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UNIVERSITAT AUTÒNOMA DE BARCELONA

FACULTAT DE BIOCIÈNCIES

Programa de Doctorat en Biologia i Biotecnologia Vegetal

Tesi Doctoral

**Unveiling the role of Phytochrome Interacting  
Factor 1 (PIF1) homologs in tomato**

Miguel Simón Moya

2020



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## **Unveiling the role of Phytochrome Interacting Factor 1 (PIF1) homologs in tomato**

Memòria de tesi presentada per Miguel Simón Moya per optar al títol de doctor per la Universitat Autònoma de Barcelona. El treball presentat s'ha dut a terme al Centre de Recerca en Agrigenòmica (CRAG).

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## SUMMARY

Light is one of the most important environmental cues influencing the plant life cycle. Plants have developed a set of complex molecular mechanisms that sense changes in light quality and quantity. PHYTOCHROME INTERACTING FACTORs (PIFs) are transcription factors that interact with the photoreceptors phytochromes (phy) and mediate the responses to red/far-red light. PIFs are involved in the regulation of a broad range of developmental processes. They have been extensively studied in *Arabidopsis thaliana*, but very little is known about their roles in other species. In this thesis, we investigate the role of the two homologs of PIF1 found in tomato (*Solanum lycopersicum*): PIF1a and PIF1b.

The analysis of *PIF1a* and *PIF1b* expression showed very different patterns, indicating a potential evolutionary divergence in their roles. Protein stability experiments in red and far-red light unveiled that PIF1b has lost its ability to interact with PhyB, while PIF1a is still able to do it, confirming the evolutionary divergence hypothesis.

On the other hand, tomato genome editing by CRISPR-Cas9 generated *pif1a* and *pif1b* loss-of-function lines, as well as double mutants *pif1a pif1b*. The phenotypic characterization of these mutants showed that both transcription factors are involved in the regulation of seed germination, synthesis of leaf pigments during de-etiolation and fruit production. Other processes are regulated just by PIF1a, such as root hair elongation, synthesis of steroidal glycoalkaloids in leaves, flowering time and fruit growth and softening. We did not identify any process regulated specifically by PIF1b alone.

Due to the central role of PIF1a, we decided to perform RNA-seq experiments in PIF1a-inducible lines. The results showed that the induction of PIF1a had a relatively minor impact in the transcriptomic profile, and that the putative gene targets of PIF1a in tomato were different from those previously identified in *Arabidopsis*.

All this data together suggests that PIF1a and, to a much lower extent, PIF1b share some roles with *Arabidopsis* PIF1, but also illustrate that neofunctionalization has taken place in tomato. Doing this, evolution managed to use the potential of these transcription factors to regulate new specific processes in this crop of agronomic interest.

## RESUMEN

La luz es una de las señales ambientales más importantes que influyen en el ciclo de vida de la planta. Las plantas han desarrollado un conjunto de complejos mecanismos moleculares que detectan cambios en la calidad y cantidad de la luz. Los PHYTOCHROME INTERACTING FACTORs (PIFs) son factores de transcripción que interactúan con los fotorreceptores fitocromos (*phy*) y median las respuestas a luz roja/roja lejana. Los PIF están involucrados en la regulación de una amplia gama de procesos del desarrollo. Se han estudiado ampliamente en *Arabidopsis thaliana*, pero se sabe muy poco sobre su papel en otras especies. En esta tesis, investigamos el papel de los dos homólogos de PIF1 presentes en tomate (*Solanum lycopersicum*): PIF1a y PIF1b.

El análisis de la expresión de *PIF1a* y *PIF1b* mostró patrones muy diferentes, lo que indica una posible divergencia evolutiva en sus roles. Los experimentos de estabilidad de las correspondientes proteínas en luz roja y roja lejana revelaron que PIF1b ha perdido su capacidad de interactuar con PhyB, mientras que PIF1a todavía puede hacerlo, confirmando la hipótesis de divergencia evolutiva.

Por otro lado, la edición del genoma de plantas de tomate por CRISPR-Cas9 generó líneas de pérdida de función *pif1a* y *pif1b*, así como mutantes dobles *pif1a pif1b*. La caracterización fenotípica de estos mutantes mostró que ambos factores de transcripción están involucrados en la regulación de la germinación de las semillas, la síntesis de pigmentos en las hojas durante la des-etiolación y la producción de frutos. Otros procesos están regulados solo por PIF1a, como el alargamiento de pelos radiculares, la síntesis de glicoalcaloides esteroideos en hojas, el tiempo de floración y el crecimiento y ablandamiento del fruto. No identificamos ningún proceso que esté regulado específicamente por PIF1b.

Debido al papel central de PIF1a, decidimos realizar experimentos de RNA-seq en líneas inducibles. Los resultados mostraron que la inducción de PIF1a tiene un impacto relativamente menor en el perfil transcriptómico, y que los posibles genes diana de PIF1a en tomate son distintos a los identificados previamente en *Arabidopsis*.

Todos estos datos en conjunto sugieren que PIF1a y, en mucho menor grado, PIF1b comparten algunas funciones con su homólogo PIF1 de *Arabidopsis*, pero también ilustran que se han producido eventos de neofuncionalización en tomate. Al hacer esto, la evolución ha podido utilizar el potencial de estos factores de transcripción para regular nuevos procesos específicos en este cultivo de interés agronómico.

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## **INTRODUCTION**



## 1. Light: Friend and Foe

Plants are photosynthetic organisms able to harvest and convert sunlight into chemical energy (ATP and NADPH) and fix atmospheric CO<sub>2</sub> into sugars. Furthermore, as a consequence of photosynthesis, O<sub>2</sub> is produced and released to the atmosphere, generating an oxidative environment in which most of current living beings thrive. Thus sunlight, through the action of plants and photosynthesis, supports life on Earth as we know it today (Hill and Scarisbrick, 1940; Armstrong et al., 1995; Reinbothe et al., 1996; Murchie and Niyogi, 2011). In addition, light is one of the external stimuli with a strongest effect on plant development. Besides photosynthesis, light influences chloroplast biogenesis, seed germination, phototropism, seedling development, and floral induction, among others. So, light is not just an energy source but it also functions as a signal regulating growth and development (Quail, 1991; Chory, 1993; Deng, 1994; Dong et al., 2015; Fernando and Schroeder, 2016).

Plants are exposed to large fluctuations of incoming sunlight during a day, a season or a year. In order to cope with this, they have optimized their photosynthetic apparatus to effectively perform two opposite functions. Firstly, the photosynthetic machinery is organized in pigment-protein complexes within the thylakoid membrane that ensure efficient *light harvesting*, so it will have enough energy for the photochemical reactions that take place in the reaction centers (RCs). Each RC is surrounded by a large peripheral antenna that absorbs photons and, in a really short time, delivers the generated electronic excitations from the pigments to the RC (Blankenship, 2002; Croce and Van Amerongen, 2013; Suga et al., 2015). A too high light intensity, however, elevates the probability that a new photon is absorbed while the RC is still not recovered from processing the previous excitation. This situation can eventually lead to the formation of chlorophyll triplets as well as Reactive Oxygen Species (ROS), such as singlet oxygen radicals (Peterman et al., 1995; Blankenship, 2002; Murchie and Niyogi, 2011; Amerongen and Chmeliov, 2019). Accumulation of ROS compounds can cause membrane peroxidation, protein denaturation, and pigment bleaching, effects that together can lead to photooxidative damage of the chloroplast and ultimately to cell death (Reinbothe et al., 1996). To cope with this problem, plants have developed *photoprotection* mechanisms that harmlessly dissipate excess excitation energy as heat by a mechanism called non-photochemical quenching (NPQ) and quench potentially harmful ROS that may eventually form.

Of the two main groups of photosynthetic pigments found in plants (chlorophylls and carotenoids), only carotenoids play roles in both light harvesting and photoprotection.

## 2. Carotenoids: pigments, signals, and light protectors

### 2.1. Biological roles of plant carotenoids

Plant carotenoids are a group of 40-carbon plastidial isoprenoids that are characterized by their colors in the yellow to red range (Ruiz-Sola and Rodríguez-Concepción, 2012). These compounds act as natural pigments in fruits and flowers, providing color to catch the attention of pollinators and seed-dispersal animals (Lord and Marshall, 2001; Bradshaw and Chemske, 2003; Tiffney, 2004; Duan et al., 2014; Llorente et al., 2016a). Carotenoids also serve as precursors of plant hormones (abscisic acid and strigolactones) and other signaling molecules with roles in chloroplast-to-nucleus (i.e. retrograde) communication and in the regulation of developmental processes (Rodríguez-Concepción et al., 2018a). However, the main roles of carotenoids in plants are those related with photosynthesis.

The maximum intensity of the solar radiation spectrum at the surface of the Earth is in the 450-550 nm region. While chlorophylls cannot absorb much light in this region, carotenoids can absorb this light strongly and transfer the excitation energy to the chlorophylls to make it available to power photosynthesis. Carotenoids hence increase the spectral range over which light can support photosynthesis, conferring to themselves the role of light-harvesting pigments (Fromme, 2008; Polívka and Frank, 2010; Magdaong et al., 2014; Hashimoto et al., 2016).

However, the essential role of carotenoids in plants is the protection against photooxidative damage. Carotenoids dissipate the excess of light energy not to be used for the RC as heat (NPQ) but they also quench triplet states of chlorophyll and, if the energy transfer to oxygen has already happened, also quench singlet oxygen (Anderson and Robertson, 1959; Demmig-Adams et al., 1996; Müller et al., 2001; Dall’Osto et al., 2007; Kim et al., 2009; Dall’Osto et al., 2012; Emiliani et al., 2018).

The roles of carotenoids are so important in plants that carotenoid biosynthesis is essential and finely regulated at multiple levels.

### 2.2. Carotenoid biosynthesis and regulation

Carotenoids are synthesized in plants by the coordinated activity of two different but connected pathways, the methylerythritol 4-phosphate (MEP) pathway and the carotenoid pathway. These two metabolic pathways are located in plastids,

where carotenoids are synthesized and accumulated. All the enzymes from both pathways are encoded by nuclear genes and targeted to plastids (Rodríguez-Concepción and Boronat, 2002; Phillips et al., 2008; Rodríguez-Concepción, 2010; Ruiz-Sola and Rodríguez-Concepción, 2012; Moise et al., 2014).

The MEP pathway is in charge of producing isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP). The addition of 3 IPP molecules to 1 DMAPP molecule produces geranylgeranyl diphosphate (GGPP), the starting precursor of most plastidial isoprenoid metabolites, including carotenoids but also the side chain of chlorophylls, tocopherols (vitamin E), phylloquinones (vitamin K) and plastoquinones (Burke et al., 1999; Beck et al., 2013; Ruiz-Sola et al., 2016a, 2016b).

Carotenoids comprise carotenes and xanthophylls. Carotenes are produced in the first steps of the carotenoid pathway. The condensation of two molecules of GGPP to form phytoene initiates the carotenoid biosynthetic pathway. After that, a series of desaturation and isomerization reactions will produce lycopene, the red carotene that gives their characteristic color to ripe tomatoes. The cyclizations of lycopene ends to produce  $\beta$ -rings or  $\epsilon$ -rings lead to  $\alpha$ -carotene (with one  $\beta$ -ring and one  $\epsilon$ -ring) or  $\beta$ -carotene (with two  $\beta$ -rings). These orange carotenes are responsible for the color of carrots. Oxidation of carotenes generates yellow-colored xanthophylls. Lutein (an abundant xanthophyll found in many flowers) is formed from  $\alpha$ -carotene whereas zeaxanthin (responsible for the color of yellow corn), violaxanthin and neoxanthin derive from  $\beta$ -carotene. These xanthophylls together with  $\beta$ -carotene are found in the chloroplasts of all plant species, where they contribute to photosynthesis and photoprotection as described above (Dogbo et al., 1988; Fraser et al., 1994; Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004; Ruiz-Sola and Rodríguez-Concepción, 2012; Moise et al., 2014).

Carotenoid synthesis is regulated at multiple levels, from the expression of biosynthetic genes to the stability of the encoded enzymes. In many cases, regulatory mechanisms act to simultaneously control important steps of both the MEP pathway and the carotenoid pathway (Rodríguez-Concepción et al., 2018a). The enzymes DEOXYXYLULOSE 5-PHOSPHATE SYNTHASE (DXS) and PHYTOENE SYNTHASE (PSY) are the ones catalyzing the first and main rate-limiting steps of the MEP pathway and the carotenoid pathway respectively. Genes encoding these two enzymes but also others from both pathways are coordinately induced during *Arabidopsis thaliana* seedling de-etiolation (Ghassemian et al., 2006; Rodríguez-Concepción, 2006; Meier et al., 2011) and tomato (*Solanum lycopersicum*) fruit ripening (Lois et al., 2000), when enhanced carotenoid production is required for photoprotection of de-etiolating seedlings and pigmentation of ripe fruit. Tomato fruit ripening is actually a really good

system to study the transcriptional regulation of carotenoid synthesis. Some of the ripening master regulators that control the most important events of ripening at transcriptional level also regulate carotenoid production. For example, the transcription factors RIPENING INHIBITOR (RIN), together with FRUITFULL1 (FUL1) and probably TOMATO AGAMOUS-LIKE1 (TAGL1), directly regulate the expression of several carotenoid biosynthetic genes, hence contributing to an orchestrated control of this pathway (Leseberg et al., 2008; Itkin et al., 2009; Fujisawa et al., 2012; Bemer et al., 2012; Fujisawa et al., 2013; Shima et al., 2013; Fujisawa et al., 2014).

Beyond transcriptional regulation, DDXS, PSY, and likely other carotenoid biosynthetic enzymes are targets of the Clp protease complex (the main plastidial proteolytic machinery) in *Arabidopsis* (Pulido et al., 2016; Welsch et al., 2017; Rodríguez-Concepción et al., 2018b). Our results suggest that DDXS and PSY are also Clp protease targets in tomato fruit ([D'Andrea et al., 2018](#)), a mechanism that likely contributes to coordinate carotenoid biosynthesis with the supply of their metabolic precursors.

In agreement with the major role of carotenoids in photosynthesis and photoprotection, light is a major regulator of carotenoid biosynthesis and accumulation at both transcriptional and post-transcriptional levels (Llorente et al., 2017). Work in *Arabidopsis* has revealed a number of light-dependent molecular factors coordinating carotenoid biosynthesis and storage, some of which were later found to also modulate carotenoid accumulation during tomato fruit ripening.

### 3. Light signaling

#### 3.1. Photoreceptors and Phytochromes

Plants are equipped with photoreceptors that detect and transduce light signals to trigger different responses (such as synthesizing carotenoids when seedlings germinated underground emerge from soil and de-etiolate). Five different types of photoreceptors have been described. Red light (R, 660 nm) and Far Red light (FR, 730 nm) are perceived by Phytochromes, UV-A light (320-390 nm) and Blue light (390-500 nm) by Cryptochromes, Phototropins and the Zeitlupe family and, finally, UV-B light (280-315 nm) by UVR-8 (Batschauer, 1999; Gyula et al., 2003; Tilbrook et al., 2013; Galvão and Fankhauser, 2015; Legris et al., 2017).

Phytochromes (phys) were first discovered in plants, but they are also present in algae, some fungi and numerous prokaryotes (Burgie and Vierstra, 2014). They

are probably the best well-known photoreceptors in plants. There are five members of the phy family in *Arabidopsis*, named phyA to phyE (Mathews, 2010; Li et al., 2015). They have partially overlapping but sometimes distinct functions throughout the plant life cycle, regulating light-dependent processes such as germination, de-etiolation, stomata development, flowering transition, senescence, shade avoidance and chloroplast development (Eisenstadt and Mancinelli, 1974; Weller et al., 2001; Stephenson et al., 2009; Burgie and Vierstra, 2014; Leivar and Monte, 2014; Sakuraba et al., 2014; Chen et al., 2014; Martínez-García et al., 2014).

Phy are synthesized in the inactive Pr state, but upon R absorption they convert to the active Pfr state. Pfr can be inactivated in the dark, after FR absorption or through thermal relaxation, a process known as dark or thermal reversion (Burgie and Vierstra, 2014; Klose et al., 2015a; Viczián et al., 2019). Following conversion to the active Pfr state, phys translocate from the cytosol to the nucleus (Sakamoto and Nagatani, 1996; Kircher et al., 1999; Nagatani, 2004). This important step is essential for almost all known roles of phy and it is conserved in all land plants as well as in marine algae (Huq et al., 2003; Chen and Chory, 2011; Possart and Hiltbrunner, 2013; Duanmu et al., 2014; Klose et al., 2015b). In the nucleus, the active Pfr forms interact with transcription factors known as Phytochrome-Interacting Factors (PIFs), triggering a rapid and global transcriptional reprogramming (Chen and Chory, 2011; Leivar and Monte, 2014; Pham et al., 2018; Shi et al., 2018). PIFs are direct regulators of carotenoid biosynthesis and accumulation, but they also participate in many other processes.

### **3.2. Phytochrome Interacting Factors (PIFs)**

PIFs are members of the basic helix-loop-helix (bHLH) transcription factor superfamily (Ni et al., 1998) that harbor short domains localized in the N-terminal part of the proteins for interaction with the active forms of phy. These transcription factors preferentially bind to G-box (CACGTG) and PBE-box (CACATG) motifs in the promotor of their target genes, even though they can bind other non-canonical motifs. The structure of target promotor regions is variable, ranging from single to multiple PIF-binding sites, each containing one or more G-box or/and PBE-box motifs (Pfeiffer et al., 2014). In *Arabidopsis* there are 8 members of the PIF family: PIF1/PIL5, PIF2/PIL1, PIF3, PIF4, PIF5, PIF6, PIF7 and PIF8. All these members have an Active phyB binding domain (APB) but only PIF1 and PIF3 present an additional Active phyA binding domain (APA) in their sequence (Leivar and Quail, 2011).

Upon interaction with phy, the members of the so-called photolabile quartet (PIFQ, which includes PIF1, PIF3, PIF4, and PIF5) are quickly phosphorylated and ubiquitinated for proteasome-mediated degradation (Shen et al., 2005, 2008; Bu et al., 2011b; Park et al., 2012; Qiu et al., 2017; Park et al., 2018). By contrast, interaction with activated phyB appears to stabilize PIF2 (Pham et al., 2018). Furthermore, PIF2 interacts with PIFQ members to prevent their binding to target genes (Pham et al., 2018). In the case of PIF7, phy interaction causes its phosphorylation and hence promotes its interaction with 14-3-3 proteins that retain the protein in the cytosol, hence preventing its transcriptional activity by a degradation-independent mechanism (Huang et al., 2018). PIF8 is only poorly characterized.

Besides phy-dependent changes in protein functionality, strong transcriptional regulation also controls PIF transcript levels, which facilitates the response to certain light conditions (Nozue et al., 2007; Bernardo-García et al., 2014; Zhang et al., 2018; Martínez et al., 2018; Galvão et al., 2019). All these regulation layers allow PIFs to regulate many different plant responses to light in *Arabidopsis* (Leivar and Quail, 2011).

### 3.3. Regulation of carotenoid accumulation by PIFs in *Arabidopsis*

As stated before, carotenoids are important for photosynthesis and photoprotection, and hence their levels change when plants are exposed to different light conditions. For example, a burst in carotenoid biosynthesis takes place during de-etiolation to protect the emerging photosynthetic apparatus from excessive light energy, whereas carotenoid biosynthesis is repressed when plants are exposed to shade (Llorente et al., 2017). Previous work in my laboratory showed that PIF1 and, to a lower extent, other PIFQ members down-regulate the accumulation of carotenoids in etiolated (i.e. dark-grown) seedlings and shade-exposed plants by specifically repressing the expression of the only PSY-encoding gene present in *Arabidopsis* (Toledo-Ortiz et al., 2010, 2014; Bou-Torrent et al., 2015). Other results have shown that PIFQ proteins also regulate the expression of genes encoding MEP pathway enzymes, with a major role for PIF1 in the regulation of the gene encoding DXS (Chenge-Espinosa et al., 2018). The underlying mechanism involves direct binding of PIF1 to specific (G-box) motifs in the promoters of *PSY* and *DXS* genes to repress their expression.

Beyond carotenoid synthesis, PIF1 regulates carotenoid contents by other mechanisms in *Arabidopsis*, including the coordination with chlorophyll biosynthesis and chloroplast development. This transcription factor was first shown to have a role in chlorophyll biosynthesis regulation during de-etiolation. In etiolated seedlings, PIF1 represses the expression of chlorophyll biosynthetic

genes to prevent deleterious accumulation of toxic chlorophyll precursors. In fact, high levels of these precursors in etiolated *pif1* mutant seedlings result in lethal bleaching upon exposure to light (Huq et al., 2004). PIF1, together again with PIF3, PIF4 and PIF5, was also found to repress the photomorphogenesis developmental program that transforms dark-grown seedlings into photosynthetically-active plants (Leivar et al., 2009). Among other processes, PIFQ members regulate the expression of genes involved in the response to ROS and oxidative stress (Chen et al., 2013), hypocotyl elongation and gravitropism (Shin et al., 2009; Leivar et al., 2012), and chloroplast development (Stephenson et al., 2009). In de-etiolating seedlings, degradation of PIFQ proteins coordinately derepress the expression of genes required for the transition to photosynthetic life, including those producing carotenoids and chlorophylls as well as those involved in chloroplast differentiation. The assembly of photosynthetic complexes and the buildup of thylakoid membranes in developing chloroplasts increase the capacity to sequester the newly synthesized carotenoid and chlorophyll molecules, but it also improves PSY enzyme activity (Welsch et al., 2000). As a result, carotenoid production and accumulation increase rapidly, protecting the emerging photosynthetic apparatus from photooxidative damage. PIF1 and other PIFQ proteins also coordinate the production of chlorophylls and carotenoids in fully-de-etiolated plants (Toledo-Ortiz et al., 2010; Bou-Torrent et al., 2015)

### 3.4. Other roles of *Arabidopsis* PIFs

PIF1 was also found to function as a key negative regulator of seed germination. Wild type (WT) seeds incubated in R germinate normally, but FR inhibits germination. *Arabidopsis pif1* mutants do not present this FR-mediated inhibition, concluding that PIF1 is the key element in the phy-dependent regulation of seed germination (Oh et al., 2004). Further studies identified that PIF1 inhibits germination through repression of gibberellin (GA) activity and promotion of abscisic acid (ABA) activity. To do so, it regulates biosynthetic and catabolic genes of both hormones to get lower levels of GAs and higher levels of ABA. Moreover, PIF1 activates the expression of two DELLA protein (GAI and RGA) to block GA signaling (Oh et al., 2006, 2007). Besides transcriptional regulation, PIFs proteins directly interact with DELLA proteins. This DELLA-PIF interaction blocks the transcriptional activity of PIFs and, in addition, mediates their degradation in order to fine-tune seed germination or hypocotyl elongation (Feng et al., 2008; De Lucas et al., 2008; Zheng et al., 2016).

Genome-wide analyses identified hundreds of differentially expressed genes when compared WT and *pif1* seed germination. Among them, many appear to be direct targets of PIF1. This result unveiled that PIF1 inhibits germination not just

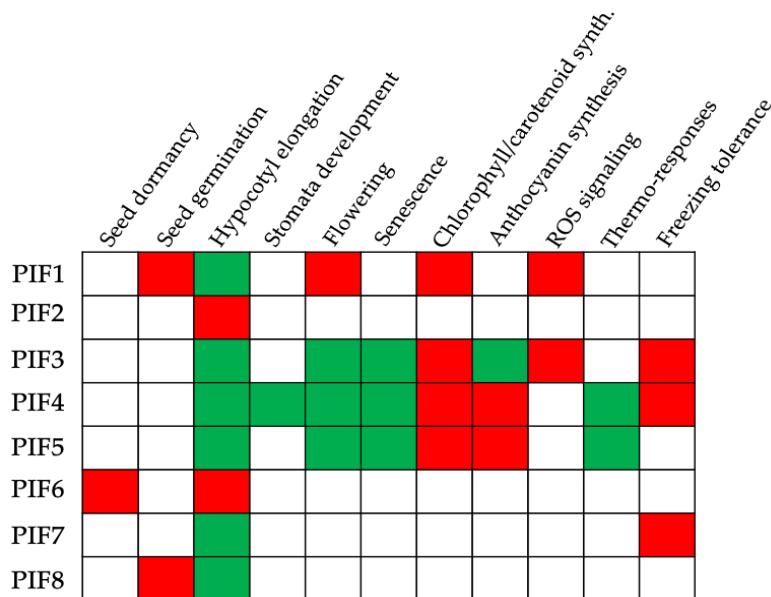
through GA and ABA, but also coordinating other hormone signals, modulating cell wall properties and regulating cell division (Oh et al., 2009; Shi et al., 2013). At the protein level, PIF1 interactors such as LEUNIG\_HOMOLOG (LUH), LONG HYPOCOTYL IN FAR-RED 1 (HFR1) or HECATE (HEC) play a really important role modulating PIF1 activity during seed germination (Shi et al., 2013; Lee et al., 2015; Zhu et al., 2016). Other nuclear proteins can interact with PIFs in order to enhance or inhibit their activity and regulate processes beyond seed germination. Well-known examples include BZR1 and PICKLE, involved in the regulation of brassinosteroid signaling (Eunyoo Oh et al., 2012; Zhang et al., 2014) or HFR1, PAR1 and PAR2, regulating PIFs activity in response to shade (Lorrain et al., 2008; Hao et al., 2012).

Recently, PIF1 was also identified as a repressor of floral transition. Mutant *pif1* plants display an early flowering phenotype in long day conditions, and genes involved in flowering initiation and GA synthesis were up-regulated in this mutant (Wu et al., 2018).

Other *Arabidopsis* PIFs have been studied intensively since the discovery of the first one, PIF3, in 1998 by the group of Dr. Peter Quail (Ni et al., 1998). Besides the previously mentioned photomorphogenic roles, PIF3, PIF4 and PIF5 have been identified as inductors of Dark Induced Senescence (DIS) in leaves (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). PIF3, PIF4 and PIF7 play a role in freezing tolerance through the transcriptional down-regulation of C-repeat binding factor (CBF) (Lee and Thomashow, 2012; Jiang et al., 2017). PIF4 and PIF5 inhibit anthocyanin biosynthesis through transcriptional repression of the anthocyanin biosynthetic genes under R conditions (Liu et al., 2015); in contrast, PIF3, collaboratively with ELONGATED HYPOCOTYL 5 (HY5), promotes anthocyanin biosynthesis (Shin et al., 2007). Oppositely to PIFQ, PIF2 seems to be a positive regulator of light-triggered seedling de-etiolation (Luo et al., 2014) and a negative regulator of shade-triggered hypocotyl elongation (Li et al., 2014). PIF6 is also a repressor of photomorphogenesis and hypocotyl elongation (Pham et al., 2018) and a regulator of primary seed dormancy (Penfield et al., 2010).

Furthermore, PIFs are integrators of multiple environmental cues. In this way, PIF4 plays an important role in high temperature-induced architectural adaptations, like rapid extension of plant axes or leaf hyponasty (Koini et al., 2009). In addition, early flowering in warm temperatures is regulated by PIF4 and PIF5 (Koini et al., 2009; Kumar et al., 2012; Thines et al., 2014).

Figure I1 summarizes schematically the main biological processes in which PIFs have been found to have a role in *Arabidopsis*. An extended revision of these roles is listed in the annexed Table A1.



**Figure I1. Schematic representation of PIF roles in *Arabidopsis*.** Green boxes indicate a promoting role of the corresponding PIF in the corresponding process, while red boxes indicate a repressing role. An extended revision of these roles can be found in annexed Table 1 in this thesis.

While most of our knowledge on PIF functions comes from work in *Arabidopsis*, some studies have been recently focused on identifying the PIF family members and their roles in other plant species.

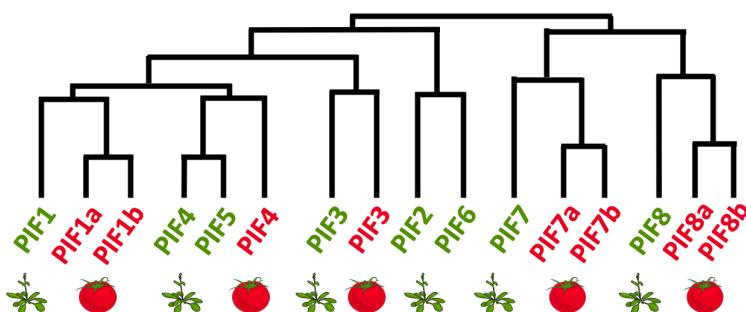
### 3.5. The tomato PIF family

The phy-PIF signaling module originated early in the evolution of plants. A single and functional copy exists for both phy and PIF proteins in the liverwort *Marchantia polymorpha* (Inoue et al., 2016), and different combinations are predicted to exist in all land plants (Lee and Choi, 2017).

PIF-LIKE (PIL) proteins have been identified in some monocots such as rice (*Oryza sativa*) and maize (*Zea mays*) (Nakamura et al., 2007; Zhou et al., 2014; Gao et al., 2015; Kumar et al., 2016; Cordeiro et al., 2016; Wu et al., 2019), but little is known about their biological roles and their functionality as real PIF transcription factors (Lee and Choi, 2017). Regarding eudicots, beyond *Arabidopsis*, the best characterized PIF family is in tomato (Fig. I2).

There are 8 tomato PIFs: PIF1a, PIF1b, PIF3, PIF4, PIF7a, PIF7b, PIF8a and PIF8b (Rosado et al., 2016; Oh et al., 2020) (Fig. I2). Compared to *Arabidopsis*, the main differences are that homologs of PIF2, PIF5 and PIF6 are not found in the tomato genome, while some member of the family seem to be duplicated (PIF1, PIF7 and PIF8). Rosado et al. explain that *Arabidopsis* PIF4 and PIF5 genes were originated by a Brassicaceae exclusive duplication, explaining the existence of a single gene in the tomato genome within the clade PIF4 (Rosado et al., 2016); in fact Llorente

et al. referred to this gene as *PIF4/5* due to the high homology with both factors (Llorente et al., 2016b). The duplication of *PIF1*, 7 and 8 is estimated to have occurred when the whole genome duplication event that preceded tomato and potato divergence took place (Wang et al., 2008; Tomato Genome Consortium, 2012). This polyploidization event has been proposed as the foundation of the PIF subfamily diversification. Positive selection following whole genome duplication would explain the existence of two *PIF1*, 7 and 8 paralogs in the tomato genome, while stochastic gene loss or no selection would explain that the others members of the family remained as single-copy genes (Rosado et al., 2016).



**Figure I2.** Phylogenetic analysis of the PIF protein family in *Arabidopsis* and tomato. Adapted figure from Oh et al., 2020.

The expression patterns of the tomato *PIF* family members in different processes have been previously reported (Rosado et al., 2016). During de-etiolation, the expression of *PIF1a*, *PIF4* and *PIF7a* increase after light exposure, while *PIF1b* and *PIF3* decrease. It is also worth noting that in terms of relative expression, *PIF1b* is the PIF-encoding gene most abundantly expressed in seedlings. Regarding circadian rhythms of expression, *PIF1a*, *PIF3* and *PIF7a* show low expression levels at the end of the day and increase their levels during the night, with a maximum 4 hours after dawn, while *PIF1b* expression progressively decreases during the night. Interestingly, *PIF1* homologs are the most highly expressed genes at the beginning of the day, showing more than 2-fold higher transcript levels in leaves than other *PIFs*. Regarding leaf senescence, *PIF1a*, *PIF3*, *PIF7a* and *PIF7b* are down-regulated during DIS, whereas *PIF1b* and *PIF4* are up-regulated. Finally, during fruit ripening, *PIF1a* expression is increased, *PIF4* decreased and *PIF1b* and *PIF3* show no major changes; transcripts of other PIF-encoding genes were not detected in this process (Rosado et al., 2016).

### 3.6. Roles of tomato PIFs

Only a few members of the tomato PIF family have been functionally characterized to date. *PIF3* was found to be a regulator of tocopherol biosynthesis in tomato fruits (Gramegna et al., 2018). Light promotes fruit tocopherol biosynthesis in a phy-dependent manner by the transcriptional regulation of biosynthetic genes, such as *GERANYLGERANYL DIPHOSPHATE REDUCTASE*

(*GGDR*), encoding the enzyme that synthesizes phytyl diphosphate (the isoprenoid moiety used for chlorophyll and tocopherol biosynthesis) from GGPP. In dark-incubated fruits PIF3 accumulates and physically interacts with the promotor of the *GGDR* gene, repressing its expression. In illuminated fruits, PIF3 is degraded upon phy activation, allowing *GGDR* de-repression and phytyl diphosphate production for tocopherol biosynthesis (Gramegna et al., 2018). Furthermore, tomato PIF3 also represses the production of another class of isoprenoid-derived metabolites called steroidal glykoalkaloids (SGAs). SGAs are produced from cholesterol by Solanaceous plant species and contribute to pathogen defense, being described as anti-nutritional compounds to animals and humans (Wang et al., 2018). PIF3 binds to the promotor of some of the SGA biosynthetic genes and down-regulates their expression (Wang et al., 2018).

PIF4 was also recently studied. RNA interference (RNAi)-mediated knockdown of *PIF4* expression resulted in a broad range of phenotypes (Rosado et al., 2019). *PIF4*-silenced lines showed impaired thermomorphogenesis and delayed leaf senescence and flowering time, confirming a functional evolutionary conservation between tomato and *Arabidopsis*. Most interestingly, transgenic lines exhibited anticipation of ripening and reduced fruit yield and size. *PIF4*-defective fruit accumulated higher carotenoid levels (Rosado et al., 2019), consistent with the negative role reported for PIFQ members on the regulation of carotenoid biosynthesis in *Arabidopsis* (Toledo-Ortiz et al., 2010, 2014; Bou-Torrent et al., 2015). Loss of *PIF4* function in tomato was also found to increase cold susceptibility, while *PIF4* overexpression enhanced cold tolerance in part by regulating GA and jasmonate biosynthesis and signaling in response to low temperature (Wang et al., 2019).

In the case of PIF1, only one of the two tomato homologs has been characterized: PIF1a (Llorente et al., 2016b). This work showed that the PIF1-PSY regulatory module controlling carotenoid biosynthesis in response to light signals in *Arabidopsis* (Toledo-Ortiz et al., 2010; Bou-Torrent et al., 2015) is evolutionary conserved in tomato and adapted to adjust carotenoid accumulation to the actual progression of ripening (Llorente et al., 2016b, 2016a). In tomato green fruits, chlorophylls adsorb R (but not FR) and cause a self-shade effect that promotes PIF1a stability and allows its binding to the promotor of *PSY1*, the gene encoding the fruit-specific PSY1 isoform (Fray and Grierson, 1993; Tomato Genome Consortium, 2012; Fantini et al., 2013; Giorio et al., 2008). Binding of PIF1a hence represses *PSY1* expression in green fruit. As ripening progresses and chlorophylls degrade, PIF1a stability declines and *PSY1* expression is derepressed to up-regulate the production of carotenoids (Llorente et al., 2016b, 2016a). Consistently, silencing of *PIF1a* using artificial microRNAs led to the accumulation of higher levels of carotenoids in ripe fruit. The PIF1a-PSY1 regulatory module was also predicted *in silico* (Koul et al., 2019), together with

other previously confirmed regulatory modules related to carotenoid biosynthesis during fruit ripening, such as RIN-PSY1 (Fujisawa et al., 2011) or TAGL1-LYC<sub>b</sub> (Vrebalov et al., 2009). It is unknown whether PIF1a plays additional roles in tomato and whether redundancy with PIF1b exists. Addressing these questions is the main goal of this doctoral thesis.





## **OBJECTIVES**



Since their discovery, PIFs have been described as key factors integrating light signaling with other internal (e.g. hormones, clock) and external (e.g. temperature) cues to regulate multiple processes in *Arabidopsis*. Complex molecular mechanisms underlying their functions have been identified in *Arabidopsis*, but relatively little is known in other plants, particularly in agronomically-interesting crops, like tomato. Previous work in the group has shown a major role for *Arabidopsis* PIF1 in the light-dependent control of the production of carotenoids and other plant isoprenoids. In this thesis, we aim to further investigate the role of the two PIF1 homologs found in the tomato genome: PIF1a and PIF1b. Specifically, we propose the following objectives:

1. Compare PIF1a and PIF1b protein features and gene expression profiles.
2. Generate tomato lines lacking PIF1a, PIF1b, or both.
3. Analyze the phenotype of mutant lines at different developmental stages and in response to different stimuli.
4. Investigate the molecular mechanism responsible for the observed phenotypes.



## **RESULTS**



## 1. Tomato PIF1 homologs show different protein features and expression profiles

It was previously reported that the tomato genome harbors two genes encoding PIF1 homologs: *PIF1a* and *PIF1b* (Llorente et al., 2016b; Rosado et al., 2016). *PIF1a* was shown to be a functional PIF whose expression is up-regulated during fruit ripening, whereas information for *PIF1b* is scarce (Llorente et al., 2016b; Rosado et al., 2016). Here we compared these two homologs in terms of subcellular localization, light-dependent protein stability, protein-protein interaction and gene expression patterns.

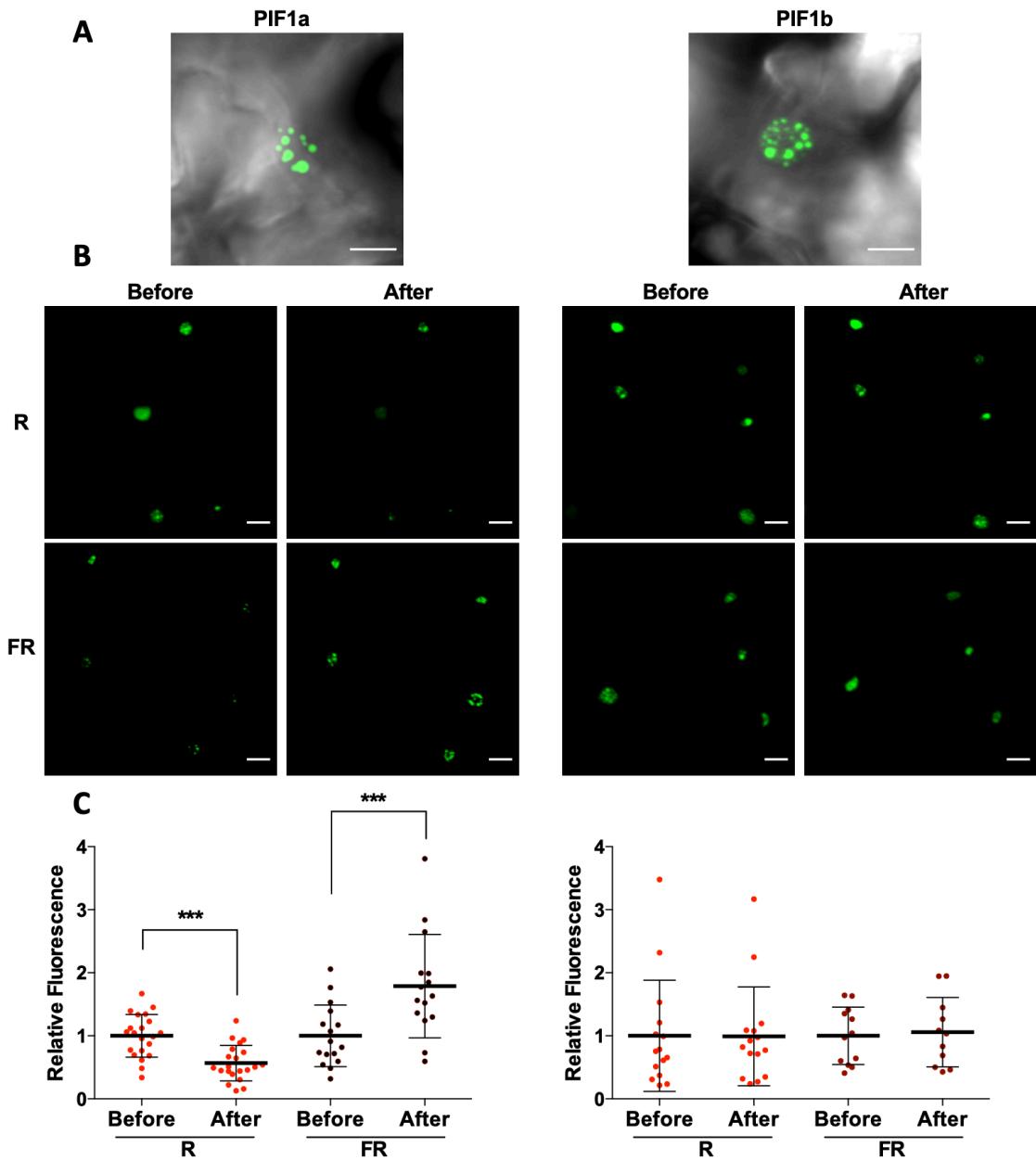
### 1.1. Protein localization and stability

*PIF1a* and *PIF1b* are very similar in their amino acid sequence (primary structure): both of them present the characteristic basic Helix-Loop-Helix (bHLH) and active phyB-binding (APB) domains found in all PIFs, and an active phyA-binding (APA) domain that in *Arabidopsis* is only present in PIF1 and PIF3. However, the APB-binding domain of *PIF1b* presents an amino acid substitution that changes a conserved Q residue to G (Rosado et al., 2016). It was proposed that this sequence change could lead to a disrupted interaction with phyB and hence an absolutely different role of *PIF1b*, as interaction with active phyB results in lower PIF activity due to protein degradation in the case of *Arabidopsis* PIF1 (Bu et al., 2011b; Park et al., 2012).

In order to experimentally confirm if *PIF1a* and *PIF1b* abundance is controlled by phyB, we tested the light-dependent stability of C-terminal GFP-tagged versions of these proteins transiently expressed in *Nicotiana benthamiana* leaves. Both *PIF1a*-GFP and *PIF1b*-GFP proteins were localized in nuclear bodies (Fig. R1A) ([Gramegna et al., 2018](#)), as expected for PIF transcription factors (Al-sady et al., 2006; Shen et al., 2005; Trupkin et al., 2014). Once we confirmed that the GFP-tagged proteins were being synthesized and correctly localized, we irradiated the agroinfiltrated leaf with R or FR for 30 min and took pictures of the same area before and after irradiation (Fig. R1B). *PIF1a*-GFP fluorescence signal decreased after the exposure to R and increased after FR, a dynamic behavior that is typical of a PIF which interacts with phyB after a prolonged illumination (Shen et al., 2008). In contrast, *PIF1b*-GFP abundance was not altered by R or FR exposure (Fig. R1C). This could indicate that, as predicted, the point amino acid mutation present in the APB domain of *PIF1b* prevents interaction with active phyB and then disrupts its light regulation, likely affecting the biological activity of the protein.

## Results

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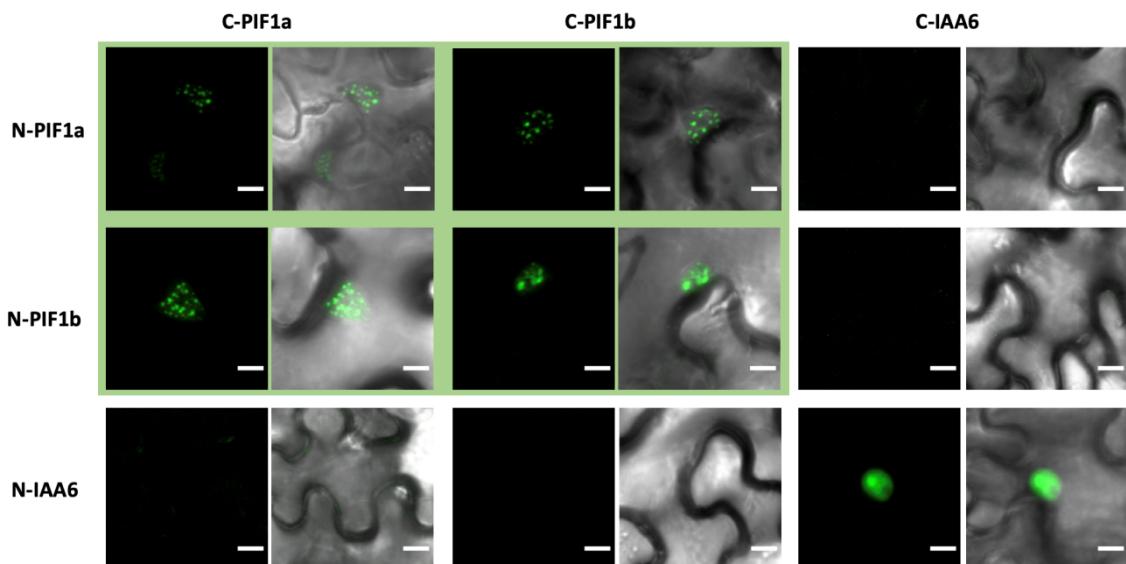


**Figure R1. Tomato PIF1a and PIF1b protein stability in response to light.**

- Confocal microscopy images of GFP fluorescence in the nucleus of a *N. benthamiana* leaf cell transiently expressing PIF1a or PIF1b fused to GFP. Scale bar = 10  $\mu$ m.
- Confocal microscopy images of leaf areas expressing the same constructs than A, before and after exposure to either R or FR for 30 min. Pictures show the same area of the leaf with the same laser conditions. Scale bar = 20  $\mu$ m.
- Quantification of GFP fluorescence of nuclei from leaf areas like those shown in B. Error bars indicate SD of at least 15 different nuclei. Asterisks mark statistically significant changes in student's t test (\*\* = p < 0.05, \*\*\* = p < 0.01)

## 1.2. Protein-protein interactions

Since *Arabidopsis* PIFs are able to interact with each other forming homodimers and heterodimers (Toledo-ortiz et al., 2003; Bu et al., 2011a), we tested if tomato PIF1a and PIF1b could form similar complexes by Bimolecular Fluorescence Complementation (BiFC). We cloned the coding sequences of PIF1a and PIF1b fused to N- and C-terminal halves of GFP and then looked for GFP fluorescence resulting from the physical interaction of the two halves in agroinfiltrated leaves. As negative control we performed the same experiment using INDOLE-3-ACETIC ACID 6 (IAA6, AT1G52830), a non-PIF interacting transcription factor involved in auxin signaling which forms homodimers (Dreher et al., 2006; Winkler et al., 2017). The result confirms that PIF1a and PIF1b are able to interact in the nucleus, more specifically in nuclear bodies, forming homodimers but also PIF1a-PIF1b heterodimers (Fig. R2).



**Figure R2. BiFC analysis of PIF1a and PIF1b protein-protein interaction.** Confocal microscopy images of GFP fluorescence in *N. benthamiana* leaf cells transiently expressing the indicated proteins fused to N- or C-terminal GFP halves for BiFC analysis. Images of representative nuclei showing either GFP fluorescence alone (left) or overlapped with bright field images (right) are shown for every combination. Scale bar = 10  $\mu$ m.

## 1.3. Gene expression patterns

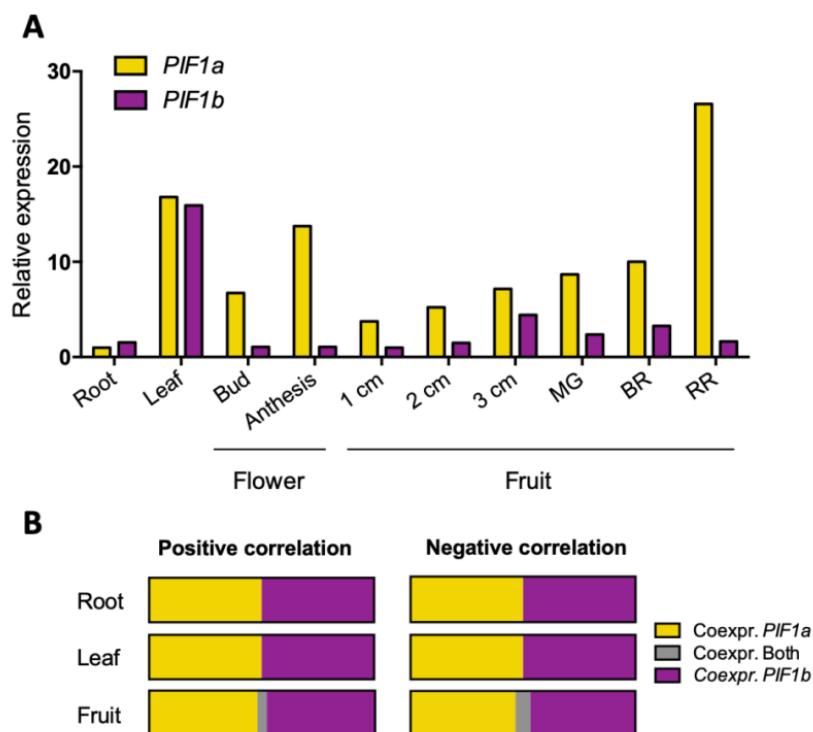
We next analyzed the expression pattern of both *PIF1a* and *PIF1b* genes by using data from the Bio-Analytic Resource for Plant Biology (BAR, University of Toronto). According to these data (Fig. R3A), both *PIF1a* and *PIF1b* are highly expressed in leaves. Transcripts encoding *PIF1a*, but not *PIF1b*, are also abundant

## Results

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in flowers. Levels of *PIF1a* transcripts are higher than those of *PIF1b* in all stages of fruit development. *PIF1b* expression increases as fruit grows until it reaches its final size at the mature green (MG) stage. During ripening, *PIF1b* expression does not change but *PIF1a* expression exhibits a strong increase as fruits move from the breaker (BR) to the red ripe (RR) stage. This result is in accordance to previously reported experimental data (Llorente et al., 2016b; Rosado et al., 2016).

Furthermore, we also analyzed the gene co-expression network (GCN) of *PIF1a* and *PIF1b* as a way of testing whether these two transcription factors might be involved in similar processes. We used data from TomExpress (Abdullah et al., 2017) to get a list of the 500 most highly co-expressed genes with *PIF1a* and *PIF1b* in three different organs: root, leaf and fruit (Fig. R3B). The comparison showed almost no overlapping of the GCNs of *PIF1a* and *PIF1b* in roots and leaves. In fruit, however, we found that about 10% of genes showed either positive (40 genes) or negative (62 genes) co-expression with both *PIF1a* and *PIF1b* (Fig. R3B).



**Figure R3. Expression analyses of *PIF1a* and *PIF1b*.**

- Levels of *PIF1a* and *PIF1b* transcripts in different plant organs. Data are obtained from BAR (University of Toronto) and expressed relative to the lowest expression level found in the study (i.e. *PIF1a* expression in roots)
- Distribution of the 500 most co-expressed genes with *PIF1a* (yellow) and *PIF1b* (purple) in roots, leaves and fruits. Overlapping genes co-expressed with both *PIF1a* and *PIF1b* are represented in grey).

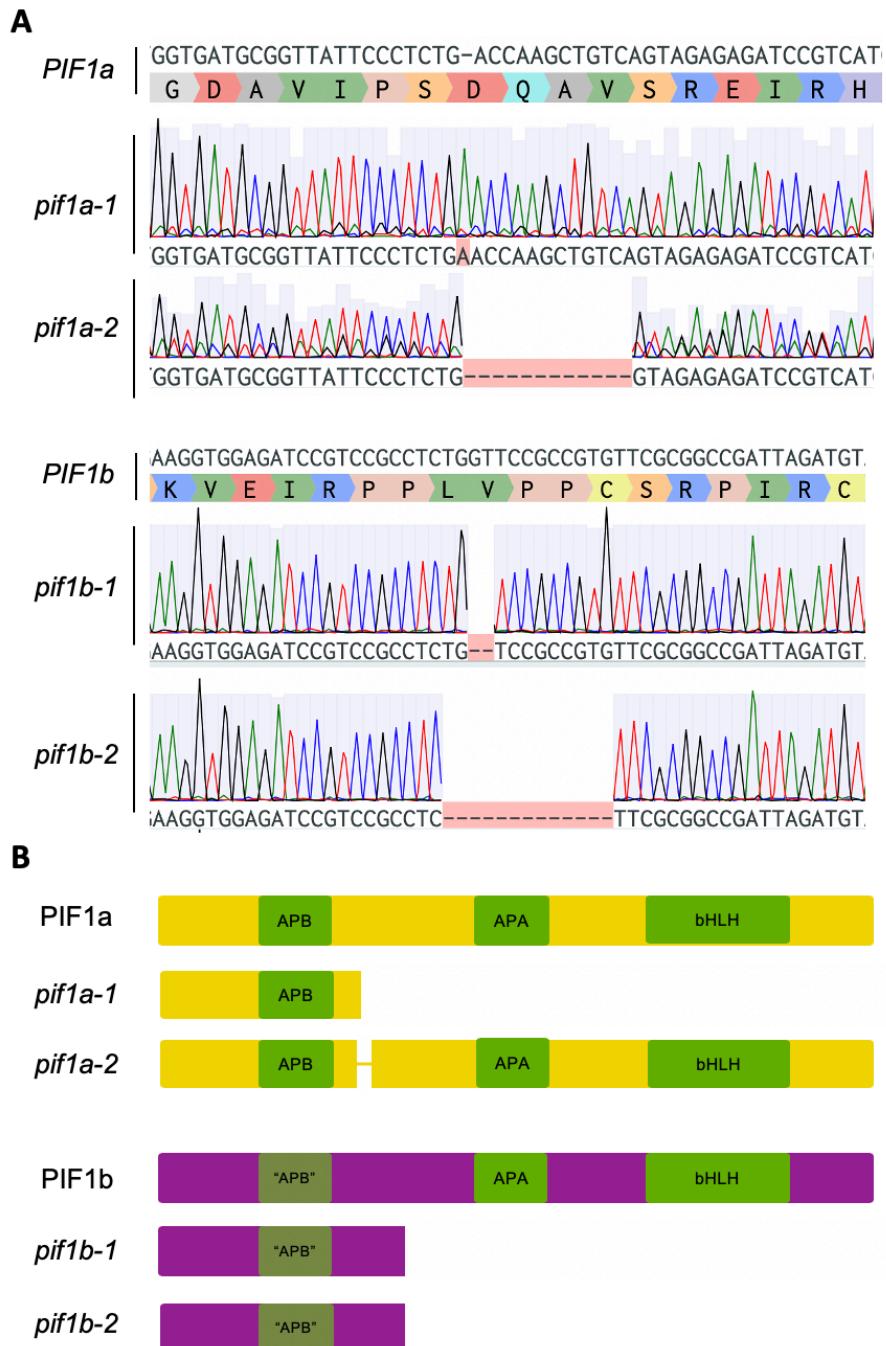
The differences observed between *PIF1a* and *PIF1b* in their expression patterns and in their GCNs (Fig. R3), together with the different behavior of the encoded proteins in response to light (Fig. R1), supports the conclusion that the two PIF1 homologs that were generated in tomato after the whole genome duplication in Solanaceae (Rosado et al., 2016) have acquired different functions.

## 2. Single and double tomato mutants defective in *PIF1a* and *PIF1b* were generated by CRISPR/Cas9

In order to test the neofunctionalization hypothesis, we collaborated with the laboratory of Dr. Alain Goossens, VIB Center for Plant Systems Biology (Ghent, Belgium), to generate tomato mutants defective in *PIF1a*, *PIF1b*, or both activities using CRISPR/Cas9. They transformed tomato (cv. Microtom) with a CRISPR/Cas9 construct harboring two guide RNAs (gRNAs), each of them targeting one of the two PIF1 homologs (see Table 3 of primers in Materials and Methods). Several T1 lines were identified that had different insertions and/or deletions (indels) in both genes. Individual T2 plants segregating from two of the transformed T1 lines were analyzed at the CRAG by PCR-based amplification of the genomic regions targeted by the gRNAs followed by sequencing of the resulting amplicons. Sequence analyses were next performed using the TIDE (Tracking of Indels by DEcomposition) web tool (Brinkman et al., 2014; Pauwels et al., 2018). This platform provides an estimation of overall editing efficiency (percentage of cells different from the WT) and the spectrum and frequency of the indel types. The result of this analysis showed that one of the T1 lines produced T2 individuals with mutations in both *PIF1a* and *PIF1b* genes (Fig. R4).

From the four identified alleles (Fig. R4), we selected individuals harboring an insertion of one nucleotide in the coding region of the *PIF1a* gene (*pif1a-1* allele, from herein *pif1a*) and a deletion of two nucleotides in the coding region of the *PIF1b* gene (*pif1b-1* allele, from herein *pif1b*) (Fig. R4A). Both mutations caused a frameshift predicted to result in shorter PIF1a and PIF1b proteins that lacked the APA and bHLH domains, likely resulting in non-functional (i.e. knock-out) mutants (Fig. R4B). From herein, genotyping of the selected alleles was routinely carried out by PCR using primers that amplified 500 bp in the mutated version. After that, since both mutations eliminated a restriction site, we digested the amplicon with the corresponding enzymes, identifying the non-digested bands as a mutant in each gene.

## Results



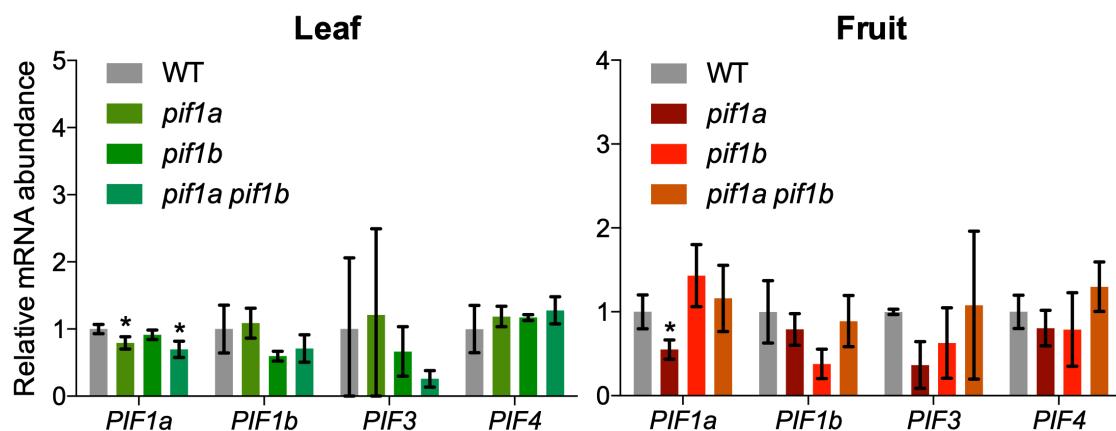
**Figure R4. Analysis of CRISPR/Cas9 mutations in *PIF1a* and *PIF1b*.**

- Chromatograms of the sequences analyzed by the TIDE platform. Indels in the identified alleles are highlighted.
- Schematic representation of PIF1a and PIF1b proteins in the CRISPR/Cas9-generated mutant alleles.

Once we identified monoallelic double *pif1a* *pif1b* mutant plants, we crossed them with untransformed (WT) plants (cv. Microtom) in order to segregate the corresponding single mutants and clean the genome from other potential off-target mutations that could have been generated by the CRISPR/Cas9 action. The

F1 population of this cross was 100% heterozygous for both genes, as expected. Analysis of the segregating F2 population then allowed to isolate single *pif1a* and *pif1b* mutants that had lost the CRISPR/Cas9 cassette. These plants were grown to produce fruit and seed. Similarly, double *pif1a pif1b* mutants and WT plants lacking the CRISPR/Cas9-associated transgenes were also selected from the segregating F2 population and used to obtain seed for future experiments.

To test whether the loss of function of PIF1a, PIF1b, or both, interfered with the expression of other PIFQ genes, we analyzed the expression of the corresponding genes in mature leaves and MG fruits of single and double mutant plants (Fig. R5). The qPCR results showed no significant differences in transcript levels between WT and mutant lines with the only exception of decreased *PIF1a* transcript levels in PIF1a-defective mutants (Fig. R5)



**Figure R5. qPCR analysis of PIF-encoding transcripts in mature leaves and MG fruit from tomato CRISPR-CAs9 mutants defective in PIF1a, PIF1b or both.** Error bars indicate SD of 3 biological replicates in the indicated tissues. Asterisks mark statistically significant changes in student's t test (\*\* = p < 0.01)

### 3. Phenotypic analysis of *pif1a* and *pif1b* mutants confirms that they play both redundant and specific roles

The selected transgene-free single and double mutants together with the WT controls were next used to investigate the physiological role of tomato PIF1 homologs based on the phenotypic characterization of the lines at different developmental stages and in response to different stimuli.

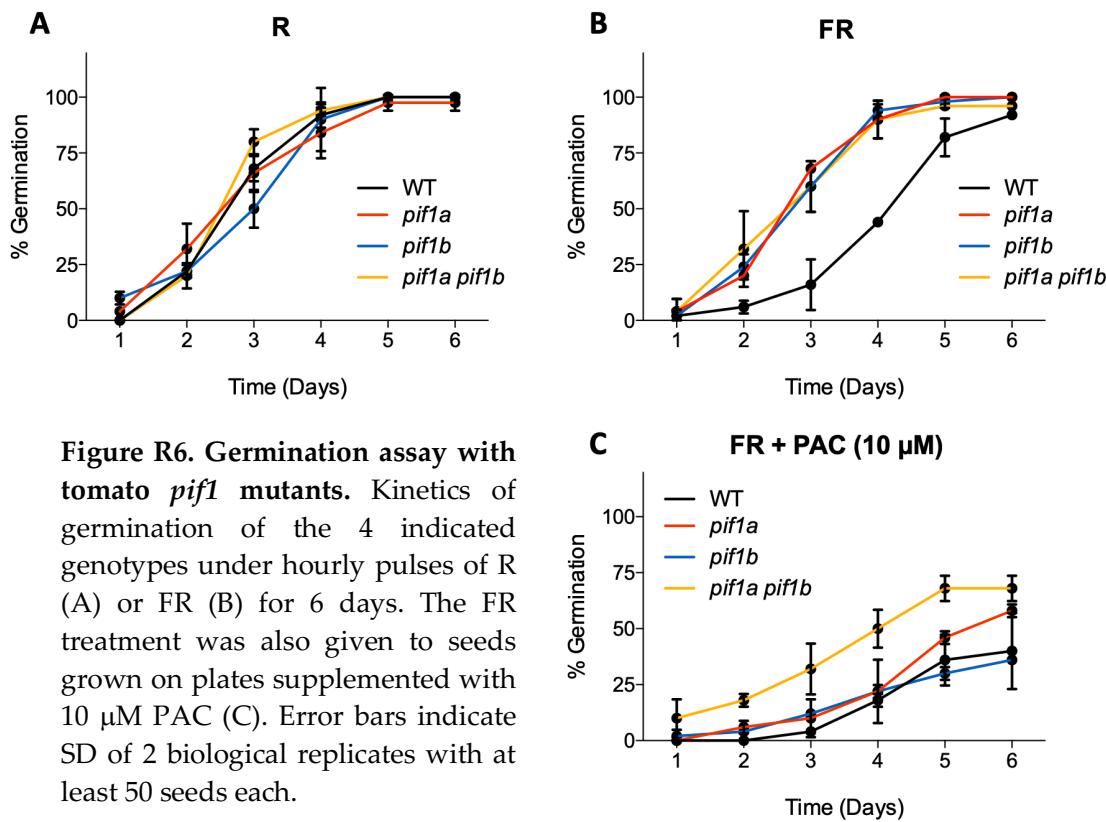
### 3.1. Seed germination

*Arabidopsis* PIF1 is an essential element in light-regulated seed germination. Under FR, PIF1 accumulates in the nucleus and represses the germination process, while in the presence of R the activated phyB degrades PIF1 and germination takes place (Oh et al., 2004; Seo et al., 2009; Shinomura et al., 1994; Lee et al., 2012; Shi et al., 2013). Light regulation of germination also occurs in tomato. Similar to *Arabidopsis*, germination of tomato seeds is repressed by FR and promoted by R (Mancinelli et al., 1966; Georghiou and Kendrick, 1991; Shichijo et al., 2001; Appenroth et al., 2006; Auge et al., 2009).

To test whether PIF1 homologs might be involved in the light-dependent regulation of seed germination in tomato, we performed a germination experiment treating the seeds from WT and mutant lines to hourly pulses of R or FR as previously described (Appenroth et al., 2006). Treatment with R resulted in similar germination rates for WT and mutant (single and double) seeds (Fig. R6A), whereas FR treatment repressed germination of WT seeds, as expected, but had little impact on the germination of mutant seeds (Fig. R6B). This indicates that both PIF1a and PIF1b repress seed germination in tomato under FR, similar to that observed for PIF1 in *Arabidopsis*. Also similar to *Arabidopsis* (Oh et al., 2006, 2007; Kim et al., 2008; Oh et al., 2009; Gabriele et al., 2010; Dirk et al., 2018), inhibition of the production of germination-promoting GAs with paclobutrazol (PAC) further repressed germination of WT seeds under FR (Fig. R6C). The inhibitory effect of PAC relative to untreated FR-exposed samples was clear in WT and mutant lines but it was much lower in the case of the double *pif1a pif1b* mutant, suggesting that both PIF1 homologs might play a redundant role in the GA-dependent regulation of seed germination. Interestingly, the single *pif1a* mutant showed some insensitivity to PAC at longer times (Fig. R6C), suggesting that, although redundant, PIF1a might have a slightly more prominent role than PIF1b in this process.

### 3.2. Seedling de-etiolation

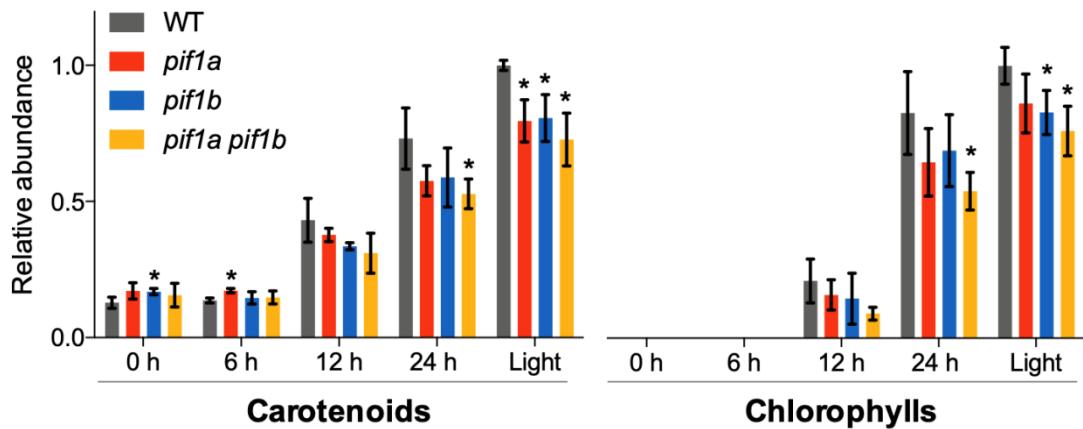
*Arabidopsis* PIF1 was also shown to regulate carotenoid and chlorophyll biosynthesis during seedling de-etiolation (Huq et al., 2004; Moon et al., 2008; Toledo-Ortiz et al., 2010). PIF1 accumulates during skotomorphogenic development in the dark, directly repressing the expression of genes supporting the production of photosynthetic pigments and the differentiation of chloroplasts. In the dark-to-light transition PIF1 is degraded, allowing the accumulation of chlorophylls and carotenoids and the transformation of etioplasts into chloroplasts (Toledo-Ortiz et al., 2010, 2014; Bou-Torrent et al., 2015).



**Figure R6. Germination assay with tomato *pif1* mutants.** Kinetics of germination of the 4 indicated genotypes under hourly pulses of R (A) or FR (B) for 6 days. The FR treatment was also given to seeds grown on plates supplemented with 10  $\mu$ M PAC (C). Error bars indicate SD of 2 biological replicates with at least 50 seeds each.

To check whether tomato PIF1 homologues contributed to carotenoid and chlorophyll production in dark-grown and de-etiolating seedlings, we germinated WT and mutant seeds in the dark for 7 days, and then illuminated them with white light for an additional day. Samples were collected at different times after illumination for measuring photosynthetic pigment levels by HPLC. Plants germinated and grown in the light were used as controls. In agreement with the results reported in *Arabidopsis*, tomato dark-grown seedlings defective in PIF1a, PIF1b, or both, accumulated higher levels of carotenoids compared with the WT (Fig. R7), even though this increase was not statistically significant in the tomato mutants. Most strikingly, the absence of PIF1a, PIF1b, or both, in tomato seedlings led to a deceleration in the light-triggered accumulation of carotenoids and chlorophylls (Fig. R7). A similar phenotype has been reported in PIF-defective *Arabidopsis* mutants when they were left for too long in the dark (Monte et al., 2004). In the case of tomato, however, reduced levels of photosynthetic pigments were also detected in mutant seedlings that were germinated and growth for 7 days in the light (Fig. R7), suggesting that the tomato PIF1 homologs might not be repressors but activators of chlorophyll and carotenoid biosynthesis during the early stages of the seedling de-etiolation process.

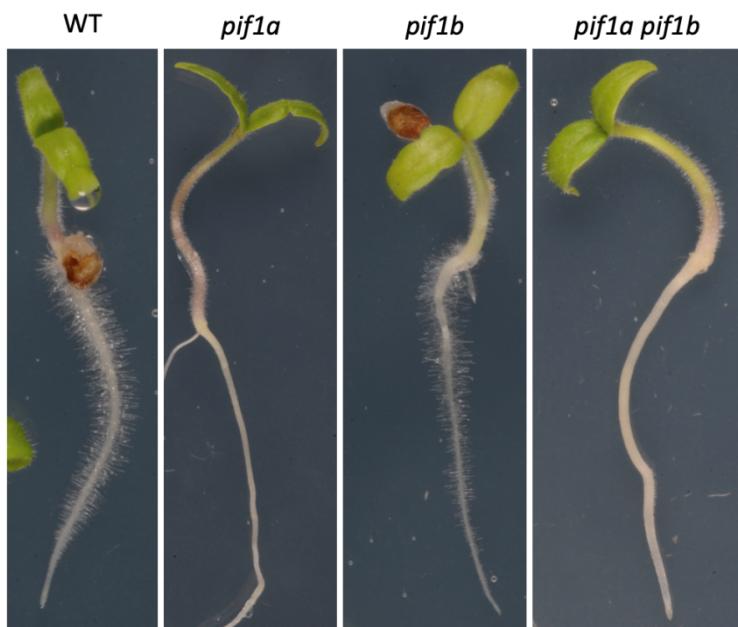
## Results



**Figure R7. Pigment quantification during seedling de-etiolation in tomato *pif1* mutants.** WT and mutant seedlings were germinated in the dark for 7 days and then illuminated with white light for the indicated times. Seedlings germinated and grown under continuous white light for 7 days were used as controls. Carotenoid and chlorophyll levels are represented relative to those in light-grown WT seedlings. Error bars indicate SD of at least 3 biological replicates. Asterisks mark statistically significant changes in the indicated genotype compared to the WT according to t-student test (\* = p < 0.05).

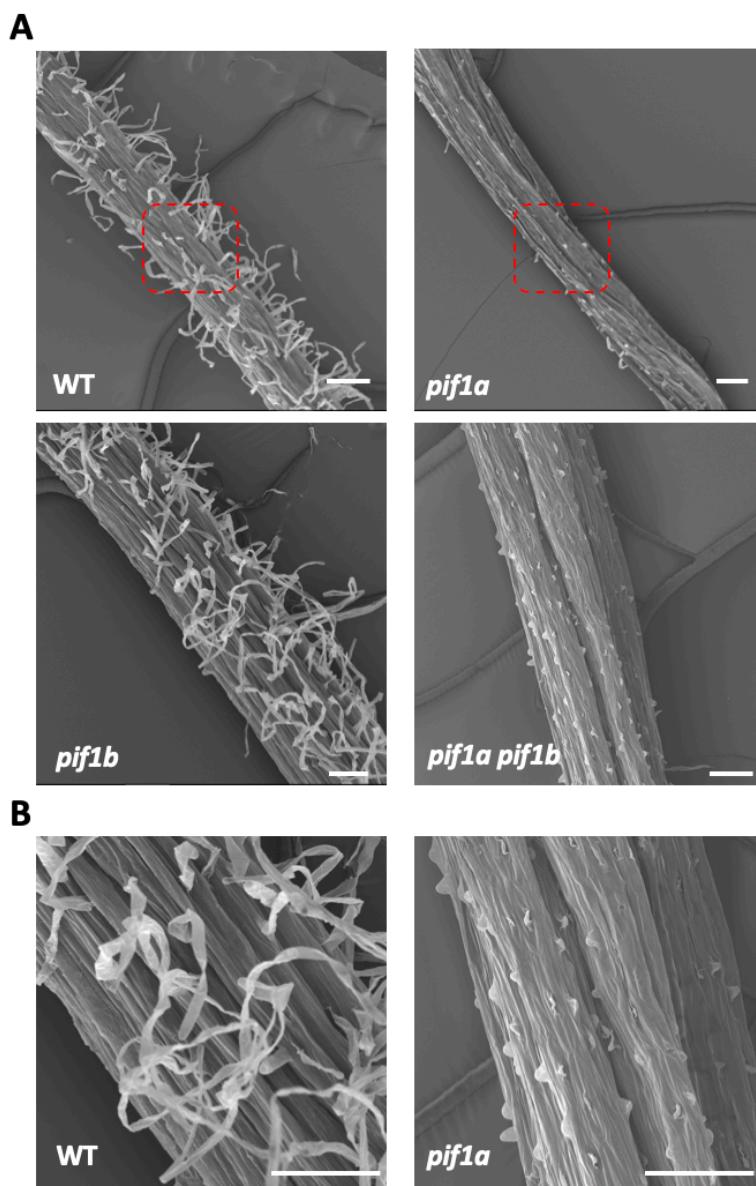
### 3.3. Root hair development

While doing the germination and de-etiolation experiments, we noticed that tomato single *pif1a* and double *pif1a pif1b* mutants had hairless roots (Fig. R8), a phenotype that has never been reported in PIF-deficient *Arabidopsis* mutants.



**Figure R8. Visual phenotype of WT and *pif1* mutant seedlings.** Seeds were germinated and grown for one week under long day conditions on MS plates.

We decided to have a closer look at this new phenotype by observing seedling roots under the Scanning Electron Microscope (SEM). Besides confirming that *pif1b* mutants had normal root hairs, SEM analysis showed that root hair primordia were indeed present in *pif1a* and *pif1a pif1b* roots, suggesting that the loss of PIF1a activity does not interfere with root hair initiation but prevents elongation (Fig. R9). This phenotype was very robust, affecting all the seedlings in homozygous populations of single *pif1a* and double *pif1a pif1b* mutants, and being absent from WT and *pif1b* mutants. Therefore, the results suggest that PIF1a, but not PIF1b, is required for root hair elongation in tomato.



**Figure R9. SEM images of tomato WT and *pif1* mutant roots.** Seedlings were grown as described in Fig. R8. Scale bar = 100  $\mu\text{m}$

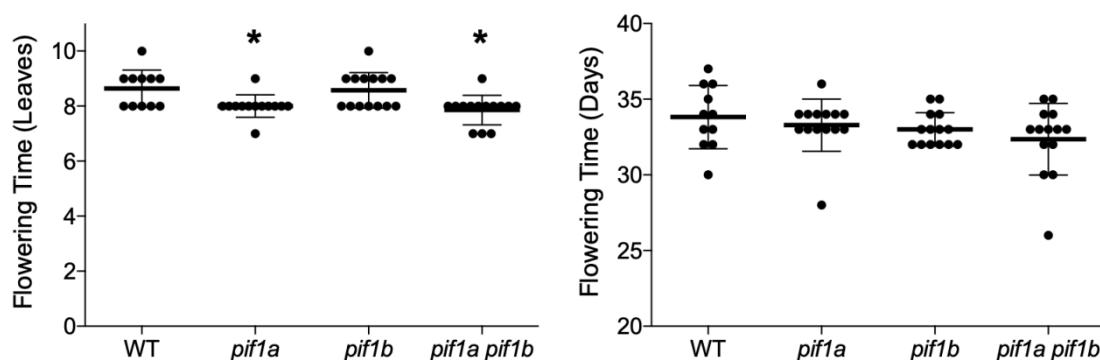
A. Overview of the roots in all the genotypes at low magnification.

B. Higher magnification detail of the WT and *pif1a* root areas marked in A.

### 3.4. Flowering time

The next phenotype analyzed was flowering time. *Arabidopsis pif1* mutants show early flowering and up-regulated expression of the major flowering-promoting genes (Wu et al., 2018). We assessed the flowering time of tomato *pif1a* and *pif1b* mutants by measuring two different parameters: (1) the number of leaves produced when the first flower reached anthesis and (2) the number of days from sowing until this event took place, following reported procedures (Dielen et al., 1998; Giliberto et al., 2005; Quinet and Kinet, 2007; Silva et al., 2018; Fantini et al., 2019).

The first parameter (number of leaves) indicated a slight but statistically significant reduction of flowering time (i.e. early flowering) in single *pif1a* and double *pif1a pif1b* mutants, but not in *pif1b* plants (Fig. R10). By contrast, the second parameter (number of days) showed no differences between genotypes (Fig. R10). A number of other studies in the literature report differences in flowering when using one of the evaluated parameters but not when using the other (Calvert, 1959; Giliberto et al., 2005).



**Figure R10. Flowering time in tomato *pif1* mutants.** Plants were grown on soil in the greenhouse under long day conditions. Error bars indicate SD of at least 11 different plants. Asterisks mark statistically significant changes in the indicated genotype compared to the WT according to one-way ANOVA (\* =  $p < 0.05$ ).

- A. Number of leaves in the plant when the first flower reached anthesis.
- B. Number of days from sowing until the first flower reached anthesis.

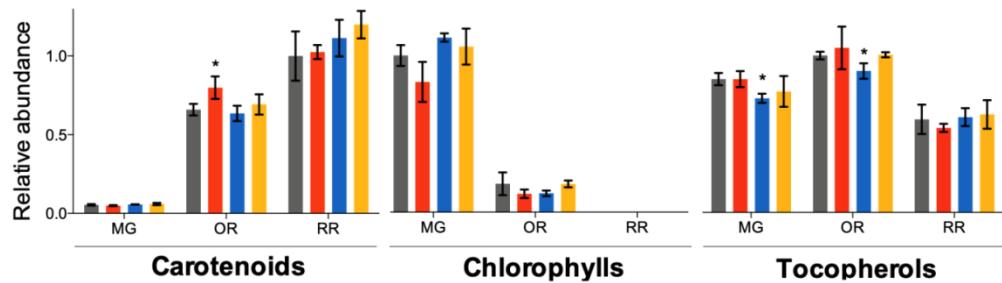
### 3.5. Fruit development

One of the most striking features of tomato is the capacity to develop fleshy fruits that acquire organoleptic and nutritional value as they ripe (Gómez et al., 2014). Despite the obvious differences between tomato and *Arabidopsis* fruits, many of the genes known to be involved in the control of tomato fruit development and

ripening are homologous to *Arabidopsis* genes controlling different developmental processes (Itkin et al., 2009; Gapper et al., 2013; Seymour et al., 2013; Pesaresi et al., 2014). In particular, genes identified to participate in light signaling in *Arabidopsis* were later found to be involved in fruit development and ripening in tomato (Monte et al., 2004; Bianchetti et al., 2018b, 2018a; Lupi et al., 2019). Using our tomato mutants defective in PIF1a and PIF1b, we aimed to investigate possible roles of these two homologs in different processes associated with tomato fruit development.

### 3.5.1. Isoprenoid accumulation

PIF1a was previously found to repress the expression of *PSY1* in green tomato fruits in order to prevent an early boost of carotenoids during ripening (Llorente et al., 2016b). Fruits from transgenic lines with a partially silenced *PIF1a* gene were found to accumulate higher levels of carotenoids when ripe (Llorente et al., 2016b), and a similar phenotype has been recently reported in *PIF4*-silenced fruits (Rosado et al., 2019). The levels of carotenoids but also other related isoprenoid metabolites reported elsewhere to be regulated by PIFs (chlorophylls and tocopherols) were quantified by HPLC in WT and mutant fruits at three different ripening stages: mature green (MG), orange (OR) and red ripe (RR). Strikingly, virtually no differences were found in the levels of any of these metabolites between WT and mutant lines in any of the stages analyzed (Fig. R11).



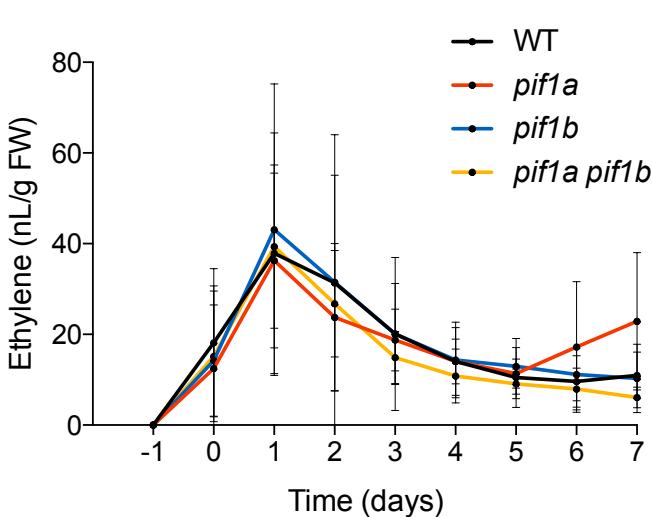
**Figure R11. Isoprenoid levels in fruits of tomato *pif1* mutants at different stages of ripening.** The indicated metabolites were quantified in 3 different pools of 4 fruits from plants grown on soil in the greenhouse under long day conditions. Error bars indicate SD of the 3 biological replicates. Asterisks mark statistically significant changes in the indicated genotype compared to the WT according to t-student test (\* =  $p < 0.05$ ).

### 3.5.2. Ethylene production

Ripening in climacteric fruits such as tomato is mainly controlled by the hormone ethylene (Gane, 1934; Giovannoni, 2004). Because an important crosstalk between PIFs and many plant hormones (including ethylene) have been

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described in *Arabidopsis* (Lau and Deng, 2010; de Lucas and Prat, 2014), we next tested whether ethylene metabolism was affected during fruit ripening of the tomato PIF1-defective mutant lines by GC-MS (Pereira et al., 2017). We found no significant differences between genotypes (Fig. 12), concluding that none of the tomato PIF1 homologs regulates ethylene production during fruit ripening.

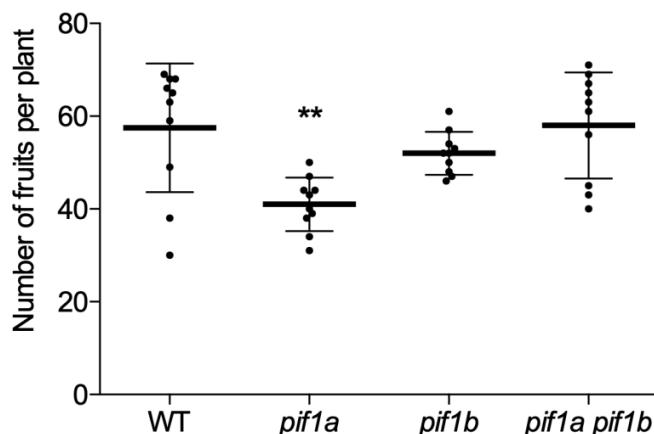


**Figure R12. Ethylene levels during fruit ripening in tomato *pif1* mutants.** Fruits from plants grown on soil in the greenhouse under long day conditions were harvested at the MG stage and transferred to sealed 50 mL tubes (one fruit per tube). Ethylene was quantified every day from the headspace inside the tube after one hour of incubation. Time was set at 0 when fruits reached the breaker (BR) stage. The graph plots results from one day before BR to 7 days after BR. Error bars indicate SD of at least 5 different fruits per timepoint.

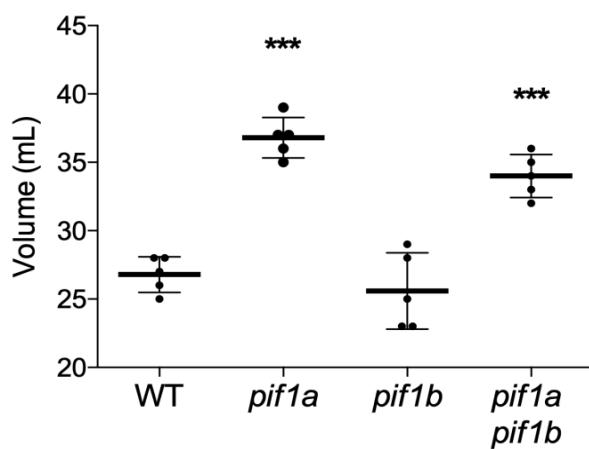
### 3.5.3. Fruit yield and size

Recently, it was shown that the down-regulation of PIF4 leads to a decrease in fruit yield and size, concluding that PIF4 is involved in the regulation of these characters (Rosado et al., 2019). In order to test whether the tomato PIF1 homologs might also participate in the regulation of fruit yield and size, we quantified the number and volume of fruits produced by the *pif1* mutant lines. Interestingly, *pif1a* mutant plants produced less fruits than WT controls, while no differences were found in the case of *pif1b* or *pif1a pif1b* lines (Fig. R13).

Fruit size was estimated by measuring the volume of groups of 10 fruits harvested from the plant at the RR stage. Opposite to that observed in the case of PIF4-silenced fruits (Rosado et al., 2019), *pif1a* and *pif1a pif1b* fruits were found to be bigger than WT or *pif1b* fruits (Fig. R14).

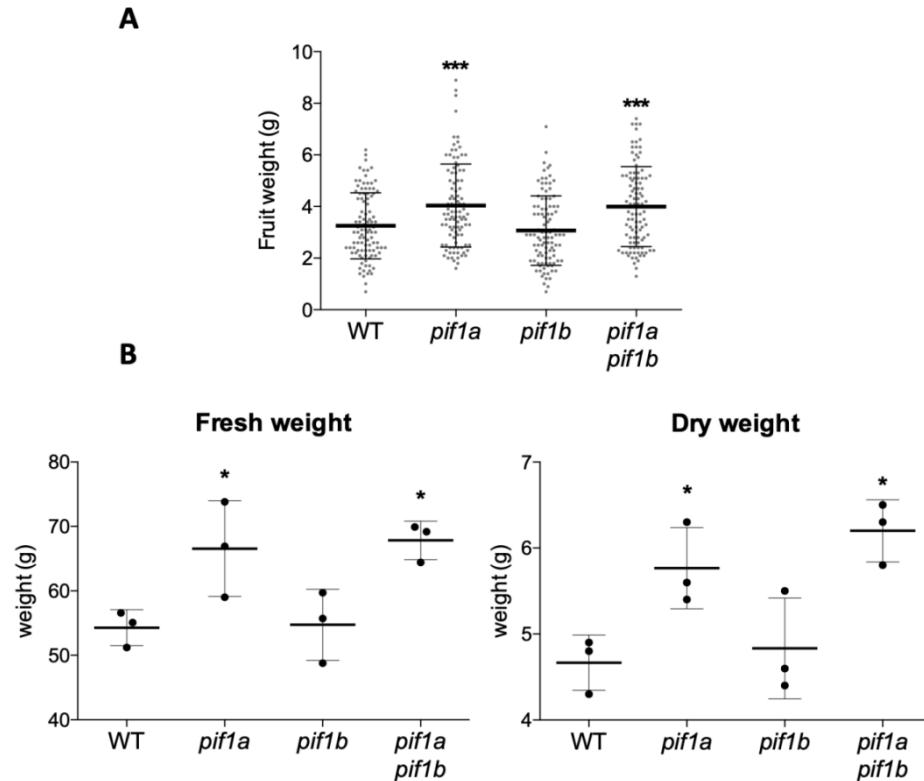


**Figure R13. Fruit yield in tomato *pif1* mutants.** Plot represents the total number of fruits produced by individual 19-week-old plants grown on soil in the greenhouse under long day conditions. Error bars indicate SD of at least 10 different plants. Asterisks mark statistically significant changes in the indicated genotype compared with WT according to one-way ANOVA (\*\* =  $p < 0.01$ )



**Figure R14. Fruit volume in tomato *pif1* mutants.** Plot represents the volume of 5 groups of 10 fruits each. Fruits were collected at the RR stage from plants grown on soil in the greenhouse under long day conditions. Error bars indicate SD. Asterisks mark statistically significant changes in the indicated genotype compared with WT according to one-way ANOVA (\*\*\* =  $p < 0.001$ )

We next measured the weight of 100 individual RR fruits from each genotype and confirmed that single *pif1a* and double *pif1a pif1b* mutant plants developed not only bigger but also heavier fruits (Fig. R15A). These differences might derive from two different mechanisms. A first option is that all genotypes produce the same number of fruit cells, but those from PIF1a-deficient fruits are bigger. In this case, it might be expected that the dry weight of *pif1a* and *pif1a pif1b* fruit would be similar to that of WT or *pif1b* fruit, assuming that the increase in cell size mostly relies on water accumulation. Another possibility is that *pif1a* and *pif1a pif1b* fruits produce more cells and hence their dry weight might be higher. By weighting groups of fresh fruits before and after drying them out in an oven, we confirmed that the difference in weight in PIF1a-defective fruits was maintained after drying the fruits (Fig. R15B), which means that it is not due to increased water content but results from an enhanced accumulation of dry matter.



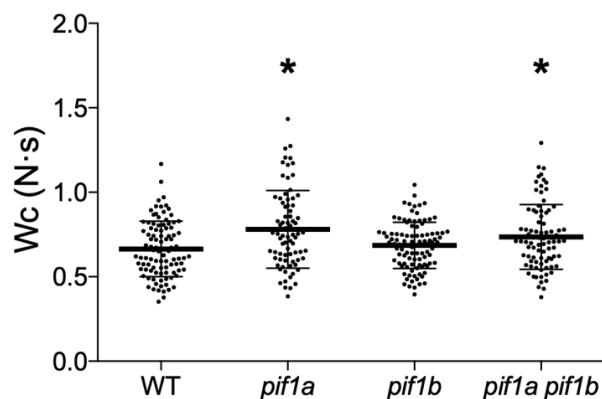
**Figure R15. Fruit weight in tomato *pif1* mutants.**

- A. Weight of 100 individual RR fruits of each genotype. Error bars indicate SD.
- B. Weight of 3 groups of 15 RR fruits before (fresh) and after (dry) incubation in an oven until complete loss of water. Error bars indicate SD.

Asterisks mark statistically significant changes in the indicated genotype compared with WT according to one-way ANOVA (\* =  $p < 0.05$ , \*\*\* =  $p < 0.001$ )

### 3.5.4. Fruit texture

The observation that *pif1a* mutants produced more and bigger fruits prompted us to investigate whether the texture of ripe PIF1a-deficient fruits was also altered. In order to measure fruit hardness, we used a texture analyzer fitted with 50 mm plate probe to perform a compression test (Kabas and Ozmerzi, 2008; Camps and Gilli, 2017). This test provides a texture parameter called Wc, which is the mechanical work needed to reach a 5% deformation of the fruit. Higher Wc values are obtained when resistance to deformation is higher, hence the fruit is harder. This is what we observed in the case of *pif1a* and *pif1a pif1b* mutants (Fig. R16). As a summary, PIF1a appears to negatively regulate fruit growth and to promote ethylene-independent fruit softening, whereas PIF1b does not have a major role in these processes.

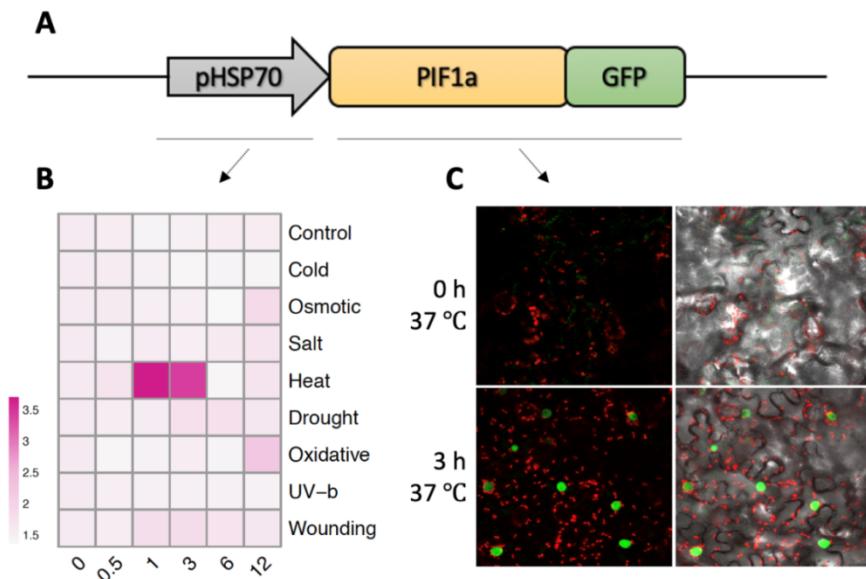


**Figure R16. Fruit texture in tomato *pif1* mutants.** Plot represents Wc measurements (compression tests) of 100 individual RR fruits of each genotype. Error bars indicate SD. Asterisks mark statistically significant changes in the indicated genotype compared with WT according to one-way ANOVA (\* =  $p < 0.05$ )

#### 4. A heat-induced construct for rapid but transient *PIF1a* overexpression in transgenic tomato lines

The phenotypic analysis of CRISPR/Cas9 mutants indicated that PIF1a is the PIF1 homolog with a more widespread role in tomato. Some processes (like germination or de-etiolation) seem to be regulated by PIF1a together with PIF1b, while others (such as root hair elongation, flowering time or fruit development) appear to be mainly controlled by PIF1a. The next step in the work was to unveil which genes are regulated by PIF1a to control these processes. Because we were interested in direct effects of PIF1a on gene expression, we designed a construct to induce *PIF1a* overexpression but only for a short period of time (Fig. R17). After thoroughly searching the literature, we decided to use the promotor of the *Arabidopsis HSP70b* (*HEAT SHOCK PROTEIN 70B*; AT1G16030) gene (Fig. R17A), which is only expressed after a heat shock (Li et al., 1999; Dong Yul Sung et al., 2001). The *HSP70b* gene is very weakly expressed under normal growth conditions (25°C). After exposure to higher temperatures (37°C), it shows an intense but transient peak of expression that is not detected with any other stress (Fig. R17B), unlike that observed with other genes of the same family (Dong Yul Sung et al., 2001). This *HSP70b* promotor has been previously used to drive transient and inducible expression in plants (Orzaez, 2005; Sarrión-Perdigones et al., 2013).

## Results



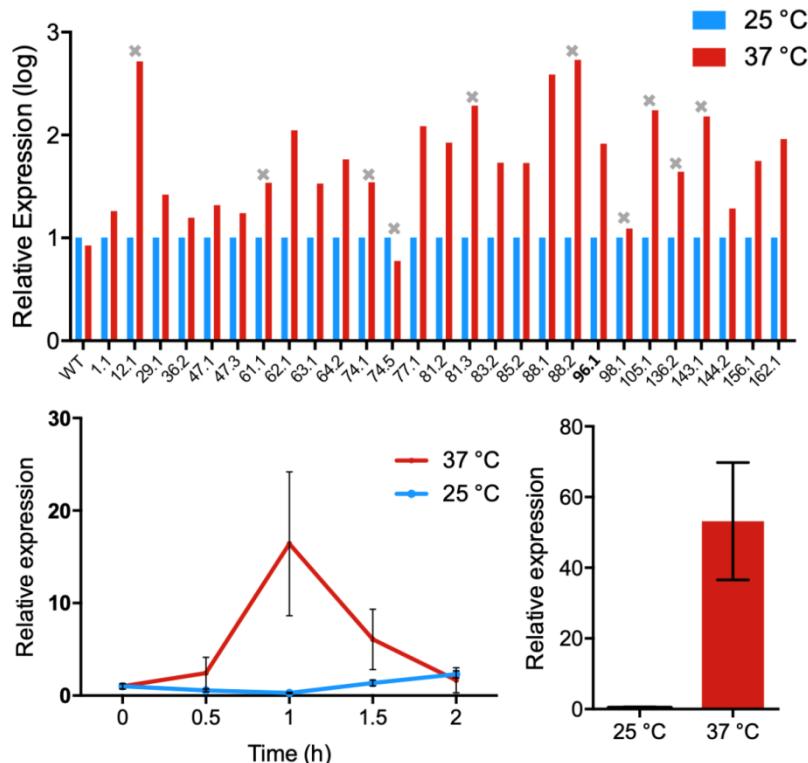
**Figure R17. Design and testing of the construct to induce PIF1a accumulation.**

- Schematic representation of the engineered construct.
- Heat map of *HSP70B* transcript levels in WT *Arabidopsis* plants before and after exposure to different stress conditions. Data were taken from the Bio-Analytic Resource for Plant Biology (BAR, University of Toronto).
- Confocal images of *N. benthamiana* leaves agroinfiltrated with the construct shown in A before and after exposing them to 37 °C for 3 hours. Note: Laser intensity was increased to identify all the fluorescent nuclei, hence the saturating signal in the picture makes nuclear bodies indistinguishable.

Additionally, we fused the sequence coding for PIF1a to GFP in order to be able to detect the transgenic protein (Fig. R17A). The resulting *HSP70b:PIF1a-GFP* construct was used to agroinfiltrate *N. benthamiana* leaves and analyze the production of the PIF1-GFP protein by confocal microscopy. After agroinfiltration, we maintained the plants in the greenhouse (i.e. long day, 25°C day and 21°C night) for 3 days. After this, we collected the agroinfiltrated leaves and observed them under a confocal microscope (Fig. R17C), as expected, no GFP fluorescence signal was detected, as the *HSP70b* promoter was supposed to be virtually silent under these conditions. Then we incubated the cut leaves for 3 hours at 37 °C. Fluorescence signal corresponding to the PIF1a-GFP protein was then detected in nuclei. These results together confirmed that the *HSP70b:PIF1a-GFP* construct was working.

The next step was to transform tomato plants with the construct in order to create transgenic lines expressing *PIF1a* in an inducible way. After the transformation we got 28 T1 independent lines that were confirmed by PCR to contain the construct. In order to screen them for *PIF1a* levels, we collected leaves from all the lines, incubated them at 25°C and 37°C for 90 min, and performed qPCR using primers to detect *PIF1a* transcripts (Fig. R18A). Expression levels of *PIF1a*

in transgenic leaves were similar to those in WT leaves when incubated at 25°C but higher when incubated at 37°C (with only two exceptions: lines 74.5 and 98.1). The induction levels were variable among lines, with some of them showing higher than 2-fold induction. Unfortunately, not all the transgenic plants produced seeds, so we only kept working with lines that exhibited a clear heat-dependent induction and produced seeds.



**Figure R18. Screening of *HSP70b:PIF1a-GFP* inducible lines.**

- qPCR analysis of *PIF1a* expression in leaves from WT plants and T1 transgenic lines upon incubation at the indicated temperatures for 90 min. Gray crosses indicate the lines that failed to produce seeds.
- Time-course induction of *PIF1a* expression in leaves of transgenic plants. Leaves were cut in two halves, each of which was incubated at the indicated temperatures for the indicated times. Error bars indicate SD of 3 biological replicates.
- Expression levels of *PIF1a* in transgenic tomato MG fruits. Fruits were cut in two halves, each of which was incubated at the indicated temperatures for 1h. Error bars indicate SD of 3 biological replicates.

After that, we performed a time-course of *PIF1a* induction in leaves and fruits from the selected lines. We divided transgenic leaves in two halves, and each of them was incubated at a different temperature: 25°C or 37°C. A representative result is shown in Fig. R18B. As observed in the case of the endogenous *Arabidopsis HSP70b* gene (Fig. R17B), a peak of expression of *PIF1a* was observed at 1 h of incubation at 37°C. In transgenic tomato leaves, the *HSP70b*-directed expression of *PIF1a* returned to basal levels only 2h after induction, while *HSP70b*

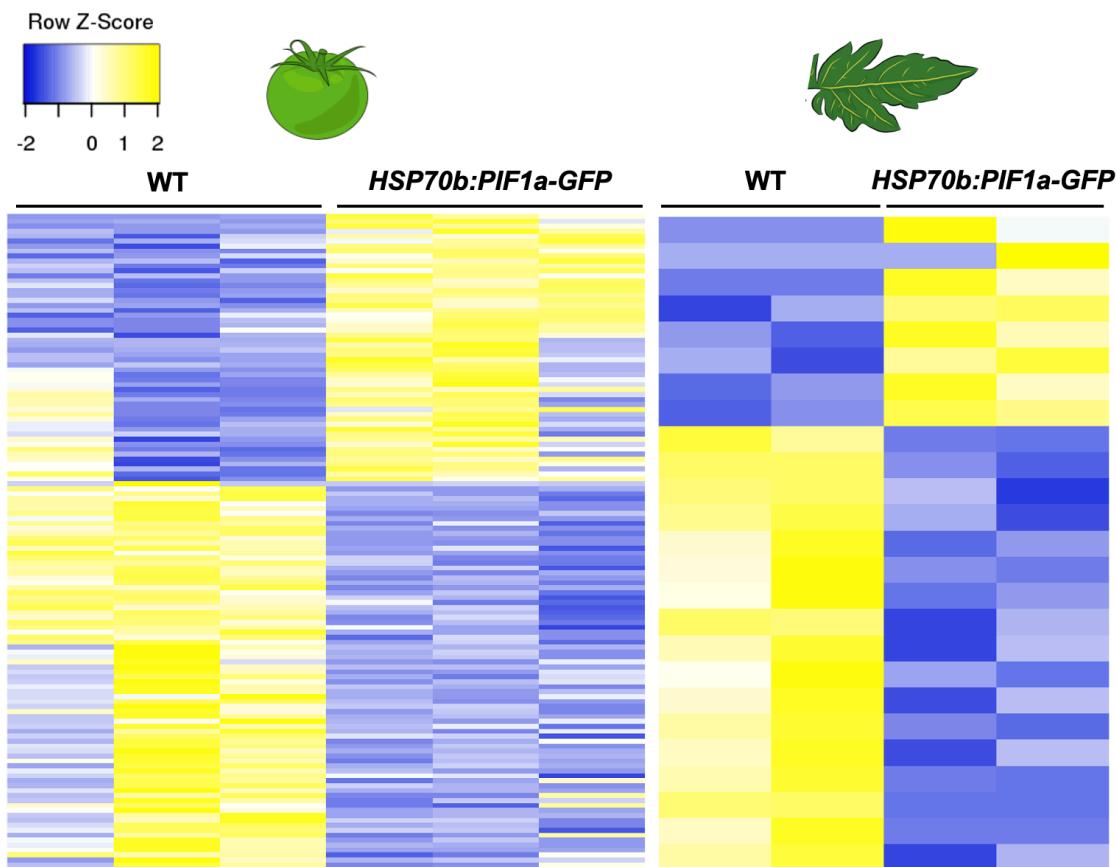
was still high 3h after induction in *Arabidopsis*. However, based on the results with *N. benthamiana* leaves (Fig. R17C), it is expected that the PIF1a-GFP protein remains in induced cells much longer than the transcripts. Similar experiments with transgenic MG fruit confirmed that the induction of *PIF1a* expression was also taking place in fruit tissues (Fig. R18C). The transgenic line that showed the most stable expression level and more consistent induction profiles and generated good amount of seeds was 96.1. The rest of the experiments were performed with this line.

## 5. RNA-seq experiments show that transient up-regulation of PIF1a only has a relatively minor impact on gene expression

To identify PIF1a target genes, we decided to perform RNA-seq experiments in both leaves and fruits from the transgenic *HSP70b:PIF1a-GFP* 96.1 line. In both cases tissues were detached from the transgenic line and WT plants and incubated for 2 h at 37 °C. We reasoned that the first hour should allow the transgene expression to peak (Fig. R18B) whereas the second hour, when the PIF1a-GFP was expected to remain active (Fig. R17C), should allow this transcription factor to bind to its target promoters and modulate the expression of its direct target genes.

### 5.1. Identification of genes regulated by PIF1a in fruit and leaf tissues

We extracted RNA from triplicates of MG fruits and duplicates of leaves from the two genotypes (WT and transgenic) after incubation at 37 °C and sent the samples to sequence polyA-containing transcripts. All the RNA quality control analyses, library generation, and mRNA sequencing was performed by Sequentia Biotech SL (Barcelona, Spain). Then, we used the Artificial Intelligence RNA-seq (AIR) platform of this company (<https://transcriptomics.sequentiabiotech.com/>) to analyze the raw data and identify differentially expressed genes (DEGs) by comparing WT and transgenic samples. By using both DEseq2 and edgeR for statistical support (both FDR < 0.05), a gene list of 132 DEGs in fruit and 25 in leaf was obtained (Fig. R19 and annexed Table A2). In fruit, 54 genes were up-regulated in the induced transgenic line and 78 were down-regulated. In leaf, 8 genes were up-regulated, while 17 were down-regulated (Fig. R20).



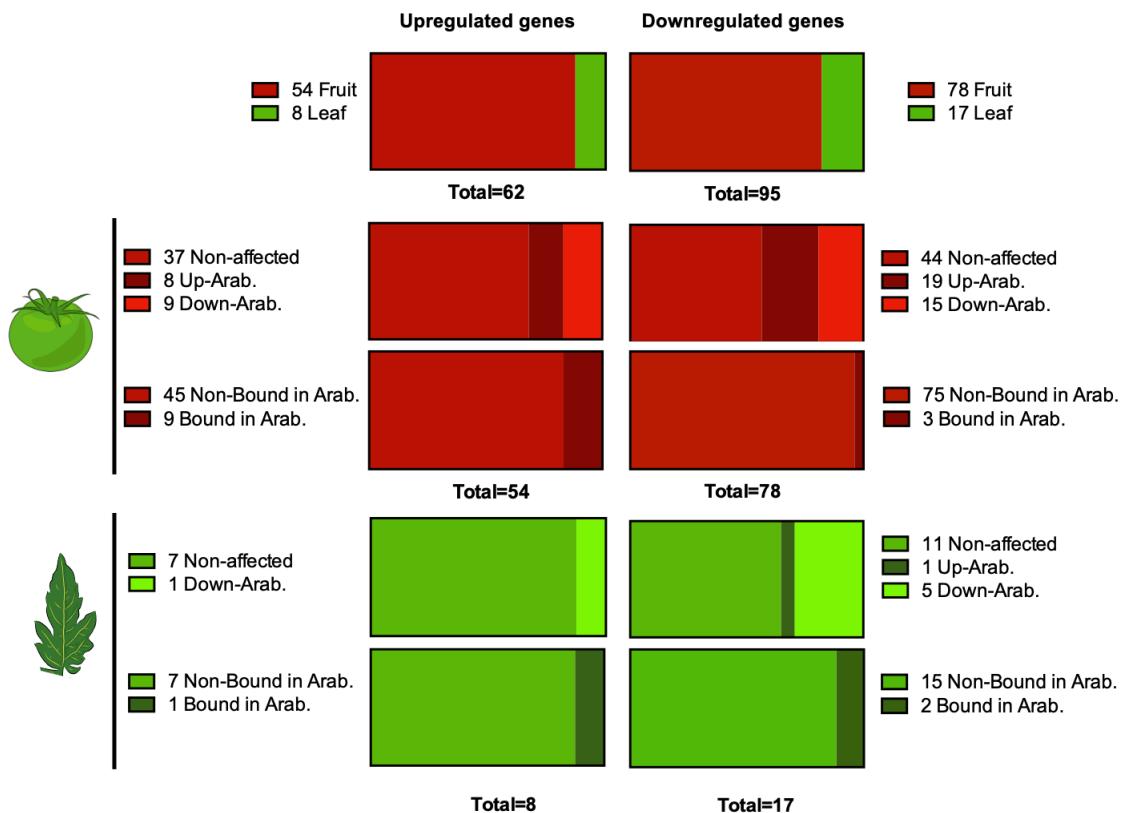
**Figure R19. DEGs identified by DEseq2 and edgeR analysis of PIF1a-overexpressing and WT samples.** Heatmaps represent the normalized expression levels (Z-score) of the identified DEGs in the three replicates from MG fruit (A) and the two replicates from leaf (B).

Since PIFs have been found to be master regulators of multiple developmental and metabolic processes, we found especially interesting the DEGs encoding transcription factors, enzymes, and hormone synthesis/response factors (Table A2). These categories include 55 genes out of 132 DEGs in fruit (41.7%) and 13 out of 25 in leaf (52%). Strikingly, no overlapping was found between DEGs identified in fruits and leaves. This could indicate very specific roles for PIF1a in each tissue.

To next investigate whether the PIF1a-regulated genes in tomato fruits and leaves were homologs of those regulated by PIF1 in *Arabidopsis* we compared our RNA-seq results with other genome-wide analyses performed with *Arabidopsis pif1* mutants (Oh et al., 2009; Chen et al., 2013; Shi et al., 2013). In order to do that, we first performed a global BLAST analysis of protein sequences that allowed to identify the *Arabidopsis* closest homologs of the identified tomato DEGs. Regarding the fruit DEGs, from the 54 genes up-regulated by PIF1a in tomato only 8 homologs were up-regulated by PIF1 in the *Arabidopsis* experiments; and just 15 out of 78 PIF1a-down-regulated genes showed a down-regulation in

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*Arabidopsis* (Fig. R20). We found a very similar proportion of genes that showed an opposite effect of PIF1a and PIF1 on the expression in tomato and *Arabidopsis* (Fig. R20). In total, 38% of the 132 tomato fruit DEGs were found to have an altered expression in *Arabidopsis pif1* mutants. We also searched the gene lists for direct target genes of *Arabidopsis* PIF1 identified by ChIP-seq (Oh et al., 2009; Pfeiffer et al., 2014). Only 18 of the 132 tomato fruit DEGs (14%) had *Arabidopsis* homologs reported to be direct PIF1 target genes (Fig. R20).

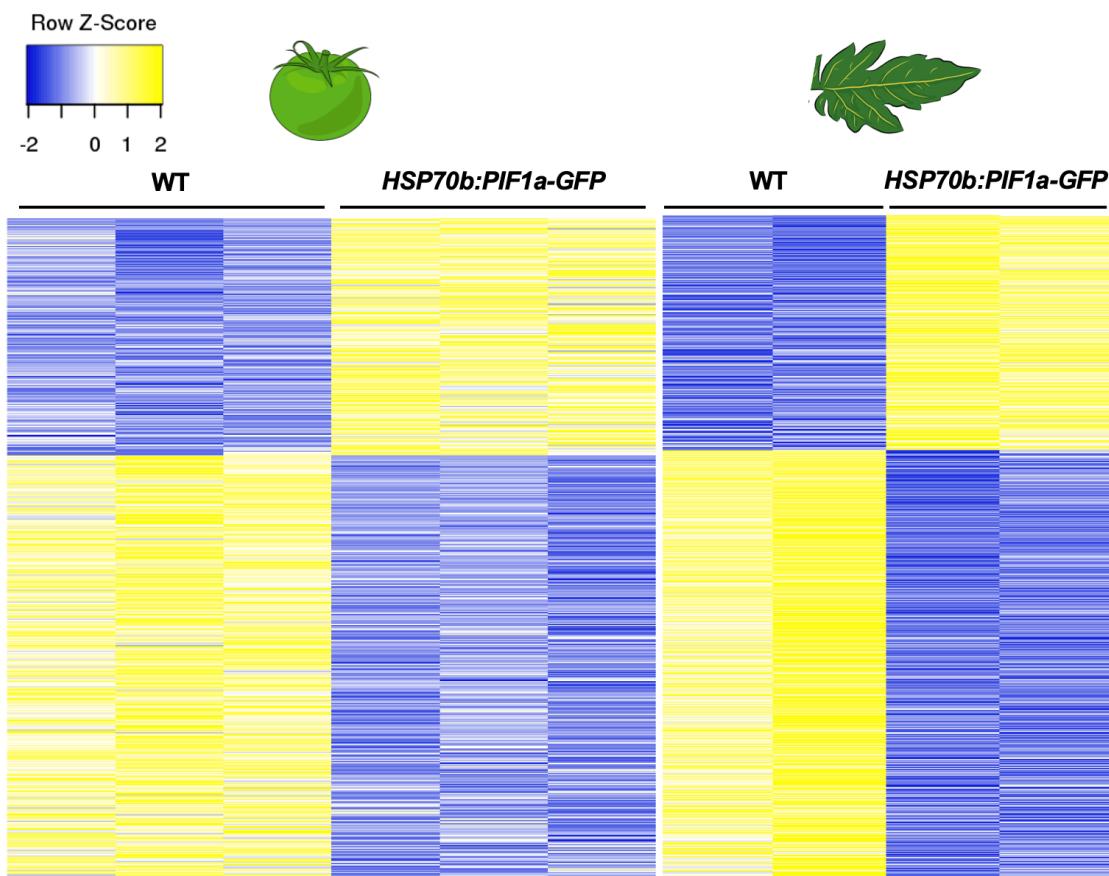


**Figure R20. Comparison between genes regulated by PIF1a in tomato (identified by DEseq2 and edgeR) and PIF1 in *Arabidopsis*.** Upper boxes represent the proportion of tomato DEGs found in fruit and leaf experiments. Lower boxes represent the proportion of tomato DEGs from fruits or leaves that are also differentially expressed in *Arabidopsis* lines with altered PIF1 levels (middle rows) or were found to be direct PIF1 targets by ChIP (bottom rows).

The same trend (although with smaller numbers) was found in leaves (Fig. R20). None of the 8 up-regulated genes in tomato was found to be up-regulated by PIF1 in *Arabidopsis* RNA-seq experiments, and just 1 of them showed a down-regulation. Regarding the 11 down-regulated genes in leaf, 4 were also down-regulated in *Arabidopsis* and 2 were up-regulated. In total, 28% of the tomato leaf DEGs were also DEGs in *Arabidopsis* and 16% (4 out of 25) were homologs of direct PIF1 targets (Fig. R20).

## 5.2. An alternative analysis to identify DEGs

Because the number of DEGs statistically supported by DEseq2 and edgeR analyses were much lower than we expected, we decided to re-analyze the RNA-seq result using a less restrictive method. First, we filtered the whole gene list to select for genes that were up- or down-regulated with a mean fold-change (FC) value of 1.5 or higher. From this list, we then discarded the genes that had overlapping FC standard deviation values. After this hand-curated analysis, we got the set of genes represented in Fig. R21 and listed in annexed Table A3. The list contained 598 DEGs in fruit (213 up-regulated and 385 down-regulated) and 1098 DEGs in leaf (388 up-regulated and 710 down-regulated). The number of overlapping DEGs between fruit and leaf samples was only 11 and 25 (up-regulated and down-regulated, respectively), which again supports the idea of very specific and different roles of PIF1a in different tissues (Fig. R22).

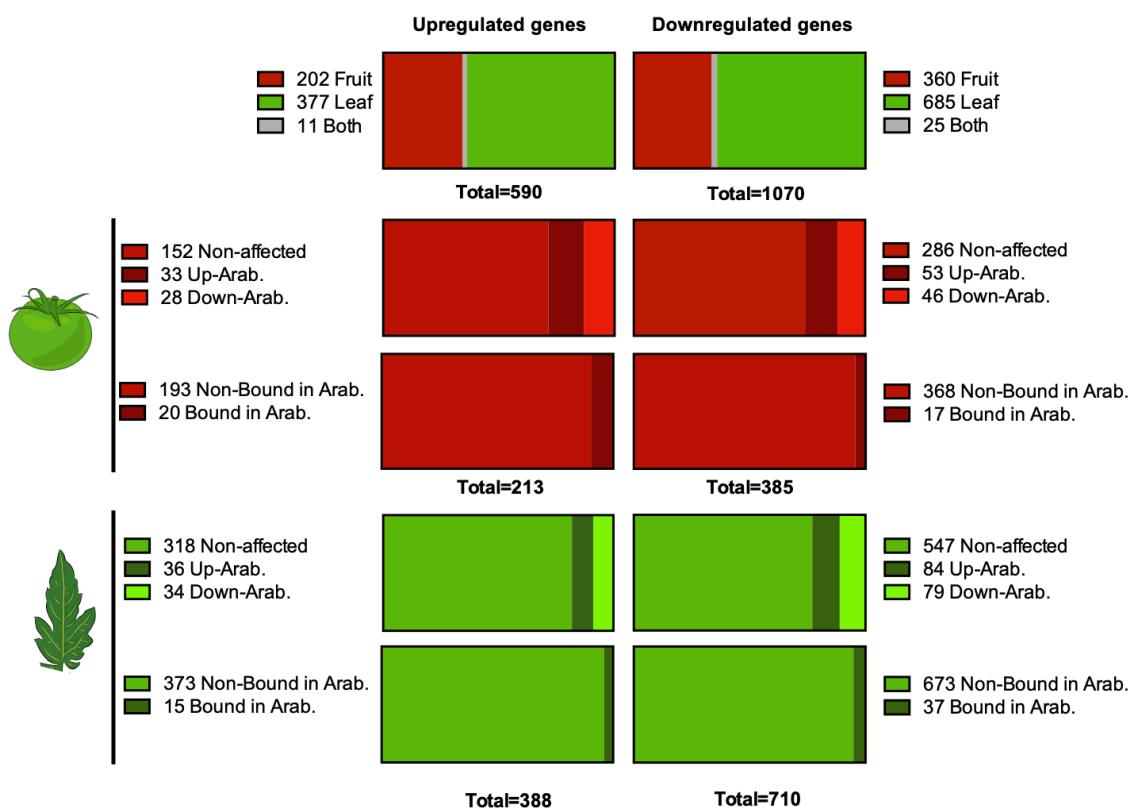


**Figure R21. DEGs identified by manually-curated analysis of PIF1a-overexpressing and WT samples.** Heatmaps represent the normalized expression levels (Z-score) of the identified DEGs in the three replicates from MG fruit (A) and the two replicates from leaf (B).

## Results

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From 213 up-regulated DEGs obtained from the fruit experiment, 33 were up-regulated and 28 were down-regulated in *Arabidopsis* experiments. And from the 286 down-regulated DEGs, 53 were up-regulated and 46 were down-regulated by *Arabidopsis* PIF1 (Fig. R22). In total, 28% of the 598 tomato fruit DEGs were found to have an altered expression in *Arabidopsis pif1* mutants and 14% had *Arabidopsis* homologs reported to be direct PIF1 target genes (Fig. R22). In leaves, from 388 up-regulated tomato DEGs 34 were up-regulated and 33 were down-regulated in *Arabidopsis*, and from the 710 down-regulated DEGs 84 were up-regulated and 79 were down-regulated (Fig. R22). These numbers represent a 24% of the 1098 tomato leaf DEGs being misexpressed in *Arabidopsis pif1* mutants, with 15% of the tomato DEGs having *Arabidopsis* homologs known to be bound by PIF1 (Fig. R22). All these proportions are very similar to those obtained with the first (short) list.

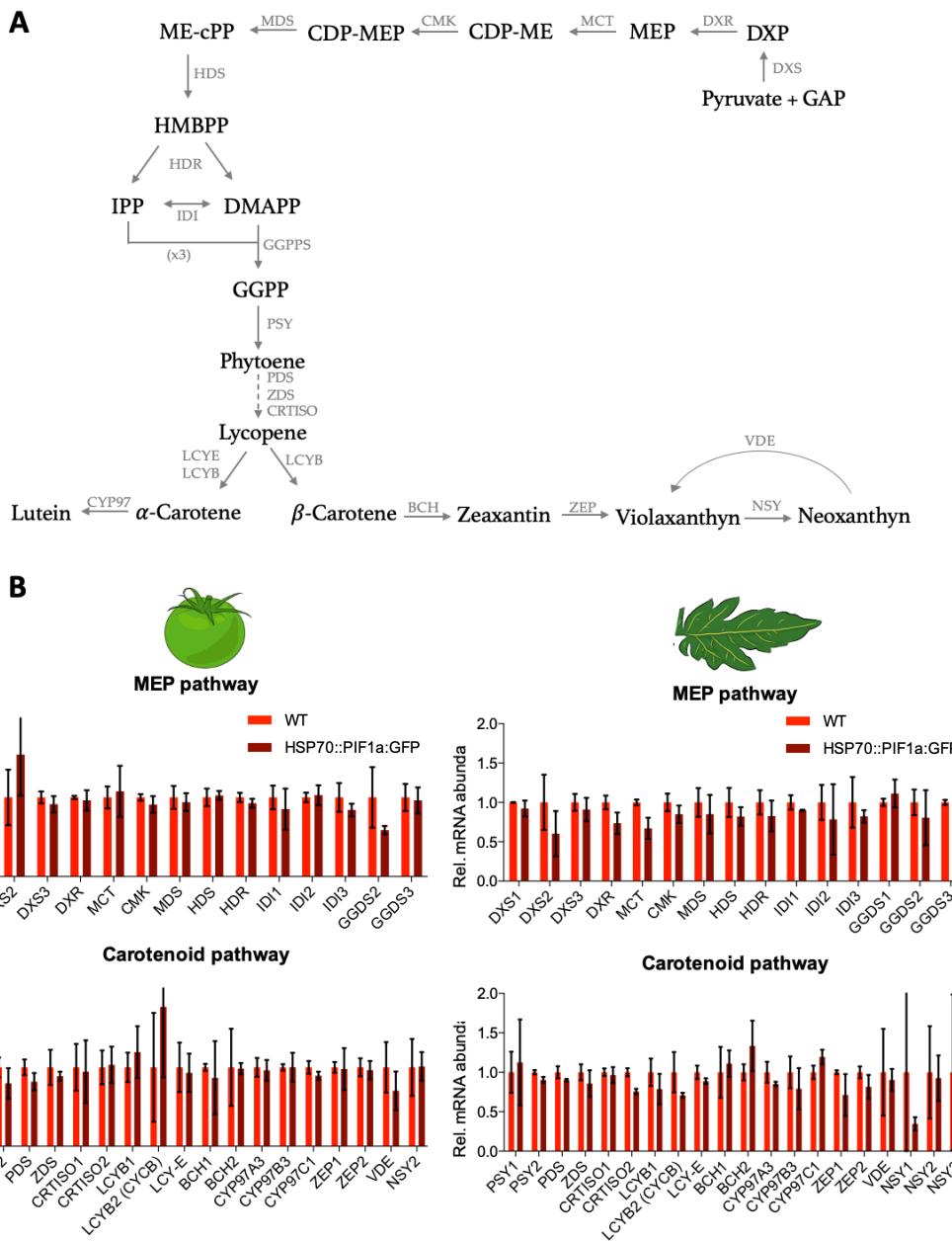


**Figure R22. Comparison between genes regulated by PIF1a in tomato (manually identified) and PIF1 in *Arabidopsis*.** Upper boxes represent the proportion of tomato DEGs found in fruit and leaf experiments. Lower boxes represent the proportion of tomato DEGs from fruits or leaves that are also differentially expressed in *Arabidopsis* lines with altered PIF1 levels (middle rows) or were found to be direct PIF1 targets by ChIP (bottom

### 5.3. Up-regulation of PIF1a has no (major) impact on genes involved in carotenoid biosynthesis

PIF1a was identified as a negative regulator of carotenoid biosynthesis in tomato (Llorente et al., 2016b). In contrast, the results obtained in this thesis showed that the *pif1a* loss-of-function mutant has WT levels of carotenoids in fruits (Fig. R11) and, most strikingly, lower levels in light-grown seedlings (Fig. R7). To complement these observations, we checked the expression levels of carotenoid biosynthetic genes (including those of the MEP pathway) in the leaf and MG fruit samples analyzed by RNA-seq (Fig. R23). The accumulation of PIF1a in the inducible lines did not significantly impact the expression of carotenoid-related genes (Fig. R23). Only in the case of some genes, such as those encoding GGDS2 and VDE in fruits or DXS2, DXR, MCT, CRTISO2 and LCYB2 in leaves, we detected a trend towards downregulation. In the case of *PSY1*, a gene reported to be repressed by PIF1a by direct binding (Llorente et al., 2016b), transcripts levels were also decreased in fruits of the induced line (Fig. R23). In any of these cases, however, the changes were found to be significant as they did not pass the threshold set for our two statistical analyses.

## Results



**Figure R23. Transcript levels of genes encoding enzymes involved in carotenoid synthesis in our RNAseq samples.**

- A. Schematic representation of MEP and carotenoid pathways. Solid lines indicate steps carried out by a single enzyme while dashed lines indicate multiple enzymatic steps.
- B. Expression levels of the indicated genes in heat-treated MG fruits and leaves from WT and *HSP70b:PIF1a-GFP* lines. Error bars indicate SD.

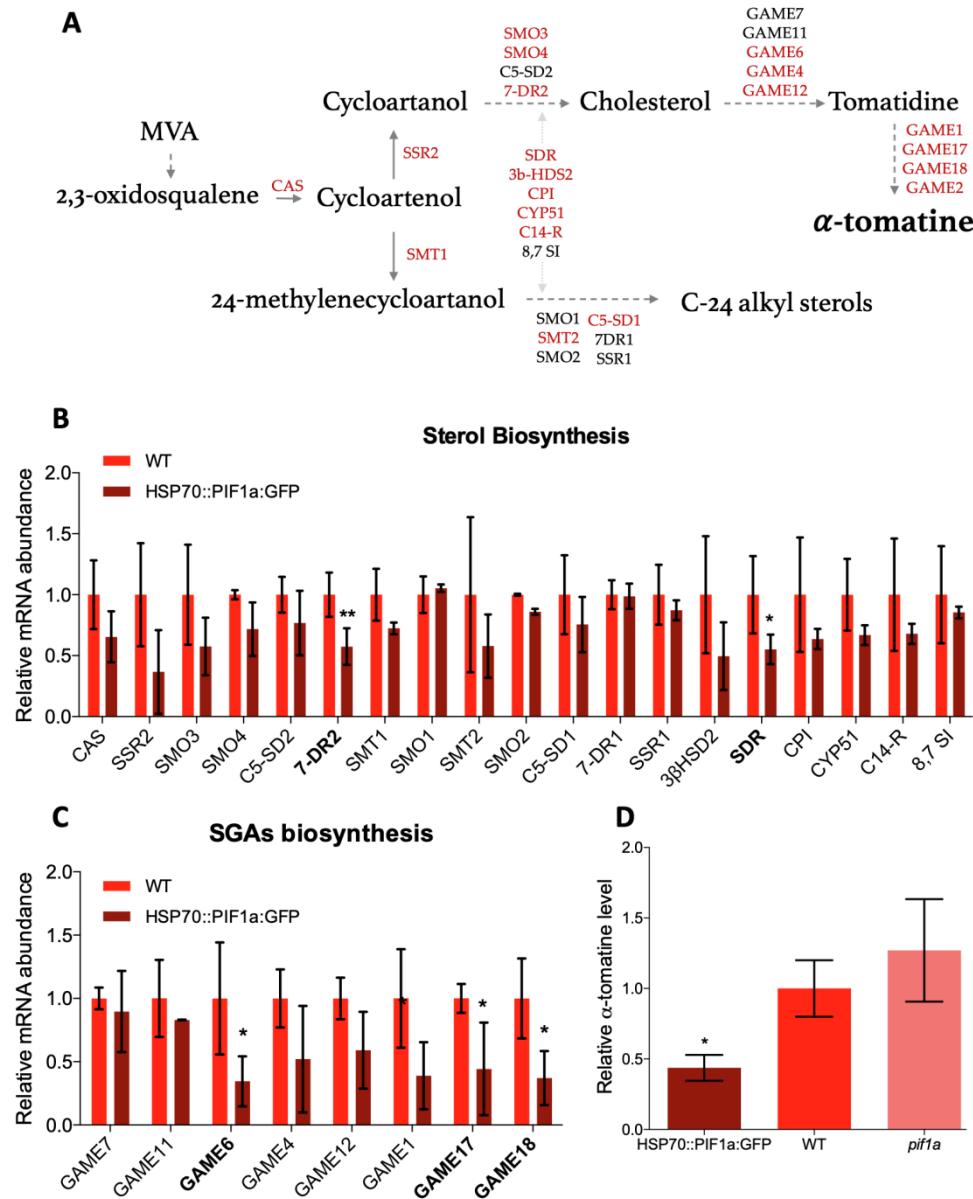
#### 5.4. PIF1a represses SGA biosynthesis

To investigate whether PIF1a had an effect on plant metabolism beyond carotenoid biosynthesis, we looked for enriched pathways in the manually-curated lists of DEGs. We used the KEGG platform (<https://www.kegg.jp>) to find metabolic pathways that could be globally altered by the PIF1a induction. We found a downregulation of several genes of the phenylpropanoid pathway by the overexpression of PIF1a in both tissues, fruits and leaves. Several steps of the pathway that converts isoprenoid precursors produced by the mevalonic acid (MVA) pathway into phytosterols and derived SGAs were also found to be downregulated in leaves.

Since PIF3 is known to regulate SGA metabolism (Wang et al., 2018), we decided to further investigate whether PIF1a might also be involved in the control of phytosterol and SGA biosynthesis. First, we checked transcript levels for all the biosynthetic enzymes of both pathways in the leaf RNA-seq dataset (Fig. R24A). We found that several genes encoding enzymes involved in the production of cholesterol, the phytosterol precursor of SGAs, were down-regulated in transgenic samples with increased PIF1a levels (Fig. R24A). Two of them, *7-DR2* and *SDR*, were identified as DEGs according to the DEseq2 and edgeR algorythms or our own manually-curated analysis (Fig. R24B). Most *GAME* genes, encoding the biosynthetic enzymes that produce SGAs from cholesterol (Sonawane et al., 2016), were also down-regulated, including DEGs identified in our analysis such as *GAME6*, *GAME17* and *GAME18* (Fig. R24C). Some of these genes are directly repressed by PIF3 (Wang et al., 2018).

To test if the PIF1a-mediated changes in gene expression resulted in reduced accumulation of SGAs in leaves, we quantified the levels of  $\alpha$ -tomatine (the main tomato SGA) in leaves from the *pif1a* mutant and the PIF1a inducible line after applying a heat shock (Fig. R24D). As expected based on the conclusion that PIF1a is a repressor of SGA biosynthesis,  $\alpha$ -tomatine levels were slightly (but not significantly) higher in PIF1a-defective leaves but significantly decreased when PIF1a levels were upregulated in heat-treated transgenic lines (Fig. R24D).

## Results



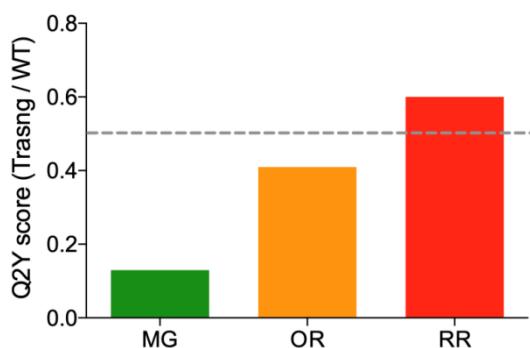
**Figure R24.** Transcript levels of genes encoding enzymes involved in phytosterol and SGA synthesis in our leaf RNAseq samples and SGAs levels in PIF1a-altered backgrounds.

- Schematic representation of phytosterol and SGA pathways from MVA to  $\alpha$ -tomatine. Solid lines indicate single enzymatic steps and dashed lines indicate multiple enzymatic steps. The enzymes indicated in red are those codified by genes that show a downregulation trend when PIF1a levels increase.
- Expression levels of cholesterol biosynthetic genes extracted from the RNA-seq data. Double asterisks (\*\*) mark statistically significant DEGs according to the DEseq2 and edgeR analysis. Single asterisk (\*) marks identified DEGs according to the manually-curated gene list (Same code in C). Error bars indicate SD.
- Expression levels of SGA biosynthetic genes extracted from the RNA-seq data.
- Quantification of the  $\alpha$ -tomatine in leaves with different PIF1a levels. Asterisk marks statistically significant changes compared with WT samples according to one-way ANOVA (\* =  $p < 0.05$ ). Error bars indicate SD.

## 6. PIF1a effect on fruit metabolism is stronger as the fruit ripens

Tomato fruit ripening is a complex developmental process from the metabolic point of view (Seymour et al., 2013; Tohge et al., 2014). Since PIF1a was found to regulate genes involved in the production of phenylpropanoids and isoprenoids in our RNAseq experiments, we next explored a possible role of this transcription factor in the fruit metabolome by performing a non-targeted metabolic profiling using Liquid Chromatography coupled to Mass Spectrometry (LC-MS). We compared the metabolic profile of fruits at MG, OR and RR stages from WT plants and the inducible lines during a 4-month research stay at Asaph Aharoni's lab, in the Weizmann Institute of Science (Rehovot, Israel).

There are two major steps in this analysis. The first one is to compare the general metabolomic profiles between samples. A statistical analysis that takes into account all the biological replicates was performed to calculate the Q2Y score. If this score is higher than 0.5, we can consider that the metabolic profiles between genotypes are different. We can also take this value as an estimation of how different the genotypes are between them (i.e. the higher the value the stronger the differences). As shown in Fig. R25, WT and transgenic lines with increased PIF1a levels were very similar at the MG stage. The same was observed in OR fruits, even though the differences between the two genotypes tended to be higher. Such differences became statistically significant in RR fruits (Fig. R25). We can therefore conclude that PIF1a has an effect on fruit metabolism that becomes more obvious as the fruit ripens, consistent with the increasing accumulation of PIF1a-encoding transcripts during fruit ripening.



**Figure R25. Statistical analysis of LC-MS experiment in different stages of fruit ripening.** Fruits at the indicated stages were collected and incubated during 3 hours at 37 °C, the first hour to reach the peak of expression and the other two to trigger the metabolomic changes due to PIF1a overaccumulation.

The second step will be to analyze which specific metabolites are differentially accumulated in each genotype in order to identify which pathways could be regulated by PIF1a during tomato fruit ripening. This step implies an extensive work in data analysis, and it is being currently performed in Asaph Aharoni's laboratory. We estimate to have these results in the following months.



## **DISCUSSION**



## 1. PIF1 homologs differentially regulate the same biological processes in tomato and *Arabidopsis*.

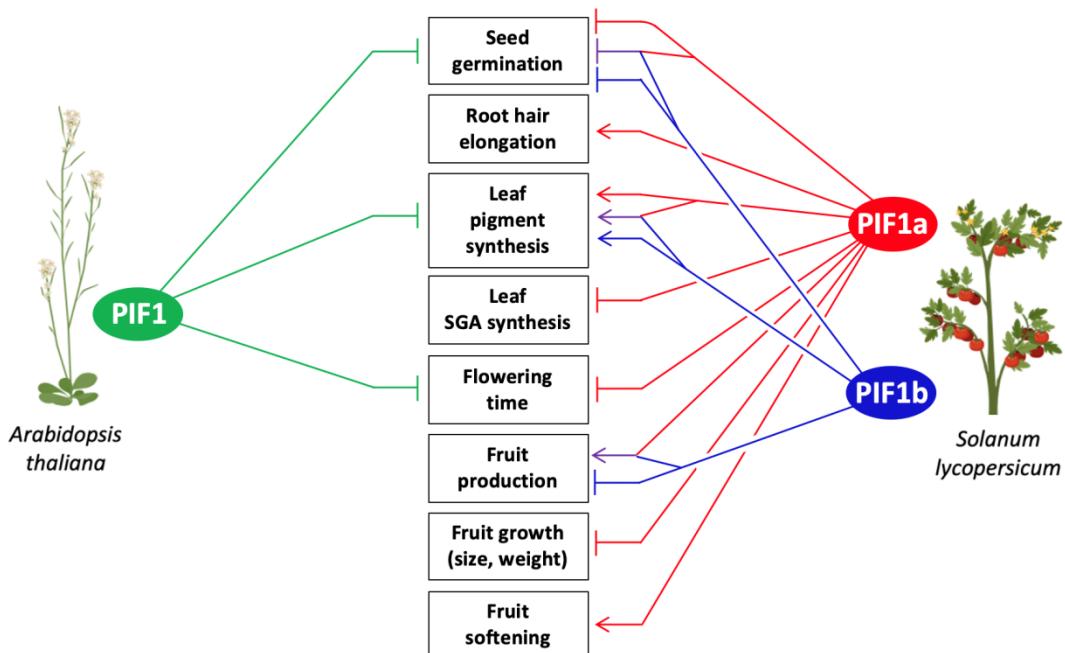
### 1.1. Seed germination and pigment biosynthesis

Several lines of evidence support the conclusion that duplicated copies of the *PIF1* gene in tomato have functionally diverged. The first one is the differential protein stability of PIF1a and PIF1b under different light conditions (Fig. R1). Our data suggest that the amino acid substitution in the APB-binding domain of PIF1b likely leads to a failure in the interaction with phyB, as PIF1b protein abundance does not decrease in R conditions or accumulates under FR. This result suggests that during evolution, PIF1b may have lost its capacity to transduce phyB-mediated light signals. Contrasting with this, PIF1b-defective lines showed light-dependent phenotypes during **seed germination** (Fig. R6) and **seedling de-etiolation** (Fig. R7).

It has been reported that phyB regulates the R-induction and FR-inhibition of seed germination in a single light pulse situation (Appenroth et al., 2006). In contrast, phyA would be involved in the induction and inhibition when the pulses were applied hourly (as we did in our experiment). Therefore, the lack of interaction with phyB would not be a problem to transduce the phyA-dependent light signal mediating the germination response under a multi-pulse experimental system. The involvement of phyA and other phys besides phyB in the control of photosynthetic pigment accumulation during seedling de-etiolation has been also described (Su et al., 2015). In fact, phyA has been described as the photoreceptor that makes a major contribution to initiating photomorphogenesis in *Arabidopsis* (Casal et al., 2003; Seo et al., 2004). This predominant role of phyA would also explain why PIF1b might regulate this process despite being unable to interact with phyB.

Light-dependent seed germination and pigment biosynthesis during seedling de-etiolation are also regulated by PIF1a. In fact, loss of any of the two PIF1 homologs produces the same phenotype than the lack of both in double mutants (Fig. R6 and R7). This made us think that both PIF1a and PIF1b are part of the same signal transduction pathway. We speculate that there might be a threshold amount of PIF1 activity needed to trigger the light signaling pathways required to inhibit germination or to accumulate pigments during seedling development. In WT lines, both PIF1a and PIF1b are required to reach this threshold. If one of the genes is mutated, the remaining amount of PIF1 activity would not be enough to normally trigger the process. Loss of all tomato PIF1 activity in double *pif1a pif1b* mutants would have the same effect observed in single mutants, i.e. when the threshold is not met by losing only one of the two genes. Another possible

scenario based on the BiFC result (Fig. R2) is that maybe PIF1a and PIF1b need to form a heterodimer to regulate the expression of the genes involved in seed germination and pigment accumulation during de-etiolation. So, when one of the elements of this complex is missing, these processes are affected (Fig. D1). This complex would be interacting with phyA through both of the elements or with phyB through just PIF1a.



**Figure D1. Schematic summary of proposed roles for PIF1 homologs in *Arabidopsis* and *tomato*.** Arrows represent induction and bars represent repression. Purple lines represent functions of PIF1a-PIF1b heterodimers as deduced from this thesis work.

The case of seed germination has another layer of complexity, because we also need to take into account the interaction between light and hormone (GA) signaling. As we indicated above, loss of PIF1 in *Arabidopsis pif1* allows germination in the presence of FR (Oh et al., 2004) and the same was observed in tomato (Fig. R6). In *Arabidopsis*, FR promotes the accumulation of PIF1, which then triggers the transcriptional activation of DELLA genes and repression of GA synthesis genes. As a result, DELLAs accumulate and repress seed germination. In absence of PIF1, DELLAs do not accumulate and seed germination is not inhibited. However, inhibition of GA synthesis with paclobutrazol (PAC) causes DELLA accumulation and hence prevents germination even in the *pif1* mutant background (Oh et al., 2006, 2007; Piskurewicz et al., 2009; Lau and Deng, 2010). In tomato, PAC treatment of FR-exposed seeds reduced but not prevented germination of the *pif1a pif1b* double mutant (Fig. R6). At long incubation times

the single *pif1a* mutant also showed an improved germination under FR and PAC compared to WT and *pif1b* lines (Fig. R6). This result suggests a differential role for PIF1 homologs in the GA-mediated control of seed germination in *Arabidopsis* and tomato. One possibility is that the target genes of PIF1 factors are different in *Arabidopsis* and tomato. This would be supported by the comparison between our RNA-seq experiments and the genome-wide analyses performed in *Arabidopsis* (Fig. R20 and R22). Indeed, we found that the vast majority of genes missregulated in tomato leaf and fruit tissues overproducing PIF1a are different from those missregulated in *Arabidopsis* lines with altered PIF1 levels.

Another conclusion based on the observed phenotypes is that PIF1 proteins may function as activators or repressors depending on the biological context. The idea of PIF1 factors being activators or repressors of the same physiological process depending on the plant species is supported by the differential phenotype of photosynthetic pigment accumulation observed during seedling de-etiolation in *Arabidopsis* and tomato. In *Arabidopsis*, the *pif1* mutant accumulates higher levels of carotenoids in the dark and produces more carotenoids and chlorophylls during de-etiolation (Huq et al., 2004; Moon et al., 2008; Toledo-Ortiz et al., 2010). In contrast, our results with tomato PIF1-defective mutants show increased levels of carotenoids in dark-grown seedlings (although the increase is not as high as in *Arabidopsis*) but decreased levels of photosynthetic pigments in de-etiolating and light-grown seedlings (Fig. R7). These results suggest that that tomato PIF1a and PIF1b homologs might not be repressors but activators of photosynthetic development and leaf pigment biosynthesis (Fig. D1). Together, the results suggest that *Arabidopsis* and tomato PIF1 factors might have strongly divergent roles in the light- and hormone-dependent control of developmental processes. Analysis of PIF1a-defective mutants of other Solanaceae species should provide valuable information on this matter.

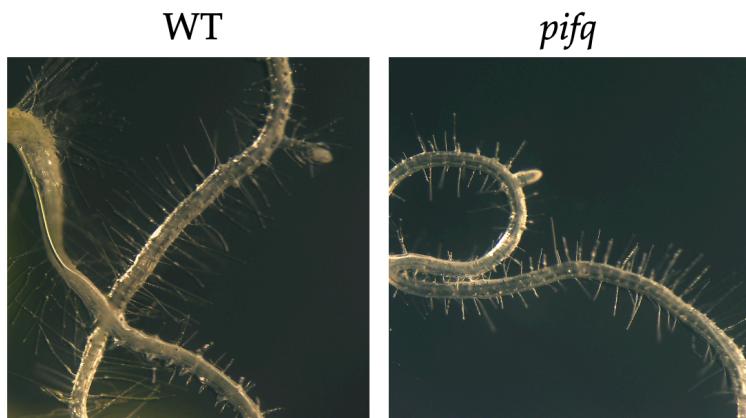
## 1.2. Root hair elongation and flowering

Seed germination and pigment accumulation in the light seem to be regulated by both PIF1a and PIF1b homologs (Fig. D1). In contrast, the phenotypic analysis of the CRISPR/Cas9 mutants unveiled that there are other processes that would be regulated by just PIF1a. A striking case is the **root hair elongation** phenotype (Fig. R8). The key feature of this phenotype is an impairment on the elongation of root hair primordia, which are initiated but do not grow in the *pif1a* mutant (Fig. R9). Nothing similar has been reported in the literature available for PIF-defective mutants in *Arabidopsis*. We actually checked the roots of *Arabidopsis pifq* mutants and confirmed that this phenotype was not present (Fig. D2). However, previous studies showed that *Arabidopsis phyB* mutant lines develop longer root

## Discussion

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hairs (Reed et al., 1993). We can speculate that this root hair phenotype of *phyB* plants might derive from increased PIF activity. The fact that no root hair phenotypes have been described in *Arabidopsis* for any combination of PIF-defective mutants (including the quadruple *pifq* mutant) suggests that *phyB*-dependent regulation of root hair elongation might depend on factors other than PIFs in *Arabidopsis*. Alternatively, scarcely explored PIFs (such as PIF2, PIF6 or PIF8) might have a major role in this process. Note that PIFs have been identified as growth regulators in other organs, specially PIF4 and PIF5 (Choi and Oh, 2016). PIF4, often together with its closest homolog PIF5, is involved in hypocotyl elongation in response to shade (De Lucas et al., 2008), blue light (Pedmale et al., 2016) or temperature (Koini et al., 2009; Thines et al., 2014). Maybe during evolution, a neofunctionalization event took place and PIF1a acquired this elongation-promoting role specifically in root hairs (Fig. D1).



**Figure D2.** *Arabidopsis* roots of WT and *pifq* mutant. Root hairs develop properly in both genotypes. Courtesy of Luca Morelli.

**Flowering time** also seems to be promoted by PIF1a, but not by PIF1b (Fig. R10). Even though the differences between PIF1a-defective and WT lines are relatively small and are only observed when counting the number of leaves from germination to anthesis (but not when counting the number of days), they are statistically significant and make sense if we consider that PIF1 in *Arabidopsis* is also described as a regulator of flowering time (Wu et al., 2018) (Fig. D1). A number of other studies in the literature report differences in flowering when using one of the evaluated parameters but not when using the other (Calvert, 1959; Giliberto et al., 2005). Note that we grew our plants in the greenhouse under standard conditions (i.e. long day photoperiod), while domesticated tomato, in contrast with *Arabidopsis*, has been described as a day-neutral species (Soyk et al., 2017).

In *Arabidopsis*, PIF1 is not the only component of light-signaling pathways described as a regulator of flowering time. Photoreceptors phyA and CRY1/2 have been identified to promote flowering by stabilizing CONSTANS (CO) (Sarid-Krebs et al., 2015), a critical gene involved in the long-day promotion of

flowering (Suárez-López et al., 2001). By contrast, phyB appears to antagonistically facilitate CO degradation in specific moments of the day (Hajdu et al., 2015). Regarding PIFs, PIF4 and PIF5 were reported as thermosensory regulators of flowering (Kumar et al., 2012; Thines et al., 2014) through the activation of FLOWERING LOCUS T (FT), a target of CO (Hanano and Goto, 2011). Despite this, tomato PIF4 seems not to be related with this process, since the down-regulation of its expression leads to a reduction in flowers per truss, but not in flowering time (Rosado et al., 2019).

## 2. PIF1a regulates biological processes in tomato that are not present in *Arabidopsis*

### 2.1. Fruit ripening

Seed germination, seedling de-etiolation, root hair development and flowering are biological processes present in both *Arabidopsis* and tomato. But the ripening of fleshy fruits is a tomato-specific process that has high agronomic interest and whose regulation has demonstrated to be really complex (Giovannoni, 2004). *PIF1a* is the only PIF-encoding gene that is up-regulated during fruit ripening (Rosado et al., 2016). One of the most characteristic features of tomato fruit ripening is the degradation of chlorophylls and the massive production of carotenoids when MG fruits start to ripe, changing their green color to orange (in the OR stage) and eventually red (in the RR stage). *PIF1a* was first identified as a regulator of fruit carotenoid synthesis during ripening (Llorente et al., 2016b). In that work, an artificial microRNA approach was used to down-regulate *PIF1a* gene expression about 75%, resulting in fruits that accumulated higher levels of total carotenoids at the ripening stages OR (~50% higher levels than WT controls) and RR (~10% increase) (Llorente et al., 2016b). In contrast with those results, WT levels of total carotenoids were detected in OR and RR fruits from the *pif1a* mutant line generated in this work (predicted to be completely devoid of functional *PIF1a* activity) (Fig. R11). The same was observed in the *pif1b* line and in the *pif1a pif1b* double mutant (Fig. R11). An explanation for this (lack of) phenotype came from the analysis of off-target effects. Recent work has shown that tomato PIF4 is a strong repressor of carotenoid biosynthesis during fruit ripening (Rosado et al., 2019). In this work they also used a gene knockdown approach to reduce the levels of PIF4, which led to an increase in total carotenoids in fruits. Likely due to off-target effects of gene knockdown approaches (Tschuch et al., 2008), the levels of transcripts encoding other PIF members were reduced in both PIF1a and PIF4 down-regulated plants. In PIF1a-silenced fruits (Llorente et al., 2016b), the most strongly down-regulated off-targeted PIF was PIF4 (~40%), while in PIF4-silenced fruits (Rosado et al., 2019) the most strongly down-

## Discussion

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regulated off-targeted PIF was PIF1a (~60%). Taking all these data together, we propose two possible explanations. First, PIF1a and PIF4 might be functionally redundant and play a similar role in modulating fruit carotenoid biosynthesis during ripening. This hypothesis involves that higher carotenoid contents would only be observed when both PIF1a and PIF4 are down-regulated. Because *PIF4* expression is not affected in the *pif1a* mutant generated in this work (Fig. R5), PIF4 levels would be high enough to repress carotenoid overaccumulation in the *pif1a* line. The second hypothesis states that PIF4, but not PIF1a, would be involved in repressing carotenoid production during ripening. Thus, reduced *PIF4* transcript levels in fruits of knock-down *PIF1a* and *PIF4* lines, but not in our CRISPR-Cas9 mutants, would lead to higher carotenoid contents.

Chlorophyll quantification in fruits also showed no differences between WT and any of the mutants defective in PIF1a or/and PIF1b (Fig. R11). It is interesting that the levels of chlorophylls and carotenoids in mutant fruits are not affected but they are decreased in young seedlings. This would indicate that the regulation of the same metabolic pathway relies on different sets of transcription factors depending on the tissue and the developmental stage. Regarding tocopherol (vitamin E) accumulation, mutant fruits defective in PIF1 homologs showed WT levels of these nutritionally-relevant metabolites (Fig. R11), suggesting that their production is not regulated by these factors. While PIF3 was found to bind to the promoter of a key tocopherol biosynthetic gene to repress expression ([Gramegna et al., 2018](#)), it was not shown whether this had an impact on fruit tocopherol levels. In any case, redundancy between PIF family members might also regulate tocopherol biosynthesis. Generating a combination of several knock-out mutant lines for different PIFs in tomato will be required to unveil this question in the future.

The metabolic profiling of *HSP70b:PIF1a-GFP* inducible lines will determine what other pathways or processes are regulated by PIF1a during fruit ripening. To date, we just learned that the metabolic profiles of RR fruits are statistically different between WT plants and the inducible line (Fig. R25), but we are still missing a list of compounds that would be differentially accumulated. In any case, the current data support our conclusion that choosing the MG stage for RNA-seq experiments aimed to identify possible PIF1a gene targets and hence regulated processes was not optimal. There are no metabolic differences between WT and inducible lines in MG fruit (Fig. R25), perhaps because up-regulation of PIF1a at that stage only has a minor effect on gene expression (Fig. R19 to R22). It is likely that, like other PIFs, PIF1a might need specific partners and cofactors to bind to the promoters of its target genes ([Pham et al., 2018](#)). If these extra components are not present, the effects of PIF1a induction on gene expression and downstream consequences (such as metabolic changes) will be less dramatic.

In addition, identification of PIF1a-regulated metabolic processes might require specific conditions for *PIF1a* overexpression to have an impact, such as appropriate supply of precursors or storage compartments. If these conditions are not met in MG fruits, increasing PIF1a levels at this stage would have little or no effect on target pathways. We therefore conclude that RNA-seq and LC-MS analyses of transcripts and metabolites after induction of *PIF1a* expression in late OR fruits from *HSP70b:PIF1a-GFP* plants would be much more informative to assess the role of PIF1a during fruit ripening.

Beyond the regulation of metabolic processes during ripening, the loss of PIF1a or/and PIF1b function had no effect on ethylene production (Fig. R12). The absence of impact on fruit ripening is consistent with that observed in the PIF1a knock-down lines, which exhibited no differences in the expression of ripening marker genes, including master regulators (Llorente et al., 2016b).

## 2.2. Fruit development

Three interesting traits of tomato as a commercial crop are fruit yield, size, and texture. We found that **fruit production** (i.e. total number of fruits per plant) was reduced in *pif1a* but not in *pif1b* or *pif1a pif1b* lines (Fig. R13). This is the only phenotype reported in the thesis that is present in one of the single mutants but not in the double mutant. The result suggests that the mutation in *PIF1b* by itself is not able to develop a phenotype different from WT, but that it is able to revert the low fruit production phenotype developed by the mutation in *PIF1a*. We hypothesize that maybe PIF1a only regulates fruit production when forming heterodimers with PIF1b. In our model, PIF1a-PIF1b heterodimers would activate fruit production whereas PIF1b-PIF1b homodimers would repress it (Fig. D1). The WT phenotype would result from a balance between activation and repression. Loss of PIF1a would lead to only repression (via PIF1b-PIF1b homodimers) in *pif1a* plants, whereas loss of PIF1b would remove both activation and repression pathways, resulting in a newly balanced situation and hence a WT phenotype in *pif1b* and *pif1a pif1b* plants. While other scenarios are possible, antagonistic roles of light signaling homologs are common. In *Arabidopsis*, PIF2 and PIF6 have antagonistic roles with PIF7 and PIFQ for the control of light-triggered seedling de-etiolation (Pham et al., 2018). In the case of PIF2, it positively regulates seedling de-etiolation and photomorphogenesis by interacting with PIF1 and other PIFQ members, preventing them to regulate their target genes (Pham et al., 2018).

On the other hand, **fruit growth** in terms of size (Fig. R14) and weight (Fig. R15A) increased in *pif1a* but also in the *pif1a pif1b* double mutant. Based on the results

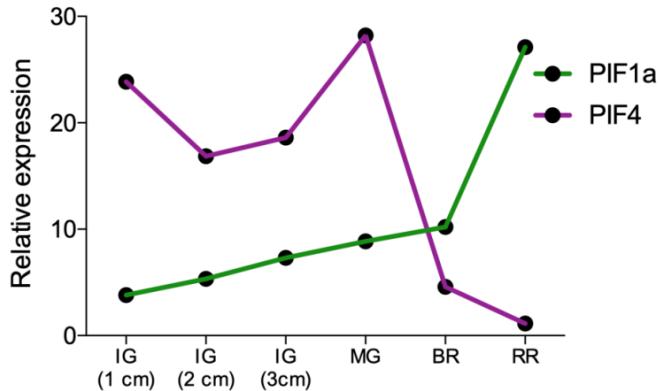
## Discussion

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of the fruit desiccation experiment (Fig. R15B) we concluded that the difference in size might be due to a difference in tissue mass, not in water content of the fruits, pointing out PIF1a as a repressor of fruit growth (Fig. D1) perhaps by down-regulating cell proliferation. However, our data do not discard that the increased fruit size and weight observed in PIF1a-deficient mutants may result from derepressed cell elongation or expansion, which also involve an increase in dry matter (e.g. cell-wall material). *Arabidopsis* PIF1 has been shown to regulate the expression of cell-wall-related genes (Oh et al., 2009; Shi et al., 2013), and the same appears to be true in the case of tomato PIF1a based on our RNA-seq data (Tables A2 and A3). Alteration of cell-wall structure by PIF1a might explain the **fruit softening** phenotype of PIF1a-defective fruits (Fig. R16). Thus, PIF1a might be involved in the loosening of the cell-wall to allow root hair elongation and fruit softening while repressing fruit growth by a different mechanism (Fig. D1). This contrasting and opposite role of PIF1a in different tissues and developmental stages can be due to different interactions with different partners depending on the tissue and the developmental stage. For instance, protein-protein interaction between PIFs and with other factors can modulate their capacity to bind to the DNA (Pham et al., 2018). In this way, PIF1a might have specific interactors to promote root hair elongation or ripe fruit softening, while a different set of interactors would repress fruit growth.

*Arabidopsis* PIF4 has an important role in promoting hypocotyl elongation in response to different light and temperature cues (Koini et al., 2009; Choi and Oh, 2016; Quint et al., 2016; Pham et al., 2018). Supporting the conclusion that PIF4 has conserved a growth-promoting role in different plant species and tissues, tomato knock-down lines for PIF4 develop smaller fruits (Rosado et al., 2019). Therefore, PIF1a and PIF4 appear to play antagonistic roles in the regulation of fruit size. A closer look at the expression patterns of the genes encoding PIF1a and PIF4 during fruit development shows that the expression of *PIF1a* is low at the early stages of fruit development (when growth takes place by cell division and proliferation) and slowly increases as cells expand (until the MG stage) and fruits begin to ripe, peaking at the RR stage (Fig. D3). In contrast, *PIF4* expression peaks at the MG stage (i.e. when fruit reaches its final size) and then drops during ripening (Fig. D3). Based on these data, we speculate that PIF4 is the main promoter of fruit growth. Similar to that discussed above on the opposite role of PIF1a-PIF1b heterodimers and PIF1b homodimers for the regulation of fruit production (Fig. D1), it is possible that PIF1a-PIF4 heterodimers could antagonize the activating role of PIF4, perhaps by preventing binding of PIF4 homodimers to fruit-promoting target genes. As PIF1a levels increase during fruit development, heterodimers become more abundant and PIF4 homodimers decrease, resulting in a progressive attenuation of growth. Then, after the MG stage, PIF4 levels drop and growth stops. The peak of *PIF1a* expression at the RR

stage is most likely unrelated to growth but required for fruit softening. Whether tomato PIF4 also interacts with PIF1a to regulate root hair elongation remains unknown.



**Figure D3. Expression profiles of tomato *PIF1a* and *PIF4* during fruit development and ripening.** Data obtained from The Bio-Analytic Resource from Plant Biology (BAR, University of Toronto).

### 2.3. SGA biosynthesis

The RNA-seq experiment unveiled that **SGA biosynthesis** in leaves would be another process regulated by PIF1a that is not naturally present in *Arabidopsis* (Fig. D1). PIF1a accumulation in heat-treated leaves of our *HSP70b:PIF1a-GFP* line led to the repression of several genes related to cholesterol and SGA biosynthesis, resulting in decreased production of  $\alpha$ -tomatine, the main SGA in leaves (Fig. R24). PIF1a is not the first tomato PIF described as a regulator of SGA synthesis. PIF3 was shown to be a repressor of SGA pathway genes, including some that are also down-regulated by PIF1a, such as GAME1 and GAME17 (Wang et al., 2018). By decreasing PIF3 expression levels using Virus Induced Gene Silencing (VIGS) they also showed that reduced PIF3 levels caused was an increase of SGAs. In addition, they performed Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP) experiments to demonstrate that PIF3 directly binds to the promoter of its target GAME genes (Wang et al., 2018). The observation that GAME genes are also rapidly down-regulated after the short-term and transient induction of *PIF1a* expression strongly suggests that PIF1a might be a direct repressor of at least some of these SGA-related genes.

SGAs are phytosterol-derived compounds, specifically cholesterol-derived, that are only found in Solanaceae (Sonawane et al., 2016). They are a family of antinutritional compounds that are abundant in leaves but decrease during fruit ripening. We speculated that PIF1a up-regulation during tomato fruit ripening (Fig. D3) might contribute to repress the expression of the SGA biosynthetic genes during this process. However, no SGA-related genes were identified as

DEGs in our fruit RNA-seq experiments. The metabolic profiling of *HSP70b:PIF1a-GFP* fruits will show if there are differences in the accumulation of SGAs during fruit ripening.

### 3. First steps to identify PIF1a targets

Identification of the genes regulated by PIF1a was expected to give valuable clues to understand the molecular basis of the mutant phenotypes but also provide information on other processes controlled by this transcription factor in different tomato tissues. That's the reason why we decided to perform RNA-seq analysis in fruits and leaves. Strikingly, the RNA-seq analysis provided a low number of differentially expressed genes (DEGs). There are different possible explanations for this surprising result.

Because our main objective was to identify the primary targets of PIF1a, we generated a heat shock induced system allowing a tight control of the *PIF1a* transgene expression and its up-regulation for a short period of time. The first hour after the heat shock induction should allow the transgene expression to peak (Fig. R18B) whereas the second hour, when the PIF1a-GFP protein was expected to accumulate (Fig. R17C), should allow this transcription factor to bind to its target promoters and modulate the expression of its direct target genes. However, the process might have occurred at a slower pace than expected, preventing a strong impact of the chimeric PIF1a-GFP protein on the expression of its target genes. Another possibility to explain the low number of DEGs is that the fusion to GFP protein would somehow interfere with the tridimensional structure of the transcription factor, inhibiting (at least partially) its capacity to bind to DNA or to interact with protein partners such as PIF1b.

As previously discussed, the presence or absence of specific protein partners can lead to an inhibition or enhancement of the PIF capacity to bind to its target genes (Pham et al., 2018). We selected mature leaves to perform the RNA-seq experiment. Maybe the protein or metabolite context of a mature leaf would not be appropriate to study the capacity of PIF1a to modify the transcriptional profile of the organ (e.g. to regulate growth or photosynthesis) because it would lack some important partners. In the case of the fruits, we selected the MG stage because we hypothesized that many target genes of PIF1a might be already activated or repressed in later stages of development, when endogenous *PIF1a* expression is highest. But maybe, like in leaves, the cellular context was not the optimal for this transcription factor to trigger the transcriptomic changes that are normally associated with naturally increased PIF1a levels. As discussed above, RNA-seq analyses in late OR fruits from *HSP70b:PIF1a-GFP* plants might be

much more informative to assess the role of PIF1a during fruit ripening. In the case of leaves, young expanding leaves might also be a better option than mature leaves.

Regardless the conclusion that the experimental design could be improved to optimize the identification of PIF1a target genes, we showed that at least some of the DEGs we identified using two alternative strategies for the analysis of RNA-seq data are indeed regulated by this transcription factor. Thus, we identified that PIF1a is involved in SGA biosynthesis as described above. Moreover, we have identified another metabolic pathway that could be regulated by PIF1a in fruits and leaves, the phenylpropanoid pathway. The metabolic profiling of transgenic fruits comparing with WT should provide us further evidence on a possible role of PIF1a on the control of this pathway, which produces metabolites important for cell wall synthesis (e.g. monolignols), photoprotection (e.g. anthocyanins and flavonoids) and defense (tannins, isoflavones, stilbenes). Interestingly, both SGA and phenylpropanoid metabolites have been proposed to have a role in during Early Blight (EB) resistance (Shinde et al., 2017). EB is a disease caused by the necrotrophic pathogen *Alternaria solani* that affects many solanaceous crops (Chaerani and Voorrips, 2006). EB symptoms in fruits and leaves develop in a wide range of environmental conditions, causing reductions in yield up to 79% (Adhikari et al., 2019), making it one of the economically most important diseases of tomato. Shinde et al., proposed that transcription factors such us WRKY1 and MYB20 might coordinate the expression of SGA and phenylpropanoid genes and that it would be an interesting strategy to fight against this infection in tomato (Shinde et al., 2017). Our results confirm that PIF1a regulates SGA accumulation in leaves and suggest that it might also regulate phenylpropanoid biosynthesis (based on the RNA-seq results). Further experiments would be worthy to study if PIF1a is a coordinator of these metabolic changes in some tissues at specific developmental stages or maybe during the infective process.

#### 4. Evolutionary implications of PIF1 duplication

The results of this thesis have provided useful data about which processes are regulated by PIF1a, PIF1b or both in tomato (Fig. D1). We can analyze these results from an evolutionary point of view. Some of the PIF1-regulated processes in *Arabidopsis* are also regulated by PIF1 homologs in tomato (including seed germination, leaf pigment accumulation and flowering time). A second group includes processes that are regulated by PIF1 homologs in tomato but not by PIF1 in *Arabidopsis*. From them, some occur in both plant species (such as root hair elongation) but others do not exist in *Arabidopsis* (fleshy fruit production and

## Discussion

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growth and leaf SGA production). Moreover, the duplication of PIF1 present in tomato led to some of the above-mentioned processes being regulated by both PIF1a and PIF1b (seed germination, leaf pigment accumulation and fruit production) while the rest became controlled just by PIF1a. Interestingly, we did not find any process that would be regulated exclusively by PIF1b (Fig. D1).

The duplication of *PIF1* has been analyzed in detail previously (Rosado et al., 2016). The *Solanum* lineage has been affected by two whole-genome duplications. The first one occurred before the divergence between *Arabidopsis* and *Solanum* more than 120 millions of years ago (MYA), while the second one preceded the divergence between tomato and potato, estimated at 71 MYA (Sato et al., 2012). The duplication of *PIF1* in *PIF1a* and *PIF1b* is predicted to have happened just before the divergence between tomato and potato (Rosado et al., 2016; Sato et al., 2012). We hypothesize that before the divergence between *Arabidopsis* and *Solanum* there were likely some processes that were already regulated by PIF1 homologs in both species (e.g., seed germination, leaf pigment contents or flowering time). During the following millions of years, the duplication of PIF1 in tomato led to newly acquired functions. While PIF1a remained as the main isoform, the presence of PIF1b provided robustness to essential processes (e.g. seed germination) and allowed to regulate new processes via heterodimerization (e.g. fruit production). On the other hand, some pre-existing processes regulated by PIF1 in *Arabidopsis* became controlled just by PIF1a in tomato, such as flowering time. The reason behind this might have been the mutation identified in PIF1b that leads to the loss of interaction with phyB. PIF1a also acquired the capacity to regulate new processes. The root hair elongation phenotype is a striking example with two possible explanations. The first one is a neofunctionalization process, by which PIF1a ended up involved in the regulation of the elongation of this cell type. A second possibility is that the original PIF1 was controlling this trait, but during evolution this role was lost in *Arabidopsis* but remained in tomato.

It is very interesting how, during evolution, some transcription factors have been recycled to regulate new processes, like fruit ripening (Gapper et al., 2013). For instance, in *Arabidopsis* SHATTERPROOF 1 (SHP1) and SHP2 are important regulators of valve margin identity and its subsequent dehiscence zone (Liljegren et al., 2000). By contrast, their closest homolog in tomato, TOMATO AGAMOUS-LIKE1 (TAGL1) controls fleshy fruit expansion (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010). Another example is SEPALLATA 4 (SEP4), which in *Arabidopsis* regulates organ identity in flowers (Bowman et al., 1991; Ditta et al., 2004). The closest homolog to SEP4 in tomato is RIPENING INHIBITOR (RIN), which is a key regulator of fruit ripening and controls climacteric respiration and

ethylene biosynthesis (Vrebalov et al., 2002; Fujisawa et al., 2011; Martel et al., 2011).

Based on the phenotypes we found, we can conclude that PIF1a might have been recycled to regulate new processes that were not present in *Arabidopsis*, such as fleshy fruit growth and leaf SGA metabolism. In the case of fruit growth, PIF1a and, likely, PIF4 might have re-adapted their already existing role in the regulation of cell elongation (Paik et al., 2017) to determine the final fruit size. The regulation of SGA metabolism is another good example of neofunctionalization. When the synthesis of these compounds was developed in the *Solanaceae* family, some previously existing factors had to be re-adapted to develop this new trait, conferring to PIF1a this role.

The comparison between our RNA-seq experiments and other genome-wide experiments performed in *Arabidopsis* showed few coincidences between the potential target genes of PIF1 homologs from both species. Since we are comparing the tomato genes, by choosing just their first hit in a BLAST search, the comparison is not perfect and maybe there is more overlapping between them than we can detect with our methodology. Nevertheless, the high proportion of divergence strongly suggests that processes of specialization and neofunctionalization besides those detected here by analyzing the phenotypes of CRISPR-Cas9 mutants likely took place during evolution after the splitting of *Arabidopsis* and *Solanum* groups. As discussed before, a deeper analysis of the potential target genes found in the RNA-seq experiments will provide some clues about which new tomato processes became regulated by PIF1a.



## **CONCLUSIONS**



1. The two PIF homologs in tomato (PIF1a and PIF1b) show different light-related protein features. PIF1b lacks a functional motif for phyB interaction.
2. PIF1a and PIF1b proteins are able to interact to form homo and heterodimers.
3. PIF1a and PIF1b show substantially different expression profiles, suggesting divergent biological functions.
4. Analysis of CRISPR/Cas9 mutants defective in PIF1a, PIF1b or both showed that the two homologs participate in the light-dependent regulation of seed germination and photosynthetic pigment accumulation during seedling de-etiolation.
5. Only PIF1a regulates root hair elongation, leaf SGA biosynthesis, flowering initiation and fruit features, such as size, weight and hardness. Loss of PIF1b has no effect in any of this processes.
6. Compared to *Arabidopsis*, the tomato PIF1 homologs have conserved functions but also appear to play opposite roles (e.g. in GA-dependent seed germination and light-triggered accumulation of carotenoids and other plastidial isoprenoids in leaves).
7. Tomato PIF1a was found to acquire new functions such as activation of root hair elongation, a feature that is not regulated by PIFs in *Arabidopsis*.
8. Neofunctionalization was also observed for PIF1a in the regulation of biological functions that are not present in *Arabidopsis* (e. g. fleshy fruit development and leaf SGA biosynthesis).
9. The analysis of transgenic lines with induced PIF1a levels showed that this transcription factor regulates different sets of genes in green fruits and leaves.
10. PIF1a-regulated genes in tomato show very little overlapping with PIF1-regulated genes in *Arabidopsis*, supporting the idea of neofunctionalization.



## **MATERIALS AND METHODS**



## 1. Plant biology techniques

### 1.1. Plant material and growth conditions

All the analyzed tomato (*Solanum lycopersicum*) transgenic lines of this thesis (listed in Table 1) were generated in cv. MicroTom background. The *HSP70b:PIF1a-GFP* line was generated in this lab, while the CRISPR/Cas9 mutant lines were generated in collaboration with the group of Dr. Alain Goossens (VIB Center for Plant Systems Biology, Ghent, Belgium). Wild type *Nicotiana benthamiana* plants were used for the experiments of subcellular localization, Bimolecular Fluorescence Complementation (BiFC) and protein stability. Both plant species were grown under standard long day greenhouse conditions (14 h light at  $27 \pm 1^\circ\text{C}$  and 10 h dark at  $24 \pm 1^\circ\text{C}$ ).

**Table 1.** Tomato transgenic lines used in this thesis.

Transgenic lines	Description
<i>pif1a</i>	CRISPR/Cas9 mutant in <i>PIF1a</i> gene
<i>pif1b</i>	CRISPR/Cas9 mutant in <i>PIF1b</i> gene
<i>pif1a pif1b</i>	CRISPR/Cas9 mutant in <i>PIF1a</i> and <i>PIF1b</i> genes
<i>HSP70::PIF1a:GFP</i>	Inducible and overexpressing <i>PIF1a</i> line

### 1.2. Phenotyping of loss-of-function lines

#### 1.2.1. Seed germination

Seed germination assay were performed as was previously described (Appenroth et al., 2006). Briefly, Tomato seeds were surface-sterilized for 10 min with 40% (v/v) NaClO, washed 3 times with sterile water and sown on sterile Murashige and Skoog (MS) medium containing 1% agar and no sucrose. After that, seeds were incubated in dark conditions and exposed hourly to 5-min R ( $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or FR ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Temperature was always kept at  $25^\circ\text{C}$ . Radicle protrusion was used as a criterion for judging seed germination.

#### 1.2.2. De-etiolation assay

Tomato seeds were surface-sterilized for 10 min with 40% (v/v) NaClO, washed 3 times with sterile water and sown on sterile wet cotton. After that, seed were incubated in dark conditions, except the Light control of the experiment, that was

incubated in continuous light conditions. 7 days after the sown, the dark-gown plants were transferred to light. Samples were collected at the indicated time points after the exposure to light. Temperature was always kept at 25 °C.

### 1.2.3. Flowering time measurement

Flowering time was assessed as was previously reported in tomato (Dielen et al., 1998). Basically, after sowing the seeds we waited until the first flower reached the anthesis stage. In that moment we measure two parameters: The number of leaves and the days post-sowing when the anthesis took place.

### 1.2.4. Fruit traits

Fruit production was measured by counting the number of produced fruits per plants in 19-weeks-old plants in greenhouse conditions.

The volume of the fruits was assessed by measuring the displaced volume in a graduated cylinder of a group of 10 representative fruits.

The weight of the fruits was assessed by two different methods. First, 100 fruits were weight individually. Second, 15 fruits were weight together as a group. This second method was used to compare the weight difference between fresh samples and dry samples. To dry the fruits, all the 15-fruits groups were incubated for 4 days at 90 °C.

Fruit hardness was measured in collaboration of the group of Dr. Jordi Gine (IRTA, Lleida, Spain). To measure it we used a texture analyzer (Stable Micro Systems, TA-XT2i) fitted with 50 mm plate probe to perform a compression test (Kabas and Ozmerzi, 2008; Camps and Gilli, 2017). The instrument was set to measure the mechanical work needed to reach 5% deformation of the original form of the fruit.

## 1.3. Transient expression in *N. benthamiana*

*A. tumefaciens* GV3101 strains were transformed with the vector of interest. Were streaked on plates with the appropriate antibiotics and grown at 28 °C for 2-3 days. A single colony (previous PCR colony corroboration) was inoculated in 5 mL YEB media, the culture was grown overnight at 28 °C with a 300 rpm rotation rate. The next day, 500 µL of the grown culture was added to 20 mL of YEB media. Culture was incubated overnight at 28 °C. OD600 values were obtained of each liquid culture with a spectrophotometer. The culture was centrifuged 10 minutes 4,000 rpm 4 °C. Then, bacteria were resuspended in the infiltration buffer (10 mM MES pH5.5.-6, 10 mM MgSO<sub>4</sub>, Acetosyringone 100

μM). Leaves from 4-week-old *N. benthamiana* plants were entirely infiltrated with the desired combination of *A. tumefaciens* strains in the infiltration buffer. After agroinfiltration, the plants were left on the bench or the greenhouse for the indicated times. Leaves were used to perform co-IP assays or confocal microscopy analysis.

In the case of BiFC experiments, equal volumes of Agrobacterium cultures were mixed to get the indicated combinations in the Results (Figure R2).

#### **1.4. Generation of transgenic tomato plants**

Tomato plants were transformed as previously described (Fernandez et al., 2009). Surface-sterile MT seeds were sown in 50% MSO medium (50% MS salts; 30g/l sucrose; Vitamin B5; agar 8 g/l; pH=5.8) and grown during 10 days at 25 °C in long day conditions (16 h light; 8 h dark). Cotyledons were cut in two halves and incubated in KCMS medium (50% MS salts; 20g/l sucrose; KH<sub>2</sub>PO<sub>4</sub> 200 mg/l; Tiamin 0.9 mg/l; 2,4 D 2 mg/l, Kinetin 1 mg/l; acetosyringone 200 μM; agar 8 g/l; pH=5.8) during 24 h. Cotyledons were incubated during 30 min with an agrobacterium suspension in liquid KCMS harboring the desired plasmid. Cotyledons were then transfer to a fresh solid KCMS medium and incubated in dark 48 h at 25 °C. Cotyledons were transferred to 2Z medium (50% MS salts; 30g/l sucrose; Nitsch vitamins; Zeatin 2 mg/l; Timentin 250 mg/l; antibiotic (pKGW plasmids is Kanamycin 100 mg/l); agar 8 g/l; pH=5.8) during 15 days in long day conditions. Every 15 days cotyledons were refreshed by transferring to new 2Z medium until regenerated plants appeared (approximately 30 days). The re-generated explants were transferred to the rooting medium (50% MS salts; 10g/l sucrose; Nitsch vitamins; Zeatin 2 mg/l; Timentin 75 mg/l; antibiotic (pKGW plasmids is Kanamycin 100 mg/l); agar 8 g/l; pH=5.8). Once roots appeared, plants were transferred to soil and acclimated at the greenhouse.

#### **1.5. Isolation of single and double CRISPR/Cas9 mutants**

As indicated previously, the CRISPR/Cas9 lines were generated in the laboratory of Dr. Alain Goossens, using 2 sgRNA, one for each gene. We received the potential double mutant plants and identified the kind of mutation for each gene. Once we obtained identified a plant that had a mutation in PIF1a and PIF1b we crossed that plant with a WT. The first generation was check by PCR and all the individuals were heterozygous, as predicted. The second generation was segregated following the mendelian rules of inheritance, so we perform a screening of 200 plants, to identify the single *pif1a* and *pif1b* mutants, the double *pif1a pif1b* mutant and also WT plants that would be exact siblings of the rest of the population, these WT plants were taken as a control for all the phenotypical analyses of this thesis.

## 2. Molecular biology techniques

### 2.1. DNA constructs

All the details about the constructs and primers used in this thesis are listed in the Tables 2 and 3, respectively.

**Table 2.** DNA constructs used in this thesis.

Construct	Vector	Bacterial Selection	Plant Selection	Reference
35S::PIF1a:GFP	pGWB405	Spect	Kan	Llorente et al., 2016
35S::PIF1b:GFP	pK7FWG2	Spect	Kan	Col. with Dr. Magdalena Rossi
35S::GFPC:PIF1a	pYFC43	Kan	Hyg	This work
35S::GFPC:PIF1b	pYFC43	Kan	Hyg	This work
35S::GFPN:PIF1a	pYFN43	Kan	Hyg	This work
35S::GFPN:PIF1b	pYFN43	Kan	Hyg	This work
pHSP70::PIF1a:GFP	pKGW	Spect	Kan	This work

GFP-tagged versions of PIF1a and PIF1b used in stability experiment were previously generated. PIF1a construct was generated in my group and it was already published (Llorente et al., 2016b). PIF1b construct was generated in Dr. Magdalena Rossi's group (Universidade de São Paulo, São Paulo, Brazil).

BiFC constructs were generated in our lab. The coding sequences of PIF1a and PIF1b were cloned into a gateway ENTRY vector (pDNOR207, Thermo Fisher). After that, using a gateway LR reaction (Thermo Fisher), the CDSs were transferred to the final expression vectors, in frame with the N- or C-terminal sequence of GFP, pYFN43 and pYFC43 respectively (Belda-Palazón et al., 2012).

CRISPR/Cas9 constructs were generated in the laboratory of Dr. Alain Goossens. Briefly, two guide RNAs (sgRNA) were cloned via cut-ligation reaction with BbsI (Thermo) and T4 DNA ligase (Thermo) in a Gateway ENTRY sgRNA shuttle vector (Ritter et al., 2017). Next, using a gateway LR reaction (Thermo Fisher), the two sgRNA modules were then combined with pDE-Cas9Km vector (Ritter et al., 2017) to yield the final expression clone.

The *HSP70b:PIF1a-GFP* construct was generated using the MultiSite Gateway technology (Thermo Fisher). Three different parts were generated independently: the promotor of *HSP70b* gene (around 2000 bp upstream of the initiation codon of AT1G16030 gene), the PIF1a CDS fused to GFP CDS and the NOPALINE SYNTHASE terminator from *Agrobacterium tumefaciens*. Each of this parts were cloned respectively in the entry vectors pDONR221 1-4, pDONR221 4r-3r and pDONR221 3-2. After that, a MultiSite Gateway LR reaction was

performed to transfer all the elements to the destination vector pKGW (Karimi et al., 2002), generating the final construct.

## 2.2. Genotyping of transgenic tomato plants

Genomic DNA from Tomato leaves was extracted according to the protocol develop by Edwards et al., 1991. Samples for PCR analysis (leaf tissue) were collected using the lid of an Eppendorf tube to pinch out a disc of material into the tube containing 3mm crystal beads. Then, 400  $\mu$ L of extraction buffer (200mM Tris HCL pH 7.5, 250 mM NaCl, 25 nM EDTA, 0.5% SDS) was added to the tube. Next, the samples were macerated (at room temperature) using a Tyssue Lyser II (QIAGEN). The extracts were centrifuged at 13,000 rpm for 1 minute and 300  $\mu$ L of the supernatant was transferred to a fresh Eppendorf tube. The supernatant was mixed with 300  $\mu$ L of isopropanol and left at room temperature for 2 minutes. Following centrifugation at 13,000 rpm for 5 minutes, the remaining liquid was discarded and the pellet was air-dried and then dissolved in 100  $\mu$ L of miliQ water.

First identification of CRISPR/Cas9 mutation was assessed thanks to the TIDE (Tracking of Indels by DEcomposition) web tool (Brinkman et al., 2014; Pauwels et al., 2018). A PCR-based amplification of the genomic region targeted by the gRNA was sequenced and analyzed in the platform. Once we identified the plants that had 100% or 50% of their molecules mutated (corresponding to non-chimeric homozygous or heterozygous plants, respectively) we analyze the chromatograms individually to identify the short insertion or deletion, using as template a chromatogram of a WT plant. The alignment was performed in Benchling ([www.benchling.com](http://www.benchling.com)) using MAFFT as an alignment program.

Once the insertions or deletions were identified, CRISPR/Cas9 lines were genotyped using a PCR to amplify 500 bp of the mutated version. During the identification of the mutation we detected that the mutation in PIF1a removed a restriction site for DrdI and the mutation in PIF1b removed a restriction site for NlaIV. The amplified fragments were then digested with the indicated enzymes. If the plant would be WT, we would see two bands (800 and 900 bp), if the plant would be homozygous for the mutation, we would see one non-digested band (1700 bp).

*HSP70b:PIF1a-GFP* lines were genotypes using a PCR that amplify specifically the chimeric construct PIF1a:GFP. All the primers used for genotyping the plants are listed in Table 3.

### 2.3. Gene expression analysis

Total RNA was extracted for tomato leaves or fruits using the Maxwell 16 LEV Plant RNA Kit (Promega). RNA was quantified using a NanoDrop (Thermo Scientific) and its integrity was analyzed by agarose gel electrophoresis. The cDNA synthesis was performed as follows according the recommendations of the Transcriptor First Strand cDNA synthesis Kit (Roche). RT-qPCR was done in a total reaction volume of 20 µL using LightCycler 480 SYBR Green I Master (Roche) on a LightCycler 480 Real-Time PCR System (Roche). The normalized expression of target genes was calculated using ACTIN4 (ACT4, Solyc04g011500) as the endogenous reference gene. Information about the primers used for qPCR is described in Table 3.

For RNA-seq experiments, RNA was extracted as mentioned above. RNA sequencing service was performed by Sequentia Biotech SL (Barcelona, Spain). RNA concentration in each sample was assayed with a ND-1000 spectrophotometer (NanoDrop) and its quality assessed with the TapeStation 4200 (Agilent Technologies). Indexed libraries were prepared from 1 µg/ea purified RNA with TruSeq Stranded mRNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quantified using the TapeStation 4200 and pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled samples of 2 nM. The pooled samples were subject to cluster generation and sequencing using an Illumina NextSeq 500 System (Illumina) in a 2x75 paired end format at a final concentration of 1.8 pmol. The raw sequence files generated (.fastq files) underwent quality control analysis using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Data analysis was performed with the online platform AIR ([www.transcriptomics.cloud](http://www.transcriptomics.cloud)) (Vara et al., 2019) using the SolGenomics Network (<https://solgenomics.net/>) *S. lycopersicum* (ITAG 2.40) reference genome.

**Table 3.** Primers used in this thesis

Primer	Gene	Use	Sequence
SIPIF1a-attB1-Nt-F	PIF1a	Cloning	GGGGACAAGTTGTACAAAAAAGCAGGC TATGAATCATCTGTCCTGATTG
SIPIF1a-attB2-R	PIF1a	Cloning	GGGGACCACTTGTACAAGAAAGCTGGG TTAACCCAGATTGATGATTGCCCTG
SIPIF1b-attB1-Nt-F	PIF1b	Cloning	GGGGACAAGTTGTACAAAAAAGCAGGC TGGATGAATTACTGTGTTGTTCTG
SIPIF1b-attB2-R	PIF1b	Cloning	GGGGACCACTTGTACAAGAAAGCTGGG TCTAAATAGTATGCTCACCAAGATTG
pHSP70-attB1-F	HSP70b	Cloning	GGGGACAAGTTGTACAAAAAAGCAGGC TTAGAACTGCGAAAAAAGGGAGC
pHSP70-attB4-R	HSP70b	Cloning	GGGGACAACCTTGTATAGAAAAGTGGGT GTGCTAAAAAAAGCTTCAGTAATTG
PIF1a-GFP-attB4r-F	PIF1a	Cloning	GGGGACAACCTTCTATAACAAAGTTGAA TGAATCATTCTGTTCTGATTITG
PIF1a-GFP-attB3r-R	PIF1a	Cloning	GGGGACAACCTTATTATACAAAGTTGTTA CTTGACAGCTCGTCCATGCC
pHSP70_seq1	HSP70b	Cloning	GCCAATCTATCCATGATGCACC
pHSP70_seq2	HSP70b	Cloning	AACCGGCAATTAGTCCGACTAAG
PIF1a_gRNA	PIF1a	Cloning	TCTACTGACAGCTGGTCAG
PIF1b_gRNA	PIF1b	Cloning	CGCGAACACGGCGGAACCAAG
PIF1a_F_CRISPR	PIF1a	Genotyping	CAGAACTAAGAAGTCCGCAATG
PIF1a_R_CRISPR	PIF1a	Genotyping	CGCGAGAACGTCCGAAG
PIF1b_F_CRISPR	PIF1b	Genotyping	CCCGAGCAATCAGCTGTAG
PIF1b_R_CRISPR	PIF1b	Genotyping	TGCCATCGTCACCTCCCT
SIPIF1a_F_qPCR2	PIF1a	qPCR	TTGCATTACCGCAGAACATCAG
SLPIF1a_R_qPCR2	PIF1a	qPCR	TTGCCTGGATTCCAACCTC
slPIF1b_F_qPCR	PIF1b	qPCR	TCAGGAAGTGGAACAGCTGAG
slPIF1b_R_qPCR	PIF1b	qPCR	TTGATGATTCCCTCTACTCCTTC
slPIF3_F_qPCR	PIF3	qPCR	TCTGGTACCCCATGTATCC
slPIF3_R_qPCR	PIF3	qPCR	AGTCCTGACCAGGATGTGC
slPIF4/5_F_qPCR	PIF4	qPCR	TTGCAGAACCCATTTC
slPIF4/5_R_qPCR	PIF4	qPCR	TGCGGTAACGCTGAGTTG
eGFP_F_qPCR2	GFP	qPCR	CACTACCAGCAGAACACCCCC
eGFP_R_qPCR2	GFP	qPCR	TCTCGTTGGGTCTTIGCTC
SIACT_F_qPCR	ACT4	qPCR	CCTTCCACATGCCATTCTCC
SIACT_R_qPCR	ACT4	qPCR	CCACGCTCGGTAGGATCT

### 3. Analytical techniques

#### 3.1. Quantification of chlorophylls, carotenoids and tocopherols by HPLC

Carotenoids, chlorophylls, tocopherols were extracted in 2 mL Eppendorf tubes from 15 mg of freeze-dried tomato pericarp tissue, using 1 mL of hexane/acetone/methanol 2:1:1 as extraction solvent and 15 µg of canthaxanthin (Sigma) as internal control. After vortexing for 10 s and lysing the tissue with 4 mm glass beads for 1 min at 30 Hz in a TissueLyser II (QIAGEN), 100 µL of water were added. Then, 1 min of TissueLyser was carried out again and samples were centrifuged for 3 min at 3,000 rpm and 4 °C. Organic phase (upper) was kept in a new tube and the rest was re-extracted with 1 mL hexane/acetone/methanol 2:1:1, 1 min of TissueLyser and centrifuging for 5 min at maximum speed and 4 °C. The new organic phase was mixed with that previously extracted and evaporated for 1 h using a SpeedVac system (Eppendorf Concentrator plus). Extracted metabolites were then completely re-dissolved in 150 µL of acetone and filtered with 0.2 µm filters into amber-colored 2 mL glass vials. Separation and detection of isolated compounds was performed from 33 µL of prepared samples using an Agilent 1200 series HPLC system (Agilent Technologies) as described previously (Fraser et al., 2000). The HPLC equipment was coupled to a Photometric Diode Array (PDA) detector, allowing the detection of the full uv-visible absorption spectra of carotenoids and chlorophylls. Tocopherols were identified using a fluorescence detector at 330 and 420 nm, respectively. Chromatogram visualization and data analysis were performed using the Agilent ChemStation software.

#### 3.2. Quantification of ethylene by GC-MS

The procedure to measure ethylene production in tomato fruit was based on previously published work in Melon (Pereira et al., 2017). Specifically, we collected tomato fruits at MG stage and incubated them in long day greenhouse conditions in opened 50-mL tubes. Once a day, the tubes were sealed with Parafilm (BEMIS) and incubated for one hour. After that hour, 30 mL of the headspace air was collected by injecting a syringe through the Parafilm layer. That samples were finally collected in a GC-MS tube and analyzed.

GC-MS analysis was performed with an Agilent 7890A gas chromatograph coupled to a 5975C mass selective detector. Using a CTC-PAL headspace sampler (Agilent Technologies), gas samples in 10 mL septum vials were incubated at 30°C with shaking for 30 sec, after which 100–500 µL was removed with a gas-tight autosampler syringe heated to the same temperature. Injection was performed on a multi-mode injector in pulsed splitless mode at 100°C with a 30-

sec pulse at 25 psi. The flow rate was otherwise held constant at 1 mL min<sup>-1</sup>. The relative detector responses of the same method run at 0.75, 1.0 and 2.0 mL min<sup>-1</sup> were also compared. The column used was an HP-PLOT Al2O3/KCl (Agilent Technologies), 0.25 mm i.d. x 30 m, with a film thickness of 5 µm. The oven temperature program was as follows: 30°C for 1.5 min, then 20°C min<sup>-1</sup> to 50°C with a 0.1-min hold time (total analysis time 2.6 min). After a solvent delay of 1.8 min to eliminate atmospheric nitrogen, the electron impact energy was set to 70 eV with the detector operating in selected ion mode. Ions at m/z 26 and 27, representing the [M-H]<sup>+</sup> and [M-2H]<sup>+</sup> ethylene fragmentation products, were monitored with a dwell time of 100 msec each at high resolution.

### 3.3. Metabolic profiling by LC-MS and data processing

Tomato fruit extracts extracts were analyzed using a high-resolution UPLC/qTOF system comprised of a UPLC (Waters Acquity) which was connected online to a photodiode array (PDA) and qTOF detectors (tandem quadrupole/time-of-flight mass spectrometer, Xyvo G2-S, Waters). Separation of metabolites was performed on the 100 x 2.1-mm i.d., 1.7- µm UPLC BEH C18 column (Waters Acquity). The mobile phase consisted of 0.1% formic acid in acetonitrile:water (5:95, v/v; phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient was as follows: from 100 to 72% phase A over 22 min, from 72 to 60% phase A over 0.5 min, from 60 to 0% phase A over 0.5 min, then held at 100% phase B for further 3.5min; and then returned to the initial conditions (100% phase A) in 0.5 min and conditioning at 100% phase A for 1.0 min. The flow rate was 0.3 mL/min, the column temperature was kept at 35°C with injection volume of 1 µL. A divert valve (Rheodyne) excluded the first 1.8 and last 4.5 min from the injection. ESI was used in positive and negative ionization mode at an m/z range from 50 to 1600 Da, and PDA was set to a range from 200 to 700 nm.

Masses of the eluted compounds were detected with the following instrumental settings: the capillary voltage was 1 kV; cone voltage - 27 V; source temperature was set to 140 °C, desolvation temperature to 500 °C, desolvation gas flow to 800 L/h with Argon used as the collision gas. Data acquisition was performed in the MSE mode with energy ramp that records an exact mass precursor and fragments ion information from every detectable component in the sample. MSE mode rapidly alternates between two functions: the first acquiring low-energy exact mass precursor ion spectra and the second acquiring elevated-energy exact mass fragment ion spectra. The collision energy was set to 4 eV for the low-energy (MS1) function and to a range of 10 to 30 eV ramp for the high-energy (MS2) function in the positive ionization mode (15 to 35 eV in the negative ionization mode). Scan time for each function was set to 0.15 s. The MS system was calibrated with sodium formate, and Leucine enkephalin was used as the lock mass reference standard. MassLynx software version 4.1 (Waters) was utilized to

control the instrument, view the raw data and calculate accurate masses and elemental compositions.

Raw data files produced as described above were converted from the vendor's format to the netCDF open source format using the MassLynx Databridge software (<http://www.waters.com>). The netCDF files, corresponding with LC-MS data in the positive and negative ionization modes, were subsequently processed in the Rsoftware environment (<https://www.r-project.org>) using open source software packages: XCMS (Smith et al. 2006) and CAMERA (Kuhl et al. 2012) which process individual raw data signals into an aligned matrix of features (i.e. a sequence of m/z – RT tuples with the integrated peak areas across the measured samples). The resulting feature matrix was extracted from the processing objects using the parameter "into" (i.e. integrate peak area) as the peak intensity measure.

Peak intensities were further scaled to the median intensity value of each sample and then log transformed. To find the differentially expressed peaks most highly correlated with the tested experimental factors, all possible contrasts between the maturation stage: mature-green ("MG"), orange ("OR"), ripe-red ("RR") and the genotype factor ("HSP70b:PIF1a-GFP" or "WT") were tested using analysis of variance (ANOVA) coupled to a fold-change filter. The ANOVA results' p-values were corrected for multiple testing using the Benjamini-Hochberg ("BH") procedure and the minimum log-fold change was set to 1.5.

Next, to account for possible interactions and intricate relationships between clusters of metabolic features and the experimental factors, the multivariate orthogonal partial least squares (OPLS) was applied for each contrast individually. The resulting OPLS models, one for each tested contrast, were inspected using the following model quality plots measures: projection of sample scores on the principal and orthogonal planes, model Q<sub>2Y</sub> and model performance in 500 iterations of cross-validation tests. Models with clear separation between replicate sample groups on the scores plot, a Q<sub>2Y</sub> score > 0.5 and significant difference between in model values on the cross-validation data were accepted and merged with the significant features detected using the univariate approach.

## 4. Imaging techniques

### 4.1. Confocal microscopy

Subcellular localization, protein stability and BiFC experiments were determined by analyzing agroinfiltrated leaf samples with an Olympus FV 1000 confocal laser-scanning microscope. GFP signal and chlorophyll autofluorescence were detected using an argon laser for excitation (at 488 nm) and a 500–510 nm filter for detection of GFP fluorescence and a 610–700 nm filter for detection of chlorophyll fluorescence.

In the case of protein stability, same conditions were used strictly to take all the images. First, a field of 20 – 30 nuclei was photographed. After that, the sample was incubated during 30 min in Red ( $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and Far Red light ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The exact same field was photographed again after that exposure, using the same parameters as before. The intensity of the fluorescence signal was measured using the ImageJ software. Individual Regions Of Interest (ROI) were created for each nucleus and identical-size ROI were used to compare the same nucleus before and after the light exposure. The intensity of fluorescence was measured in each pixel of all the ROI and each ROI was considered an individual replicate within one population.

### 4.2. SEM

Root samples were washed 2 washes on 10 min in absolute ethanol and after that they were dry by critical point using an equipment Emitech K850. Then, the samples were mounted on the supports of the SEM suing a biadhesive conductive disk and coated with a thin layer of gold (about 60nm) to make them conductive electrical. This was done with the SEM coating system model SC510 (Fisons Instruments). The study was carried out using a Field Emission Scanning Electron Microscope J-7001F (Jeol) using a secondary electron detector to analyze the topology of the samples.

### 4.3. Photography

Photographs were done using a Nikon D7000 camera coupled to the objective AF-S NIKOR 18-70 MM 1:3.5-4.5G and AF-S MICRO NIKKOR 105 mm 1:2.8G.

## 5. Expression data from Databases

Expression data of different tissues were retrieved from *the Bio-Analytic Resource for Plant Biology* (BAR, University of Toronto), using the tomato tool available in that website and *PIF1a* to *PIF1b* Solyc IDs.

Co-expression analysis was performed as previously described (Ahrazem et al., 2018). Briefly, tomato *PIF1a* to *PIF1b* Solyc IDs were used to retrieve all the expression data available for different cultivars/tissues/treatment in the *Tomexpress* database (Maza et al., 2013). Subsequently, global gene co-expression (GCN) analysis was carried out by calculating pairwise Pearson correlation coefficients between each PIF gene against the tomato genome was computed, and Fisher's Z-transformation was used to test the statistical significance of the pairwise correlations.





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## **ANNEXES**



**Table A1. Described PIF roles in *Arabidopsis*.** Green/Red color indicates the promoting/inhibiting role in the mentioned process.

PIF1		
Red	Seed germination	Oh et al., 2004
Red	Chlorophyll and carotenoid biosynthesis	Huq et al., 2004
Green	Hypocotyl elongation	Shin et al., 2009
Red	ROS signaling	Chen et al., 2013
Red	Flowering time	Wu et al., 2018
PIF2		
Red	Hypocotyl elongation	Luo et al., 2014
PIF3		
Green	Hypocotyl elongation	Ni et al., 1998
Green	Anthocyanins biosynthesis	Shin et al., 2007
Red	Chlorophyll and carotenoid biosynthesis	Shin et al., 2009
Red	ROS signaling	Chen et al., 2013
Green	Leaf senescence	Song et al., 2014
Green	Flowering time	Costa Galvao et al., 2015
Red	Freezing tolerance	Jiang et al., 2017
PIF4		
Green	Hypocotyl elongation	Huq et al., 2002
Green	Thermo-responses	Koini et al., 2009
Green	Stomata development	Casson et al., 2009
Red	Carotenoid biosynthesis	Toledo-Ortiz et al., 2010
Red	Freezing tolerance	Lee et al., 2012
Green	Leaf senescence	Sakuraba et al., 2014
Green	Flowering time	Costa Galvao et al., 2015
Red	Anthocyanins biosynthesis	Liu et al., 2015
PIF5		
Green	Hypocotyl elongation	Fujimori et al., 2004
Red	Chlorophyll and carotenoid biosynthesis	Shin et al., 2009
Green	Thermo-responses	Thines et al., 2014
Green	Leaf senescence	Sakuraba et al., 2014
Green	Flowering time	Costa Galvao et al., 2015
Red	Anthocyanins biosynthesis	Liu et al., 2015
PIF6		
Red	Seed dormancy	Penfield et al., 2010
Red	Seed dormancy	Penfield et al., 2010
PIF7		
Green	Hypocotyl elongation	Leivar et al., 2008
Red	Freezing tolerance	Lee et al., 2012
PIF8		
Green	Hypocotyl elongation	Oh et al., 2020
Red	Seed germination	Oh et al., 2020







































