana*) and other species. Since all except one of the phyllobilins that have

¹ This work was supported by the Swiss National Science Foundation (grant no. 31003A–132603 to S.H.).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Stefan Hörtensteiner (shorten@botinst.uzh.ch).

[W] The online version of this article contains Web-only data.

[OPEN] Articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.114.239541

Figure 1. Structural outline of the PAO/phyllobilin pathway of chlorophyll breakdown showing the chemical constitutions of chlorophyll *a* and of selected chlorophyll catabolites that are relevant for this work. R¹ to R⁴ indicate sites of modifications that are found in nonfluorescent phyllobilins of different plant species (Kräutler and Hörtensteiner, 2013). Relevant reactions (PPH, CLH, PAO, and RCCR) are indicated. Note that dephytylation by PPH was shown to be the major reaction of pheophorbide *a* formation during leaf senescence in *Arabidopsis* (Schelbert et al., 2009). The inset indicates that conversion of chlorophyll to pFCC requires the concerted action of different CCEs and of SGR.

been characterized structurally are derived from chlorophyll *a* (Hörtensteiner and Kräutler, 2011), the reductive part of the chlorophyll cycle that converts chlorophyll *b* into chlorophyll *a* has been considered an integral part of senescence-related chlorophyll breakdown (Tanaka et al., 2011).

The magnesium- and phytol-free intermediate of chlorophyll *a*, pheophorbide *a*, is a genuine breakdown product of chlorophyll (Langmeier et al., 1993). However, the means of pheophorbide formation during leaf senescence was (and still is) controversial, because the order of reactions—that is, dechelation versus dephytylation—was unclear (Amir-Shapira et al., 1987), although the favored hypothesis was that dephytylation by CHLOROPHYLLASE (CLH) would precede magnesium dechelation (Tanaka and Tanaka, 2006). We recently showed that the two CLHs of *Arabidopsis* are dispensable for leaf senescence (Schenk et al., 2007). Instead, we and others identified a novel esterase, PHEOPHYTINASE (PPH), which specifically dephytylates pheophytin, but not chlorophyll, and is required for chlorophyll breakdown in *Arabidopsis* and rice (*Oryza sativa*; Morita et al., 2009; Schelbert et al., 2009; Ren et al., 2010). Thus, PPH-deficient mutants exhibit a stay-green phenotype, which is characterized by a high retention of chlorophyll together with the accumulation of significant amounts of pheophytin during leaf senescence. This indicates that dechelation precedes dephytylation, at least during leaf senescence. By contrast, CLHs have been implicated in the postharvest senescence of broccoli (*Brassica oleracea* var *italica*) and citrus (*Citrus* spp.) fruit ripening (Jacob-Wilk et al., 1999; Azoulay Shemer et al., 2008; Chen et al., 2008; see below). Pheophorbide *a*, the last chlorin-type

intermediate of chlorophyll breakdown, is oxygenolytically opened by PHEOPHORBIIDE A OXYGENASE (PAO) to yield a red chlorophyll catabolite, which is further reduced to pFCC by RED CHLOROPHYLL CATABOLITE REDUCTASE (RCCR; Rodoni et al., 1997). PAO is responsible for the open tetrapyrrolic backbone of the phyllobilins. For this reason, the pathway described above is now termed the PAO/phyllobilin pathway of chlorophyll breakdown (Kräutler and Hörtensteiner, 2013).

Recently, it was shown that the chloroplast-localized chlorophyll catabolic enzymes (CCEs) physically interact at the thylakoid membrane, most likely to allow metabolic channeling of the breakdown intermediates upstream of pFCC that are potentially phototoxic (Sakuraba et al., 2012). STAY-GREEN (SGR), a chloroplast-localized protein (Hörtensteiner, 2009), is critical for these interactions; *nonyellowing1-1*, an *Arabidopsis* SGR mutant (Ren et al., 2007), is defective in CCE protein interaction (Sakuraba et al., 2012). This indicates that, rather being biochemically active itself, SGR may function as a scaffold protein to recruit CCEs for protein complex formation during chlorophyll breakdown. As a consequence, mutants that are deficient in SGR exhibit a stay-green phenotype (Barry, 2009; Hörtensteiner, 2009). In addition, SGR (negatively) regulates carotenoid biosynthesis during tomato fruit ripening (Luo et al., 2013) and (positively) regulates root nodule senescence in *Medicago truncatula* (Zhou et al., 2011), implying that SGR has diverse functions that are not restricted to chlorophyll degradation.

The PAO/phyllobilin pathway has largely been elucidated through investigations that focused on leaf

senescence. Nevertheless, chlorophyll breakdown during fruit ripening was considered to be identical to the mechanism occurring during leaf senescence (Hörtensteiner and Kräutler, 2011). Deficiency of SGR, as for example in the tomato *green flesh* (*gf*) and the red pepper (*Capsicum annuum*) *chlorophyll retainer* mutants, causes a stay-green phenotype of these mutants in leaves and fruits (Barry et al., 2008; Borovsky and Paran, 2008), indicating that SGR is required for chlorophyll breakdown in both tissues. Similarly, PAO and RCCR were found to be active in chromoplast membranes isolated from tomato and red pepper fruits (Moser and Matile, 1997; Akhtar et al., 1999), and recently, different fluorescent and nonfluorescent phyllobilins were shown to occur in ripening apple (*Malus domestica*), pear (*Pyrus communis*), and banana (Kräutler, 2008; Moser et al., 2009). Finally, SGR and PAO have been identified in a recent proteome analysis of tomato chromoplasts (Barsan et al., 2010). In summary, these data indicate that the pathways of chlorophyll breakdown during fruit ripening and leaf senescence are identical. Yet, the identification of PPH as the major dephytylating enzyme of leaf senescence (Schelbert et al., 2009) challenges this view, because, contrary to the situation in leaves, CLH was shown to be involved during ethylene-induced ripening of citrus fruits (Jacob-Wilk et al., 1999; Harpaz-Saad et al., 2007; Azoulay Shemer et al., 2008).

The aim of this work, therefore, was to investigate whether PPH, besides its requirement for leaf senescence, is also involved in chlorophyll breakdown during fruit ripening. Using tomato as a model, we show that the PAO/phyllobilin pathway is active both during fruit ripening and leaf senescence, because genes encoding CCEs and SGR are transcriptionally up-regulated in

both ripening fruits and senescing leaves. However, lines silenced in tomato *PPH* (*SIPPH*) were specifically deficient in leaf senescence-related chlorophyll breakdown, while the involvement of PPH in fruit ripening-related breakdown seems to be less important. Although our data show a transient delay of chlorophyll breakdown in the absence of PPH, *SIPPH*-silencing fruits ultimately degrade chlorophyll like the wild type. Pheophytin-specific phytol hydrolysis was reduced in chromoplasts of *SIPPH*-silencing lines, but substantial enzyme activity remained in these lines, which leads us to speculate that other hydrolases are important (in addition to PPH). The identity of these activities remains elusive.

RESULTS

The PAO/Phyllobilin Pathway Is Active during Chlorophyll Degradation in Tomato Leaves and Fruits

To enlighten whether the PAO/phyllobilin pathway is responsible for the loss of chlorophyll in tomato, CCE gene expression was analyzed during leaf senescence and fruit ripening. Yellowing was observed during the progression of natural senescence of tomato leaves starting at 60 d after germination (Fig. 2A), and within 23 d, the content of chlorophyll *a* and *b* decreased to around 30% of the initial amount (Fig. 2C). As shown in Figure 2, B and D, the chlorophyll content of tomato fruits at the breaker stage was reduced within 4 d of ripening, and red and yellow pigments, mainly carotenoids (Egea et al., 2010), became visible. Gene expression levels of *SISGR* and *SIPAO*, as analyzed by semiquantitative reverse transcription (RT)-PCR, increased during both leaf senescence and fruit ripening (Fig. 2, E and F).

Figure 2. The PAO/phyllobilin pathway is active during chlorophyll degradation in tomato leaves and fruits. A, Phenotypic appearance of the first true leaves from wild-type tomato during natural senescence starting from 60 d after germination. B, Phenotypes of fruits during ripening. GM, Green mature; B, breaker. C and D, Quantification of total chlorophyll during natural leaf senescence (C) and fruit ripening (D). Total leaves and fruit exocarp and mesocarp tissues at the indicated times were used for chlorophyll quantification. Data represent means of three technical replicates $\pm$ SD. FW, Fresh weight. E and F, Analysis of gene expression during natural leaf senescence (E) and fruit ripening (F). *SITIP41* was used as a control (Expósito-Rodríguez et al., 2008). Expression was analyzed with the number of PCR cycles as indicated. PCR products were separated on agarose gels and visualized with ethidium bromide.

These results confirmed published quantitative PCR (qPCR) data on CCE gene expression (Lira et al., 2014) and indicated that the PAO/phyllobilin pathway is activated during chlorophyll breakdown in tomato and that chlorophyll is degraded in a similar manner

in tomato leaves and fruits. Nevertheless, it remained to be demonstrated whether the core phytol hydrolytic enzyme during chlorophyll degradation is PPH, as demonstrated in *Arabidopsis* leaves (Schelbert et al., 2009).

Figure 3. Analysis of PPH proteins from different plant species. A, Maximum likelihood phylogenetic tree of PPH proteins from different higher plant species. Branch support values are based on 100 bootstrap replicates and are indicated when higher than 0.6. Aegta, *Aegilops tauschii*; Ambtr, *Amborella trichopoda*; Araly, *Arabidopsis lyrata*; Arath, *Arabidopsis*; Bradi, *Brachypodium distachyon*; Capru, *Capsella rubella*; Cicar, *Cicer arietinum*; Citcl, *Citrus clementina*; Citsi, *Citrus sinensis*; Cucsa, *Cucumis sativus*; Eutsa, *Eutrema salsugineum*; Frave, *Fragaria vesca*; Genau, *Genlisea aurea*; Glyma, soybean; Horvu, barley; Lotja, *Lotus japonicus*; Medtr, *Medicago truncatula*; Nicta, *Nicotiana tabacum*; Orybr, *Oryza brachyantha*; Orysa, rice; Phavu, common bean; Poptr, *Populus trichocarpa*; Prupe, *Prunus persica*; Setit, *Setaria italica*; Solly, tomato; Soltu, *Solanum tuberosum*; Sorbi, *Sorghum bicolor*; Theca, *Theobroma cacao*; Triur, *Triticum urartu*; Vitvi, *Vitis vinifera*; Zeama, *Zea mays*. B, Alignment of PPH proteins from *Arabidopsis* (Arath) and tomato (Solly). Two potential start Met residues are underlined. Cleavage sites of the chloroplast transit peptide sequences as predicted by ChloroP (Emanuelsson et al., 1999) are indicated with arrows. The PPH motif (Schelbert et al., 2009) containing the active-site Ser residue (arrowhead) is boxed. Identical amino acids are shaded in gray.

SIPPH Is Expressed in Tomato and Localizes to Chloroplasts

BLASTP searches (Altschul et al., 1997) for PPH protein homologs in tomato identified SIPPH (Solyc01g088090). Highly homologous PPH proteins were present in all sequenced plant genomes as single proteins, except for soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*), with three and two PPHs, respectively (Fig. 3A). PPHs of species within different plant families, including Fabaceae, Brassicaceae, Solanaceae, and Gramineae, clustered into separate clades. Overall protein sequence identity within families was between 65% and 96%, and even the most divergent PPH from *Genlisea aurea* was more than 58% identical to the other protein sequences. An alignment of SIPPH and AtPPH, which exhibits 62.8% sequence identity, is shown in Figure 3B. The conserved PPH domain (Schelbert et al., 2009) with its proposed active-site Ser residue (boxed in Fig. 3B) was present in all PPH proteins included in the phylogenetic tree of Figure 3A. Expression of SIPPH, as analyzed by semiquantitative RT-PCR, increased with the onset of leaf senescence and fruit ripening and correlated with the transcript levels of *SIPAO* and *SISGR* (Fig. 2, E and F). From these results, we concluded that SIPPH is involved in chlorophyll breakdown and likely acts as the phytol hydrolytic enzyme in leaves and fruits. In order to analyze the subcellular localization of SIPPH, which based on its proposed function was expected to localize to plastids, we constructed C-terminal GFP fusions (SIPPH-GFP). The sequence of the predicted SIPPH complementary DNA (cDNA) contained two possible in-frame start codons (underlined Met residues in Fig. 3B); however, none of these encoded a PPH version that would contain an N-terminal chloroplast transit peptide according to the prediction by ChloroP (Emanuelsson et al., 1999). Therefore, both varieties, SIPPH(long) and SIPPH(short), were cloned. The fusion proteins were transiently expressed in senescing *Arabidopsis* mesophyll protoplasts and analyzed by confocal laser-scanning microscopy. As shown in Figure 4, the overlay of GFP

fluorescence and chlorophyll autofluorescence indicated that the long SIPPH version localized to the chloroplast, while the GFP signal of the short version was detected in the cytosol. From these results, we conclude that SIPPH is indeed located in the chloroplast and that SIPPH(long) represents the full-length SIPPH version, with a likely 61-amino acid chloroplast transit peptide as predicted by ChloroP (Emanuelsson et al., 1999; Fig. 3B).

SIPPH Is a Genuine PPH

Phylogenetic analysis and sequence alignment of PPH homologs revealed the PPH motif including the proposed active-site Ser residue to be present in SIPPH (Fig. 3B). This indicated that SIPPH is a genuine PPH and, thus, an ortholog of *Arabidopsis* PPH (Schelbert et al., 2009). To confirm this, the *Arabidopsis pph-1* mutant was complemented with an SIPPH cDNA construct (long version) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. As shown earlier, *pph-1* is impaired in chlorophyll breakdown and shows a stay-green phenotype (Schelbert et al., 2009). To induce senescence, detached T1 leaves of three independent complementation lines (*pph-1/35S::SIPPH_1*, *pph-1/35S::SIPPH_2*, and *pph-1/35S::SIPPH_10*) were dark incubated for 7 d. Indeed, ectopic expression of SIPPH complemented the *pph-1* phenotype, and leaves of all three tested lines showed leaf yellowing comparable to the wild type (Fig. 5A). To further verify the function of SIPPH as PPH, we examined the enzymatic activity of a recombinant truncated version of SIPPH devoid of the predicted chloroplast transit peptide (Δ SIPPH). Δ SIPPH was expressed in *Escherichia coli* as an N-terminal maltose-binding protein fusion (MBP- Δ SIPPH). The recombinant fusion protein was highly stable and largely located in the soluble bacterial cell fraction (Fig. 5B). Using chlorophyll *a* or pheophytin *a*, or mixtures of both as substrate, we could confirm SIPPH to be highly specific for pheophytin *a* (Fig. 5, C and D), comparable to its *Arabidopsis* ortholog (Schelbert et al., 2009). These

Figure 4. Subcellular localization of SIPPH. Two SIPPH varieties, SIPPH(long) and SIPPH(short), were transiently expressed as GFP fusions in *Arabidopsis* protoplasts isolated from senescent leaves. GFP fluorescence (GFP) and chlorophyll autofluorescence (chlorophyll) were examined by confocal laser-scanning microscopy. Merged images show the overlay of GFP and autofluorescence. Bars = 10 μ m.

Figure 5. Confirmation of SIPPH as a genuine PPH. A, Complementation of Arabidopsis *pph-1* with SIPPH. Detached leaves of 4-week-

data strongly support the assumption that SIPPH acts as genuine PPH.

SIPPH Catalyzes the Cleavage of Phytol in Senescing Tomato Leaves

To analyze whether SIPPH is required for in vivo chlorophyll breakdown in tomato, transgenic tomato plants were generated that harbored an *SIPPH*-silencing construct expressed under the control of the CaMV 35S promoter (*SIPPHi*). Levels of *SIPPH* expression of several independent transgenic tomato lines were determined in leaf and fruit tissues by semiquantitative RT-PCR and qPCR (Supplemental Fig. S1). Several independent RNA interference (RNAi) lines displayed strongly reduced *SIPPH* expression as compared with the wild type, and lines *SIPPHi_17* and *SIPPHi_27*, with expression levels of less than 16% and 7%, respectively, in leaves and fruits at breaker + 1 d were chosen for further analysis.

To elucidate whether the absence of SIPPH causes a stay-green phenotype during chlorophyll breakdown in leaves as described for Arabidopsis (Schelbert et al., 2009), senescence was initiated in detached leaves of the wild type, *gf*, *SIPPHi_17*, and *SIPPHi_27* by dark incubation for up to 10 d in the presence of 1 mM ethephon, a precursor of ethylene. After 6 d, visual yellowing (Fig. 6A) and decrease of chlorophyll *a* and *b* (Fig. 6B) were observed in wild-type leaves, while leaves of *gf* and the two silencing lines still appeared green and chlorophyll degradation was significantly delayed. Thus, after 10 d, chlorophyll content was decreased to less than 50% in the wild type, whereas in *gf*, *SIPPHi_17*, and *SIPPHi_27*, approximately 70% of the initial chlorophyll was still present. In addition, HPLC analysis of pigment extracts showed that pheophytin accumulated in both analyzed RNAi lines after 6 and 10 d of dark incubation (Fig. 6C). By contrast, pheophytin was detected in only marginal amounts in wild-type and *gf* leaves. This was in agreement with the in vitro substrate specificity of SIPPH for

old plants of three independent transformants (*pph-1/35S::SIPPH_1*, *pph-1/35S::SIPPH_2*, and *pph-1/35S::SIPPH_10*) in the T1 generation were dark incubated for 7 d. Col-0, Columbia-0. B to D, Analysis of recombinant SIPPH. B, Heterologous expression of MBP and MBP-ΔSIPPH fusion proteins in *E. coli*. U, Cells before induction with isopropylthio-β-galactoside; I, cells after isopropylthio-β-galactoside induction for 3 h; S, soluble cell fraction after lysis. Note that MBP-ΔSIPPH was largely retained in the soluble cell fraction. Molecular size markers (kD) are indicated on the left. C, HPLC analysis of 60-min assays employing soluble *E. coli* lysates expressing MBP-ΔSIPPH or MBP alone with mixtures of chlorophyll *a* and pheophytin *a* as substrate. Note that SIPPH specifically hydrolyzed pheophytin *a* to pheophorbide *a*, although chlorophyll *a* was present in excess. Arrows indicate HPLC retention times of substrates and the respective dephytylated products. D, Time-dependent formation of pheophorbide *a* and chlorophyllide *a* from pheophytin *a* and chlorophyll *a*, respectively, in assays with MBP-ΔSIPPH. Note that the activity of MBP-ΔSIPPH with chlorophyll *a* as substrate is marginal. Data are means ± SD of three assays.

Figure 6. Silencing of *SIPPH* results in a stay-green phenotype in senescing tomato leaves. A, Leaf phenotype after 0, 6, and 10 d of ethylene-induced senescence in the dark. B to D, Pigment composition in senescing tomato leaves. B, Quantification of total chlorophyll. C, Quantification of pheophytin *a*. Note that pheophytin *a* was not detected (n.d.) in the wild type (WT) after 6 d of dark incubation. D, Quantification of phyllobilins after 10 d of dark incubation. All data are means of three biological replicates $\pm$ sd. FW, Fresh weight.

pheophytin (Fig. 5) and comparable to the effect in the *Arabidopsis pp1-1* mutant (Schelbert et al., 2009).

In *Arabidopsis* and many other species, nonfluorescent phyllobilins have been shown to constitute final catabolites of chlorophyll breakdown (Hörtensteiner and Kräutler, 2011; Kräutler and Hörtensteiner, 2013). Tomato wild-type leaves accumulated large quantities of phyllobilins after 10 d of dark incubation (Fig. 6D). By contrast, in *SIPPHi_17* and *SIPPHi_27* as well as in *gf*, phyllobilins did not accumulate to the same extent (Fig. 6D), confirming the impairment of chlorophyll degradation in these lines. In summary, we conclude that *SIPPH* is the core hydrolytic enzyme during chlorophyll breakdown in tomato leaves and that its absence blocks the overall process of chlorophyll degradation. As a consequence, chlorophyll is retained, pheophytin accumulates, and phyllobilin abundance is largely diminished.

***SIPPH* Is Active during Fruit Ripening, But Other Unknown Hydrolases Are Active in Parallel**

As shown in Figure 2, chlorophyll breakdown in tomato occurs during both leaf senescence and fruit ripening. Hence, we were interested in whether dephytylation in tomato fruits was also catalyzed by *SIPPH*, as shown for tomato leaves (Fig. 6). For this, we analyzed pigment composition in fruits of the wild type, *gf*, and the two RNAi lines *SIPPHi_17* and *SIPPHi_27* during the process of ripening at four different ripening stages: green mature, breaker, breaker + 2 d, and breaker + 4 d (Fig. 7). When compared with the wild type, the two *SIPPH*-silencing lines were retarded in chlorophyll breakdown and showed higher chlorophyll levels at the onset of ripening (breaker) and the half-ripe stage (breaker + 2 d). However, at the full-ripe stage (breaker + 4 d), the RNAi

lines had lost chlorophyll comparable to the wild type. This indicated that the absence of *SIPPH* caused a transient retention of chlorophyll during fruit ripening but did not result in a true stay-green phenotype, as in *gf* fruits (Fig. 7A; Barry et al., 2008). The transient retardation of chlorophyll degradation in the silencing lines was accompanied by a transient accumulation of pheophytin *a*, the substrate of *SIPPH*, while wild-type and *gf* fruits did not accumulate pheophytin *a* at any stage of ripening (Fig. 7B). Thus, the RNAi lines accumulated up to 13-fold levels of pheophytin *a* at the breaker stage as compared with the controls. However, pheophytin *a* quantities were largely reduced at the breaker + 4 d stage in *SIPPH*-silencing fruits and were comparable to the wild type and *gf* (Fig. 7B). This transient accumulation of pheophytin *a* during the fruit ripening process implied an involvement of *SIPPH* in chlorophyll breakdown also during fruit ripening on the one hand; on the other hand, however, it indicated that other phytol hydrolytic activities may be involved and may compensate for the absence of *PPH* in the silencing lines. To address this, we performed *in vitro* activity assays using chromoplasts of wild-type and RNAi lines at the breaker + 2 d stage, thereby comparing pheophytin-specific activities in solubilized and non-solubilized chromoplast membranes. For different plant species, including citrus fruits, membrane solubilization has been shown to be a prerequisite for the activation of CLHs (and possibly other dephytylating activities), which are present in membranes in a latent form (Amir-Shapira et al., 1986; Matile et al., 1999). Dephytylation of pheophytin was significantly reduced by about 25% in non-solubilized chromoplasts of *SIPPHi_17* and *SIPPHi_27* when compared with the wild type (Fig. 7C). These differences likely reflect the absence of *SIPPH* in the RNAi lines; in addition, other dephytylating activities are present in chromoplasts. Furthermore, after solubilization,

Figure 7. Analysis of SIPPH during fruit ripening. A and B, Analysis of pigment composition during fruit ripening in *SIPPH*-silencing lines. A, Quantification of total chlorophyll. Note that silencing of *SIPPH* causes a transient delay of chlorophyll degradation. B, Quantification of pheophytin a. Note that *SIPPH*-silencing lines transiently accumulate

overall activity in the wild type was about twice that compared with nonsolubilized chromoplasts, but it was not different between the wild type and the silencing lines for both chlorophyll and pheophytin (Fig. 7D). This indeed supports the assumption that, besides PPH, major additional activities are present in ripening tomato fruit chromoplasts that are capable of dephytylation of either chlorophyll or pheophytin.

To test whether CLHs could be important, we analyzed tomato *CLH* (*SICLH*) expression during leaf senescence and fruit ripening. The tomato genome contains four *CLH* genes. The deduced proteins of two of them (Solyc06g053980 = *SICLH1* and Solyc09g082600 = *SICLH3*) clustered with Arabidopsis *CLH2* in a phylogenetic tree, while Solyc09g06520 (*SICLH2*) and Solyc12g005300 (*SICLH4*) were more similar to *AtCLH1* (Supplemental Fig. S2A; Lira et al., 2014). With the exception of a slight up-regulation of *SICLH1* during leaf senescence, the expression of none of the *SICLHs* as analyzed by semi-quantitative RT-PCR correlated with the progression of leaf senescence (Supplemental Fig. S2B) or fruit ripening (Fig. 8). Transcripts for *SICLH3* were hardly detectable. This confirmed published qPCR data on *SICLH* expression (Lira et al., 2014). It is interesting that these results reflect the situation in Arabidopsis, where *CLH1* expression decreases during leaf senescence (Zimmermann et al., 2004; Winter et al., 2007) and PPH represents the major dephytylating activity (Schelbert et al., 2009).

DISCUSSION

The identification of pheophorbide a as an intermediate of chlorophyll breakdown (Hörtensteiner et al., 1995) demonstrated that dephytylation is an early step of breakdown and occurs within plastids. Phytol removal is important for two reasons: (1) it renders chlorophyll breakdown products water soluble (that is, a prerequisite for their ultimate storage in the vacuole as phyllobilins; Matile et al., 1988; Kräutler and Hörtensteiner, 2013); and (2) removal of phytol is regarded as a prerequisite for the degradation of chlorophyll-binding proteins during senescence. Thus, mutants that are incapable of phytol hydrolysis, such as Arabidopsis *pph-1* and rice *nonyellow coloring3* (*nyc3*), exhibit a stay-green phenotype during leaf senescence and retain large quantities of light-harvesting complex subunits (Morita et al., 2009; Schelbert et al., 2009). Likewise, mutations in

pheophytin a. GM, Green mature; B, breaker. C and D, Phytol hydrolytic activities of tomato chromoplasts at the breaker + 2 d stage. Pheophytin a + b or chlorophyll a + b was used as substrate, and the formation of the respective products (pheophorbide a or chlorophyllide a) was analyzed by HPLC. Note that, because the b forms of substrates were present in only small quantities in the assays, their products were not quantified. C, Hydrolytic activities in non-solubilized chromoplasts (– Triton X-100). D, Total hydrolytic activities in solubilized chromoplasts (+Triton X-100). Data are means of three biological replicates $\pm$ SD. FW, Fresh weight; WT, wild type.

Figure 8. Gene expression analyses of *SICLH1* to *SICLH4* during fruit ripening in wild-type tomato. *SITIP41* was used as a control (Expósito-Rodríguez et al., 2008). Expression was analyzed with the number of PCR cycles as indicated. PCR products were separated on agarose gels and visualized with ethidium bromide. PCR on genomic DNA (gDNA) was performed to test the efficacy of the primers used for gene expression analyses. The sizes of the fragments amplified with genomic DNA are indicated on the right. GM, Green mature; B, breaker.

steps upstream of dephytylation, such as SGR and NYC1 (that is, a CCE involved in chlorophyll *b*-to-chlorophyll *a* reduction), also result in stay-greenness coupled to apoprotein retention (Kusaba et al., 2007; Park et al., 2007; Aubry et al., 2008; Barry et al., 2008; Horie et al., 2009).

Pigment dephytylation was considered for more than a century to be catalyzed by CLHs (Willstätter and Stoll, 1913) that are able to hydrolyze both chlorophyll and pheophytin (Schelbert et al., 2009). However, their molecular identification in 1999 (Jacob-Wilk et al., 1999; Tsuchiya et al., 1999) was puzzling, since, in contrast with the predicted localization within plastid membranes, some of the cloned CLHs were suggested to localize extraplastidically and all of the identified genes encoded predicted soluble rather than membrane-localizing proteins (Takamiya et al., 2000; Hörtensteiner, 2006). Several reports that address the subcellular localization of CLHs have been published with conflicting results. Thus, the two *Arabidopsis* CLHs were shown to reside in the cytosol (Schenk et al., 2007), while the CLHs of citrus and *Ginkgo biloba* localize within plastids (Okazawa et al., 2006; Azoulay Shemer et al., 2008). The conflicting subcellular localization of CLHs prompted the hypothesis that additional extraplastidial breakdown pathways for chlorophyll may exist (Takamiya et al., 2000). However, demonstration that chloroplast-localized PAO, acting downstream of dephytylation, is involved in chlorophyll breakdown (Hörtensteiner et al., 1995; Sakuraba et al., 2012) and the finding that the absence of both *Arabidopsis* CLHs had only a marginal effect on chlorophyll breakdown (Schenk et al., 2007) challenged this idea and questioned whether CLHs are involved at all. The identification of PPH as a pheophytin-specific phytol hydrolase (Schelbert et al., 2009) supported this view, and now it is commonly accepted that PPHs rather than CLHs are responsible for leaf senescence-related chlorophyll breakdown (Tanaka et al., 2011), at least in *Arabidopsis* and rice. The results of this study extend

this assumption to tomato, because, as in *Arabidopsis* *pph* mutants (Schelbert et al., 2009), leaf yellowing was largely blocked in *SIPPH*-silencing lines and significant amounts of pheophytin *a* accumulated upon senescence induction in the dark (Fig. 6). Furthermore, genes encoding highly conserved PPHs are commonly present in higher plants (Fig. 3), allowing the extrapolation that pheophytin-specific dephytylation by PPHs may be a common feature of chlorophyll breakdown during leaf senescence.

Chlorophyll breakdown, however, not only occurs during leaf senescence but also, for example, during leaf desiccation in resurrection plants (*Craterostigma pumilum* and *Xerophyta viscosa*), during fruit ripening and seed maturation (Armstead et al., 2007; Delmas et al., 2013; Christ et al., 2014). Analysis of the dephytylation step in ripening fruits has been limited nearly exclusively to *Citrus* spp. (Amir-Shapira et al., 1987; Trebitsh et al., 1993; Jacob-Wilk et al., 1999; Azoulay Shemer et al., 2008), where leaf senescence-related chlorophyll breakdown has not been studied in detail (Katz et al., 2005). We chose tomato as a model because, besides a rather short life cycle, it offers established genetic tools as well as well-defined methods for fruit ripening and leaf senescence analysis (Akhtar et al., 1999; Barry et al., 2008) and, thus, allowed the simultaneous analysis of dephytylation during leaf senescence and fruit ripening (Figs. 6 and 7). With the *SIPPH*-silencing lines produced here, we are able to demonstrate that PPH surely participates in chlorophyll breakdown also during tomato fruit ripening, but its contribution is limited. Based on activity measurements on isolated chromoplast membranes (Fig. 7), we conclude that other phytol hydrolytic activities are present in ripening tomato fruits that either naturally participate in dephytylation as well or compensate for the absence of PPH in the silencing lines. The nature of these activities remains elusive; however, CLHs appeared as possible candidates. CLHs have been shown to dephytylate chlorophyll and pheophytin *in vitro* (Schelbert et al., 2009). Furthermore, CLHs exhibit an intriguing latency, which requires their *in vitro* activation by detergents or high concentrations of solvents (Amir-Shapira et al., 1986; Matile et al., 1999). In our assays, solubilization of chromoplasts with Triton X-100 increased the overall pheophytin hydrolytic activity by about 2-fold, indicating that CLHs contribute to the overall activity. This view that tomato CLHs may participate in dephytylation and/or may substitute for PPH seems to be in agreement with studies in citrus, where CLH was shown to play a major role in fruit ripening (Trebitsh et al., 1993; Brandis et al., 1996; Jacob-Wilk et al., 1999). Thus, citrus CLH was detected in chloroplasts by *in situ* immunofluorescence labeling. Furthermore, the enzyme is proteolytically processed at the N- and C termini, posttranslational modifications that are unrelated to chloroplast targeting but were shown to be important for activity (Harpaz-Saad et al., 2007; Azoulay Shemer et al., 2008; Azoulay-Shemer et al., 2011). Finally, citrus CLH is transcriptionally up-regulated during ethylene-induced citrus ripening (Jacob-Wilk et al., 1999). Because of the

presence of four *CLH* genes in the tomato genome, analysis of *CLH* function during fruit ripening was beyond the scope of this work and needs to be addressed in a separate study in the future. Nevertheless, we analyzed *CLH* expression, but in contrast to *PPH* expression (Fig. 2), *CLH* transcript levels were rather low and did not correlate with the progression of fruit ripening or leaf senescence (Fig. 8; Supplemental Fig. S2B). We cannot exclude, however, the possibility that, also in tomato, *CLHs* may be regulated posttranscriptionally rather than at the expression level. Nevertheless, it is interesting that *CLHs* have not been identified in proteome analyses of tomato chromoplasts, in contrast to many CCEs, such as *PPH*, *SGR*, *PAO* and *RCCR* (Barsan et al., 2010, 2012; Wang et al., 2013), pointing to their presence, if at all, in rather low abundance.

Thus, despite the implication that *CLHs* may contribute to the overall phytol hydrolytic activity observed in tomato fruit chromoplasts, other explanations are possible as well. The genome of tomato, like other species (Schelbert et al., 2009), encodes several hundred α/β -hydrolases, many of which are predicted to localize to plastids. The common feature of such hydrolases is the presence of a catalytic triad with a conserved Ser residue (Tsuchiya et al., 2003), but they group into distinct protein families based on sequence similarity. As an example, both tomato *PPH* and *CLHs* belong to the α/β -hydrolases, but their overall sequence identity is below 27%. It is possible that one or several other, so far unidentified, plastid-localizing hydrolases are involved in dephytylation during chlorophyll breakdown in tomato fruits. These activities may also contribute to the remaining chlorophyll degradation activities observed in leaves of *SIPPH*-silencing lines (Fig. 6B) and *Arabidopsis ppH* mutants (Schelbert et al., 2009).

This view is supported from investigations in *Arabidopsis*, where VITAMIN E5 (VTE5) has been shown to be responsible for the biosynthesis of 80% of α -tocopherol present in seeds (Valentin et al., 2006). VTE5 catalyzes the phosphorylation of phytol to phytol phosphate (i.e. the first of two phosphorylation steps required to synthesize phytol pyrophosphate for salvage into tocopherol; DellaPenna and Last, 2006; Ischebeck et al., 2006). It is commonly accepted that phytol hydrolysis of chlorophyll is a major source of phytol for tocopherol biosynthesis. Surprisingly, however, the absence of *PPH*, the two *CLHs*, or all three genes in a triple mutant does not affect seed tocopherol content in *Arabidopsis* (Zhang et al., 2014), pointing to a different phytol hydrolytic activity. Furthermore, triple *ppH-1 clh1 clh2* mutants do not show an embryo stay-green phenotype (Zhang et al., 2014), contrary to mutants deficient in *SGR* or *NYC1* (Nakajima et al., 2012; Delmas et al., 2013). Thus, it appears that *SGR* and some CCEs, such as *NYC1* and *PAO*, are commonly active during chlorophyll degradation in different plant tissues, while *PPH* is active in leaf senescence but plays only a minor role during fruit ripening and seed development.

MATERIALS AND METHODS

Plant Material and Senescence Induction

Seeds of tomato (*Solanum lycopersicum*) ecotype Ailsa Craig wild type and *gf* were obtained from Yoram Eyal (Volcani Center). For analysis of fruit ripening, plants were grown in soil under nutrient-sufficient conditions; plants were kept in small pots with limited nutrient supply to induce timely leaf senescence. Growth was under long-day conditions in a greenhouse with fluence rates of 100 to 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 25°C and 60% humidity. Alternatively, sterilized seeds were placed on one-half-strength Murashige and Skoog (MS) medium (2.2 g L⁻¹ MS basal salt mixture, 10 g L⁻¹ Suc, and 0.6% [w/v] phytagar), and plants were grown for 4 to 6 weeks at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 21°C. Plants were subsequently transferred to soil and grown for another 5 to 6 weeks in a phytotron (12-h/12-h light/dark cycle [40 to 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$], 60% humidity, and 22°C). For induction of senescence with ethylene, leaves of phytotron-grown plants were placed on filter paper soaked with 1 mM ethephon and incubated in the dark at room temperature. Likewise, leaves of *Arabidopsis* (*Arabidopsis thaliana*) Columbia-0 and *ppH-1* (Schelbert et al., 2009) were placed on wet filter paper and incubated in the dark.

Analysis of Chlorophyll and Catabolites

For the determination of chlorophyll and pheophytin concentrations, pigments were extracted from tomato leaf tissue and flavedo of fruits by homogenization in liquid nitrogen and subsequent extraction into 90% (v/v) acetone and 10% (v/v) 0.2 M Tris-HCl, pH 8 (Schelbert et al., 2009; Christ et al., 2012). After centrifugation (2 min, 16,000g, and 4°C), supernatants were used for spectrophotometric analysis (Strain et al., 1971) or for reverse-phase HPLC (C18 Hypersil ODS column [125 × 4.0 mm, 5 μm], Linear 206 PHD-diode array detector [365–700 nm], and ChromQuest version 2.51 software [Thermo Fisher Scientific]) as described (Langmeier et al., 1993). Phyllobilins were extracted and analyzed by HPLC as described (Christ et al., 2012).

Biocomputational Methods and Phylogenetic Analysis

SIPPH (Solyc01g088090.2) and *SICLHs* (*SICLH1*, Solyc06g053980.2; *SICLH2*, Solyc09g065620.2; *SICLH4*, Solyc12g005300.1; and *SICLH3*, Solyc09g082600.1) were identified by BLASTP searches (Altschul et al., 1997) with the Sol Genomics Network database (<http://solgenomics.net/>) using *Arabidopsis PPH* (AtPPH) and *CLH1* (AtCLH1), respectively, as queries. Full-length protein sequences of *PPH* homologs from other species were identified by BLASTP searches at the National Center for Biotechnology Information (<http://ncbi.nlm.nih.gov/>). Phylogenetic trees (Fig. 3A; Supplemental Fig. S2A) were estimated using the maximum likelihood method (<http://phylogeny.fr>; Dereeper et al., 2008). Branch support values of the phylogram are based on 100 nonparametric bootstrap replicates. The sequence alignment between *SIPPH* and AtPPH (Fig. 3B) was performed using the program DIALIGN (<http://bibiserv.techfak.uni-bielefeld.de/dialign/submission.html>; Morgenstern, 2004).

Generation of Transgenic Tomato Lines and *ppH-1* Complementation

cDNA derived from mature green tomato fruits was obtained from Yoram Eyal and was used to clone the full-length sequence of *SIPPH* [*SIPPH*(long)]. For silencing of *SIPPH* by RNAi, a 400-bp cDNA sense and antisense fragment of *SIPPH* was amplified using Pfu polymerase (Promega) with gene-specific primers as listed in Supplemental Table S1 and cloned in the silencing vector pHannibal (Wesley et al., 2001). A *NotI* fragment containing the silencing construct between the CaMV 35S promoter and an OCTOPINE SYNTHASE terminator was excised and subcloned into pGreen0029 (Hellens et al., 2000). For ectopic complementation of *ppH-1*, full-*SIPPH*(long) was cloned in a pGreen0029-derived vector (pGr-At-RCCR; Průžinská et al., 2007) that harbors a CaMV 35S promoter and a CaMV poly(A) terminator. For that, the *NdeI*/*EcoRI* insert of pGr-At-RCCR was replaced with a PCR-amplified (for primers, see Supplemental Table S1), *NdeI*/*EcoRI*-restricted fragment containing *SIPPH*(long). After verifying the inserts by sequencing, both constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 together with pSOUP (Hellens et al., 2000). *Arabidopsis ppH-1* mutants were transformed by the floral dip method (Clough and Bent, 1998). Transformants were selected on kanamycin, and plants of the T1 generation were used for further experiments.

To generate *SIPPH*-silencing tomato lines, seeds were sterilized with 1.2% (v/v) sodium hypochlorite for 15 min. Seeds were rinsed three times with sterile water

and placed on medium (one-half-strength MS, 1.5% [w/v] Suc, and 0.8% [w/v] phytagar) in 10-cm-high sterile glass pots. After 9 to 12 d of growth under long-day conditions in a culture room at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 21°C, cotyledons were excised by removing 2 to 3 mm of the leaf blades from both the proximal and distal ends. Cotyledons were placed upside down in petri dishes containing D1 medium (4.4 g L⁻¹ MS salts including B5 vitamins, 30 g L⁻¹ Glc, 1 mg L⁻¹ zeatin, 0.1 mg L⁻¹ naphthyl acetic acid, 1 mg L⁻¹ folic acid, 2 mM MES-KOH, pH 5.6–5.7, and 8 g L⁻¹ phytagar) and incubated in the culture room for 2 d. *A. tumefaciens* cells harboring the silencing construct were grown overnight at 28°C. Cells of a 20-mL culture were collected by centrifugation (6,000g for 15 min), and the pellet was resuspended in MSO-KOH, pH 5.6 (4.4 g L⁻¹ MS salts including B5 vitamins and 20 g L⁻¹ Suc) to an optical density at 600 nm of 0.4 to 0.5. Acetosyringone (100 μM) was added, and the culture was grown for another 2 h at 28°C. For transformation, cotyledons were incubated with the bacterial culture for 2 h in the dark. After another 2 to 3 d of cultivation on D1 medium, the cotyledons were transferred to D1 medium containing kanamycin (75 mg L⁻¹) and timenten (100 mg L⁻¹). Shoot regeneration was detected after about 30 d, and respective plantlets were then transferred to DL medium (4.4 g L⁻¹ MS salts including B5 vitamins, 20 g L⁻¹ Glc, 2 mg L⁻¹ indole-3-butyric acid, 1 mg L⁻¹ folic acid, 2 mM MES-KOH, pH 5.6–5.7, and 8 g L⁻¹ agar) for root induction. Rescued transformants were transferred to soil.

GFP Fusion Protein Analysis

Both *SIPPH* cDNA varieties, *SIPPH*(long) and *SIPPH*(short), were amplified using PCR Extender polymerase (5Prime) with the gene-specific primers listed in Supplemental Table S1. After restriction digestion with *Xma*I, the fragment was cloned into the corresponding site of pUC18-spGFP6 (Meyer et al., 2006), thereby producing C-terminal fusions of *SIPPH* with GFP (*SIPPH*-GFP). Sequence accuracy was confirmed by sequencing. Mesophyll protoplasts were isolated from leaves of *Arabidopsis* (Columbia-0) grown under short-day conditions according to published procedures (Endler et al., 2006). Leaves were incubated in the dark for 3 d prior to protoplast isolation. Cell numbers were quantified with a Neubauer chamber, and density was adjusted to 2×10^6 protoplasts mL⁻¹. Transformation of protoplasts was performed with 20% (w/v) polyethylene glycol as published (Meyer et al., 2006). Transformed protoplasts were incubated in the dark at room temperature for 24 to 48 h prior to confocal laser-scanning microscopic analysis (Leica TCS SP5; Leica Microsystems). GFP fluorescence was imaged at an excitation wavelength of 488 nm, and the emission signal was detected between 495 and 530 nm for GFP and between 643 and 730 nm for chlorophyll autofluorescence.

RNA Isolation, Semiquantitative RT-PCR, and qPCR

For semiquantitative RT-PCR, total RNA was extracted from leaf tissues or the flavedo of fruits using TRIzol according to the manufacturer's instructions (Life Technologies). Polyvinylpolypyrrolidone was added to ground tissue for extraction. After DNA digestion with RQ1 DNase (Promega), first-strand cDNA was synthesized from total RNA using either the RETROscript kit (Life Technologies) or Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT)₁₅ primers (Promega). PCR was performed with gene-specific primers as listed in Supplemental Table S1. To control primer suitability for RT-PCR analysis, PCR was run with genomic DNA extracted from tomato fruits. Tomato *type 2A-interacting protein41* (*SITIP41*) (Solyc10g049850.1) was used as the control gene (Expósito-Rodríguez et al., 2008).

RNA extraction for qPCR analysis and qPCR were performed as described (Quadrana et al., 2013). The PCR primers used are listed in Supplemental Table S1. All reactions were performed with two technical replicates and at least three biological replicates. mRNA levels were quantified using the 7500 Real-Time PCR system (Applied Biosystems) and SYBR Green Master Mix (Applied Biosystems). Data were analyzed with LinRegPCR software (Ruijter et al., 2009) to obtain cycle threshold values and to calculate primer efficiency. Expression values were normalized to the mean of two constitutively expressed genes, *TIP41* and *EXPRESSED* (Solyc07g025390.2.1; Expósito-Rodríguez et al., 2008). A permutation test, which lacks sample distribution assumptions (Pfaffl et al., 2002), was used to detect statistical ($P < 0.05$) differences in expression levels between samples using the algorithms in the *fgStatistics* software (<http://sites.google.com/site/fgStatistics/>).

Analysis of Recombinant SIPPH

For heterologous expression of *SIPPH* in *Escherichia coli*, a truncated cDNA fragment, lacking the 61 5'-terminal amino acids encoding the likely chloroplast transit peptide, was produced by PCR using Extender polymerase (5Prime) with

primers as listed in Supplemental Table S1. After restriction digestion with *Eco*RI, the fragment was cloned into pMal_c2 (New England Biolabs), producing a truncated MBP-*SIPPH* fusion (MBP- Δ *SIPPH*). After verifying the insert by sequencing, the construct was transformed into *E. coli* BL21(DE3). Recombinant *SIPPH* protein was expressed and cells were lysed as described (Schelbert et al., 2009). PPH activity assays (300 μL) were performed with 15 μL of crude protein extract (approximately 130 μg of soluble protein), 0.1 mM pheophytin *a* and/or chlorophyll *a* (final acetone concentration, 6.7% [v/v]), and 0.1 M HEPES-KOH, pH 8, containing 1 mM EDTA. In assays with substrate mixtures, pheophytin *a* and chlorophyll *a* were present at concentrations of 35 and 65 μM , respectively. After incubation at 34°C for various time periods, reactions were stopped by adding 2 volumes of acetone and analyzed by reverse-phase HPLC as described (Schelbert et al., 2009). Pheophytin *a* was produced from pure chlorophyll *a* (LivChem) by acidification as described (Schelbert et al., 2009).

Chromoplast Isolation and Activity Measurements

Chromoplasts of tomato mesocarp tissue at the breaker + 2 d stage were isolated as published for red pepper (*Capsicum annuum*; Christ et al., 2012) with some modifications. Mesocarp tissue was blended in a Sorvall mixer three times for 5 s with isolation buffer (1 mL g⁻¹ fresh weight) containing 400 mM Suc, 50 mM Tris-MES, pH 8, 2 mM EDTA, 10 mM polyethylene glycol 4000, 5 mM dithiothreitol, and 5 mM L(+)-ascorbic acid. Subsequently, the suspension was filtered through two layers of gauze and centrifuged (10 min at 12,000g). The pellet was carefully resuspended in isolation buffer (1 mL g⁻¹ fresh weight). After repeating the centrifugation step, chromoplasts were resuspended in Tris-MES buffer (0.05 mL g⁻¹ fresh weight) containing 25 mM Tris-MES, pH 8, and 5 mM L(+)-ascorbic acid. Isolated chromoplasts were divided into two fractions and either supplemented with 0.1 volume of Tris-MES buffer containing 10% (v/v) Triton X-100 to obtain a final Triton X-100 concentration of 1% (v/v) (+Triton X-100) or chromoplasts were supplemented with 0.1 volume of Tris-MES buffer (–Triton X-100). Both chromoplast fractions were incubated with rotation in the dark at 4°C for 30 min. Aliquots of isolated chromoplasts were frozen in liquid nitrogen and stored at –80°C. Phytol hydrolysis assays (total volume of 100 μL) consisted of 10 μL of chromoplasts (corresponding to 0.2 g fresh weight), 70 μM pheophytin *a/b* or chlorophyll *a/b*, with about 10-fold excess of the *a* pigment in both cases (3% [v/v] final acetone concentration) and reaction buffer (0.1 M HEPES-KOH, pH 8, and 1 mM EDTA). After incubation at 34°C for 45 min, reactions were stopped by adding 2 volumes of acetone. After centrifugation (16,000g for 2 min), samples were analyzed by reverse-phase HPLC as described (Langmeier et al., 1993). Substrate production and quantification were performed as described (Schelbert et al., 2009).

GenBank or Sol Genomics Network (<http://solgenomics.net/>) identification numbers for the DNA/protein sequences used in this work are as follows. PPH sequences: *Aegilops tauschii*, 475611823; *Amborella trichopoda*, 548840076; *Arabidopsis lyrata*, 297811489; *Arabidopsis*, 15240707 (AtPPH, At5g13800); *Brachypodium distachyon*, 357123819; *Capsella rubella*, 565459260; *Cicer arietinum*, 502127590; *Citrus clementina*, 567892823; *Citrus sinensis*, 568858818; *Cucumis sativus*, 449436343; *Eutrema salsugineum*, 567173584; *Fragaria vesca*, 470134497; *Genlisea aurea*, 527208569; soybean, 356539136 (Glyma1), 356531629 (Glyma2), 356542875 (Glyma3); barley, 326498881; *Lotus japonicus*, 388497996; *Medicago truncatula*, 357458507; *Nicotiana tabacum*, 156763846; *Oryza brachyantha*, 573959173; rice, 115467988; common bean, 561022305 (Phavu1), 561004436 (Phavu2); *Populus trichocarpa*, 224106163; *Prunus persica*, 462415467; *Setaria italica*, 514804304; tomato, 460367643 (*SIPPH*, Solyc01g088090.2); *Solanum tuberosum*, 565357100; *Sorghum bicolor*, 242060434; *Theobroma cacao*, 508704687; *Triticum urartu*, 473998920; *Vitis vinifera*, 225449963; and *Zea mays*, 226530215. Additional sequences for *Arabidopsis*: AtCLH1, 30912637 (At1g19670); AtCLH2, 30912739 (At5g43860); SGR, 75100772 (At4g22920); and PAO, 41688605 (At3g44880). Additional sequences for tomato: SICLH1, 460390857 (Solyc06g053980.2); SICLH2, 460403437 (Solyc09g065620.2); SICLH4, 460412186 (Solyc12g005300.1); SICLH3 (Solyc09g082600.1); SITIP41, 460406627 (Solyc10g049850.1); and EXPRESSED, 460394765 (Solyc07g025390.2.1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression analysis of *SIPPH* in *SIPPH*-silencing lines.

Supplemental Figure S2. Analysis of tomato CLHs.

Supplemental Table S1. List of primers used in this study.

ACKNOWLEDGMENTS

We thank Yoram Eyal for providing tomato seeds and cDNA, and gardeners Christian Frey and Kari Huwiler, for taking care of the plants.

Received March 14, 2014; accepted July 3, 2014; published July 17, 2014.

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Supplemental Table S1: List of Primers

<i>Gene/construct</i>	<i>Primer name</i>	<i>Sequence (5'-3')</i>
<i>RT-PCR</i>		
<i>SISGR</i>	SISGR_S	AACTCCCTGTGTTCTCAAG
	SISGR_AS	GGAAAGCAACAGGCACAAGC
<i>SIPAO</i>	SIPAO_S	GCATTCCGAAATTGGCTTAGAC
	SIPAO_AS	GCTAATCCAGCACTTATAATTGC
<i>SIPPH</i>	SIPPH_S	GTGTGGAATGAACAATGTACC
	SIPPH_AS	CCATTGAGAAGTCATTGATCC
<i>SICLH1</i>	SICLH1_S	GGTAGACTTGCTAGTGACCTG
	SICLH1_AS	CAAGCTGGCTTGCAACATTGC
<i>SICLH2</i>	SICLH2_S	CTCTAAAATTCTCAGCACTCC
	SICLH2_AS	GACCATAATCCTTAGCAAGG
<i>SICLH3</i>	SICLH3_S	CTCATGTTGGGCCAAATTTG
	SICLH3_AS	ACCATAAGTTGCCTTTCTC
<i>SICLH4</i>	SICLH4_S	GCTGAGTTTTTCAACGAGAG
	SICLH4_AS	CAGGATCAAGTTTAATAGGAC
<i>TIP41</i>	TIP41_S	GCTGCGTTTCTGGCTTAGG
	TIP41_AS	ATGGAGTTTTTGAGTCTTCTGC
<i>qPCR</i>		
<i>SIPPH</i>	RT-PPH-F	TATGGAGGGAGCAAGTACGC
	RT-PPH-R	TGGAGGGCAGAGGAAAAGTAC
<i>Expressed</i>	Expressed F	GCTAAGAACGCTGGACCTAATG
	Expressed R	TGGGTGTGCCTTTCTGAATG
<i>TIP41</i>	TIP41 F	ATGGAGTTTTTGAGTCTTCTGC
	TIP41 R	GCTGCGTTTCTGGCTTAGG
<i>cloning SIPPH-GFP</i>		
<i>SIPPH(long)</i>	SIPPH(long)_XmaI_f	GGCCCGGGATGGAATTTTGTCTTTCTATTCTG
	SIPPH_XmaI_r	GGCCCGGGCTGGAGAGTAACTCCATCTTG
<i>SIPPH(short)</i>	SIPPH(short)_XmaI_f	GGCCCGGGATGTTTTCTAGACTAAAAGAAAG
	SIPPH_XmaI_r	GGCCCGGGCTGGAGAGTAACTCCATCTTG
<i>cloning MBP-ΔSIPPH</i>		
<i>SIPPH</i>	SIPPH_EcoRI_LP	GGAATTCGCTTCTGTTAAGGGGGTTGAC
	SIPPH_EcoRI_RP	GGAATTCCTATGGAGAGTAACTCCATCTTG
<i>cloning 35S::SIPPH</i>		
<i>SIPPH</i>	SIPPH_Eco_RP	GGAATTCCTATGGAGAGTAACTCCATCTTG
	SIPPH_LPNde	GTTCCATATGGAATTTTGTCTTTCTATTCTG
<i>cloning SIPPHi</i>		
<i>SIPPH</i>	SIPPH_XhoI_SacII_LP	CCCTCGAGCCGCGGACAATAATTTTAAAGAG
	SIPPH_KpnI_RP	GGGGTACCAGATTTTAGAAACATGGAAAG
	SIPPH_BamHI_LP	CGGGATCCGGACAATAATTTTAAAGAG
	SIPPH_ClaI_RP	CCATCGATAGATTTTAGAAACATGGAAAG

Supplemental Figure S1. Analysis of *SIPPH* expression of in *SIPPH*-silencing lines. A, Semi-quantitative gene expression analysis in fruits. B, Semi-quantitative gene expression analysis in leaves. *SITIP41* was used as a control (Expósito-Rodríguez et al., 2008). PCR products were separated on agarose gels and visualized with ethidium bromide. Transgenic lines 17 and 27 were chosen for further investigations. C, Expression of *SIPPH* in lines 17 (*SIPPHi_17*) and 27 (*SIPPHi_27*), used in this study, was analyzed by qPCR in leaves and fruits at breaker + 1. Expression levels were normalized against two constitutively expressed genes *SITIP41* and *Expressed* (Expósito-Rodríguez et al., 2008). Data are mean $\pm$ SE of at least three biological replicates with each two technical replicates.

Supplemental Figure S2. Analysis of tomato chlorophyllases. A, Maximum likelihood phylogenetic tree of CLH proteins from Arabidopsis (AtCLHs) and tomato (SICLHs). Branch support values are based on 100 bootstrap replicates. B, Analysis of gene expression of *SICLH1-4* during natural leaf senescence in wild-type tomato starting from 60 days after germination. *SITIP41* was used as a control (Expósito-Rodríguez et al., 2008). Expression was analyzed with the number of PCR cycles as indicated. PCR products were separated on agarose gels and visualized with ethidium bromide. PCR on genomic DNA (gDNA) isolated from tomato fruits was performed to test efficacy of the primers used for gene expression analyses. Size of the fragments amplified with gDNA is indicated on the right. Note that the results on gDNA shown here are identical to the ones shown in Figure 8.

ATTACHMENT IV

Supplemental Figure S1

Supplemental Figure S1. Phylogenetic representation of the NAC subfamily of *Solanum lycopersicum* and *Arabidopsis thaliana*. Phylogenetic reconstruction obtained from the alignment of all NAC transcription factors of *A. thaliana* and *S. lycopersicum* obtained from the Plant Transcription Factor Database. Three putative orthologs (in blue) were chosen, namely SIORE1S02, SIORE1S03 and SIORE1S06 accordingly to the chromosome position. Marked with green or red circles are the functionally characterised proteins from *A. thaliana* or *S. lycopersicum*, respectively

Supplemental Figure S2

Supplemental Figure S2. Alignment of SLORE1s, AtORE1 and AtORS1. The subdomains A-E of the NAC domain are underlined. Shading threshold = 100%

Supplemental Figure S3

Supplemental Figure S3. Chlorophyll and senescence marker transcript levels after 48 hours of senescence-inducing treatments. Images of the leaves after 48 hours of senescence-inducing treatments. Heat map indicating the relative transcript ratio of *SIGLK1* (Soly07g053630) and *SISAG12* (Soly02g076910) normalized against values from samples harvested 0h and 48h after ethylene treatment, respectively. Values represent the mean from at least three biological replicates. Black colour = not detected. Statistically significant differences compared with the corresponding controls ($P < 0.05$) are coloured accordingly to the scale. Chlorophyll content in leaf samples. Values represent the mean $\pm$ SE from at least three biological replicates. Statistically significant differences compared to not-treated control are marked with asterisks ($P < 0.05$).

Supplemental Figure S4

Supplementary Figure S4. Nuclear subcellular localization of SLGLKs and SIOR1s proteins. GFP fusion proteins were transiently expressed in *Nicotiana tabacum* leaves by infiltration with *Agrobacterium tumefaciens*. GFP, DAPI nuclear marker, bright-field and merged signals are indicated above the panels. Bars, 20 μ m.

Supplemental Figure S5

Supplemental Figure S5. *SIORE1S02* knockdown effectiveness. *SIORE1S02* mRNA level in young plant leaves (YPL), mature plant leaves (MPL), mature green fruits (MG), breaker +1 fruits (BR1) and breaker +6 fruits (BR6) of untransformed and *SIORE1S02* knockdown plants. Value mean $\pm$ SE of at least three biological replicates normalised against the respective sample from MT control genotype. Asterisks denote statistically significant differences between MT control and the transgenic lines ($P < 0.05$).

Supplemental Figure S6

Supplemental Figure S6. *SIORE1S02* knockdown effect over *SIORE1S03* and *SIORE1S06* transcript abundance. Top: *SIORE1S03* and *SIORE1S06* mRNA level in young plant leaves (YPL) and mature plant leaves (MPL) from control genotype (MT) and *SIORE1S02*-knockdown plants. Value mean $\pm$ SE of at least three biological replicates normalised against the respective sample from MT control genotype. Asterisks denote statistically significant differences between MT control and the transgenic lines ($P < 0.05$). Bottom: detail of the alignment of the coding sequence of *SIOREs*, the blue boxes highlight the region of *SIORE1S02* targeted by the RNAi construct. Each coloured arrow pair indicates the primers used for qPCR analysis. Threshold for shading = 100%.

Supplemental Figure S7

Supplemental Figure S7. Chloroplast ultrastructure of MPL leaves of *SIOR1502*-knockdown and MT control genotype. PG, plastoglobulus; G, granum.

Supplemental Figure S8

Supplemental Figure S8. *SIORE1S02*-knockdown effects on fruit morphology and size. Morphology, diameter and weight of breaker+6 fruits from MT and *SIORE1S02*-knockdown genotypes. Values represent the mean $\pm$ SE from at least forty four biological replicates. Asterisks denote statistically significant differences to the MT control genotype ($P < 0.05$). Bar = 0.7 cm.

Supplemental Figure S9

Supplemental Figure S9. Effect of *SIORE1S02* knockdown on fruit development and metabolism. **A**, Number of days from anthesis to reach breaker stage. Value mean ± SE of at least ten biological replicates. Asterisk denote statistically significant differences compared to the corresponding sample from MT control ($P < 0.05$). **B**, Total soluble protein (TSP) of mature green fruits (MG), breaker +1 fruits (BR1) and breaker +6 fruits (BR6) from MT control genotype and *SIORE1S02*-knockdown plants. Value mean ± SE of at least three biological replicates. Asterisk denote statistically significant values compared to the corresponding sample from MT control ($P < 0.05$).

Supplemental Table S1. NAC sequence accessions used for phylogenetic reconstruction.

Locus	Species	Name
At1g01010	<i>Arabidopsis thaliana</i>	ANAC001
AT1G02210	<i>Arabidopsis thaliana</i>	ANAC110
At1g02220	<i>Arabidopsis thaliana</i>	ANAC003
At1g02230	<i>Arabidopsis thaliana</i>	ANAC004
At1g02250	<i>Arabidopsis thaliana</i>	ANAC005
AT1G03490	<i>Arabidopsis thaliana</i>	ANAC006
At1g12260	<i>Arabidopsis thaliana</i>	VND4/EMB2749
AT1G19040	<i>Arabidopsis thaliana</i>	ANAC111
AT1G25580	<i>Arabidopsis thaliana</i>	SOG1
At1g28470	<i>Arabidopsis thaliana</i>	SND3
At1g32510	<i>Arabidopsis thaliana</i>	ANAC011
At1g32770	<i>Arabidopsis thaliana</i>	SND1/NST3
At1g32870	<i>Arabidopsis thaliana</i>	ANAC013
At1g33060	<i>Arabidopsis thaliana</i>	ANAC014
At1g34180	<i>Arabidopsis thaliana</i>	ANAC016
At1g34190	<i>Arabidopsis thaliana</i>	RAO2
At1g52890	<i>Arabidopsis thaliana</i>	ANAC019
At1g54330	<i>Arabidopsis thaliana</i>	ANAC020
AT1G60240	<i>Arabidopsis thaliana</i>	ANAC112
At1g60280	<i>Arabidopsis thaliana</i>	ANAC023
At1g60300	<i>Arabidopsis thaliana</i>	ANAC107
At1g60340	<i>Arabidopsis thaliana</i>	ANAC108
At1g60350	<i>Arabidopsis thaliana</i>	ANAC024
At1g60380	<i>Arabidopsis thaliana</i>	ANAC109
At1g61110	<i>Arabidopsis thaliana</i>	ANAC025
At1g62700	<i>Arabidopsis thaliana</i>	VND5
AT1G64100	<i>Arabidopsis thaliana</i>	ANAC113
AT1G64105	<i>Arabidopsis thaliana</i>	ANAC027
At1g65910	<i>Arabidopsis thaliana</i>	ANAC028
At1g71930	<i>Arabidopsis thaliana</i>	VND7
At1g77450	<i>Arabidopsis thaliana</i>	ANAC032
At1g79580	<i>Arabidopsis thaliana</i>	SMB/URP7
At2g17040	<i>Arabidopsis thaliana</i>	ANAC036
At2g18060	<i>Arabidopsis thaliana</i>	VND1
At2g24430	<i>Arabidopsis thaliana</i>	ANAC038
At2g27300	<i>Arabidopsis thaliana</i>	NTL8
At2g33480	<i>Arabidopsis thaliana</i>	ANAC041
AT3G01600	<i>Arabidopsis thaliana</i>	ANAC044
At3g03200	<i>Arabidopsis thaliana</i>	ANAC045
At3g04060	<i>Arabidopsis thaliana</i>	ANAC046
At3g04070	<i>Arabidopsis thaliana</i>	SHYG
At3g04410	<i>Arabidopsis thaliana</i>	ANAC106
At3g04420	<i>Arabidopsis thaliana</i>	ANAC048
AT3G04430	<i>Arabidopsis thaliana</i>	ANAC049
At3g10480	<i>Arabidopsis thaliana</i>	ANAC050
At3g10490	<i>Arabidopsis thaliana</i>	ANAC051/52

AT3G12910	<i>Arabidopsis thaliana</i>	ANAC114
AT3G12977	<i>Arabidopsis thaliana</i>	ANAC115
At3g15500	<i>Arabidopsis thaliana</i>	ANAC055
At3g17730	<i>Arabidopsis thaliana</i>	ANAC057
At3g18400	<i>Arabidopsis thaliana</i>	ANAC058
At3g29035	<i>Arabidopsis thaliana</i>	AtORS1
At3g44290	<i>Arabidopsis thaliana</i>	ANAC060
At3g44350	<i>Arabidopsis thaliana</i>	ANAC061
At3g49530	<i>Arabidopsis thaliana</i>	NTL6
At3g55210	<i>Arabidopsis thaliana</i>	ANAC063
AT3G56520	<i>Arabidopsis thaliana</i>	ANAC116
At3g56530	<i>Arabidopsis thaliana</i>	ANAC064
At3g56560	<i>Arabidopsis thaliana</i>	ANAC065
AT4G01520	<i>Arabidopsis thaliana</i>	ANAC067
AT4G01540	<i>Arabidopsis thaliana</i>	NTM1
AT4G01550	<i>Arabidopsis thaliana</i>	NTM2
At4g17980	<i>Arabidopsis thaliana</i>	ANAC071
At4g27410	<i>Arabidopsis thaliana</i>	RD26
At4g28500	<i>Arabidopsis thaliana</i>	SND2
At4g28530	<i>Arabidopsis thaliana</i>	ANAC074
AT4G29230	<i>Arabidopsis thaliana</i>	ANAC075
At4g35580	<i>Arabidopsis thaliana</i>	NTL9/CBNAC
At4g36160	<i>Arabidopsis thaliana</i>	VND2
At5g04400	<i>Arabidopsis thaliana</i>	ANAC077
At5g04410	<i>Arabidopsis thaliana</i>	ANAC078
At5g09330	<i>Arabidopsis thaliana</i>	VNI1
At5g13180	<i>Arabidopsis thaliana</i>	VNI2
AT5G18037	<i>Arabidopsis thaliana</i>	ANAC117
At5g24590	<i>Arabidopsis thaliana</i>	TIP
At5g39610	<i>Arabidopsis thaliana</i>	AtORE1
At5g62380	<i>Arabidopsis thaliana</i>	VND6
AT5G64530	<i>Arabidopsis thaliana</i>	XND1
At5g66300	<i>Arabidopsis thaliana</i>	VND3
Solyc00g255510	<i>Solanum lycopersicum</i>	SNAC001
Solyc01g009860	<i>Solanum lycopersicum</i>	SNAC002
Solyc01g021730	<i>Solanum lycopersicum</i>	SNAC003
Solyc01g094490	<i>Solanum lycopersicum</i>	SNAC004
Solyc01g102740	<i>Solanum lycopersicum</i>	SNAC005
Solyc01g104900	<i>Solanum lycopersicum</i>	SNAC006
Solyc02g036430	<i>Solanum lycopersicum</i>	SNAC007
Solyc02g061780	<i>Solanum lycopersicum</i>	SNAC008
Solyc02g061870	<i>Solanum lycopersicum</i>	SNAC009
Solyc02g061900	<i>Solanum lycopersicum</i>	SNAC010
Solyc02g061910	<i>Solanum lycopersicum</i>	SNAC011
Solyc02g062060	<i>Solanum lycopersicum</i>	SNAC012
Solyc02g062070	<i>Solanum lycopersicum</i>	SNAC013
Solyc02g062090	<i>Solanum lycopersicum</i>	SNAC014
Solyc02g062210	<i>Solanum lycopersicum</i>	SNAC015
Solyc02g069960	<i>Solanum lycopersicum</i>	SNAC016
Solyc02g070140	<i>Solanum lycopersicum</i>	SNAC017

Solyc02g077610	<i>Solanum lycopersicum</i>	SNAC018
Solyc02g081270	<i>Solanum lycopersicum</i>	SNAC019
Solyc02g084350	<i>Solanum lycopersicum</i>	SNAC020
Solyc02g087920	<i>Solanum lycopersicum</i>	SNAC021
Solyc02g088180	<i>Solanum lycopersicum</i>	SNAC022
Solyc02g093420	<i>Solanum lycopersicum</i>	SNAC023
Solyc03g059300	<i>Solanum lycopersicum</i>	SNAC024
Solyc03g062670	<i>Solanum lycopersicum</i>	SNAC025
Solyc03g062750	<i>Solanum lycopersicum</i>	SNAC026
Solyc03g078120	<i>Solanum lycopersicum</i>	SNAC027
Solyc03g080090	<i>Solanum lycopersicum</i>	SNAC028
Solyc03g083880	<i>Solanum lycopersicum</i>	SNAC029
Solyc03g097650	<i>Solanum lycopersicum</i>	SNAC030
Solyc03g098190	<i>Solanum lycopersicum</i>	SNAC031
Solyc03g114220	<i>Solanum lycopersicum</i>	SNAC032
Solyc03g114260	<i>Solanum lycopersicum</i>	SNAC033
Solyc03g115850	<i>Solanum lycopersicum</i>	SINAM2
Solyc04g005610	<i>Solanum lycopersicum</i>	SNAC035
Solyc04g009440	<i>Solanum lycopersicum</i>	SINAC1
Solyc04g015960	<i>Solanum lycopersicum</i>	SNAC037
Solyc04g025760	<i>Solanum lycopersicum</i>	SNAC038
Solyc04g072220	<i>Solanum lycopersicum</i>	SNAC039
Solyc04g078670	<i>Solanum lycopersicum</i>	SNAC040
Solyc04g079940	<i>Solanum lycopersicum</i>	SNAC041
Solyc05g007550	<i>Solanum lycopersicum</i>	SNAC042
Solyc05g007770	<i>Solanum lycopersicum</i>	SINAC2
Solyc05g009840	<i>Solanum lycopersicum</i>	SNAC044
Solyc05g010740	<i>Solanum lycopersicum</i>	SNAC045
Solyc05g021090	<i>Solanum lycopersicum</i>	SNAC046
Solyc05g026180	<i>Solanum lycopersicum</i>	SNAC047
Solyc05g055470	<i>Solanum lycopersicum</i>	SNAC048
Solyc05g055480	<i>Solanum lycopersicum</i>	SNAC049
Solyc06g008360	<i>Solanum lycopersicum</i>	SNAC050
Solyc06g034340	<i>Solanum lycopersicum</i>	SNAC051
Solyc06g060230	<i>Solanum lycopersicum</i>	SNAC052
Solyc06g061080	<i>Solanum lycopersicum</i>	SNAC053
Solyc06g063380	<i>Solanum lycopersicum</i>	SNAC054
Solyc06g063430	<i>Solanum lycopersicum</i>	SNAC055
Solyc06g065410	<i>Solanum lycopersicum</i>	SNAC056
Solyc06g068580	<i>Solanum lycopersicum</i>	SNAC057
Solyc06g069100	<i>Solanum lycopersicum</i>	SNAC058
Solyc06g069710	<i>Solanum lycopersicum</i>	SNAC059
Solyc06g073050	<i>Solanum lycopersicum</i>	SNAC060
Solyc06g074170	<i>Solanum lycopersicum</i>	SNAC061
Solyc06g083840	<i>Solanum lycopersicum</i>	SNAC062
Solyc06g083850	<i>Solanum lycopersicum</i>	SNAC063
Solyc07g006840	<i>Solanum lycopersicum</i>	SNAC064
Solyc07g045030	<i>Solanum lycopersicum</i>	SNAC065
Solyc07g053590	<i>Solanum lycopersicum</i>	SNAC066
Solyc07g053680	<i>Solanum lycopersicum</i>	SNAC067

Solyc07g062240	<i>Solanum lycopersicum</i>	SNAC068
Solyc07g062840	<i>Solanum lycopersicum</i>	GOB
Solyc07g063410	<i>Solanum lycopersicum</i>	SNAC070
Solyc07g063420	<i>Solanum lycopersicum</i>	SINAC3
Solyc07g066330	<i>Solanum lycopersicum</i>	SNAC072
Solyc08g006020	<i>Solanum lycopersicum</i>	SNAC073
Solyc08g007020	<i>Solanum lycopersicum</i>	SNAC074
Solyc08g008660	<i>Solanum lycopersicum</i>	SNAC075
Solyc08g028850	<i>Solanum lycopersicum</i>	SNAC076
Solyc08g068380	<i>Solanum lycopersicum</i>	SNAC077
Solyc08g074300	<i>Solanum lycopersicum</i>	SNAC078
Solyc08g077110	<i>Solanum lycopersicum</i>	SNAC079
Solyc08g079120	<i>Solanum lycopersicum</i>	SNAC080
Solyc09g010160	<i>Solanum lycopersicum</i>	SNAC081
Solyc09g025310	<i>Solanum lycopersicum</i>	SNAC082
Solyc10g005010	<i>Solanum lycopersicum</i>	SNAC083
Solyc10g006880	<i>Solanum lycopersicum</i>	NOR
Solyc10g047060	<i>Solanum lycopersicum</i>	SNAC085
Solyc10g055760	<i>Solanum lycopersicum</i>	SNAC086
Solyc10g079220	<i>Solanum lycopersicum</i>	SNAC087
Solyc10g083450	<i>Solanum lycopersicum</i>	SNAC088
Solyc11g005920	<i>Solanum lycopersicum</i>	SNAC089
Solyc11g008000	<i>Solanum lycopersicum</i>	SNAC090
Solyc11g008010	<i>Solanum lycopersicum</i>	SNAC091
Solyc11g017470	<i>Solanum lycopersicum</i>	SINAC4
Solyc11g018660	<i>Solanum lycopersicum</i>	SNAC093
Solyc11g022480	<i>Solanum lycopersicum</i>	SNAC094
Solyc11g065540	<i>Solanum lycopersicum</i>	SNAC095
Solyc11g068620	<i>Solanum lycopersicum</i>	SNAC096
Solyc11g068750	<i>Solanum lycopersicum</i>	SNAC097
Solyc12g013620	<i>Solanum lycopersicum</i>	SNAC098
Solyc12g017400	<i>Solanum lycopersicum</i>	SNAC099
Solyc12g036480	<i>Solanum lycopersicum</i>	SNAC100
Solyc12g056790	<i>Solanum lycopersicum</i>	SNAC101

Supplemental Table S2. Relative transcript values of genes addressed in leaves of *OEmiR164a* lines.

		<i>SlmiR164</i>	<i>SISAG12</i>	<i>SIGLK1</i>	<i>SIOR1S02</i>	<i>SIOR1S03</i>	<i>SIOR1S06</i>
WT	NS	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.07
	ES	0.81 ± 0.07	10.50 ± 1.82	0.82 ± 0.04	2.09 ± 0.21	3.01 ± 0.24	1.92 ± 0.25
	LS	0.42 ± 0.05	207.27 ± 26.26	0.52 ± 0.07	9.86 ± 0.77	6.72 ± 0.83	2.72 ± 0.41
miR_2	NS	6.77 ± 0.53	0.23 ± 0.02	1.68 ± 0.13	1.10 ± 0.06	0.32 ± 0.03	0.65 ± 0.07
	ES	8.10 ± 0.74	1.19 ± 0.09	1.17 ± 0.08	1.04 ± 0.08	0.31 ± 0.02	0.60 ± 0.06
	LS	7.06 ± 0.55	5.41 ± 0.42	0.81 ± 0.06	1.14 ± 0.09	0.40 ± 0.03	0.63 ± 0.03
miR_3	NS	8.34 ± 0.61	0.11 ± 0.01	1.45 ± 0.15	1.09 ± 0.11	0.28 ± 0.03	0.64 ± 0.04
	ES	8.70 ± 0.88	1.18 ± 0.05	1.20 ± 0.09	1.03 ± 0.04	0.24 ± 0.01	0.53 ± 0.03
	LS	6.14 ± 0.48	14.17 ± 1.11	0.97 ± 0.08	0.99 ± 0.05	0.32 ± 0.02	0.47 ± 0.06

NS: non-senescent leaves; ES: early senescing leaves; LS: late senescing leaves. Values represent mean of at least three biological replicates normalised against the NS WT sample. Statistically significant differences in comparison to the NS WT sample are indicated in bold ($P < 0.05$).

Supplemental Table S3. Carotenoid, tocopherol and chlorophyll content in <i>SIORE1502</i> knockdown lines									
	Carotenoids				Tocopherols			Total	Chlorophyll
	Lutein	Lycopene	Neurosporene	β-Carotene	Alpha	Beta	Gamma	Delta	
<i>Mature Green</i>									
MT	1.42 ± 0.12	ND	0.20 ± 0.01	0.49 ± 0.05	118.74 ± 2.62	0.82 ± 0.03	2.61 ± 0.16	ND	121.98 ± 2.84
L1	1.47 ± 0.07	ND	0.22 ± 0.01	0.46 ± 0.03	151.87 ± 1.15	1.06 ± 0.02	4.43 ± 0.55	ND	157.38 ± 1.42
L3	1.42 ± 0.22	ND	0.20 ± 0.02	0.75 ± 0.22	146.57 ± 1.39	1.02 ± 0.01	3.66 ± 0.40	ND	152.86 ± 0.86
L6	1.42 ± 0.14	ND	0.21 ± 0.01	0.53 ± 0.14	155.65 ± 5.02	1.18 ± 0.17	3.75 ± 0.25	ND	166.08 ± 2.30
<i>Breaker +1 day</i>									
MT	0.86 ± 0.02	ND	0.24 ± 0.01	0.53 ± 0.05	152.03 ± 3.31	1.62 ± 0.07	20.75 ± 1.47	1.45 ± 0.34	175.39 ± 4.55
L1	0.78 ± 0.02	ND	0.20 ± 0.01	0.43 ± 0.02	179.38 ± 1.94	1.52 ± 0.06	26.33 ± 0.92	0.99 ± 0.12	208.33 ± 3.15
L3	0.64 ± 0.06	ND	0.20 ± 0.01	0.29 ± 0.10	182.92 ± 7.83	1.96 ± 0.08	34.09 ± 3.96	2.47 ± 0.20	224.79 ± 8.34
L6	0.89 ± 0.01	ND	0.22 ± 0.01	0.48 ± 0.05	176.62 ± 4.55	1.82 ± 0.08	28.95 ± 0.76	1.07 ± 0.18	209.00 ± 3.34
<i>Breaker +6 days</i>									
MT	0.54 ± 0.07	25.36 ± 1.26	0.35 ± 0.01	0.94 ± 0.05	137.83 ± 7.64	1.78 ± 0.22	33.51 ± 3.07	1.85 ± 0.25	174.83 ± 10.74
L1	0.54 ± 0.08	19.56 ± 0.43	0.59 ± 0.11	0.78 ± 0.07	158.09 ± 3.23	2.02 ± 0.09	38.56 ± 4.89	1.55 ± 0.26	199.15 ± 8.59
L3	0.56 ± 0.02	28.37 ± 1.03	0.42 ± 0.06	0.96 ± 0.03	181.24 ± 5.79	3.20 ± 0.26	54.95 ± 5.95	3.20 ± 0.12	248.57 ± 12.64
L6	0.62 ± 0.05	36.19 ± 3.14	0.35 ± 0.01	0.85 ± 0.05	175.59 ± 6.25	2.20 ± 0.17	29.45 ± 1.33	1.66 ± 0.13	214.06 ± 11.36
<i>Young Plant Leaf</i>									
MT	46.43 ± 2.23	ND	0.59 ± 0.02	36.08 ± 2.14	253.88 ± 4.51	ND	0.78 ± 0.03	ND	254.83 ± 4.48
L1	48.90 ± 3.72	ND	0.66 ± 0.02	37.72 ± 2.77	279.38 ± 8.03	ND	1.01 ± 0.06	ND	280.46 ± 8.11
L3	40.85 ± 2.19	ND	0.57 ± 0.01	31.78 ± 1.75	249.76 ± 1.50	ND	0.85 ± 0.10	ND	250.61 ± 1.58
L6	51.85 ± 5.25	ND	0.61 ± 0.05	38.12 ± 3.81	263.51 ± 7.50	ND	0.80 ± 0.04	ND	252.95 ± 15.82
<i>Mature Plant Leaf</i>									
MT	31.94 ± 2.18	ND	0.37 ± 0.03	16.56 ± 0.43	408.78 ± 7.21	ND	1.84 ± 0.17	ND	415.99 ± 6.40
L1	25.69 ± 1.21	ND	0.33 ± 0.01	16.73 ± 1.15	357.92 ± 3.03	ND	1.98 ± 0.14	ND	362.17 ± 3.41
L3	24.57 ± 1.56	ND	0.42 ± 0.01	18.35 ± 0.29	338.40 ± 5.19	ND	1.62 ± 0.10	ND	333.62 ± 7.38
L6	29.08 ± 0.48	ND	0.40 ± 0.04	17.10 ± 1.89	373.63 ± 2.17	ND	1.49 ± 0.04	ND	375.26 ± 2.08

Statistically significant differences between the control genotype (MT) and transgenic lines are indicated in bold ($P < 0.05$). Values represent means ± SE from at least three biological replicates. Values of carotenoids are expressed in $\mu\text{g g}^{-1}$ FW, of tocopherol in $\mu\text{g g}^{-1}$ DW, of chlorophyll of YPL and MPL in mg g^{-1} FW and of MG and BR1 in $\mu\text{g g}^{-1}$ FW. ND, not detected.

Supplemental Table S4. Relative transcript values of genes addressed in leaves and along fruit ripening of *SOREIS02* knockdown lines.

		<i>SGDC1</i>	<i>SGDC2</i>	<i>SOREIS02</i>	<i>SOREIS03</i>	<i>SOREIS06</i>	<i>SGAG12</i>	<i>SVTE1</i>	<i>SVTE2</i>	<i>SVTE3(1)</i>	<i>SVTE3(2)</i>	<i>SVTE4</i>	<i>SVTE5</i>	<i>SVTE6</i>	<i>SPPH</i>	<i>SPPH1</i>	<i>SICHG</i>	<i>SGOR</i>	<i>SPDS</i>	<i>SPSY1</i>	<i>SPSY2</i>	<i>SLCY8</i>	<i>SLCY9</i>	
YPL	MT	1.00 ± 0.04	NA	1.00 ± 0.12	1.00 ± 0.03	1.00 ± 0.08	ND	1.00 ± 0.05	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.15	1.00 ± 0.04	1.00 ± 0.13	1.00 ± 0.17	1.00 ± 0.02	1.00 ± 0.10	
	L1	1.72 ± 0.28	NA	0.18 ± 0.05	0.49 ± 0.09	0.66 ± 0.05	ND	1.19 ± 0.08	1.18 ± 0.05	1.13 ± 0.03	0.90 ± 0.01	1.20 ± 0.06	0.89 ± 0.06	1.19 ± 0.07	2.02 ± 0.23	1.53 ± 0.14	1.05 ± 0.04	1.13 ± 0.31	1.29 ± 0.04	1.00 ± 0.13	1.43 ± 0.07	1.09 ± 0.12	1.01 ± 0.12	1.07 ± 0.17
	L3	1.71 ± 0.27	NA	0.39 ± 0.16	0.67 ± 0.07	0.67 ± 0.02	ND	1.01 ± 0.25	1.17 ± 0.07	1.02 ± 0.07	0.86 ± 0.09	1.18 ± 0.14	0.74 ± 0.06	1.02 ± 0.09	1.35 ± 0.20	1.02 ± 0.38	0.83 ± 0.10	1.09 ± 0.25	1.22 ± 0.08	1.00 ± 0.19	0.94 ± 0.12	0.88 ± 0.07	0.90 ± 0.25	
MPL	L6	1.36 ± 0.16	NA	0.24 ± 0.03	0.51 ± 0.15	0.43 ± 0.19	ND	1.07 ± 0.16	0.92 ± 0.12	1.34 ± 0.05	1.00 ± 0.09	1.03 ± 0.06	0.84 ± 0.07	0.76 ± 0.11	1.33 ± 0.14	0.65 ± 0.29	0.61 ± 0.11	0.63 ± 0.16	1.11 ± 0.06	1.34 ± 0.09	0.55 ± 0.20	1.10 ± 0.05	0.68 ± 0.18	
	MT	1.00 ± 0.17	NA	1.00 ± 0.11	1.00 ± 0.16	1.00 ± 0.13	1.00 ± 0.14	1.00 ± 0.04	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.19	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.01	
	L1	2.28 ± 0.13	NA	0.09 ± 0.01	0.45 ± 0.01	0.39 ± 0.02	0.35 ± 0.01	1.29 ± 0.01	1.35 ± 0.09	1.24 ± 0.07	1.38 ± 0.02	1.00 ± 0.01	1.22 ± 0.02	1.02 ± 0.02	0.96 ± 0.16	1.73 ± 0.23	1.31 ± 0.03	3.95 ± 0.15	3.29 ± 0.15	1.00 ± 0.02	1.01 ± 0.04	1.20 ± 0.05	1.08 ± 0.08	0.64 ± 0.05
NPL	L3	1.64 ± 0.17	NA	0.15 ± 0.07	0.50 ± 0.17	0.47 ± 0.03	0.24 ± 0.04	1.20 ± 0.13	1.29 ± 0.05	0.93 ± 0.09	1.27 ± 0.15	0.99 ± 0.01	1.26 ± 0.08	0.93 ± 0.08	0.82 ± 0.03	1.54 ± 0.33	1.32 ± 0.12	2.41 ± 0.41	1.19 ± 0.06	1.00 ± 0.10	1.04 ± 0.07	1.00 ± 0.10	1.02 ± 0.06	0.51 ± 0.07
	L6	1.34 ± 0.04	NA	0.13 ± 0.03	0.50 ± 0.02	0.51 ± 0.08	0.32 ± 0.02	1.12 ± 0.08	1.19 ± 0.17	1.16 ± 0.11	1.16 ± 0.06	1.03 ± 0.09	1.15 ± 0.03	1.03 ± 0.06	0.96 ± 0.07	1.38 ± 0.15	1.07 ± 0.03	1.76 ± 0.13	0.93 ± 0.05	1.07 ± 0.20	1.08 ± 0.09	1.08 ± 0.12	0.68 ± 0.07	
	MT	NA	1.00 ± 0.09	1.00 ± 0.24	NA	NA	NA	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.14	NA	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.05	NA	1.00 ± 0.36	NA	1.00 ± 0.04	1.00 ± 0.21	1.00 ± 0.18	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.04	
MG	L1	NA	1.22 ± 0.23	0.05 ± 0.01	NA	NA	NA	1.72 ± 0.13	0.86 ± 0.05	1.03 ± 0.04	NA	1.16 ± 0.05	1.05 ± 0.15	1.70 ± 0.11	NA	3.17 ± 0.24	NA	0.72 ± 0.13	1.02 ± 0.10	0.62 ± 0.16	1.72 ± 0.02	0.75 ± 0.02	0.82 ± 0.22	
	L3	NA	1.67 ± 0.10	0.05 ± 0.01	NA	NA	NA	1.92 ± 0.15	0.87 ± 0.03	1.23 ± 0.07	NA	1.28 ± 0.03	1.28 ± 0.05	1.58 ± 0.03	NA	8.33 ± 0.66	NA	2.10 ± 0.23	1.03 ± 0.04	0.45 ± 0.02	1.65 ± 0.12	1.10 ± 0.02	0.78 ± 0.16	
	L6	NA	1.89 ± 0.23	0.04 ± 0.01	NA	NA	NA	1.76 ± 0.13	0.87 ± 0.07	1.33 ± 0.05	NA	1.32 ± 0.01	1.20 ± 0.03	1.86 ± 0.02	NA	4.55 ± 0.14	NA	1.36 ± 0.07	1.22 ± 0.07	0.36 ± 0.01	1.60 ± 0.12	0.81 ± 0.02	0.79 ± 0.05	
BRL	MT	NA	1.00 ± 0.05	1.00 ± 0.07	NA	NA	NA	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.02	NA	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	NA	1.00 ± 0.21	NA	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.02	
	L1	NA	0.97 ± 0.14	0.10 ± 0.02	NA	NA	NA	0.88 ± 0.04	1.23 ± 0.04	1.07 ± 0.06	NA	0.94 ± 0.03	0.87 ± 0.01	0.88 ± 0.01	NA	1.12 ± 0.04	NA	1.57 ± 0.19	1.12 ± 0.04	1.06 ± 0.04	1.58 ± 0.05	1.04 ± 0.06	1.28 ± 0.07	
	L3	NA	0.65 ± 0.17	0.06 ± 0.03	NA	NA	NA	1.02 ± 0.08	1.15 ± 0.01	0.94 ± 0.03	NA	0.98 ± 0.07	0.82 ± 0.07	0.79 ± 0.02	NA	1.04 ± 0.10	NA	0.70 ± 0.13	0.96 ± 0.08	0.77 ± 0.04	0.69 ± 0.16	0.63 ± 0.03	1.18 ± 0.10	
BRL	L6	NA	1.17 ± 0.10	0.09 ± 0.01	NA	NA	NA	0.91 ± 0.02	1.20 ± 0.07	1.11 ± 0.02	NA	1.07 ± 0.02	0.93 ± 0.03	0.79 ± 0.04	NA	1.01 ± 0.05	NA	1.55 ± 0.28	1.07 ± 0.03	0.98 ± 0.03	1.29 ± 0.16	0.93 ± 0.01	0.93 ± 0.12	
BRL	MT	NA	1.00 ± 0.09	1.00 ± 0.24	NA	NA	NA	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.14	NA	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.05	NA	1.00 ± 0.36	NA	1.00 ± 0.04	1.00 ± 0.21	1.00 ± 0.18	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.04	
	L1	NA	1.22 ± 0.23	0.05 ± 0.01	NA	NA	NA	1.72 ± 0.13	0.86 ± 0.05	1.03 ± 0.04	NA	1.16 ± 0.05	1.05 ± 0.15	1.70 ± 0.11	NA	3.17 ± 0.24	NA	0.72 ± 0.13	1.02 ± 0.10	0.62 ± 0.16	1.72 ± 0.02	0.75 ± 0.02	0.82 ± 0.22	
	L3	NA	1.67 ± 0.10	0.05 ± 0.01	NA	NA	NA	1.92 ± 0.15	0.87 ± 0.03	1.23 ± 0.07	NA	1.28 ± 0.03	1.28 ± 0.05	1.58 ± 0.03	NA	8.33 ± 0.66	NA	2.10 ± 0.23	1.03 ± 0.04	0.45 ± 0.02	1.65 ± 0.12	1.10 ± 0.02	0.78 ± 0.16	
BRL	L6	NA	1.89 ± 0.23	0.04 ± 0.01	NA	NA	NA	1.76 ± 0.13	0.87 ± 0.07	1.33 ± 0.05	NA	1.32 ± 0.01	1.20 ± 0.03	1.86 ± 0.02	NA	4.55 ± 0.14	NA	1.36 ± 0.07	1.22 ± 0.07	0.36 ± 0.01	1.60 ± 0.12	0.81 ± 0.02	0.79 ± 0.05	
BRL	MT	NA	1.00 ± 0.05	1.00 ± 0.07	NA	NA	NA	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.02	NA	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	NA	1.00 ± 0.21	NA	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.02	
	L1	NA	0.97 ± 0.14	0.10 ± 0.02	NA	NA	NA	0.88 ± 0.04	1.23 ± 0.04	1.07 ± 0.06	NA	0.94 ± 0.03	0.87 ± 0.01	0.88 ± 0.01	NA	1.12 ± 0.04	NA	1.57 ± 0.19	1.12 ± 0.04	1.06 ± 0.04	1.58 ± 0.05	1.04 ± 0.06	1.28 ± 0.07	
	L3	NA	0.65 ± 0.17	0.06 ± 0.03	NA	NA	NA	1.02 ± 0.08	1.15 ± 0.01	0.94 ± 0.03	NA	0.98 ± 0.07	0.82 ± 0.07	0.79 ± 0.02	NA	1.04 ± 0.10	NA	0.70 ± 0.13	0.96 ± 0.08	0.77 ± 0.04	0.69 ± 0.16	0.63 ± 0.03	1.18 ± 0.10	
BRL	L6	NA	1.17 ± 0.10	0.09 ± 0.01	NA	NA	NA	0.91 ± 0.02	1.20 ± 0.07	1.11 ± 0.02	NA	1.07 ± 0.02	0.93 ± 0.03	0.79 ± 0.04	NA	1.01 ± 0.05	NA	1.55 ± 0.28	1.07 ± 0.03	0.98 ± 0.03	1.29 ± 0.16	0.93 ± 0.01	0.93 ± 0.12	
BRL	MT	NA	1.00 ± 0.09	1.00 ± 0.24	NA	NA	NA	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.14	NA	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.05	NA	1.00 ± 0.36	NA	1.00 ± 0.04	1.00 ± 0.21	1.00 ± 0.18	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.04	
	L1	NA	1.22 ± 0.23	0.05 ± 0.01	NA	NA	NA	1.72 ± 0.13	0.86 ± 0.05	1.03 ± 0.04	NA	1.16 ± 0.05	1.05 ± 0.15	1.70 ± 0.11	NA	3.17 ± 0.24	NA	0.72 ± 0.13	1.02 ± 0.10	0.62 ± 0.16	1.72 ± 0.02	0.75 ± 0.02	0.82 ± 0.22	
	L3	NA	1.67 ± 0.10	0.05 ± 0.01	NA	NA	NA	1.92 ± 0.15	0.87 ± 0.03	1.23 ± 0.07	NA	1.28 ± 0.03	1.28 ± 0.05	1.58 ± 0.03	NA	8.33 ± 0.66	NA	2.10 ± 0.23	1.03 ± 0.04	0.45 ± 0.02	1.65 ± 0.12	1.10 ± 0.02	0.78 ± 0.16	
BRL	L6	NA	1.89 ± 0.23	0.04 ± 0.01	NA	NA	NA	1.76 ± 0.13	0.87 ± 0.07	1.33 ± 0.05	NA	1.32 ± 0.01	1.20 ± 0.03	1.86 ± 0.02	NA	4.55 ± 0.14	NA	1.36 ± 0.07	1.22 ± 0.07	0.36 ± 0.01	1.60 ± 0.12	0.81 ± 0.02	0.79 ± 0.05	
BRL	MT	NA	1.00 ± 0.05	1.00 ± 0.07	NA	NA	NA	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.02	NA	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	NA	1.00 ± 0.21	NA	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.02	
	L1	NA	0.97 ± 0.14	0.10 ± 0.02	NA	NA	NA	0.88 ± 0.04	1.23 ± 0.04	1.07 ± 0.06	NA	0.94 ± 0.03	0.87 ± 0.01	0.88 ± 0.01	NA	1.12 ± 0.04	NA	1.57 ± 0.19	1.12 ± 0.04	1.06 ± 0.04	1.58 ± 0.05	1.04 ± 0.06	1.28 ± 0.07	
	L3	NA	0.65 ± 0.17	0.06 ± 0.03	NA	NA	NA	1.02 ± 0.08	1.15 ± 0.01	0.94 ± 0.03	NA	0.98 ± 0.07	0.82 ± 0.07	0.79 ± 0.02	NA	1.04 ± 0.10	NA	0.70 ± 0.13	0.96 ± 0.08	0.77 ± 0.04	0.69 ± 0.16	0.63 ± 0.03	1.18 ± 0.10	
BRL	L6	NA	1.17 ± 0.10	0.09 ± 0.01	NA	NA	NA	0.91 ± 0.02	1.20 ± 0.07	1.11 ± 0.02	NA	1.07 ± 0.02	0.93 ± 0.03	0.79 ± 0.04	NA	1.01 ± 0.05	NA	1.55 ± 0.28	1.07 ± 0.03	0.98 ± 0.03	1.29 ± 0.16	0.93 ± 0.01	0.93 ± 0.12	
BRL	MT	NA	1.00 ± 0.09	1.00 ± 0.24	NA	NA	NA	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.14	NA	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.05	NA	1.00 ± 0.36	NA	1.00 ± 0.04	1.00 ± 0.21	1.00 ± 0.18	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.04	
	L1	NA	1.22 ± 0.23	0.05 ± 0.01	NA	NA	NA	1.72 ± 0.13	0.86 ± 0.05	1.03 ± 0.04	NA	1.16 ± 0.05	1.05 ± 0.15	1.70 ± 0.11	NA	3.17 ± 0.24	NA	0.72 ± 0.13	1.02 ± 0.10	0.62 ± 0.16	1.72 ± 0.02	0.75 ± 0.02	0.82 ± 0.22	
	L3	NA	1.67 ± 0.10	0.05 ± 0.01	NA	NA	NA	1.92 ± 0.15	0.87 ± 0.03	1.23 ± 0.07	NA	1.28 ± 0.03	1.28 ± 0.05	1.58 ± 0.03	NA	8.33 ± 0.66	NA	2.10 ± 0.23	1.03 ± 0.04	0.45 ± 0.02	1.65 ± 0.12	1.10 ± 0.02	0.78 ± 0.16	
BRL	L6	NA	1.89 ± 0.23	0.04 ± 0.01	NA	NA	NA	1.76 ± 0.13	0.87 ± 0.07	1.33 ± 0.05	NA	1.32 ± 0.01	1.20 ± 0.03	1.86 ± 0.02	NA	4.55 ± 0.14	NA	1.36 ± 0.07	1.22 ± 0.07	0.36 ± 0.01	1.60 ± 0.12	0.81 ± 0.02	0.79 ± 0.05	
BRL	MT	NA	1.00 ± 0.05	1.00 ± 0.07	NA	NA	NA	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.02	NA	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	NA	1.00 ± 0.21	NA	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.02	
	L1	NA	0.97 ± 0.14	0.10 ± 0.02	NA	NA	NA	0.88 ± 0.04	1.23 ± 0.04	1.07 ± 0.06	NA	0.94 ± 0.03	0.87 ± 0.01	0.88 ± 0.01	NA	1.12 ± 0.04	NA	1.57 ± 0.19	1.12 ± 0.04	1.06 ± 0.04	1.58 ± 0.05	1.04 ± 0.06	1.28 ± 0.07	
	L3	NA	0.65 ± 0.17	0.06 ± 0.03	NA																			

Statistically significant differences between the 4 transcript ratios of form genetic and MT control plants are indicated in bold letters ($P < 0.05$). Values represent mean ± 1st from at least three biological replicates. ND: Not detected. NA: Not analyzed.

Supplemental Table S5. Primers used in the experiments.			
Gene Name	Locus	Sequence	
SIORE1S02- knockdown construct			
SIORE1S02	Solyc02g088180	F	CACCCATCATCTTCTATCAAGTTCCCC
		R	GTCATGATCTTCGTAACCTCTC
SIORE1 s overexpression construct			
SIORE1S02	Solyc02g088180	F	CACCATGGAAATTGTGTGTGGATTGGTGGTC
		R	TCAATAATTCCATAGACAATCGATGTCCACTGG
SIORE1S03	Solyc03g115850	F	CACCATGATGATGGAGAATTTTTCTGCAAGTGTTAAGATG
		R	TCAGTAAGTCCAGAAGCAATCAAGATCTTGTTG
SIORE1S06	Solyc06g069710	F	CACCATGGAGAATTATTCAGGAGTTGTTAAGG
		R	TCAGCAACTCCAGAGACAATC
BiFC and confocal construct			
SIORE1S02	Solyc02g088180	F	ATGGAAATTGTGTGTGGATTGGTGGTC
		R	CCCAGTGGACATCGATTGTCTATGGAATTAT
SIORE1S03	Solyc03g115850	F	ATGATGATGGAGAATTTTTCTGCAAGTGTTAAGATG
		R	CAACAAGATCTTGATTGCTTCTGGACTTAC
SIORE1S06	Solyc06g069710	F	ATGGAGAATTATTCAGGAGTTGTTAAGGATGATGATC
		R	GGGTCTTGATTGTCTCTGGAGTTGC
SIGLK1	Solyc07g053630	F	ATGCTAGTTGTGTACCTTTTAGTAACACAACG
		R	GGGTACCTAAAATACCACCAACTTGTCGA
SIGLK2	Solyc10g008160	F	ATGCTTGCTCTATCTTCATCATTG
		R	GATTACCAAAAATACCCCAACT
AtORE1	At5G39610	F	ATGGATTACG AGGCATCAAGAATCG
		R	CAGAAGAATTGGATTGCGTTTGGAATTC
AtGLK2	At5G44190	F	ATGTTAACTGTTTCTCCGGCTCCAG
		R	CAAGGAGTTTCT AATGTTCTCTCTTCTCT
Stem-loop RT-PCR			
SlmiR164	NA	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACTGCACG	
qPCR primers			
SIORE1S02	Solyc02g088180	F	ACAACAGCGAGAAGTAGTGG
		R	GCATCAATCCAGAATCTCCATAC
SIORE1S03	Solyc03g115850	F	ACATTTCAAGGCTTGAGAGA
		R	AGGTGAATTGTTGAAGGAATTGAT
SIORE1S06	Solyc06g069710	F	GATTCTGCTACTGCTACTGCTT
		R	GGATCTTGAACCCCAATGAAG
SIGLK1	Solyc07g053630	F	GCTGTAGAGCAACTAGGTGTAGATAAGG
		R	CAACTCGCTGCCTCCACTTC
SISAG12	Solyc02g076910	F	ATGTCCTCCTCAAAGCCAAA
		R	TTTCAGTTGGTGTAGCCCTT
SlmiR164	NA	F	ACTGATGTGGAGAAGCAGGGCA
		R	GTGCAGGGTCCGAGG
SIVTE1	Solyc08g068570	F	CGAACTCCTCATAGCGGGTATC
		R	CACGCCAGTAAACCGAGGC
SIVTE2	Solyc07g017770	F	CAATTCAGTTCCTGCTGAG
		R	CCTCCAACATGCTCTTGCGTG
SIVTE3(1)	Solyc09g065730	F	CTTGACCAATCTCCTCATC
		R	GCACGCCTTCTCCAGG
SIVTE3(2)	Solyc03g005230	F	GCTAAGGCTAGGCAGAAGGAG
		R	CAGGCAACCCACCTATGG
SIVTE4	Solyc08g076360	F	CAGATCATCGTGCTGCTCAG
		R	CCTCTCTGCTTGACAGGAC
SIVTE5	Solyc03g071720	F	CGTATCAGGACGGGCTCGC
		R	TCACCACCACACATCATTGCTAATG

<i>SIVTE6</i>	Solyc07g062180	F	AGCACAAGCATCAGTGTCTG
		R	AAGAAAGCAGCCGCAATACC
<i>SIPPH</i>	Solyc01g088090	F	TATGGAGGGAGCAAGTACGC
		R	TGGAGGGCAGAGGAAAAGTAC
<i>SIPPHL1</i>	Solyc02g062610	F	GATTTGGTGCTTCTGCCTTTC
		R	GCTGTTTCTTCAGTTCCTTC
<i>SIPSY1</i>	Solyc03g031860	F	CGATGGTGCTTTGTCCGATAC
		R	CTCATCAACCCAACCGTACC
<i>SIPSY2</i>	Solyc02g081330	F	GCATCACATAACTCCACAAGC
		R	CGCATTCCTTCAACCATATCTCTG
<i>SILCY8</i>	Solyc06g074240	F	GCACCCACATCAAAGCCAGAG
		R	GCCACATGGAGAGTGGTGAAG
<i>SICYC8</i>	Solyc04g040190	F	TTGACTTAGAACCTCGTTATTGG
		R	AACAGTTCCTTTGTCTATTATCT
<i>SIPDS</i>	Solyc03g123760	F	CGTTCCTGTCTTCTCCGC
		R	CTAGAACATCCCTTGCCTCCAG
<i>SIGGDR</i>	Solyc03g115980	F	CAGAGACGCTCGCTAAGG
		R	GCTTCAGAGTCTGTCCGATATC
<i>SICHLG</i>	Solyc09g014760	F	CCAATTCCTTCAGGTGCGGT
		R	CCCACCAAGGCAAGCTGATA
<i>Expressed</i>	Solyc07g025390	F	TGGGTGTGCCTTTCTGAATG
		R	GCTAAGAACGCTGGACCTAATG
<i>TIP41</i>	Solyc10g049850	F	GCTGCGTTTCTGGCTTAGG
		R	ATGGAGTTTTTGAGTCTTCTGC