Short title: SlPIF4 regulates plant development and fruit yield

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Title: Downregulation of PHYTOCHROME-INTERACTING FACTOR 4 influences plant development and fruit production

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One-sentence summary: Silencing of a light-signaling factor reveals developmental trade-offs between tomato plant growth, senescence, fruit yield and nutritional quality.

FOOTNOTES

Author contributions: DR performed most of the experiments and analyzed the data; BT and RB performed experiments; RZ, FRRA, EP and EISF provided technical assistance; FTSN contributed to experimental design; DR, MR and LF conceived the project, designed experiments, wrote the paper and collected contributions of all authors. MR agrees to serve as the author responsible for contact and ensures communication.

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ABSTRACT

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

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

INTRODUCTION

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

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

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

have been limited to impacts on isoprenoid metabolism in fruits (Llorente et al., 2016; Gramegna et al., 2019).

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

RESULTS

Constitutive silencing of tomato *SlPIF4*

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

SlPIF4 regulates fruit ripening and quality

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

Interestingly, *SlPIF4*-silenced fruits not only accumulated more carotenoids but also ripened faster than control fruits considering the time from anthesis to BR stage (Figure 2D). We further confirmed this phenotype by analyzing colorimetric parameters of detached fruits throughout ripening (Supplemental Figure S1). In accordance with the observed advance in ripening, the color change was initially faster from BR to BR2 (2 days after BR) in fruits from L20 homozygous *SlPIF4*-silenced plants. In line with their higher lycopene content, *SlPIF4*-silenced ripe fruits showed more intense red color in comparison to the WT (Supplemental Table S1). Higher transcript abundance of the ripening master

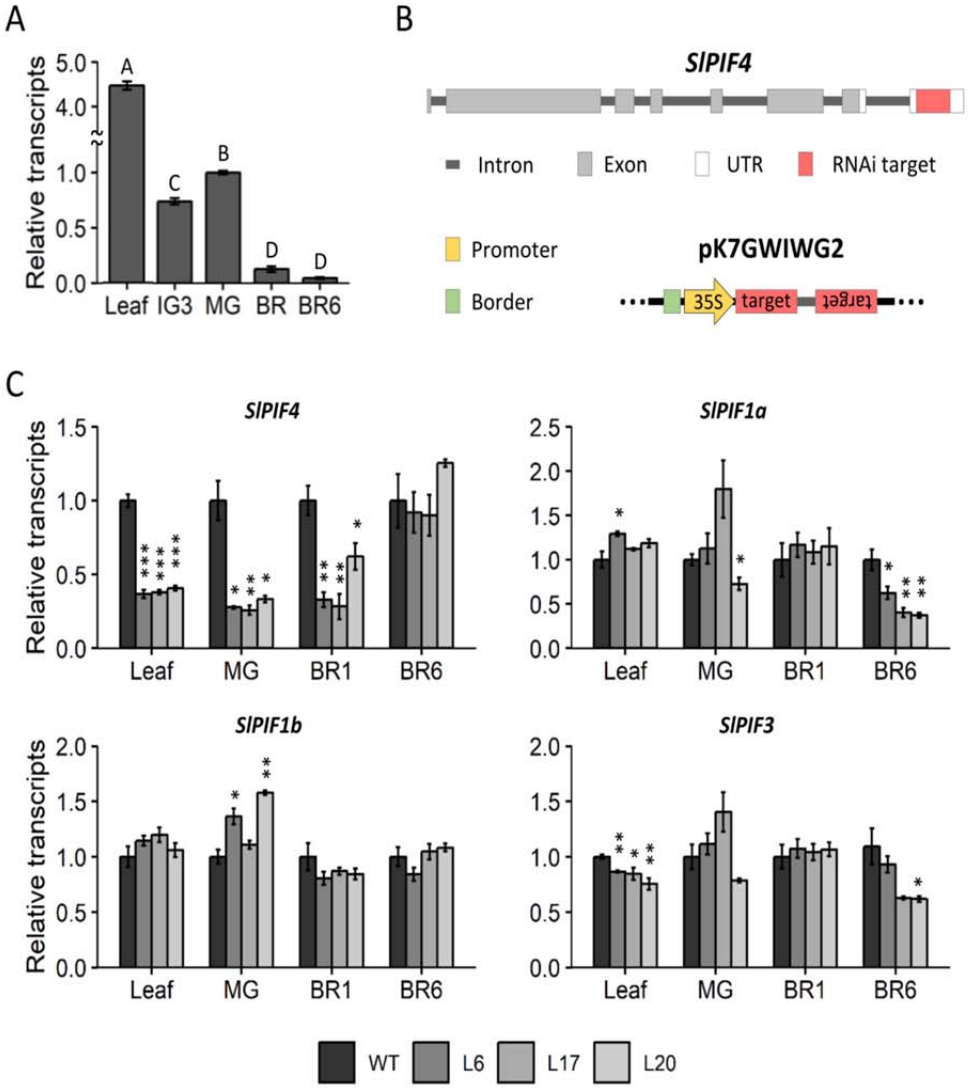

Figure 1. Expression profile of *SlPIF4* in wild-type plants and of *SlPIF* genes in *SlPIF4*-silenced plants.

A, Transcript profile of *SlPIF4* in wild-type plants. B, *SlPIF4* gene structure showing the RNAi target sequence on the 3' UTR in red. Construct used for silencing in pK7GWIWG2 vector. C, mRNA abundance of *SlPIF* genes in *SlPIF4*-silenced plants. Data shown are mean ± SE of at least three biological replicates (each composed of four fruits or two leaves) normalized against the MG stage (A) or the wild-type control (C). Significant differences with wild-type control are denoted by letters (ANOVA followed by Fisher's LSD test) and asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: Leaf: the fourth leaf of 90 days old plants; IG3, immature-green; MG, mature-green; BR, breaker stage; BR1, one day after BR stage; BR6: 6 days after BR stage; WT, wild type; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20.

131 regulator *RIPENING INHIBITOR* (*SlRIN*) and the key genes involved in carotenoid biosynthesis, namely

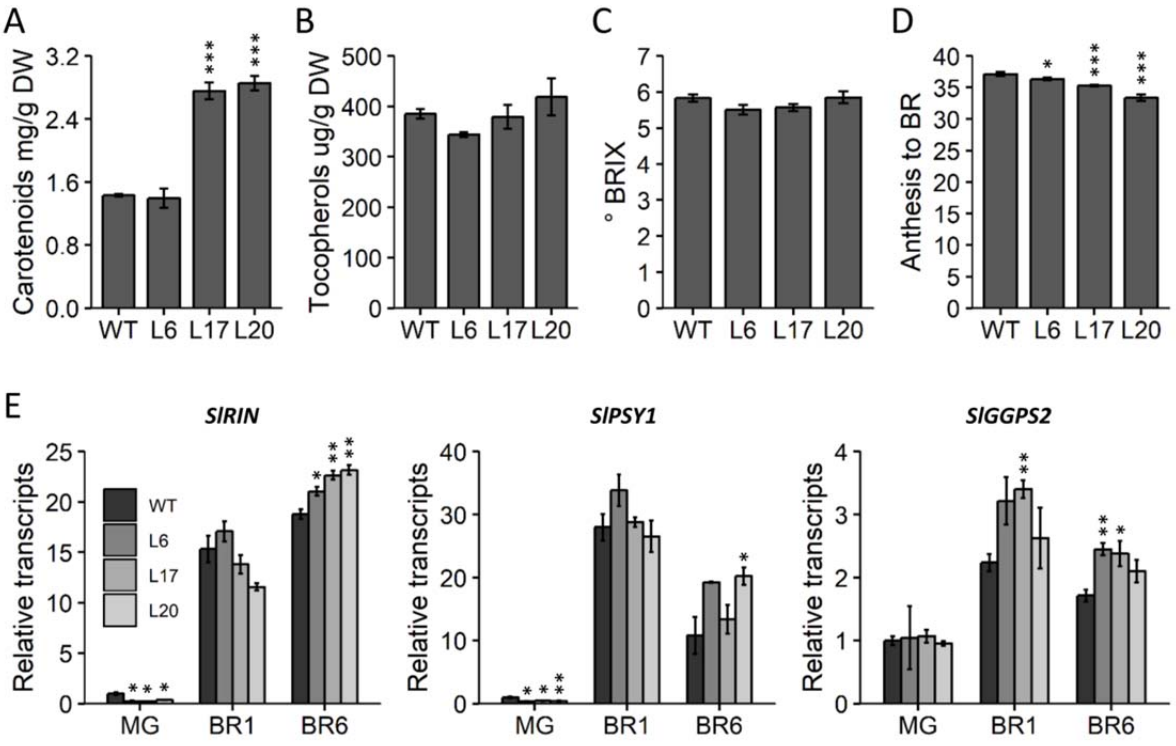

Figure 2. *SlPIF4* silencing affects tomato fruit quality.
A to C, Carotenoid (A), tocopherol (B), and ° BRIX (C) in ripe fruits (12 days post breaker stage; BR). D, Ripening time from anthesis to BR stage. E, mRNA abundance relative to wild type MG of differentially expressed genes involved in carotenogenesis.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

GERANYL GERANYL DIPHOSPHATE SYNTHASE (*SlGGPS2*) and *PHYTOENE SYNTHASE* (*SlPSY1*), detected in the transgenic lines explains, at least in part, these phenotypes and suggests a role of SlPIF4 in the regulation of fruit ripening and carotenogenesis (Figure 2E).

***SlPIF4* silencing impacts flowering and fruit production**

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

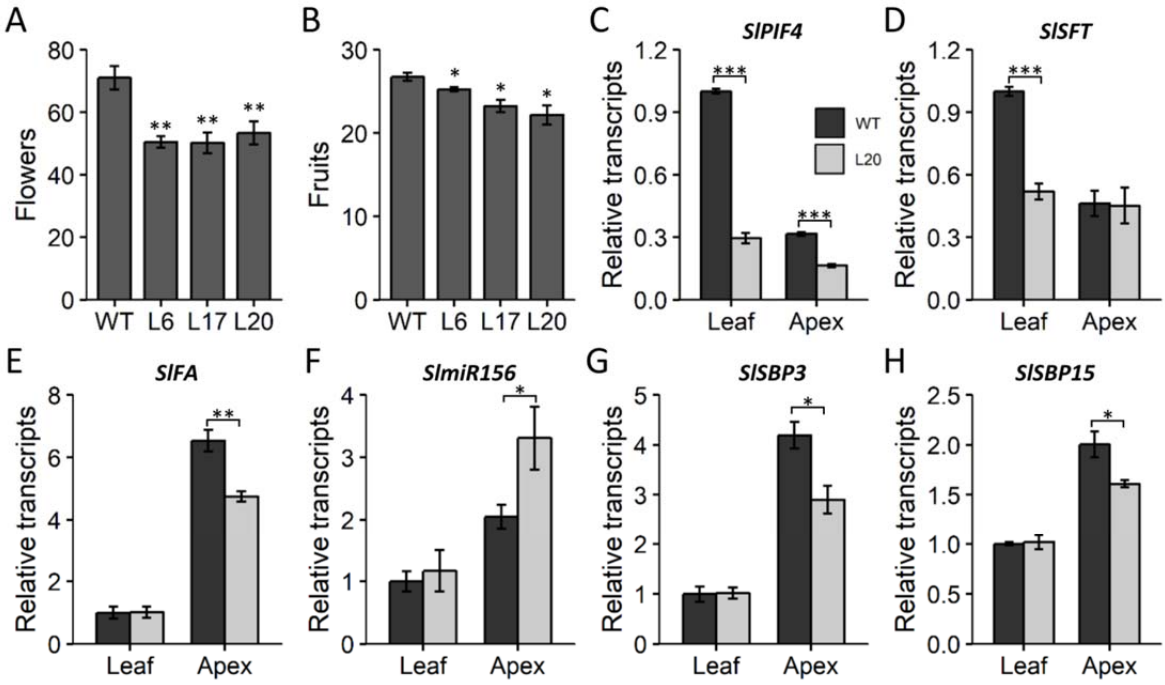

Figure 3. *SlPIF4* silencing affects plant development and fruit yield.
A and B, Total flower and fruit number produced by T2 18-week-old plants. C to H, Transcript profile of flowering genes in 30-day-old T4 plants. Values represent means ± SE of at least six different plants (A and B) or three biological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

and indirectly by repressing *microRNA156* (*AtmiR156*) expression. Flowering in tomato is regulated similarly; *SlmiR156* represses the expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* and *15* (*SlSPB3* and *SlSBP15*) in both apex and leaf. In turn, these proteins induce the expression of *SINGLE FLOWER TRUSS* (*Sl*SFT), the *AtFT* ortholog, in leaves and *FALSIFLORA* (*SlFA*) in shoot apices (Silva et al., 2019). SlSFT protein is translocated to the apex and together with FA induces flowering (Molinero-Rosales et al., 2004). However, the role of SlPIF4 in this regulatory network has not been addressed yet. Thus, we tested whether flowering was also affected by reduced *SlPIF4* expression in tomato. In silenced lines, a significant reduction in flower number was observed, which was reflected by reduced fruit production in 18-weeks old plants (Figure 3A-B). Interestingly, individual transgenic trusses produced fewer flowers than in WT, as addressed for the two first flowering trusses (Supplemental Figure S2 A).

No changes in flowering time were observed between the studied genotypes, when either the number of leaves until the first truss or the number of days until the anthesis of the first flower per plant were scored (Supplemental Figure S2 B, C). In order to understand the molecular mechanism behind this phenotype, the miR156-SPB-SFT/FA module that regulates flowering in tomato (Silva et al., 2019) was profiled in leaves and shoot apices harvested from WT and L20 30-day-old young plants (Figure 3C-H). *SlPIF4* was shown to be under-expressed in apex in comparison to leaves, and silencing was confirmed in both organs. Downregulation of *SlSFT* and *SlFA* florigens was observed in leaves and apices from *SlPIF4*-silenced plants, respectively. Moreover, the abundance of miR156 increased in the apex of transgenic plants, which negatively correlated with its targets *SlSBP3* and *SlSBP15* in the same organ. These data demonstrated that SlPIF4 regulates flowering in tomato reinforcing the hypothesis that members of the PIF4 clade play a conserved role in angiosperms.

SlPIF4 silencing impacts vegetative growth and fruit size

To better understand to what extent constitutive silencing affected fruit yield, we compared different growth and production parameters in WT and L20 homozygous plants. At an early age (5-week-old), *SlPIF4*-silenced plants were visually smaller than WT (Figure 4A). Differences in size were accentuated during the life cycle, and 18-week-old plants showed clear differences in size and fruit production (Figure 4B), with *SlPIF4*-silencing causing an observed 15% reduction in vegetative weight and 23% reduction in fruit weight, accounting for a total reduction of 21% in plant aerial mass (Supplemental Table S2). Interestingly, fruit production was affected beyond number, as *SlPIF4*-silenced individual red fruits were smaller in mass and diameter compared to WT (Figure 4B and Supplemental Table S2). These developmental differences could not be attributed to altered carbon assimilation rates since no alterations in photosynthesis were detected (Supplemental Table S3). Instead, the observed phenotype is most likely caused by reduction of auxin levels (Figure 4C) and aggravated by the reduction in overall carbon assimilation due to lowered leaf area (Figure 4A) and number (Supplemental Table S2).

On the other hand, although differences in size appeared early in fruit development (Figure 4D), impaired fruit growth in *SlPIF4*-silenced plants could not be directly explained by the reduction of auxin levels, since no differences were detected in immature fruits (Figure 4C). Interestingly, carbohydrate profiling revealed a shift in sugar partitioning in the silenced lines. Whereas no changes in soluble sugars were observed (Supplemental Table S4), starch was accumulated at higher levels in leaves and reduced in fruits from the L20 homozygous transgenic plants (Figure 4E). These observations were in accordance

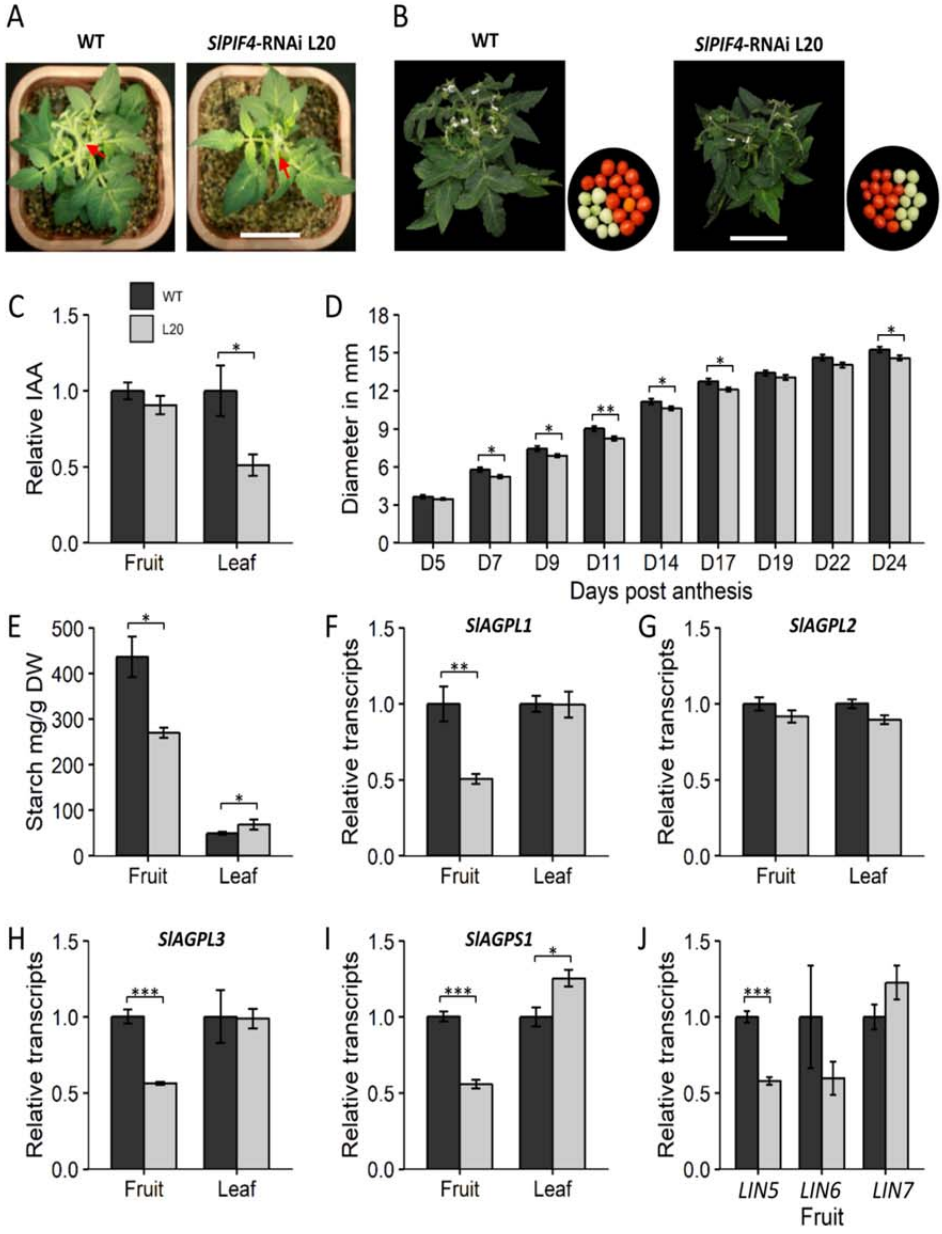

Figure 4. *SlPIF4* silencing affects growth and source-sink relationship.

A and B, Representative 5- (A) and 15- (B) week-old plants. A, Developmental delay in *SlPIF4*-silenced line; red arrow indicates the first inflorescence. Bar=5 cm. B, Differences in size and fruit production. Bar=10 cm. C and E to J, Relative auxin level (C), starch content (E), and transcript profile of starch biosynthetic and cell wall invertase genes (F to J) in immature green fruits and source leaves. D, Size differences between wild-type and *SlPIF4*-silenced fruits of 5–24 days post anthesis (D5 to D24). Values represent means ± SE of at least three biological replicates composed of two leaves or four fruits (C and E to J), or 20 individual fruits (D). Significant differences with wild-type control are denoted by asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: WT, wild type; L20, homozygous T4 35S::SlPIF4-RNAi L20; *AGPL1-3*, *ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1-3; AGPS1, ADP-GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1; LIN5-7, LYCOPERSICUM INVERTASE 5-7.*

181 with the expression profile of ADP-GLUCOSE PYROPHOSPHORYLASE (AGPase) large and small subunit-

encoding genes (*SlAGPL1*, *SlAGPL2*, *SlAGPL3*, and *SlAGPS1*), involved in starch biosynthesis, in both organs (Figure 4F-I). Additionally, expression of the flower- and fruit-specific invertase encoding gene *LYCOPERSICUM INVERTASE 5* (*SlLIN5*) (Fridman et al., 2004) was reduced in transgenic fruits (Figure 4J), which could be indicative of reduced sink strength caused by *SlPIF4* silencing. Thus, these further support the functional conservation of PIF4 in regulating plant growth and auxin biosynthesis but also illustrate a new role for SlPIF4 protein in fruit yield.

SlPIF4 participates in thermomorphogenesis

Beyond light, temperature is a key factor regulating plant growth and development (Kami et al., 2010; Quint et al., 2016), and many studies performed in *A. thaliana* have placed AtPIF4 as an integrator of light and temperature responses (Franklin et al., 2011; Sun et al., 2012; Gangappa and Kumar, 2017). To address whether tomato SlPIF4 also participates in temperature perception, hypocotyl elongation was analyzed in seedlings maintained for 3 days in either ambient- (25°C) or high-temperature (30°C) conditions under a day-neutral photoperiod. Only WT seedlings responded to the treatment and showed longer hypocotyls at 30°C, whereas the hypocotyl length of *SlPIF4*-silenced seedlings remained unchanged (Figure 5A-B). Expression analysis revealed the up-regulation of *YUCCA FLAVIN MONOOXYGENASES*, *SlYUC8A*, and *SlYUC8C*, in WT seedlings in high-temperature conditions compared to that in *SlPIF4*-silenced seedlings (Figure 5C), suggesting that the observed high temperature-associated elongation is the consequence of auxin biosynthesis enhancement, demonstrating SlPIF4 involvement in temperature responsiveness.

Tomato PIF4 promotes age-induced leaf senescence

In *A. thaliana*, AtPIF4 and AtPIF5 promote both age- and dark-induced senescence by activating *ORESARA 1* (*ORE1*) transcription factors, genes involved in chlorophyll breakdown, such as *STAYGREEN* (*SGR*), and repressing chloroplast maintainer *GOLDEN2-LIKE 1* (*GLK1*) (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). Although the same downstream effectors are involved in leaf senescence in tomato (Lira et al., 2017), the role of SlPIF4 in this signaling pathway has not been addressed so far. To investigate its involvement, leaves without any signs of senescence (non-senescent, NS), with initial yellowing (early-senescent, ES), and advanced yellowing (late-senescent, LS) were harvested from WT plants. Leaves from corresponding phytomeres from *SlPIF4*-silenced plants were also collected. Visually,

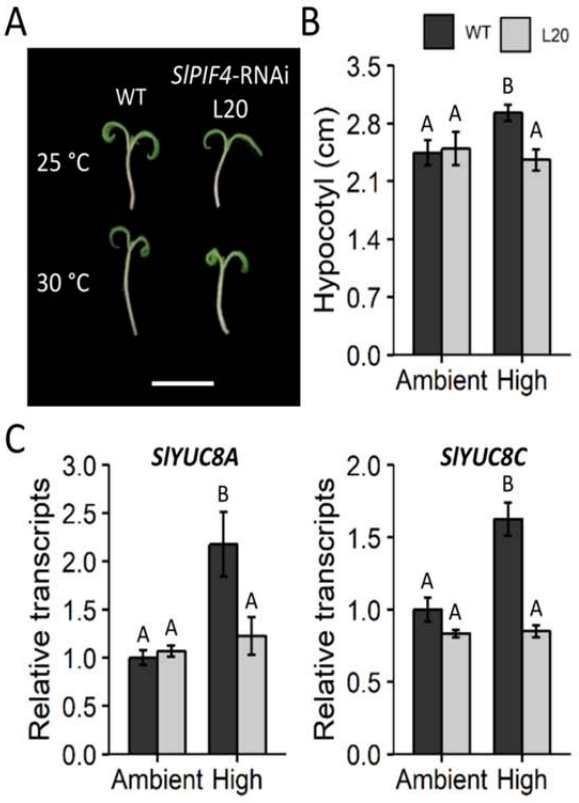

Figure 5. SlPIF4 participates in high temperature-induced hypocotyl elongation.
A, Composite image of representative seedlings depicting differences in size in response to temperature. Bar=2 cm. B, Hypocotyl length. C, Relative transcript profile of auxin biosynthetic genes. Data shown are mean ± SE of at least 14 seedlings (B) or three (C) biological replicates, composed of five seedlings each. Ambient: 25°C, and high: 30°C. Significant differences with wild-type control are denoted by different letters (ANOVA followed by Fisher's LSD test). Abbreviations denote the following: WT, wild type; L20, T4 homozygous 35S::SlPIF4-RNAi L20; *YUC8A-C, YUCCA FLAVIN MONOOXYGENASE 8A-C.*

212 silenced leaves remained greener than control leaves (Figure 6A), suggesting that senescence was

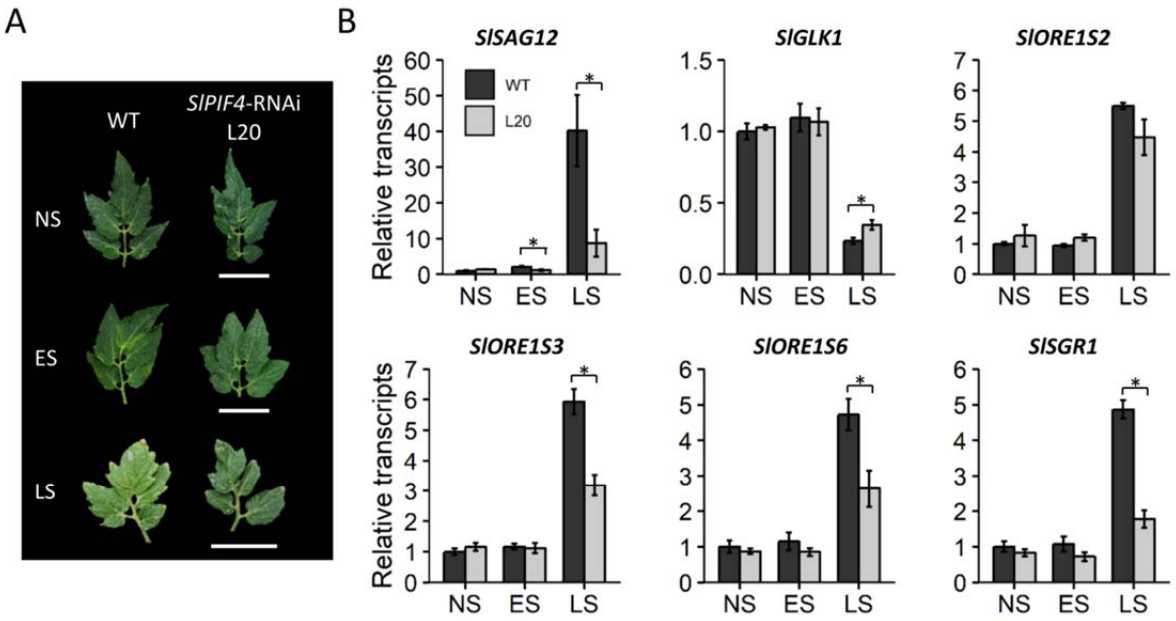

Figure 6. *SlPIF4* silencing delays age-induced leaf senescence.
A, Composite image of representative non-senescent (NS, 6th phytomer), early-senescent (ES, 4th phytomer), and late-senescent (LS, 1st, and 2nd phytomers) leaves from wild-type and *SlPIF4*-silenced 18-week-old plants. Bars=5 cm. B, Transcript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

213 delayed in these plants. Lowered expression of senescence marker *SENESCENCE ASSOCIATED GENE 12*
214 (*SlSAG12*) confirmed this hypothesis. Also, higher expression of chloroplast maintainer *SlGLK1* and
215 reduced levels of senescence-associated transcription factors *SlORE1S23* and *SlORE1S26* as well as
216 *SlSGR1* possibly contributed to the observed staygreen phenotype (Figure 6B). Thus, similarly to that
217 described in *A. thaliana,* SlPIF4 participates in the senescence-inducing pathway.

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DISCUSSION

Although *PIF* genes have been extensively studied in *A. thaliana* for over twenty years (Ni et al., 1998), only recently they have been identified in tomato (Rosado et al., 2016). The only *SlPIFs* studied so far are *SlPIF1a* and *SlPIF3*, which have been demonstrated to regulate fruit nutraceutical value (Llorente et al., 2016; Gramegna et al., 2019). With the aim of expanding our knowledge on the potential biotechnological use of the PIF protein family in crop species, here we comprehensively characterized *SlPIF4*-silenced tomato plants taking into account the role of PIF4 in flowering time and fruit setting in *A. thaliana* (Brock et al., 2010) and, more recently, in biomass production in switchgrass (*Panicum virgatum*; Yan et al., 2018).

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

Interestingly, although *SlPIF4* is poorly expressed in WT ripening fruits (Figure 1A), the silencing of this gene had a considerable effect on ripening process (Figure 2, Supplemental Figure S1), uncovering a role for tomato PIFs in regulating ripening time. The late increase in expression of the master ripening regulator *SlRIN* observed in BR6 transgenic fruits cannot explain the ripening advance, but may contribute to carotenoid accumulation and induction of *SlPSY1* in ripe fruits (Fujisawa et al., 2011) (Figure 2). The upregulation of *SlPSY1* and *SlGGPS2* expression, whose encoded enzymes act upstream in carotenogenesis, explains the accumulation of all carotenoid forms in ripe fruits (Supplemental Table S1). It is unlikely, though, that *SlPIF4* directly regulates carotenogenesis, because no differential carotenoid accumulation occurred in *SlPIF4*-silenced MG fruits, when *SlPIF4* is normally highly expressed (Supplemental Table S1, Figure 1A). Previous work showed that changes in pigment composition during ripening alter the quality of the light filtered through the fruit pericarp, increasing the relative red/far-red ratio (Llorente et al., 2016). As a consequence, PIF degradation increases, enhancing *SlPSY1* expression and carotenoid accumulation. The reduced *SlPIF1a* mRNA levels, which

inversely correlates with *SlPSY1* expression in transgenic BR6 fruits, might additionally contribute to the observed phenotypes at this stage (Figure 1C, Figure 2). Since no changes in tocopherols were detected in *SlPIF4*-silenced fruits (Figure 2B), it is possible that only *SlPIF3* participates in the regulation of tocopherol biosynthesis as previously proposed (Gramegna et al., 2019). Nevertheless, the presented data demonstrate a functional convergence of tomato PIFs in regulating fruit nutritional quality (Llorente et al., 2016; Bianchetti et al., 2018; Gramegna et al., 2019), and suggest a unique role of SlPIF4 in the regulation of ripening time and progression, which should be further dissected in future research.

Manipulation of flowering-related traits is a key strategy to improve fruit yield in tomato (Krieger et al., 2010). Indeed, *SlPIF4* silencing had an impact on fruit production derived from lowered flower number (Figure 3, Supplemental Table S2). Considering that altered PIF4 levels in other species, such as *A. thaliana*, rice, and maize, also affect flowering, our data agree with the notion of a conserved function of PIF4 angiosperm homologs (Kumar et al., 2012; Kudo et al., 2017; Shi et al., 2018). Accordingly, both tomato and Arabidopsis PIF4 induce flowering via miR156-SPB-florigen (SlSFT or AtFT) module (Figure 3) (Xie et al., 2017). However, *SlPIF4* silencing only affected flower number (Figure 3), but not flowering time (Supplemental Figure S2 B, C), in opposition to findings in the *A. thaliana pif4* mutant (Brock et al., 2010; Thines et al., 2014; Galvão et al., 2015). We attribute this difference to two facts. First, these species have different requirements for flowering: whereas domesticated tomato is a day-neutral species (Soyk et al., 2017), *A. thaliana* is a long-day plant (Cho et al., 2017); therefore, alterations of light-sensing in tomato are not expected to affect flowering time. Second, whereas mutations in *SlSFT* affect flowering time by changing the rate of truss production (Molinero-Rosales et al., 2004), reduction of *SlSFT* transcripts, as observed in *SlPIF4*-silenced plants, are expected to have only mild effects on flowering and would not necessarily affect flowering time. However, downregulation of *SlSFT* caused a reduction in the number of flowers produced per truss (Supplemental Figure S2 A), in agreement with observations in *sft* mutants displaying altered inflorescence development (Molinero-Rosales et al., 2004). On the other hand, it is possible that manipulation of *SlPIF4* in wild tomato species would bear different results since wild tomatoes flower earlier under short days (Soyk et al., 2017). Loss of photoperiod sensitivity in domesticated varieties has been associated with mutations at *SELF PRUNING 5G* (*SP5G*) locus, an anti-florigen. Such mutations reduce the expression of this gene under long-day conditions, therefore attenuating the photoperiodic response (Cao et al., 2016; Soyk et al., 2017; Zhang et al., 2018). Interestingly, SlPHYB1 regulates *SP5G* expression (Cao et al., 2018), which makes this gene a likely target of regulation by SlPIF4 and reinforces the idea of a possible effect of

SlPIF4 on flowering time in wild species. Recent work in tomato has shown that the tomato DELLA, PROCERA, which is involved in gibberellin signaling, induces flowering via the miR156-SPB-SFT module (Silva et al., 2019). However, in *A. thaliana*, DELLA proteins are flowering repressors, which inhibit the activity of AtPIF4 (De Lucas et al., 2008; Xu et al., 2016). In this sense, investigation of tomato PROCERA-PIF4 interaction could reveal new layers of species-specific regulation of flowering.

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

The results showed here for adult plants (Figure 4) and seedlings (Figure 5) suggest that SlPIF4 regulates growth by inducing auxin biosynthesis, which is in agreement with observations in *A. thaliana* (Nozue et al., 2007; Niwa et al., 2009; Kunihiro et al., 2011; Nieto et al., 2015), rice (*Oryza sativa*; Todaka et al., 2012), and maize (*Zea mays*; Shi et al., 2018) reporting PIF4 as growth regulator. Additionally, loss of temperature responsiveness in *SlPIF4*-silenced seedlings reveals another conserved feature for member of the PIF4 clade in angiosperms (Figure 5) (Koini et al., 2009). Recent works have placed PHYTOCHROME B (PHYB) as an integrator of light and temperature perception and PIF4 as a key protein in mediating the responses to both signals in *A. thaliana* (Jung et al., 2016; Legris et al., 2016). In this context, future studies investigating interactions between SlPIF4, SlPHYB1, and SlPHYB2 will add invaluable information to understanding photo- and thermomorphogenesis in tomato.

Finally, *SlPIF4*-silenced plants displayed a delay in age-induced leaf senescence (Figure 6), explained by the downregulation of SlORE1 transcription factor-encoding genes. These senescence-associated proteins negatively regulate chloroplast maintainer SlGLK1 and upregulate the expression of

chlorophyll degradation enzymes, such as *SlSGR* (Lira et al., 2017), resulting in the staygreen phenotype observed in the old transgenic leaves (Figure 6). It has been demonstrated that, in *A. thaliana*, AtPIF4 regulates *SGR*, *ORE*, and *GLK* by directly binding to specific motifs in their promoter regions (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). Moreover, the overexpression of maize *ZmPIF4* and *ZmPIF5* accelerates leaf senescence in *A. thaliana* (Shi et al., 2018). These data strongly support the previously suggested functional conservation of PIF4 among angiosperms (Rosado et al., 2016).

In summary, besides the functions previously described for PIF4, our results present additional roles for this protein, including the regulation of sugar partitioning, fruit production and ripening. Overall, the pleiotropic effects observed in *SlPIF4*-silenced plants not only highlight the importance of PIFs in plant development, but also suggest that manipulation of light signaling is an efficient strategy to improve tomato yield and quality.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Sampling

SlPIF4-silenced lines were generated by constitutively expressing an intron-spliced hairpin RNA construct containing a 180-bp fragment of the 3'UTR region of *SlPIF4 locus* (Solyc07g043580). To avoid off-target effects, the construct was designed to have minimal complementarity with other genes, especially other *SlPIFs*, and then the sense/antisense fragment was used as query for a BLAST search against the Sol Genomics Network database (www.solgenomics.net). The fragment was amplified from cDNA with primers listed in Supplemental Table S5, cloned into pK7GWIWG2(I) (Karimi et al., 2002) and introduced into tomato (*Solanum lycopersicum* L.) cv. Micro-Tom (MT) harboring the wild-type *SlGLK2* allele (Carvalho et al., 2011) via *Agrobacterium*-mediated transformation according to Pino et al. (2010), with modifications described in Bianchetti et al. (2018). Despite its partial impairment in brassinosteroid biosynthesis due to the weak mutation *d*, MT cultivar has been extensively demonstrated to represent a convenient and adequate model system to study fruit biology (Campos et al., 2010). The presence of the transgene was confirmed by PCR using the primers 35S forward and RNAi-specific reverse (Supplemental Table S5). After silencing verification by RT-qPCR analysis, three transgenic lines with a reduction of approximately 60% in *SlPIF4* mRNA level were selected for further analyses: 35S::SlPIF4-RNAi L6, L17, and L20. Two different generations of silenced plants were used in this work: L6, L17, and L20 segregating lines in T2 and; L20 homozygous line in T4.

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

Reverse transcription quantitative PCR (RT-qPCR)

RNA extraction, cDNA synthesis and qPCR were performed as described by Quadrana et al. (2013) following MIQE guidelines (Bustin et al., 2009). Stem-loop pulse reverse transcription for *SlmiR156* quantification was performed as described previously by Varkonyi-Gasic et al. (2007). qPCR was carried out in QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) using 2X Power SYBR Green Master Mix (Life Technologies) in a 10-µL final volume. Quantitation cycle values and PCR efficiencies were obtained from absolute fluorescence data analyzed in LinRegPCR software package

(Ruijter et al., 2009). Expression values were normalized with *TIP41* and *EXPRESSED* reference genes (Expósito-Rodríguez et al., 2008). All primers and accession numbers can be found in Supplemental Table S5.

Fruit color, carotenoid, tocopherol, and BRIX determination

Fruit color and intensity (Hue angle and Chroma) were determined using a Konica Minolta CR-400 colorimeter as described in Su et al. (2015). Carotenoid extraction was carried out as described in Bianchetti et al. (2018) with modifications. Briefly, 20 mg of freeze-dried fruit pericarps were homogenized sequentially with 100 µl of saturated NaCl, 200 µl of dichloromethane, and 1 ml of hexane: diethyl ether (1:1, v/v). Supernatant was collected after centrifugation and pellets were re-extracted additional three times with 500 µl hexane: diethyl ether mixture. Supernatant fractions were combined, vacuum-dried, suspended in 200 µl of acetonitrile, and filtered through a 0.45-µm membrane. Tocopherol extraction was performed as described by Lira et al. (2016). Briefly, 25 mg of freeze-dried fruit pericarps were homogenized sequentially in 1.5 mL methanol, 1.5 mL chloroform, and 2.5 mL Tris NaCl (Tris 50 mM pH 7.5, NaCl 1M) solution. Following centrifugation, the organic fraction was collected and samples were re-extracted in 2 mL chloroform. Fractions were combined, then 3 mL of combined samples was vacuum dried, suspended in 200 µl of hexane:tert-butyl methyl ether (90:10, v/v), and filtered through a 0.45-µm membrane. Carotenoids and tocopherol levels were determined by high-performance liquid chromatography (HPLC) in an Agilent 1100 as described in Lira et al. (2017). Total soluble sugars measured as ° BRIX were determined in ripe (BR12) fruits as follows. Fresh pericarp tissue was homogenized with metallic beads and briefly span. ° BRIX of resulting juice was measured in a portable digital refractometer NR151 (J.P. Selecta).

Hormone analysis

3-Indoleacetic acid (IAA) was extracted and quantified as in Silveira et al. (2004). Briefly, 1 g of powdered tissue was homogenized in a buffer containing 80% ethanol v/v, 1% polyvinylpyrrolidone-40 w/v and [^{3}H]IAA, used as an internal standard. Samples were incubated and subsequently centrifuged. The supernatant was collected and freeze-dried. Volume was adjusted to 3 mL with water, and the pH adjusted to 2.5. The organic fraction, obtained following double extraction with ethyl ether, was completely vacuum-dried, redissolved in 150 µL methanol and filtered through a 0.45-µm membrane.

Auxin levels were determined by high-performance liquid chromatography (HPLC) in 5 lm C18 column (Shimadzu Shin-pack CLC ODS), with a fluorescence detector (excitation at 280 nm, emission at 350 nm). Fractions containing IAA were collected and analyzed in the scintillation counter (Packard Tri-Carb) to estimate losses during the procedure.

Leaf Gas-Exchange and Fluorescence Measurements

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

Starch and soluble sugar quantification

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

Statistical analyses

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

ACCESSION NUMBERS

Sequence data from this article can be found in the Solgenomics database (solgenomics.net) under accession numbers listed in Supplemental Table S5.

SUPPLEMENTAL DATA

Supplemental Figure S1. Off-vine ripening is affected in *SlPIF4*-silenced fruits.

Supplemental Figure S2. *SlPIF4* silencing affects the number of flowers per truss, but not flowering time.

Supplemental Table S1. Carotenoid content in fruits.

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

Supplemental Table S3. Photosynthesis parameters.

Supplemental Table S4. Soluble sugars.

Supplemental Table S5. Primers used in this work.

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FIGURE LEGENDS

Figure 1. Expression profile of *SlPIF4* in wild-type plants and of *SlPIF* genes in *SlPIF4*-silenced plants.

A, Transcript profile of *SlPIF4* in wild-type plants. B, *SlPIF4* gene structure showing the RNAi target sequence on the 3' UTR in red. Construct used for silencing in pK7GWIWG2 vector. C, mRNA abundance of *SlPIF* genes in *SlPIF4*-silenced plants. Data shown are mean ± SE of at least three biological replicates (each composed of four fruits or two leaves) normalized against the MG stage (A) or the wild-type control (C). Significant differences with wild-type control are denoted by letters (ANOVA followed by Fisher's LSD test) and asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: Leaf: the fourth leaf of 90 days old plants; IG3, immature-green; MG, mature-green; BR, breaker stage; BR1, one day after BR stage; BR6: 6 days after BR stage; WT, wild type; L6, 35S::SlPIF4-RNAi L6; L17, 35S::SlPIF4-RNAi L17; L20, 35S::SlPIF4-RNAi L20.

Figure 2. *SlPIF4* silencing affects tomato fruit quality.

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

Figure 3. *SlPIF4* silencing affects plant development and fruit yield.

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

Figure 4. *SlPIF4* silencing affects growth and source-sink relationship.

A and B, Representative 5- (A) and 15- (B) week-old plants. A, Developmental delay in *SlPIF4*-silenced line; red arrow indicates the first inflorescence. Bar=5 cm. B, Differences in size and fruit production. Bar=10 cm. C and E to J, Relative auxin level (C), starch content (E), and transcript profile of starch biosynthetic and cell wall invertase genes (F to J) in immature green fruits and source leaves. D, Size differences between wild-type and *SlPIF4*-silenced fruits of 5–24 days post anthesis (D5 to D24). Values represent means ± SE of at least three biological replicates composed of two leaves or four fruits (C and E to J), or 20 individual fruits (D). Significant differences with wild-type control are denoted by asterisks (two-tailed *t*-test; P<0.05 *; P<0.01 **; P<0.001 ***). Abbreviations indicate the following: WT, wild type; L20, homozygous T4 35S::SlPIF4-RNAi L20; *AGPL1-3*, *ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1-3; AGPS1, ADP-GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1; LIN5-7, LYCOPERSICUM INVERTASE 5-7.*

Figure 5. SlPIF4 participates in high temperature-induced hypocotyl elongation.

A, Composite image of representative seedlings depicting differences in size in response to temperature. Bar=2 cm. B, Hypocotyl length. C, Relative transcript profile of auxin biosynthetic genes. Data shown are mean ± SE of at least 14 seedlings (B) or three (C) biological replicates, composed of five seedlings each. Ambient: 25°C, and high: 30°C. Significant differences with wild-type control are denoted by different letters (ANOVA followed by Fisher's LSD test). Abbreviations denote the following: WT, wild type; L20, T4 homozygous 35S::SlPIF4-RNAi L20; *YUC8A-C*, *YUCCA FLAVIN MONOOXYGENASE 8A-C.*

Figure 6. *SlPIF4* silencing delays age-induced leaf senescence.

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

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