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Guard cell activity of PIF4 and HY5 control transpiration

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ABSTRACT

Whole-plant transpiration, controlled by plant hydraulics and stomatal movement, is regulated by endogenous and environmental signals, with the light playing a dominant role. Stomatal pore size continuously adjusts to changes in light intensity and quality to ensure optimal CO_2 intake for photosynthesis on the one hand, together with minimal water loss on the other. The link between light and transpiration is well established, but the genetic knowledge of how guard cells perceive those signals to affect stomatal conductance is still somewhat limited. In the current study, we evaluated the role of two central light-responsive transcription factors; a bZIP-family transcription factor *ELONGATED HYPOCOTYL5* (*HY5*) and the basic helix-loop-helix (BHLH) transcription factor *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*), in the regulation of steady-state transpiration. We show that overexpression of *PIF4* exclusively in guard cells (GCPIF4) decreases transpiration, and can restrain the high transpiration of the *pif4* mutant. Expression of *HY5* specifically in guard cells (GCHY5) had the opposite effect of enhancing transpiration rates of WT- Arabidopsis and tobacco plants and of the *hy5* mutant in Arabidopsis. In addition, we show that GCHY5 can reverse the low transpiration caused by guard cell overexpression of the sugar sensor *HEXOKINASE1* (*HXK1*, GCHXK), an established low transpiring genotype. Finally, we suggest that the GCHY5 reversion of low transpiration by GCHXK requires the auto-activation of the endogenous *HY5* in other tissues. These findings support the existence of an ongoing diurnal regulation of transpiration by the light-responsive transcription factors HY5 and PIF4 in the stomata, which ultimately determine the whole-plant water use efficiency.

1. Introduction

Maintaining water homeostasis is critical for plants' normal growth and productivity, temporal drought episodes, resilience, and survival (Claeys and Inzé, 2013; von Caemmerer and Baker, 2007; Resco de Dios et al., 2016). Water management is regulated mainly by transpiration, which consists of stomatal conductance and hydraulic dynamics of roots, stem, and leaves (Sack and Holbrook, 2006). Whole-plant transpiration is regulated by endogenous as well as environmental signals, with light playing a dominant role (Yang et al., 2020; McAusland et al., 2016; Lawson and Matthews, 2020; Wang et al., 2010; Shimazaki et al., 2007; Matthews et al., 2018). Following lights on, the light signals stimulate stomatal opening and initiate the daily gas exchange between the leaf and the atmosphere. Light continuously determines stomatal conductance throughout the day, thereby shaping the daily rate of gas exchange (i.e., H_2O and CO_2 diffusion) (Lawson and Matthews, 2020; O'Carrigan et al., 2014; Matthews et al., 2020; Grondin et al., 2015).

Stomatal opening is primarily initiated by monochromatic blue light and, to a lesser extent, by red and far-red lights (Mao et al., 2005). Red light supports the synergistic action of stomatal opening in the background of weak blue light, suggesting that red and blue light work together to open stomata (Ogawa et al., 1978). Adding blue light to a red light background can increase the magnitude of stomatal conductance over the diurnal period but hampers the overall water use efficiency compared with red light alone (Matthews et al., 2020). Saturation of the response to blue light occurs at low flux rates and is independent of mesophyll photosynthesis. Red light responses, however, saturate at relatively high flux rates, similar to mesophyll photosynthetic fluxes

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(Shimazaki et al., 2007; Ando and Kinoshita, 2019). A greater stomatal conductance may cause excessive water loss that can lead to water limitation. Therefore, the evolution of a mechanism for limiting maximal stomatal conductance and transpiration in response to light (e.g., drought avoidance) is not surprising.

Blue and red light receptors control stomatal movement and conductance, in response to changes in light (Wang et al., 2010; Matthews et al., 2020; Mao et al., 2005), but the genetic knowledge concerning the downstream elements involved in the process emanates mainly from studies focusing on photomorphogenesis at the seedlings stage (Delker et al., 2014; Nemhauser and Chory, 2002; Quint et al., 2016). Several transcription factors involved in the light signal transduction were shown to act as key regulatory junctions in photomorphogenesis, such as the *PHYTOCHROME INTERACTING FACTOR*s (PIFs). PIFs are central players in transducing light signals perceived by the light-sensing PHYTOCHROME (PHY) photoreceptors (Pacin et al., 2016; Castillon et al., 2007; Bae and Choi, 2008; Fraser et al., 2016; Leivar and Monte, 2014; Leivar and Quail, 2011; Wang et al., 2022a). PHYB, the upstream regulator of PIFs, reduces water loss and improves drought tolerance by regulating stomatal conductance and density (Boccalandro et al., 2009, 2003; GONZáLEZ et al., 2012). The stomata of the *phyB* mutants are more closed, and those of the *pif3 pif4* mutants are more open compared with the wild-type, indicating that PIFs may act downstream of PHYB in regulating stomatal conductance in response to light (Wang et al., 2010). Recently, a role for PIF4 as a negative regulator for stomatal development under high temperatures was shown (Lau et al., 2018), further supporting the potential link between PIFs and stomatal conductance. Additionally, PIFs effect on plant growth under different light regimes was also reported (Leivar et al., 2020).

In the context of light-dependent hypocotyl elongation, a central transcription factor that antagonizes the effects of PIFs is the *ELONGATED HYPOCOTYL5* (*HY5*) (Quint et al., 2016; Gangappa and Botto, 2016; Gangappa and Kumar, 2017). While the absence of light promotes elongation via the PIFs, light prevents the degradation of HY5, which targets *PIF4* for degradation, thereby preventing elongation (Quint et al., 2016; Gangappa and Botto, 2016; Xu, 2018; Xu, 2019; Toledo-Ortiz et al., 2014). Extensive genetic and biochemical studies have established that HY5 controls light-regulated transcriptional changes, with up to 1/3 of the genome being putatively regulated by HY5 (Xiao et al., 2021; Burko et al., 2020a). However, the involvement of HY5 in whole-plant transpiration management in response to light changes is so far unexplored.

While transpiration data for HY5 is lacking, changes in stomatal development were recently reported. In this study, the authors found that HY5 binds to, and promotes the *STOMAGEN*, which triggers stomatal developmental events in the epidermal layer, leading to increased stomatal density and stomatal index (the stomata to epidermal cells ratio (Wang et al., 2021)). However, while it is suggested that HY5 controls stomatal development in response to light signals under long-day, it has no effect under short-day growth conditions (Zoulias et al., 2020). These inconclusive results highlight the complex nature of stomatal sensing of light signals and HY5 signal in particular, and provide extra motivation for exploring the role of HY5 in transpiration.

Researchers have long wondered in which tissues and cell types the light signal of HY5 is perceived. HY5 act as a shoot-to-root mobile transcription factor that, when expressed in the mesophyll, or even when is confined to guard cells, is translocated to the roots (Chen et al., 2016; Kelly et al., 2021). Recently, we found that modulated expression of *PIF4* and *HY5*, specifically in guard cells, is sufficient to promote or terminate hypocotyl elongation, respectively, uncovering a putative role for these genes in guard cells (Kelly et al., 2021).

In addition to light, sugars were also shown to affect hypocotyl elongation. *HEXOKINASE1* (*HXK1*) is a glucose-phosphorylating enzyme that mediates sugar-sensing in addition to its catalytic role (Kelly et al., 2013; Granot and Kelly, 2019) and supports the sugar-induced hypocotyl growth (Kelly et al., 2021). When *HXK1* is expressed specifically in guard cells, the hypocotyls are three times longer than those of WT seedlings (Kelly et al., 2021). In addition to promoting hypocotyl growth, the expression of *HXK1*, specifically in guard cells (GCHXK), reduces transpiration by about 20% (Kelly et al., 2013, 2019), linking developmental with physiological effects, emanating from stomata.

Overall, it seems intuitive that light transducing pathways should cross-talk with transpiration and stomatal conductance. However, the direct link between light-transducing transcription factors; PIF4 and HY5 and the regulation of steady-state transpiration was not tested so far. Moreover, the specific contribution of the guard cell tissue to the PIFs/HY5 effect on transpiration has never been addressed.

In this study, we aim to explore how light signals that are perceived in guard cells by PIF4 and HY5 shape whole-plant transpiration. Our findings support the existence of an ongoing diurnal regulation of whole plant transpiration by the light-responsive transcription factors; HY5 and PIF4 in a stomata-specific manner. Moreover, as was recently shown for hypocotyl elongation, we report a cross-talk in the stomata between PIF4, HY5, and HXK1 to affect daily transpiration.

2. Material and methods

2.1. Plant material and growth conditions

The Arabidopsis plants used in this study were of the *Arabidopsis thaliana* Col-0 ecotype. The *pif4–101* (Pacin et al., 2016), *hy5* (Kelly et al., 2021), *GCHXK and GCGFP* (Kelly et al., 2013, 2017a), *GCPIF4/-pif4*, GCHY5/*hy5*, GCHXK/GCHY5, GCHXK/GCHY5/*hy5*, GCHXK/*hy5*, GCHY5-GFP, GCHXK/GCHY5-GFP plants have been described previously (Kelly et al., 2021). The generation of transgenic tobacco (*N. tabacum* cv. *Samsun NN*) GCHY5 plants is described below (Section 2.2). The Arabidopsis plants were grown in a growth chamber under short-day conditions (10 h light, 150 μmol m^{-2} s-1) at a controlled temperature of 20–22 °C with ~50–60% humidity. All plants were grown in a potting mix containing (w/w) 30% vermiculite, 30% peat, 20% tuff, and 20% perlite (Even Ari, Israel). The tobacco plants were grown in a mixture of 70% tuff and 30% peat (Even Ari). For the whole plant transpiration measurements, the Arabidopsis and tobacco plants were grown in a greenhouse under semi-controlled, natural light conditions in Rehovot, Israel, from January to February of 2020 (Arabidopsis) and during September of 2019 (tobacco).

2.2. Generation of transgenic tobacco plants

To generate transgenic GCHY5 tobacco plants, we used a vector previously described (Kelly et al., 2021) that contains the HY5 coding sequence under the control of a guard cell-specific promoter *KST1* (*KSTppro::AtHY5*). Electrocompetent *Agrobacterium tumefaciens* (GV3101 strain) was transformed using a 100 ng plasmid by electroporation. Tobacco was transformed using *Agrobacterium*-mediated transformation (Horsch et al., 1985; Gallois and Marinho, 1995). Leaf discs of sterile leaves were placed in an induction medium that contained Murashige and Skoog (MS, Duchefa Biochemie, The Netherlands), 3% sucrose (Duchefa), 1 mg l^{-1} 6-benzylaminopurine (BA; Sigma-Aldrich, Israel), 2 mg l^{-1} naphthalene acetic acid (NAA; Duchefa), and 100 μM acetosyringone (AS; Sigma-Aldrich) for 24 h at 25 °C in the dark. Leaf discs were then immersed with *Agrobacterium* for 2 min, dried, and transferred back to induction medium for another three days. Explants were then shifted to selection medium (MS containing 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 500 mg l^{-1} Claforan (Cla; Cefotaxim, Duchefa), and 200 mg l^{-1} kanamycin (Kan; Duchefa) for selection. Small plantlets appeared after ~1 month and were shifted to MS medium containing 0.1 mg l^{-1} BA, 500 mg l^{-1} Cla, and 200 mg l^{-1} Kan. Developed plantlets (2 cm long) were transferred to rooting medium (MS+500 mg l^{-1} Cla, and 200 mg l^{-1} Kan). Rooted plantlets were transferred to soil and were kept for 10 d in high humidity before being transferred to the greenhouse.

2.3. Whole plant transpiration

Whole-plant daily transpiration rate was determined using the functional phenotyping system Plantarray 3.0 platform (Plant-Ditech, https://www.plant-ditech.com/), as detailed by (Halperin et al., 2016; Dalal et al., 2020). Individual WT and transgenic/mutant plants were used as described for each experiment. A 3.9-l pot with a single tobacco plant or with four Arabidopsis plants was grown under semi-controlled conditions. Each pot was placed on a scale load unit and sealed to prevent evaporation from the surface of the growth medium. Each scale contains a control unit connected to a serial data logger for data collection. The output of the load units was monitored every 10 s, and the average readings over 3-min periods were collected in a data logger for further analysis. Whole-plant transpiration was calculated as a numerical derivative of the load cell output following a data-smoothing process (Dalal et al., 2020). Each plant's transpiration rate was normalized to its weight or total leaf area, as described for each experiment. Environmental conditions of photosynthetically active radiation (PAR) and vapor pressure deficit (VPD) were monitored throughout the experiment by an atmospheric probe. At the end of the experiment, plants were taken for dry weight measurements.

2.4. Gas-exchange analysis

Transpiration and stomatal conductance was measured using LI-600 (LI-COR, Lincoln, NE, USA) Porometer under ambient growth conditions. The gas-exchange analysis described in Figs. 4 and 5 was conducted using the LI-6800 (LI-COR), where photosynthesis was induced under light (200 µmol m-2 s-1) with 400 µmol mol-1 CO_2 surrounding the leaf (Ca). The amount of blue light was set to 5% of the photosynthetically active photon flux density. The flow rate was set to 150 µmol air s-1, and the leaf-to-air vapor pressure deficit was kept around 1–1.1 kPa during the measurement. Leaf temperature was 22 °C.

2.5. Leaf area and whole rosette measurements

For leaf area measurements described in Figs. 1 and 4, leaves were removed, images were taken and leaf area was determined using the ImageJ (http://rsb.info.nih.gov/ij/) software's wand (tracing) tool. For the whole rosette area described in Fig. 3, the whole rosette was removed and area was analyzed using the LI-COR 3100 area meter (LI-COR).

2.6. Determination of K_{leaf}

K_{leaf} was calculated for each individual leaf by dividing the whole-leaf transpiration rate, E (measured with LI-600 under PAR of 180 µE $m^{-2}s^{-1}$), by the leaf water potential, Δ_{leaf}. In our calculation, $\Delta_{leaf} = \Psi_{leaf,light} - \Psi_{leaf,dark}$ as leaves remain intact.

Immediately following E measurement, the leaf was transferred to a pressure chamber (ARIMAD-3000; MRC Israel) to determine $\Psi_{leaf,light}$ and next an additional leaf from the same plant, fully covered with tin foil (where $E = 0$), was transferred to the pressure chamber to determine $\Psi_{leaf,dark}$. These measurements were conducted between 10:00 and 13:00 (1–4 h after the lights on).

2.7. Confocal microscopy imaging

Image acquisition of the GCHY5-GFP mature plants was done using a Leica SP8 laser scanning microscope (Leica, Wetzlar, Germany)

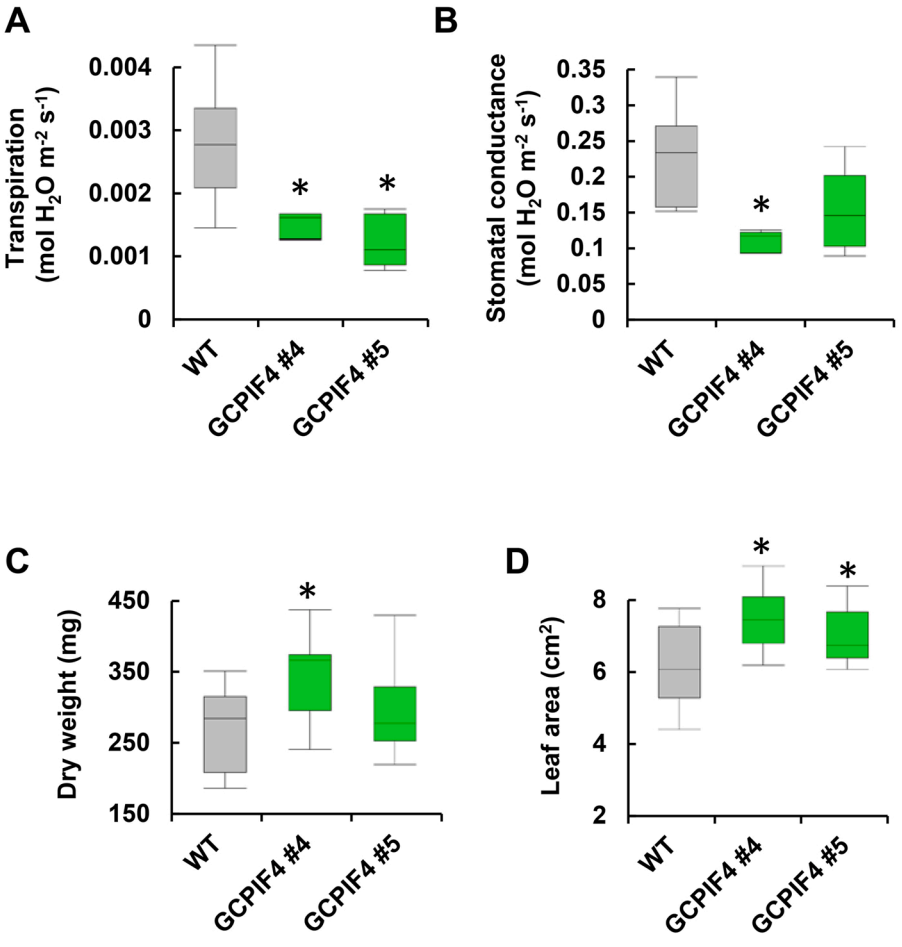

Fig. 1. Guard cell-specific expression of *PIF4* reduces transpiration of WT plants. (A-B) Transpiration rate (A) and stomatal conductance (B) of WT and two independent transgenic Arabidopsis lines expressing *AtPIF4* specifically in guard cells (GCPIF4). (C-D) Dry weight (C) and leaf area (D) of WT and GCPIF4 plants. (A-D) The box plots extend from the first to third quartiles, and the whiskers extend from the minimum to the maximum levels. Lines within the boxes signify median values ($n = 8$–10). Asterisks indicate significant differences relative to the WT (Dunnett's test, $P < 0.05$).

equipped with a solid-state laser with 488 nm light, a HC PL APO CS 63 × /1.2 water immersion objective (Leica) and Leica Application Suite X software (LASX, Leica). Images of GFP signal were acquired using the 488-nm laser light and the emission was detected with HyD (hybrid) detector in a range of 500–525 nm. Autofluorescence of the chloroplasts was detected in a range of 650–750 nm with a PMT detector.

2.8. Stomatal measurements

For the stomatal densities and index measurements, epidermal peels were removed and immediately taken for imaging. Three random fields were selected and averaged for every biological repetition (n = 10 overall). In each area, all stomata and epidermal cells were imaged under a bright-field inverted microscope (1M7100; Zeiss; Jena, Germany), on which a Hitachi HV-D30 CCD camera (Hitachi; Tokyo, Japan) was mounted. The Stomatal index was calculated as the number of stomata/number of epidermal cells). A microscopic ruler (Olympus; Tokyo, Japan) was used for size calibration.

2.9. Statistical analysis

Statistical analysis was performed using the JMP 16 software. Box plots were prepared using the graph builder tool. Means were compared using Student's *t*-test, Tukey's HSD test or Dunnett's method, as described for each experiment. Means were considered significantly different at $P < 0.05$.

3. Results

3.1. Overexpression of PIF4 and HY5 in guard cells modifies transpiration of Arabidopsis and tobacco plants

Plant responses to light are mediated by regulatory factors such as the *PIF4* and *HY5* transcription factors. These factors regulate contrasting developmental effects; while *PIF4* supports hypocotyl elongation, the HY5 ceases elongation. The specific counter responses of *HY5* and *PIF4* to light, together with the general effect of light on transpiration has led us to explore the potential involvement of *PIF4* and *HY5* in transpiration. We hypothesized that transpiration is affected by the activity of PIF4 and HY5 in an antagonist manner, and that guard cells are sufficient to stimulate this effect. To address this hypothesis, we assayed the transpiration rate of plants expressing *AtPIF4* (*GCPIF4*) or *AtHY5* (*GCHY5*) specifically in the guard cells of Arabidopsis WT plants using gas-exchange analysis. The *GCPIF4* plants displayed lower stomatal

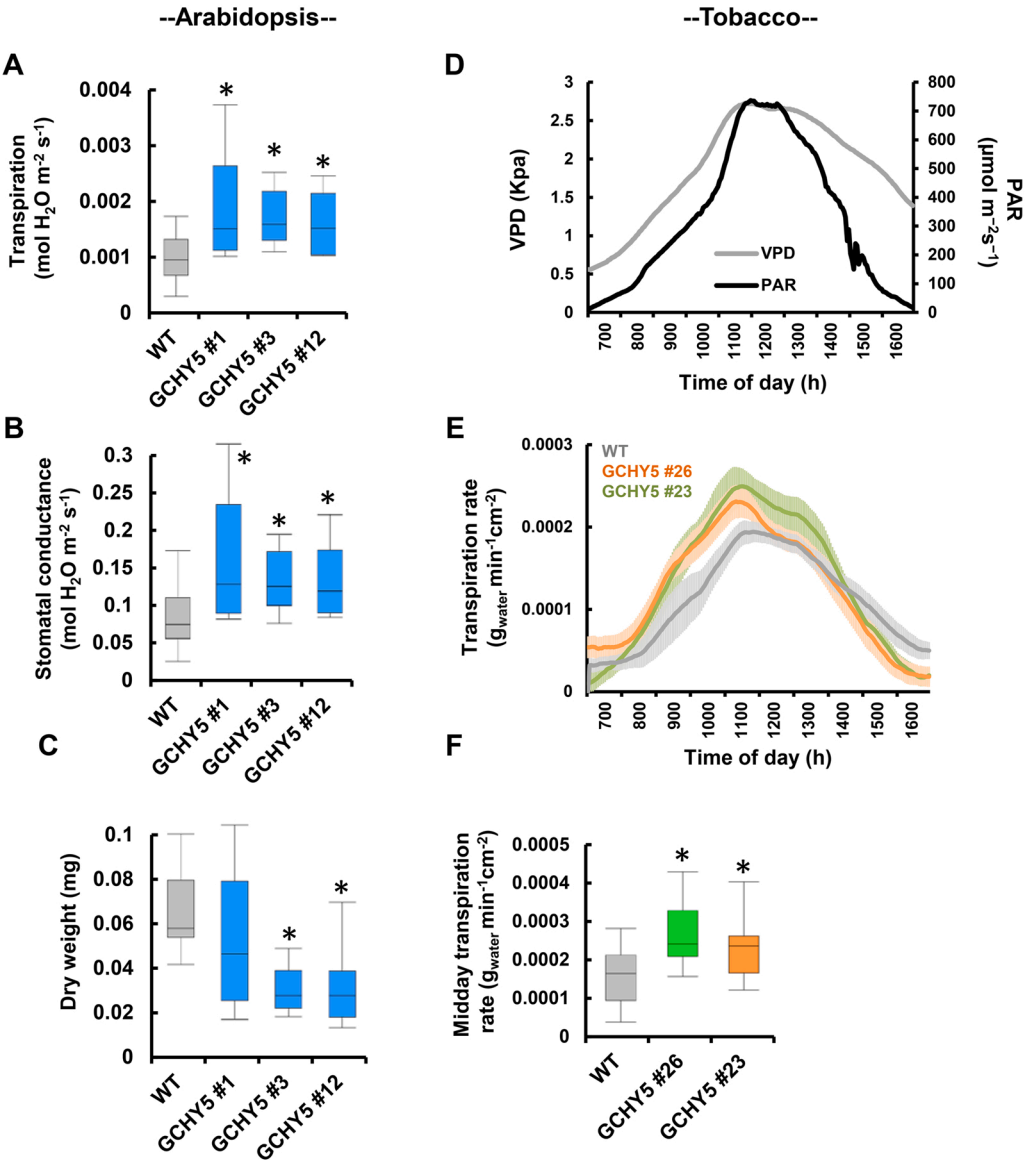

Fig. 2. Elevated expression of *HY5* in guard cells of Arabidopsis and tobacco enhances transpiration rate. (A-B) Transpiration rate (A) and stomatal conductance (B) of WT and three independent transgenic Arabidopsis lines expressing *AtHY5* specifically in guard cells (GCHY5). (C) The dry weight of GCHY5 plants. (D-F) Diurnal transpiration rate of wild type and two independent tobacco GCHY5 lines, measured using the lysimeter system. (D) Diurnal vapor pressure deficit (VPD) and photosynthetically active radiation (PAR). (E) Diurnal transpiration rate normalized to leaf area and monitored simultaneously and continuously throughout the day. Data are means ± SE ($n = 17$–18). (F) Midday transpiration rate averaged at midday (10:00 h-12:00 h). (A-C, F) The box plots extend from the first to third quartiles, and the whiskers extend from the minimum to the maximum levels. Lines within the boxes signify median values ($n = 8$–12 for A-C, $n = 17$–18 for F). Asterisks indicate significant differences relative to the WT (Dunnett's test, $P < 0.05$).

conductance and transpiration rates (Fig. 1A-B). While it was expected that low transpiration would result in growth inhibition, we did not observe any growth penalty in the GCPIF4 plants (Fig. 1C-D). Contrary to GCPIF4, the GCHY5 plants displayed higher transpiration rates and stomatal conductance (Fig. 2A-B), together with growth inhibition, when compared to WT (Fig. 2C). This pattern was also observed for plants expressing HY5-GFP fusion in their guard cells (GCHY5-GFP, Fig. 3). In these plants, the GFP signal was observed in the guard cells of mature leaves (Fig. 3A), together with higher stomatal conductance (Fig. 3C) and growth inhibition (Fig. 3D-E). These results further confirmed the effect of GCHY5 in promoting transpiration.

To further explore the role of HY5 and its effect on transpiration, we generated tobacco plants expressing HY5 under the control of a guard cell-specific promoter (*GCHY5*). We examined the whole-plant diurnal transpiration behavior of the *GCHY5* plants by performing continuous measurements of transpiration rate using the precise and sensitive lysimeter-scale system that allows continuous water loss measurements (Dalal et al., 2020). We monitored transpiration rate in addition to changes in light intensity and vapor pressure deficit (VPD, Fig. 2D). Transpiration rate, normalized to the leaf area (Fig. 2E), followed the diurnal changes in radiation and VPD throughout the day (Fig. 2D). As in the case of Arabidopsis (Fig. 2A), the transpiration rate of the *GCHY5* tobacco plants was significantly higher than WT, mainly during the first part of the day (Fig. 2E-F). These results further support the role of HY5 in enhancing the transpiration rate when expressed specifically in the guard cells.

3.2. *Guard cell-specific complementation of PIF4 in a pif4 mutant is sufficient to reduce transpiration and increase water-use efficiency*

Arabidopsis *pif4* null-mutant plants displayed higher stomatal conductance and transpiration rates than WT (Fig. 4A-B). However, complementation of *PIF4* expression, exclusively in guard cells (GCPIF4*/pif4*), restrained the high transpiration and stomatal conductance imposed by the *pif4* mutation. (Fig. 4A-B). Next, we measured photosynthesis in GCPIF4*/pif4* plants using the gas-exchange system and

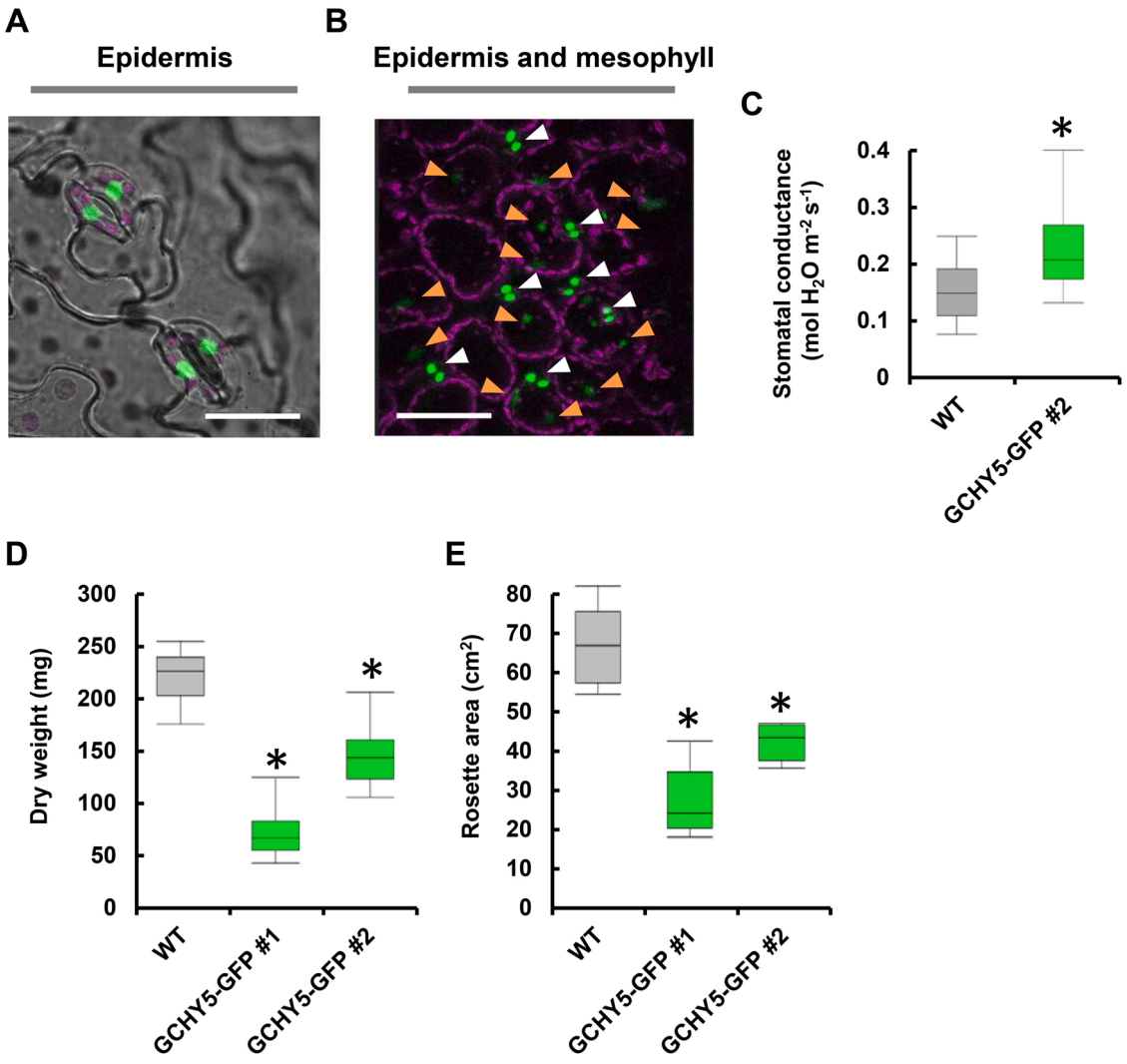

Fig. 3. HY5 produced within guard cells is translocated to mesophyll cells of mature leaves, increases stomatal conductance, and inhibits growth. (A-B) GFP distribution in mature leaves of plants expressing HY5-GFP in guard cells (GCHY5-GFP). (A) Distribution of HY5-GFP in the epidermal peel. The panel is a merged images of white light, chlorophyll-autofluorescence (stained magenta), and GFP-fluorescence (stained green). Scale bar (white) = 20 μm. (B) Distribution of HY5-GFP in mature leaf composed of epidermis and mesophyll cells. The panel is a merged images of chlorophyll-autofluorescence (stained magenta) and GFP-fluorescence (stained green). White arrows indicate the location of GFP in guard cells, and orange arrows indicate the location of GFP in mesophyll cells. Scale bar (white) = 50 μm. (C) Stomatal conductance of WT and GCHY5-GFP plants. (D-E) The dry weight (D) and rosette area (E) of GCHY5-GFP plants. (C-E) The box plots extend from the first to third quartiles, and the whiskers extend from the minimum to the maximum levels. Lines within the boxes signify median values ($n = 25$ for C, $n = 10$ for D and E). Asterisks indicate significant differences relative to the WT (*t*-test, $P < 0.05$ for C, Dunnett's test, $P < 0.05$ for D and E).

found it was similar to WT and *pif4* (Fig. 4C). Thus, an average photosynthesis value, combined with lower transpiration, resulted in improved water-use efficiency (*I*WUE) of the GCPIF4/pif4 plants, calculated as the rate of photosynthesis divided by the transpiration rate (Fig. 4D). As in the case of PIF4 overexpression in the WT background (*GCPIF4* plants; Fig. 1), the reduction in transpiration did not result in impaired growth when GCPIF4 was expressed under the *pif4* mutant background (Fig. 4E-F). These results suggest a central role for the light-sensitive transcription factor PIF4 within guard cells in regulating transpiration. Moreover, the results indicate that the effect of *GCPIF4* is independent of *PIF4* expression in other tissues.

3.3. Overexpression of HY5 in guard cells enhances transpiration of the *hy5* mutant

Next, we assayed the transpiration of plants overexpressing *AtHY5*, specifically in the guard cells, in the *hy5* mutant background (GCHY5/*hy5*). We performed continuous measurements of the whole plant transpiration rate using the lysimeter-scale system. The GCHY5/*hy5* plants displayed a notable increase in transpiration compared to the *hy5* mutant plants and compared to WT throughout the day (Fig. 5A). Midday transpiration rate (averaged between 12:00 h-14:00 h), while the transpiration is at its peak, revealed transpiration rates that are 25% and 9% higher compared to *hy5* in lines 7 and 12, respectively (Fig. 5B). These results support a role for HY5 in guard cells as a positive regulator of transpiration. The photosynthesis of the GCHY5/*hy5* plants was similar to *hy5*, and together with the high transpiration results in a reduction in *IWUE* (Fig. 5C-D). Nevertheless, even though transpiration was significantly higher and *IWUE* was lower, growth inhibition was observed only in the case of GCHY5/*hy5* #12 (Fig. 5E). Hence, the GCHY5 effect on growth in the GCHY5/*hy5* plants was only mild compared to the effect observed when it was expressed under the WT background (GCHY5 and GCHY5-GFP, Fig. 2C, 3D-E).

3.4. GCHY5 counteracts the reduced transpiration effect of GCHXK in an *AtHY5*- dependent manner

The results so far demonstrate that the effect of HY5 in guard cells promotes transpiration (Figs. 2, 3 and 5). Recently, it was shown that GCHY5 counteracted the elongated hypocotyl effect in seedlings, stimulated by *HEXOKINASE1* (HXK) - a glucose phosphorylating/sensor enzyme (Kelly et al., 2021). While the GCHY5 terminates hypocotyl growth, the GCHXK seedlings that express HXK specifically in guard cells have the opposite effect of promoting hypocotyl growth (Kelly et al., 2021). On top of promoting hypocotyl elongation at the seedlings stage, GCHXK reduces transpiration rates of mature plants by about 20% (Kelly et al., 2019). Given these findings, we wished to explore whether GCHY5 can reverse the low transpiration imposed by GCHXK. We thus used the GCHXK plants as a system for mimicking low basal transpiration via reduced stomatal conductance. We evaluated the transpiration of Arabidopsis plants co-expressing GCHY5 and GCHXK (GCHY5/GCHXK). We tested the GCHY5/GCHXK effect in the WT background (GCHY5/GCHXK/WT) and under the *hy5* mutant background (GCHY5/GCHXK/*hy5*). The transpiration of GCHY5/GCHXK in the WT background (GCHY5/GCHXK/WT) was higher than the low transpiration imposed by GCHXK, suggesting that GCHY5 can enhance the low transpiration of GCHXK when expressed in the WT background (Fig. 6A-B). However, under the *hy5* mutant background, the transpiration of GCHXK/GCHY5/*hy5* remained low as in GCHXK (Fig. 6A-B). The low transpiration of GCHXK/GCHY5/*hy5* plants indicate that having HY5 only in the guard cells is insufficient to complement the low transpiration induced by GCHXK, and that the enhanced transpiration

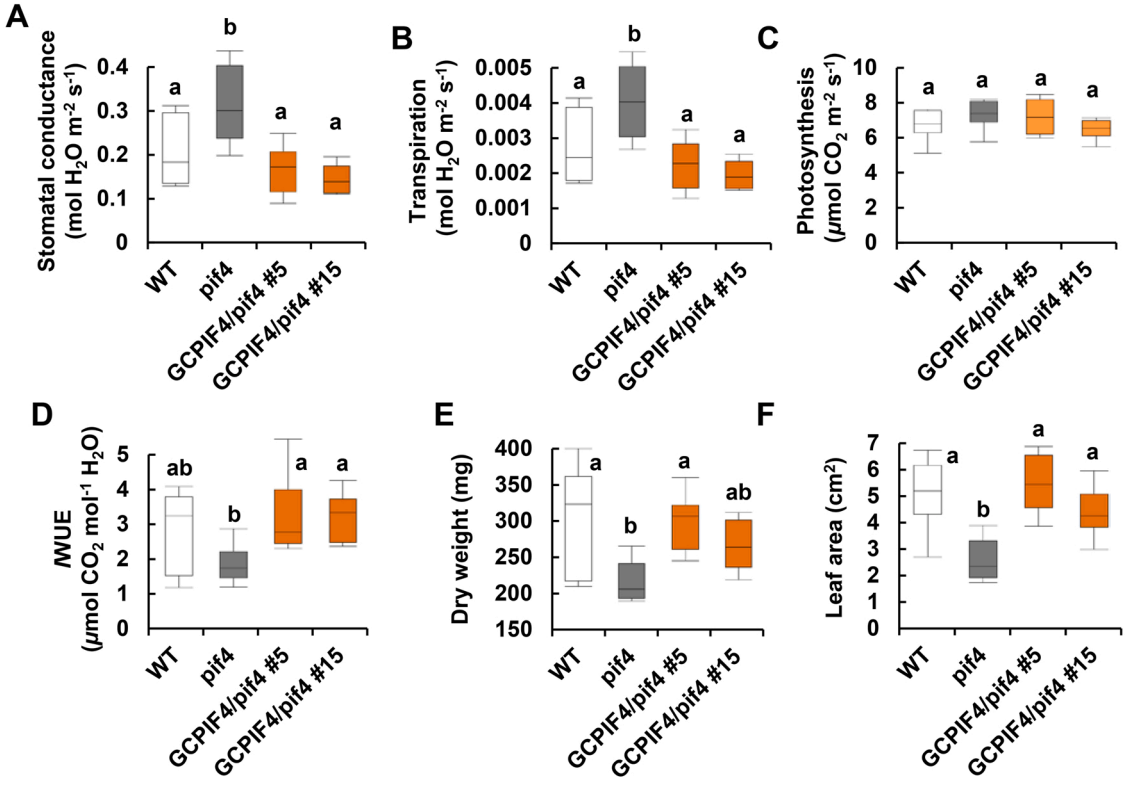

Fig. 4. GCPIF4 complements the high transpiring *pif4* mutants and increases water use efficiency. (A-D) Gas exchange analysis of WT, *pif4*, and two independent transgenic lines expressing *AtPIF4* specifically in guard cells under the *pif4* mutant background (GCPIF4/*pif4*). Stomatal conductance (A), transpiration rate (B), photosynthesis (C), and instantaneous water-use efficiency (*I*WUE, D) of the GCPIF4/*pif4* plants. (E-F) Dry weight (E) and leaf area (F) of WT, *pif4*, and GCPIF4/*pif4* plants. (A-F) The box plots extend from the first to third quartiles and the whiskers extend from the minimum to the maximum levels. Lines within the boxes signify median values ($n = 6$–12 for A-D, $n = 8$–12 for E-F). Different letters indicate significant differences (Tukey's HSD test, $P < 0.05$).

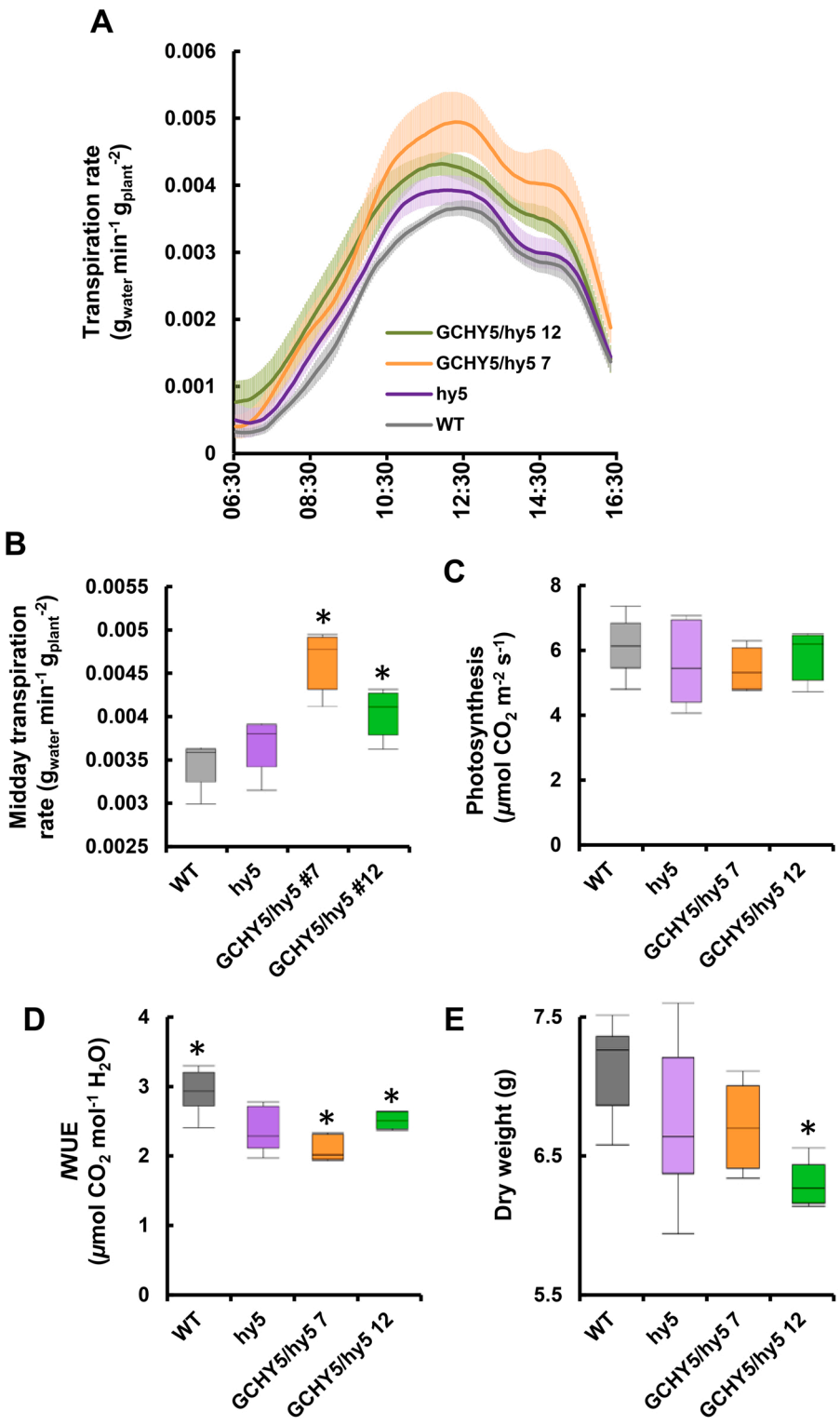

Fig. 5. GCHY5 promotes transpiration under the *hy5* mutant background. (A) Transpiration rate of Arabidopsis WT, the mutant *hy5*, and two independent lines expressing HY5 specifically in guard cells under the *hy5* mutant background (GCHY5/*hy5*). The transpiration rate was normalized to the plant's fresh weight and was monitored simultaneously and continuously throughout the day. Data are means ± SE ($n = 6$). (B) Midday transpiration rate of (A), averaged at midday (12:00 h-14:00 h). (C) Photosynthesis (D), and instantaneous water-use efficiency (*I*WUE) of GCHY5/*hy5*. (E) The dry weight of GCHY5/*hy5* plants. The box plots extend from the first to third quartiles, and the whiskers extend from the minimum to the maximum levels. Lines within the boxes signify median values ($n = 6$ for B and E, $n = 6$–8 for C and D). Asterisks indicate significant differences relative to the *hy5* (Dunnett's test, $P < 0.05$).

by GCHY5 probably requires the presence of *AtHY5* in tissues other than guard cells when low basal transpiration is imposed by GCHXK (Fig. 6A-B). To test whether this phenomenon is related to the leaf water flux throughout the plant (from xylem to mesophyll), we have characterized the leaf hydraulic conductivity (K_{leaf}) of all genotypes and found no significant contribution of K_{leaf} to the GCHXK /GCHY5/WT high transpiration or GCHXK /GCHY5/*hy5* low transpiration (Fig. 6C). GCHXK presented higher K_{leaf} as was reported previously (Kelly et al., 2017b), but the GCHY5 had no effect, suggesting that the role of HY5 that is required for increasing transpiration is not related to leaf hydraulics.

4. Discussion

4.1. GCPIF4, GCHY5, and transpiration

The stomatal conductance is continuously adjusted to meet the plant's gas-exchange requirements by responding to various

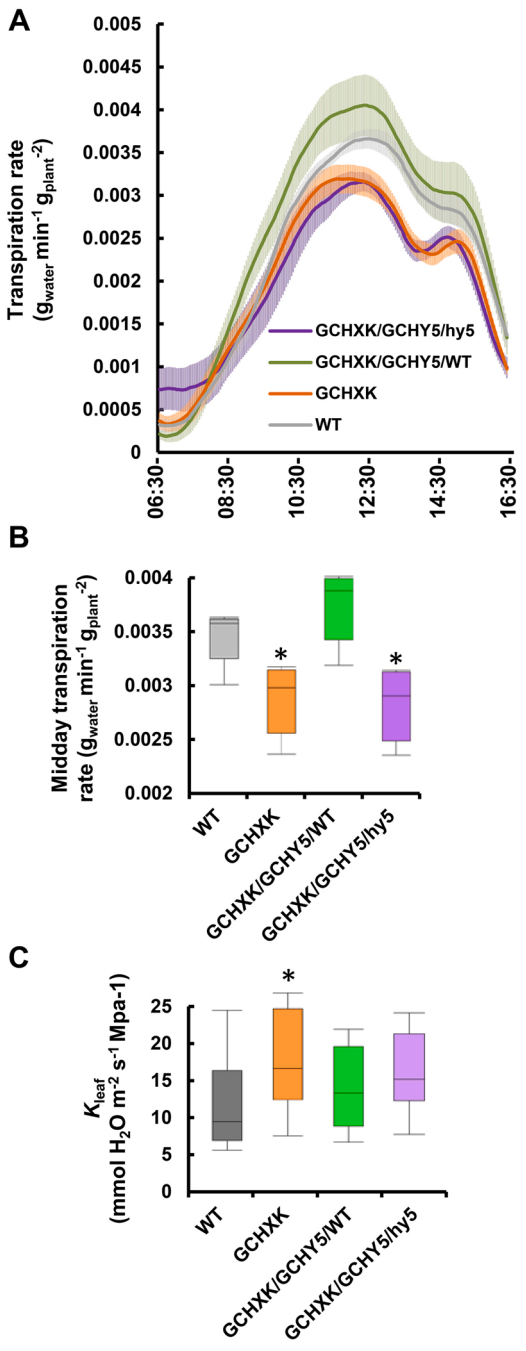

Fig. 6. The ability of GCHY5 to reverse the low transpiration of GCHXK depends on the endogenous *AtHY5* and does not affect leaf hydraulics. (A) Transpiration rate of Arabidopsis WT, GCHXK, GCHXK/GCHY5/WT (WT background), and GCHXK/GCHY5/*hy5* (*hy5* mutant background) plants, normalized to the plant's fresh weight was monitored simultaneously and continuously throughout the day. Data are means ± SE ($n = 6$). (B) Midday transpiration rate of (A), measured between 12:00 h-14:00 h. (C) Leaf hydraulic conductivity (K_{leaf}) of WT, GCHXK, GCHXK/GCHY5/WT, and GCHXK/GCHY5/*hy5* plants. (B-C) The box plots extend from the first to third quartiles, and the whiskers extend from the minimum to the maximum levels. Lines within the boxes signify median values ($n = 6$ for B, $n = 10$–14 for C). Asterisks indicate significant differences relative to the WT (Dunnett's test, $P < 0.05$).

environmental signals, with light playing a dominant role. Light, which triggers the light-reaction step of photosynthesis to produce energy in an autotrophic manner, also mediates transpiration, determined by a combination of light intensity and quality. Hence, guard cells respond to changes in light and the resulting effect on transpiration is critical for the plant's day-to-day activity, production, and survival. While it is well-established that blue and red light receptors control transpiration in response to changes in light (Wang et al., 2010; Matthews et al., 2020; Mao et al., 2005), the knowledge concerning the downstream elements participating in the response, is relatively limited. In the present study, we demonstrated that *PIF4* and *HY5* play a key role in regulating plant transpiration. When specifically expressed in the guard cells, *PIF4* reduces the transpiration rate, while *HY5* has the opposite effect of enhancing transpiration (Figs. 1–5). Using a guard cell- specific promoter enabled us to demonstrate that guard cells are sufficient to perceive light signals and stimulate changes that affect transpiration.

Recent studies have shown that PIFs affect transpiration when expressed under a global, non-specific promoter. In maize and carrot, overexpression of *ZmPIF3*, *ZmPIF1*, and *DcPIF3* resulted in lower transpiration and promoted drought tolerance (Gao et al., 2018a, 2018b; Wang et al., 2022b). In addition, the Arabidopsis *pif3 pif4* double mutant's stomatal aperture size is larger than WT (Wang et al., 2010), suggesting that global expression of PIFs can alter stomatal aperture and whole-plant transpiration. Nevertheless, the *GCPIF4* highlights the impact of PIF4 on transpiration, even when its expression is confined to a specific (guard) cell type. Compared to PIFs, the knowledge concerning HY5 and stomatal movement is minimal, and the effect itself is less pronounced. By using the *hy5* mutant, its homolog *hyh*, and the double *hy5/hyh* mutants, HY5 was shown to affect stomatal aperture under UV-B light, while no effects were observed under white light (Tossi et al., 2014). However, using guard cells overexpression, we show that *HY5* clearly affects transpiration under normal growth conditions (Figs. 2, 3, and 5).

The effect of stomata on transpiration is driven by stomatal movement, as well as from stomatal development, where changes in stomatal density (SD, number of guard cells pairs per unit area) define transpiration rate. The final stomatal density of a mature leaf is determined by stomatal initiation (differentiation) at the first stages of leaf ontogenesis and, at later stages, by epidermal cell expansion. Recent studies have shown that *pif4* and *hy5* mutants affect stomatal development and that this effect depends on day length (Lau et al., 2018; Xu, 2018; Wang et al., 2021; Zoulias et al., 2020). For the question of whether *GCHY5* and *GCPIF4* transpiration involve changes in SD to affect the overall transpiration, we measured SD and SI (ratio of guard cells to epidermal cells). We found that the GCPIF4/*pif4* and GCHY5-GFP plants display lower and higher SD, respectively (Fig. S1A and C), which correlates with the direction of transpiration in those plants. Compared to SD, the stomatal index remain unaffected (Fig. S1B and D). This finding indicates that the differences observed for SD are not due to changes in stomatal differentiation (Fig. S1B and D) but rather the result of changed epidermal cell expansion that eventually defines growth. Taken together, it is clear that GCPIF4 and GCHY5 plants' overall transpiration is also affected by changes in SD.

Moreover, these results suggest that guard cells are sufficient to trigger signals exported from the guard cells to affect the overall epidermal cell growth and alter the transpiration rate. It is well-established that PIF4 promotes auxin biosynthesis and signaling (Leivar and Monte, 2014; Legris et al., 2019). Hence, it is possible that within guard cells, PIF4 stimulates auxin (or auxin-related genes) production that is exported to affect cell expansion in other tissues. In support of this possibility, *GCPIF4* was recently suggested to promote hypocotyl growth via the auxin pathway in a similar way (Kelly et al., 2021).

Our finding supports previous knowledge concerning the conflicting roles of PIFs and HY5 in light responses. During seedling's development, HY5 antagonizes the function of PIF4 (and PIFs in general) that is essential for hypocotyl elongation (Delker et al., 2014; Quint et al., 2016). The genetic factors that are involved in the light-induced hypocotyl elongation were primarily verified by using knock-out mutants and global overexpression lines, but also when they are confined to a specific tissue, such as epidermis, mesophyll, phloem, or guard cells (Kelly et al.,

2021; Savaldi-Goldstein et al., 2007; Burko et al., 2020b; Kim et al., 2020; Procko et al., 2016). In addition to the previously known effects, we have established here that guard cell activity of PIF4 and HY5 is sufficient to control PIF4/HY5-dependent transpiration (Figs. 1–5) and all together, suggesting that stomata act as central developmental- and physiological photo-sensory cells. Moreover, these findings suggest a novel, unestablished link between hypocotyl length and transpiration rate for genes expressed in guard cells (for a detailed discussion, see Section 4.4).

4.2. GCPIF4 as a potential tool for improving plant water-use efficiency

Our work demonstrates a key role for *PIF4* in restricting transpiration rates. The high transpiration of the *pif4* mutant was complemented by the addition of GCPIF4 (Fig. 4). The low transpiration did not result in impaired growth as expected when transpiration is reduced (Figs. 1 and 4). Lower transpiration together with similar biomass gain and leaf area suggest that GCPIF4 can improve WUE (Fig. 4D). These results imply that normal-to-improved growth can co-occur with reduction in transpiration. It is generally accepted that reduced stomatal conductance (g_s) will lower the amount of CO_2 absorbed, consequently lowering biomass production and yield (Lawson and Blatt, 2014; Lawson et al., 2014). However, several studies demonstrated it is not always the case, showing improved WUE when g_s is reduced without compromising photosynthesis and growth. For example, manipulating malate metabolism by overexpressing nicotinamide adenine dinucleotide phosphate (NADP)-malic enzyme (NADP-ME) in guard cells and vascular cells decreased stomatal aperture but improved photosynthesis and biomass (Müller et al., 2018). High sugar sensing activity in guard cells by HXK1 had a similar effect of decreased stomatal conductance without impairing photosynthesis or biomass (Kelly et al., 2019, 2017b). More examples include manipulating the photosynthetic system in tobacco, overexpression of TaPYL4 ABA receptor in wheat, and expressing a synthetic light-responsive potassium channel in Arabidopsis guard cells (Mega et al., 2019; Głowacka et al., 2018; Papanatsiou et al., 2019). Other results showing reduced g_s with normal photosynthesis rates come from targeting stomatal density-related genes. Plants harboring a mutation in *GT-2 LIKE 1* (*GTL1*) and overexpressing *EPIDERMAL PATTERNING FACTOR 2* (*EPF2*) plants, for example, have reduced stomatal density together with reduced g_s values but display standard photosynthesis rate and growth while improving WUE (Yoo et al., 2010, 2011; Doheny-Adams et al., 2012). The GCPIF4 trait provides additional support for breaking the g_s /*A* positive correlation for achieving high WUE. Regarding the underlying mechanism, we suggest that in *GCPIF4* plants the g_s is reduced to levels that do not limit CO_2 uptake, while reducing transpiration, thus improving WUE.

Several studies have demonstrated that PIFs act via the abscisic acid (ABA) responsive pathways. In Arabidopsis, PIF4 and PIF5 induce leaf senescence by triggering ABA signaling (Sakuraba et al., 2014). In addition, PIFs interact with the PYL8 and PYL9 ABA receptors to induce ABA signaling in the dark (Qi et al., 2020). Finally, DNA-affinity purifications found that PIF3 and PIF5 directly bind to ABA-responsive genes, such as ABI5 and several ABA receptors (Liang et al., 2020). Taken together, overproduction of PIF4 in the stomata may set higher basal ABA levels, thus maintaining partial closure of the stomata at all times compared to the WT. In addition, ABA stimulation by *GCPIF4* may have a dual effect, where in addition to the direct effect on stomatal movement, it may pose a long-term effect of reducing stomatal density to limit transpiration. Finally, ABA overproduction in the whole plant tissues is known to enforce growth arrest and pleiotropic effects by withholding major pathways, such as photosynthesis (Thompson et al., 2007; He et al., 2018; Estrada-Melo et al., 2015). Expressing *PIF4* exclusively in the stomata allows us to avoid those inhibitory effects and specifically target guard cells to prevent water loss, which eventually improves the overall plant WUE.

4.3. GCHY5 elicits a long-distance signal to affect transpiration

We have demonstrated that under steady-state conditions, the specific expression of *HY5* in guard cells (GCHY5) is sufficient to enhance transpiration in both WT and *hy5* genetic backgrounds (Figs. 2, 3, and 5). Interestingly, this is not the case when the genetic background is GCHXK (Fig. 6). Under the presence of GCHXK, GCHY5 counteracts the low transpiration effect imposed by GCHXK, but it does so only when a functional HY5 is present in other tissues (under the WT background, GCHXK /GCHY5/WT). When HY5 is absent (under the *hy5* mutant background, GCHXK/GCHY5/*hy5*), GCHY5 cannot reverse the low transpiration of GCHXK (Fig. 6A-B). Thus, when GCHXK reduces transpiration, the execution of the GCHY5-induced transpiration response depends on the presence of *AtHY5* in other tissues. GCHXK was recently shown to mediate the sucrose-induced hypocotyl elongation in Arabidopsis seedlings in a PIF4-dependent manner. Transcriptional analysis at this early stage revealed that GCHXK suppresses *HY5*. At the same time, *PIF1*, *PIF3*, and *PIF4* expressions are induced (Kelly et al., 2021), implying that the low transpiration imposed by GCHXK at the mature stage might directly involve PIF4 and HY5. An alternative explanation is that GCHXK and GCHY5 produce two independent conflicting signals, summing up to a certain degree of transpiration rate.

HY5 acts as a long-distance signal, which moves from the shoot to the roots and is required for carbon-to-nitrogen synchronization (Chen et al., 2016). HY5 was also shown to exit the guard cells of young seedlings and translocate to other tissues, where it can auto-activate the expression of *AtHY5* in mesophyll and the phloem of roots (Chen et al., 2016; Kelly et al., 2021; Abbas et al., 2014; Binkert et al., 2014). By using GCHY5-GFP plants, we show here that HY5 exits the guard cells of mature leaves as well (Fig. 3A-B), demonstrating that long-distance translocation of HY5 occurs at all developmental stages. While it is clear that under the WT, as well as under the *hy5* mutant backgrounds, GCHY5 promotes transpiration (Figs. 2, 3, and 5), the effect on growth is less pronounced in the GCHY5/*hy5* plants (Fig. 5E). This is most likely since the growth inhibitory effect of GCHY5 relies on auto-activation of the local *AtHY5*. Under the *hy5* mutant background, the translocation of HY5, generated within guard cells and exported out, fails to meet the local *AtHY5* in its destination; auto-activation does not occur, and the overall response of HY5 is therefore attenuated.

For the question of how HY5 promotes overall transpiration when activated outside the guard cells, we raised the possibility that HY5 may enhance plant hydraulic activity to affect whole-plant water loss. Leaf hydraulic conductance (*K*leaf) of the GCHXK/GCHY5/WT plants remained as in WT (Fig. 6 C), indicating that *K*leaf is not involved in the HY5- non-stomatal effect on transpiration. The endogenous HY5 likely activates a yet unknown signal to increase transpiration. Recently, it was shown that HY5 binds and activates- or represses a large group of genes (Xiao et al., 2021; Burko et al., 2020a), which may raise several theories regarding the nature of this signal. Due to the significant overlap between light, HY5, and hormonal pathways at the seedlings stage (Gangappa and Botto, 2016), it seems reasonable to assume that hormonal cues are generated in response to HY5 to affect transpiration. HY5 produces a signal that eliminates the effects of both PIF4 and GCHXK (under WT background). Since PIF4 and GCHXK stimulate ABA responses (Kelly et al., 2013; Sakuraba et al., 2014; Qi et al., 2020; Liang et al., 2020), the HY5 activity may involve limiting the ABA responses. Yet, further research is required to understand better the nature of the signal triggered by the long-distance effect of HY5, leading to higher transpiration.

4.4. Linking hypocotyl length with transpiration

This study shows that the expression of the light-responsive transcription factors *HY5* and *PIF4* in guard cells is sufficient to modify transpiration rates. In a previous study, we uncovered a role for guard cells in adjusting the hypocotyl length of young seedlings (Kelly et al.,

2021). Joining these two studies together uncovers an interesting correlation. We noticed that manipulation of gene expression exclusively in guard cells changes hypocotyl length, which is negatively correlated to the level of transpiration (Fig. S2, Table S1). When the hypocotyls are long, the transpiration is low, and on the contrary, when hypocotyls are short, the transpiration is high. For example, the *pif4* mutant has short hypocotyls and high transpiration (short/high, (Delker et al., 2014), Fig. 4). However, when *GCPIF4* is expressed under the *pif4* background (GCPIF4*/pif4*), this phenotype is reversed; hypocotyls are long, and transpiration is low (long/low, Fig. 4B, (Kelly et al., 2021)). This negative correlation also occurs when *GCHY5* is expressed under the *hy5* mutant background (GCHY5*/hy5*), where the hypocotyls are short, and the transpiration is high (short/high, Fig. 5, (Kelly et al., 2021)). This novel correlation of long/low or short/high (hypocotyl/transpiration) is observed in other genetic backgrounds as well. The *phyB*, *phyA*, *phyA-phyB*, *cry1* and *cry1cry2* mutants that are impaired in phytochrome and cryptochrome light-receptors all have long hypocotyls together with reduced stomatal aperture size and g_s (Fig. S2A-B, Table S1). Overexpression of *PHYB* and *CRY1* has the opposite effect; short hypocotyls and open stomata (Wang et al., 2010; Mao et al., 2005; Chory et al., 1996), (Table S1). Mutants in the following genes; *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*cop1*), *DE-ETIOLATED 1* (*det1*), *PHOSPHO-ENOLPYRUVATE CARBOXYLASE KINASE 1* (*pck1*), *CARBONIC ANHYDRASE 1, 4* (*ca1ca4*) and *AUXIN RESISTANT 1* (*axr1*) display a similar correlation as well. These mutants exhibit short hypocotyls together with higher stomatal conductance or open stomata, thus providing additional support for the negative correlation between hypocotyl length and transpiration rate ((Wang et al., 2010; Mao et al., 2005; Delker et al., 2014; Eckert and Kaldenhoff, 2000; Moazzam-Jazi et al., 2018; Fernando and Schroeder, 2015; Penfield et al., 2012, 2004; Hu et al., 2010; Engineer et al., 2014, 2015), Fig. S2C-F, Table S1). The effect on transpiration and on hypocotyl length might be a result of changes in stomatal movement or from a combined effect of stomatal movement and stomatal development, as found in the current study and in the case of *ca1ca4*, and *axr* mutants (Engineer et al., 2014; Balcerowicz et al., 2014). The biological relevance of this correlation may raise diverse hypotheses linking environmental conditions during seedlings' development with their mature physiological behavior (risk-taking levels). Naturally, the idea that hypocotyl length is related to the level of transpiration in mature stages merits further studies by which large natural population accession panels are characterized.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111583.

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