Molecules and Cells

High Ambient Temperature Accelerates Leaf Senescence via PHYTOCHROME-INTERACTING FACTOR 4 and 5 in *Arabidopsis*

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Leaf senescence is a developmental process by which a plant actively remobilizes nutrients from aged and photosynthetically inefficient leaves to young growing ones by disassembling organelles and degrading macromolecules. Senescence is accelerated by age and environmental stresses such as prolonged darkness. Phytochrome B (phyB) inhibits leaf senescence by inhibiting phytochrome-interacting factor 4 (PIF4) and PIF5 in prolonged darkness. However, it remains unknown whether phyB mediates the temperature signal that regulates leaf senescence. We found the light-activated form of phyB (Pfr) remains active at least four days after a transfer to darkness at 20°C but is inactivated more rapidly at 28°C. This faster inactivation of Pfr further increases PIF4 protein levels at the higher ambient temperature. In addition, *PIF4* mRNA levels rise faster after the transfer to darkness at high ambient temperature via a mechanism that depends on ELF3 but not phyB. Increased PIF4 protein then binds to the *ORE1* promoter and activates its expression together with ABA and ethylene signaling, accelerating leaf senescence at high ambient temperature. Our results support a role for the phy-PIF signaling module in integrating not only light signaling but also temperature signaling in the regulation of leaf senescence.

Keywords: Arabidopsis, PIF4, phytochrome, senescence, temperature

INTRODUCTION

Plants display various adaptive responses to survive at different temperatures. For example, as an adaptation to low temperatures under weak blue light, plant chloroplasts move from periclinal cell surfaces to anticlinal cell surfaces (Kodama et al., 2008). This presumably avoids photodamage induced by light exposure that exceeds the plant's photosynthetic capacity at low temperatures. Plant thermomorphogenesis is another example of plant adaptation to high ambient temperature. This process is characterized by the elongation of hypocotyls and stems, and the production of hyponastic leaves with elongated petioles (Casal and Balasubramanian, 2019; Gray et al., 1998; Koini et al., 2009; Lippmann et al., 2019; Quint et al., 2016). Hypocotyl elongation is thought to be an adaptation that avoids heat absorbed by the soil in the

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seedling stage, while hyponastic leaves with elongated petioles help dissipate heat more efficiently by opening a rosette structure in the vegetative stage. High ambient temperature also reduces leaf size and thickness, reduces leaf stomatal density, and hastens flowering (Blázquez et al., 2003; Crawford et al., 2012; Halliday et al., 2003; Kumar et al., 2012). It is particularly important to understand these adaptive changes in growth pattern and flowering time because they reduce overall plant productivity. As the globe continues to warm, this reduced productivity may exacerbate global food shortages (Bita and Gerats, 2013).

To adapt to changing temperatures, plants must first be able to sense temperature. Any biological molecule, in principle, could serve as a thermosensor because temperature affects all molecular kinetics. Indeed, different classes of biological molecule including DNA, RNA, protein, and membrane have been identified as thermosensors for different temperature responses (Casal and Balasubramanian, 2019; Chung et al., 2020; Vu et al., 2019). Plant photoreceptors have also been suggested as potential thermosensors. Phytochrome B (phyB), which shifts from its biologically active Pfr form to its inactive Pr form upon the exposure to high ambient temperature and far-red light, acts as a thermosensor for plant thermomorphogenesis (Jung et al., 2016; Legris et al., 2016). Phototropin, which is also deactivated thermally, acts as a thermosensor for cold temperature-induced chloroplast movement in *Marchantia polymorpha* (Fujii et al., 2017). Both of these photoreceptors are thought to confer temperature sensitivity because higher ambient temperatures induce more rapid reversions of their biologically active forms to their inactive forms.

Phytochrome-interacting bHLH transcription factor 4 (PIF4) is one of the key factors in *Arabidopsis* that promotes thermomorphogenesis at high ambient temperatures (Koini et al., 2009; Kumar et al., 2012). High ambient temperature increases PIF4 activity partly by elevating *PIF4* mRNA levels (Casal and Balasubramanian, 2019; Ding et al., 2020; Koini et al., 2009; Stavang et al., 2009). Several proteins are implicated in the increase in *PIF4* mRNA levels at high ambient temperatures. One, EARLY FLOWERING 3 (ELF3), is a multifunctional nuclear protein that normally represses *PIF4* mRNA expression, but increasing ambient temperatures inhibit ELF3 to prevent this repression (Box et al., 2015; Nusinow et al., 2011). ELF3 forms the evening complex with ELF4 and LUX ARRHYTHMO (LUX). This represses the expression of *PIF4* mRNA by binding to the *PIF4* promoter in the early evening. Consistent with this, three evening complex mutants, *elf3, elf4*, and *lux*, express high levels of *PIF4* mRNA in a long day (LD) condition regardless of the ambient temperature (Mizuno et al., 2014). UV RESISTANCE LOCUS 8 (UVR8), a UVB photoreceptor, also represses *PIF4* mRNA expression independent of ELONGATED HYPOCOTYL 5 (HY5) (Hayes et al., 2017). Several other factors also activate *PIF4* mRNA expression at high ambient temperature. For example, high ambient temperature increases *PIF4* mRNA via DE-ETIOLATED 1 (DET1) and CONSTITUTIVE PHOTOMORPHOGENIC 1/SUPPRESSOR OF PHYA-105 1 to 4 (COP1/SPAs). These substrate adaptor proteins in the CULLIN 4 (CUL4) E3 ligase complex activate *PIF4* mRNA expression at high ambient temperatures by either degrading HY5, a bZIP transcription factor that represses *PIF4* mRNA by directly binding to its promoter (Delker et al., 2014; Lee et al., 2007), or independent of HY5 (Gangappa and Kumar, 2017). Three TEOSINE BRANCHED 1/CYCLOIDEA/PCF transcription factors, TCP5, TCP13, and TCP17 also increase *PIF4* mRNA expression at high ambient temperature by directly targeting the *PIF4* promoter (Zhou et al., 2019).

High ambient temperature can also increase PIF4 activity partly by elevating PIF4 protein stability. PIF4 protein is degraded by light in part via its interaction with phyB and BLADE ON PETIOLE 1 and 2 (BOP1/2), which are BTB-domain containing substrate adaptor proteins in the CUL3 E3 ligase complex (Zhang et al., 2017). In addition to phyB and BOPs, UVR8 destabilizes PIF4 protein in UV-B-treated plants grown at high ambient temperature (Hayes et al., 2017), whereas DET1 and COP1/SPAs stabilize PIF4 protein regardless of temperature (Gangappa and Kumar, 2017). In a third and final mechanism, high ambient temperature regulates PIF4 protein activity via the activity of other proteins. LONG HYPOCOTYL IN FAR-RED 1 (HFR1), an atypical HLH protein that inhibits PIF4 DNA binding by forming heterodimers with PIF4, is stabilized by high ambient temperature, mitigating high temperature responses by PIF4 (Foreman et al., 2011; Hayes et al., 2017; Hornitschek et al., 2009; Ma et al., 2016). FCA, an RNA binding protein, is recruited more strongly to the *YUCCA 8 (YUC8)* promoter at high ambient temperature, where it reduces H3K4me2 levels, leading to the dissociation of PIF4 from the *YUC8* promoter (Lee et al., 2014). Cryptochrome 1 (cry1), a blue light photoreceptor, is recruited more strongly to its target promoters at high ambient temperature by PIF4 where it inhibits PIF4 transcriptional activity (Ma et al., 2016). TCP5 and TCP17 also interact with PIF4 and enhance PIF4 transcriptional activity (Han et al., 2019; Zhou et al., 2019).

Leaf senescence is the final stage of leaf development characterized by the degradation of chlorophyll molecules and internal organs followed by the remobilization of nutrients to younger organs in plants. Leaf senescence is accelerated by endogenous factors such as leaf age and by environmental stresses such as prolonged darkness, drought, salt stress, and pathogen attack (Lim et al., 2007). Many plant hormones have shown to regulate leaf senescence either promotively (ABA, brassinosteroids, ethylene, jasmonic acid, salicylic acid) or repressively (auxin, cytokinin, gibberellic acids) in conjunction with intricate networks of various transcription factors including bZIP, NAC, and WRKY transcription factor families. Ethylene is a plant hormones that promotes leaf senescence. Ethylene binds to and inhibits the ethylene receptor-kinase complex, ETRs-CTR1, resulting in the activation of EIN2. Activated EIN2 stabilizes EIN3 and related EIL transcription factors (Dubois et al., 2018). This promotes leaf senescence either by activating the expression of senescence promoting other transcription factors such as ORE1 and AtNAP or by repressing miR164, which targets ORE1 (Li et al., 2013). ABA is another plant hormone that promotes leaf senescence. The core ABA signaling comprise the SnRK2s that activate group A bZIP transcription factors including ABI5, the group A PP2Cs that inhibit SnRK2s, and PYR/PYLs that bind to ABA. ABA binding to PYR/PYLs promotes the binding of PYR/PYLs

to PP2Cs, inhibiting their activities. The inhibition of PP2Cs liberates SnRK2s allowing the phosphorylation and activation of the group A bZIP transcription factors that promote leaf senescence. These group A bZIPs do this by activating ORE1 and chlorophyll degradation genes (Cutler et al., 2010; Sakuraba et al., 2014). In leaf senescence induced by prolonged darkness, PIF4 and its homologs, PIF3 and PIF5, promote leaf senescence by directly activating the expression of senescence-associated downstream target genes such as *ETHYLENE INSENSITIVE 3 (EIN3), ABA INSENSITIVE 5 (ABI5), ORESARA 1 (ORE1), NON-YELLOWING 1 (NYE1), and GOLDEN2-LIKE 2 (GLK2)*, whereas phyB and ELF3 suppress senescence either by inhibiting PIF proteins or by repressing the expression of *PIF4* mRNA, respectively (Sakuraba et al., 2014; Song et al., 2014).

High ambient temperature may also regulate senescence. Among animals, the lifespan of *Drosophila* doubles at 21°C compared to 27°C (Miquel et al., 1976) and *C. elegans* live four times longer at 10°C than at 25°C (Van Voorhies and Ward, 1999). Ectotherms also live longer at lower temperatures (Finch, 1990; Munch and Salinas, 2009). In contrast, although human women have slightly higher average body temperature than men, they live longer (Keil et al., 2015; Waalen and Buxbaum, 2011). High ambient temperature has shown to accelerate senescence in sunflower primary leaves, in wheat flag leaves, in creeping bentgrass, and in sorghum (Chauhan et al., 2009; De la Haba et al., 2014; Djanaguiraman et al., 2014; Jespersen et al., 2016). Since high ambient temperature accelerates the thermal reversion of the Pfr form of phyB and increases PIF4 levels, it seems likely that high ambient temperature accelerates leaf senescence by increasing PIF4 and its homologs via the acceleration of phyB thermal reversion.

In this study, we asked whether high ambient temperature promotes leaf senescence in *Arabidopsis* via phyB, PIF4, and PIF5. We report that high ambient temperature does indeed accelerate leaf senescence via PIF4 both dependent on and independent of phyB.

MATERIALS AND METHODS

Plant materials and growth conditions

For general growth and seed harvest, *Arabidopsis thaliana* were grown at 22°C to 24°C under a LD condition (16-h light/8-h dark) with white light (100 μmol m^{-2} s^{-1}). Mutants including *pif4* (SAIL_1288_E07), *pif5-3* (SALK_087012), *ein2-1* (CS3071), *ore1* (SAIL_694_C04), *aba2-1* (CS156), *lrb1-1* (SALK_145146), *lrb2-1* (SALK_001013), *cop1-4* and *elf3-8* (CS3794) were obtained from the Arabidopsis Biological Resource Center (ABRC) and the Nottingham Arabidopsis Stock Center (NASC). Double mutants, *pif4 pif5* and *lrb1-1 lrb2-1*, were generated by crossing the corresponding single mutants. To produce the PIF4pro:PIF4-FLAG/pif4 lines, a *PIF4* promoter fragment was amplified with a specific primer set and cloned into pCAMBIA1300 with an adaptor encoding a FLAG tag. Subsequently, the PIF4 coding region was amplified with a specific primer set and cloned into the resulting vector. The PIF4pro:PIF4-FLAG construct was then transformed into the *pif4* mutant by *Agrobacterium*-mediated transformation and homozygous lines were established. For *UBQ10pro:YFP-PIF4* lines, the PIF4 coding region was amplified with a specific primer set and cloned into the GW vector (CD3-1948) with an N-terminal YFP fusion protein. The UBQ10pro:YFP-PIF4 construct was transformed into Col-0 by Agrobacterium-mediated transformation and homozygous lines were established. Two wild type phyB overexpression lines, *35Spro:PHYB-GFP*, *35Spro:PHYB-MYC* and two phyB variant overexpression lines, *35Spro:PHYB^{Y276H}-FLAG*, *35Spro:PHYB^{S584F}-GFP* were transformed into *phyb-9* by Agrobacterium-mediated transformation and homozygous lines were established. The EIN3-overexpressing line (*35Spro:EIN3-FLAG/Col-0*; Sakuraba et al., 2014) and the ABI5-overexpressing line (*35Spro:ABI5-FLAG/Col-0*; Lim et al., 2013) were previously reported. The specific primer sets used are listed in Supplementary Table S1.

Leaf senescence assay

Arabidopsis seedlings were grown on half Murashige and Skoog (MS) agar plates (1/2 MS, 0.8% phytoagar, 0.05% MES, pH 5.7) at 20°C for 7 days under continuous white light (100 μmol m^{-2} s^{-1}). For the senescence assays with whole seedlings, plates with 7-day-old light-grown seedlings were transferred to darkness either at 20°C or at 28°C for the indicated times. For the senescence assays with detached leaves, cotyledons of 7-day-old light grown seedlings were cut, floated on water, and transferred to darkness either at 20°C or at 28°C for the indicated times. For the senescence assays under dim light, cotyledons of 7-day-old white light grown seedlings were cut, floated on water in the presence or absence of 100 μM fluridone, and incubated under dim white light (5 μmol m^{-2} s^{-1}) for 9 days. For the *Solanum lycopersicum* senescence experiments, seedlings were grown at 22°C for 12 days under continuous white light (100 μmol m^{-2} s^{-1}) and transferred to darkness for 7 days either at 20°C or at 28°C. For the age-dependent natural leaf senescence experiments, *Arabidopsis* were grown at 22°C under a LD condition until the third and fourth leaves reached 16 to 32 days of leaf age. Then, the plants were transferred to the same light condition at either 22°C or 29°C. The photochemical efficiency of photosystem II and the chlorophyll content of the third and fourth leaves were measured on the indicated days.

To measure chlorophyll levels in senescent leaves, chlorophylls were extracted from 10 detached cotyledons or from cotyledons cut from seedlings in 100% ethanol for 1 day at 4°C in the dark. Then, the extracted chlorophyll levels were determined using a spectrophotometer (Lichtenthaler, 1987). All handling of the plates was done under a green safety light. Chlorophyll levels were normalized by the fresh weight of the cotyledons.

To inactivate Pfr before the transfer to darkness, the seedlings or detached cotyledons were irradiated with far-red light (2.5 μmol m^{-2} s^{-1}) for 10 min or for the indicated times.

mRNA expression analysis

To measure the expression of *PIF4* and PIF4 target gene mRNAs during senescence, 7-day-old light-grown seedlings were transferred to darkness at either 20°C or 28°C for the indicated times and then sampled for RNA purification. The

presence of active Pfr during senescence was also determined by measuring PIF target gene mRNA levels after a far-red light pulse. To inactivate Pfr in the middle of the period of darkness, seedlings were transferred to darkness either at 20°C or 28°C with or without a pre-FRp. Then, they were irradiated with a far-red light (2.5 μmol m^{-2} s^{-1}) for 10 min (mid-FRp) in the middle of the period of darkness at the indicated times, incubated 1 h in the darkness at either 20°C or 28°C, and then sampled for RNA purification. All handling of the plates was done under a green safety light.

Total RNA was isolated from 80 seedlings using the MiniBEST Plant RNA Extract Kit (Takara, Japan). Total RNA (2 μg) was used to synthesize cDNAs using oligo (dT) 18 primers and the M-MLV reverse transcriptase (Promega, USA). Expression of specific mRNAs was determined via quantitative real-time polymerase chain reaction (PCR) with specific primer sets (Supplementary Table S1) using the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The expression of specific mRNAs was normalized by the expression levels of *PP2AA3* (AT1G13320) mRNAs.

ChIP assay

Seven-day-old light-grown seedlings were transferred to darkness for 1 day either at 20°C or 28°C and sampled for chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed as described (Oh et al., 2007). Briefly, seedlings were cross-linked with a crosslinking buffer (0.4 M Sucrose, 10 mM Tris pH 8.0, 1 mM EDTA, 2.7% formaldehyde, 0.8% MG-132, 1 mM PMSF) for 20 min under vacuum, frozen with liquid nitrogen, and ground with a mortar and pestle. The ground samples were resuspended in nuclei isolation buffer (0.25 M Sucrose, 15 mM PIPES pH 6.8, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 0.1 mM $CaCl_2$, 9% Triton X-100, 1 mM PMSF) and debris was removed with a miracloth. Nuclei were isolated by centrifugation (9,800*g* at 4°C for 10 min). Then, isolated nuclei were resuspended in nuclei lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na-deoxycholate, 1% SDS, 1 mM PMSF). The DNA was sheared via 15 sonication cycles with 30 s on and 30 s off in iced water using a Bioruptor (Cosmo Bio, Japan) and the supernatant was collected after centrifugation (9,800*g* at 4°C for 10 min). FLAG-tagged PIF4, EIN3, and ABI5 were immunoprecipitated using EZview Red anti-FLAG M2 Affinity gel (F2426; Sigma-Aldrich, USA) from *PIF4pro:PIF4-FLAG/pif4*, *35S:EIN3-FLAG/Col*, and *35S:ABI5-FLAG/Col* seedling samples, respectively. After reverse cross-linking and protein digestion, the precipitated DNA was purified using the HighGene PCR purification system (BioFACT, Korea). The level of each precipitated DNA fragment was determined by quantitative real-time PCR with the specific primer sets listed in Supplementary Table S1 and normalized as a percentage of the input DNA fragments. Enrichment at the ORE1 promoter was determined using the *ORE1-2* primer set for *PIF4pro:PIF4-FLAG/pif4* and *35Spro:EIN3-FLAG/Col* and the *ORE1-1* primer set for *35Spro:ABI5-FLAG/Col* (Sakuraba et al., 2014).

Immunoblot analysis

Seedlings were sampled and frozen immediately with liquid nitrogen in a dark room under a green safety light. Frozen seedlings were ground and resuspended in UREA protein extraction buffer (100 mM NaH2PO4 pH 7.8, 10 mM Tris-HCl pH 8.0, 1 mM PMSF, 8 M urea with the Calbiochem Protease Inhibitor Cocktail) and the debris was removed by centrifugation (9,800*g* at 4°C for 10 min). The supernatant was then mixed with an SDS-PAGE sample buffer and boiled at 100°C for 5 min. For the immunoblot analyses, boiled samples were loaded on an 8% SDS-PAGE gel and the separated proteins were transferred to nitrocellulose membranes (Amersham Hybond ECL; GE Healthcare Life Science, USA) with a transfer buffer (5.8 g/L Tris base, 29 g/L glycine, 20% methanol, and 0.01% SDS). Endogenous PIF4 protein levels were detected using a rabbit polyclonal anti-PIF4 antibody (AS163157; Agrisera, Sweden) with the Clarity Max Western ECL substrate (Bio-Rad). FLAG-tagged PIF4 protein was detected using a rabbit polyclonal anti-FLAG antibody (F7425; Sigma-Aldrich) and YFP-tagged PIF4 protein was detected using a rabbit anti-GFP antibody (ab290; Abcam, England) with EzWest-LumiOne (ATTO, Japan). RPT5 was detected using a rabbit anti-RPT5 antibody (PM8245; Enzo, USA).

phyB photobody imaging

Four-day-old light-grown *35Spro:PHYB-GFP* seedlings were transferred to darkness at either 20°C or 28°C for the indicated times. Seedlings were fixed with 4% formaldehyde for 15 min under vacuum. Fluorescence images were obtained using a fluorescence microscope (Nikon A1 HD25; Nikon, Japan).

Accession numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this paper are as follows: PHYB (AT2G18790); PIF4 (AT2G43010); PIF5 (AT3G59060); PIL1 (AT2G46970); HFR1 (AT1G02340); EIN2 (AT5G03280); EIN3 (AT3G20770); ABI5 (AT2G36270); ABA2 (AT1G52340); ORE1 (AT5G39610); NYE1 (AT4G22920); UBQ10 (AT4G05320); LRB1 (AT2G46260); LRB2 (AT3G61600); COP1 (AT2G32950); ELF3 (AT2G25930); and PP2AA3 (AT1G13320).

RESULTS

High ambient temperature accelerates leaf senescence via PIF4 and PIF5 in prolonged darkness

Leaf senescence is accelerated both by internal aging programs and environmental stresses such as darkness, drought, high salinity, and pathogen attacks (Lim et al., 2007). High ambient temperature, because it affects so many plant processes (e.g., seed germination, stem elongation, and flowering) may be another critical environmental factor that accelerates leaf senescence.

We examined leaf senescence by transferring light-grown seedlings or detached leaves to a dark condition at either 20°C or 28°C. Leaf senescence is marked by a gradual change of leaf color from green to yellow—due to reduced chlorophyll—and increased expression of senescence marker genes such as *ORE1* and *NYE1*. *Arabidopsis* seedlings become yellow faster and retain less chlorophyll when transferred to

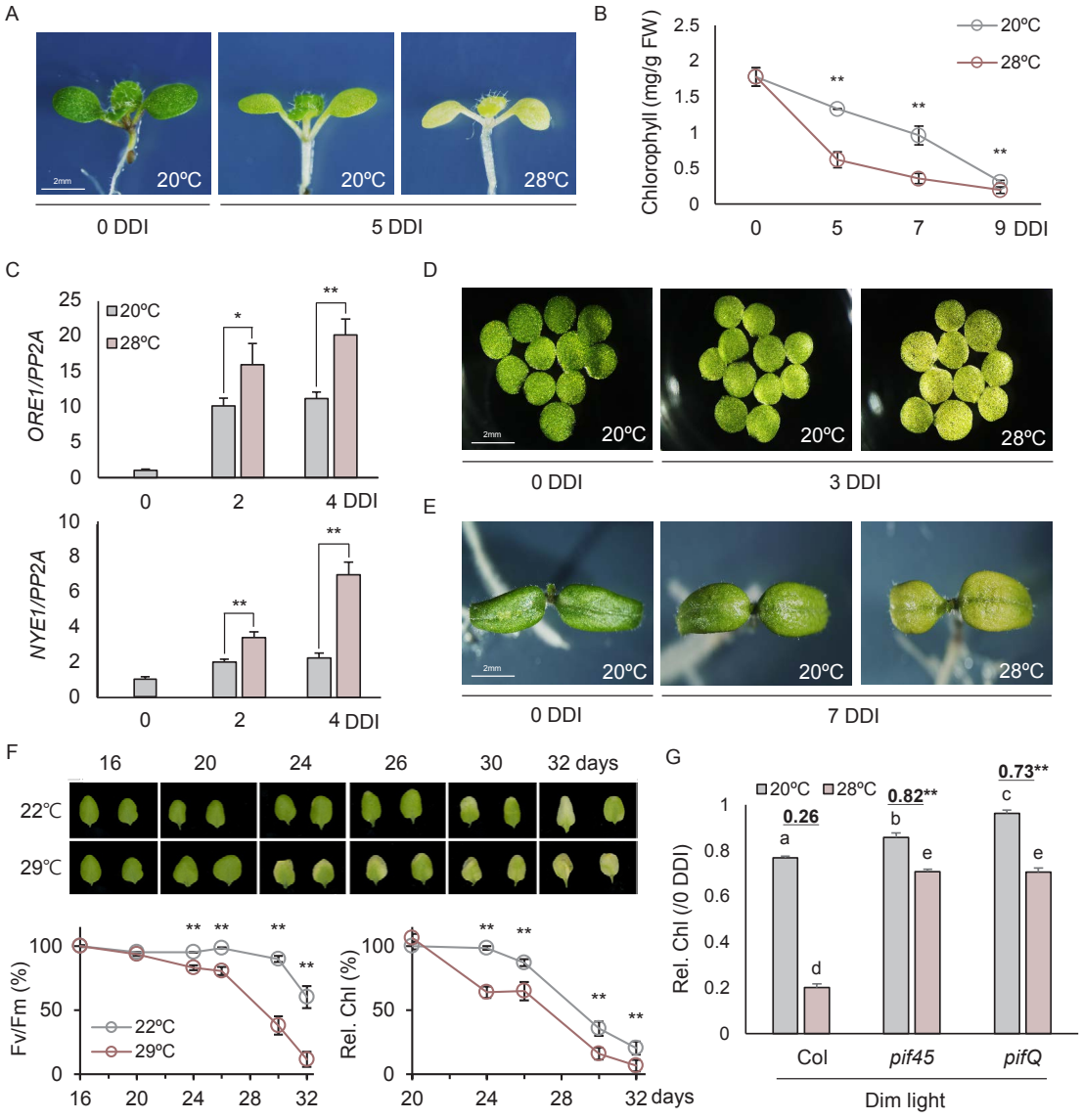

Fig. 1. High ambient temperature accelerates leaf senescence. Leaf senescence was induced by transferring 7-day-old seedlings grown at ambient temperature under continuous white light to darkness for varying numbers of days (DDI, days of dark incubation) either at ambient temperature (20°C) or at high ambient temperature (28°C). For *Arabidopsis*, 7-day-old light-grown seedlings were transferred to darkness for the assay. The degree of senescence was determined by quantifying leaf yellowing, chlorophyll levels, and the mRNA levels of senescence marker genes. (A) Accelerated leaf senescence in *Arabidopsis* seedlings at high ambient temperature after 5 DDI. (B) High ambient temperature accelerates the reduction in chlorophyll levels after the transfer to darkness. Chlorophyll levels (Chl) were normalized by seedling fresh weight (FW). (C) Higher expression of two senescence marker genes, *ORE1* and *NYE1*, at high ambient temperature after 2 and 4 DDI. mRNA levels were normalized by *PP2AA3* mRNA levels. For (B) and (C), error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences between corresponding values at 20°C and 28°C. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (D) Accelerated leaf senescence of detached cotyledons at high ambient temperature after 3 DDI. (E) Accelerated leaf senescence of 12-day-old tomato seedlings at high ambient temperature after 7 DDI. Scale bars = 2 mm. (F) Accelerated natural leaf senescence of *Arabidopsis* at high ambient temperature. Plants grown at 22°C were transferred to high ambient temperature (29°C) when the 3rd and 4th leaves were 16-32 days of leaf age. Photochemical efficiency of photosystem II and chlorophyll levels of the 3rd and 4th leaves were determined at the indicated days of leaf age and normalized by that of plants grown at 22°C for 16 days (Fv/Fm) or 20 days (chlorophyll). Error bars indicate SE (n = 4-20 leaves). Asterisks indicate statistically significant differences between corresponding values of 22°C and 29°C. $**P < 0.01$ (Student's *t*-test). (G) Leaf senescence of detached cotyledons at two different temperatures under dim white light after 9 days. Relative chlorophyll levels (Rel. Chl) were normalized by the chlorophyll level of each line at 0 DDI. Ratios of chlorophyll levels between 28°C and 20°C were indicated above graph bars. Col, Col-0 ecotype. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences between corresponding values of wild type and mutants. $**P < 0.01$ (Student's *t*-test). Different letters indicate statistically significant differences (two-way ANOVA, Tukey's HSD, $P < 0.05$).

darkness at 28°C rather than 20°C (Figs. 1A and 1B). We also found increased expression of senescence marker genes at 28°C rather than 20°C (Fig. 1C), indicating that the seedlings senesce faster at 28°C than 20°C. We also observed this accelerated senescence at high ambient temperature in detached cotyledons, not just whole seedlings (Fig. 1D), and in tomato, *Solanum lycopersicum*, seedlings, not just *Arabidopsis* (Fig. 1E). High ambient temperature also accelerates the natural senescence of *Arabidopsis* grown in soil (Fig. 1F). Not only in darkness, high ambient temperature also accelerates the senescence of detached cotyledons under dim white light (Fig. 1G). Together, our results indicate high ambient temperature accelerates leaf senescence in plants.

Since PIF4 and PIF5 promote senescence at ambient temperature, we asked whether PIF4 and PIF5 also accelerate leaf senescence at high ambient temperatures. We found the *pif4 pif5* (*pif4/5*) double mutant is greener and retains more chlorophyll than wild type seedlings either when transferred to darkness at both 28°C and 20°C (Figs. 2A and 2B) or under dim white light (Fig. 1G). When we compared the relative reduction of chlorophyll at these two different temperatures, we found *pif4/5* seedlings retain relatively more chlorophyll than wild type at 28°C than 20°C. The *pifQ* mutant seedlings entered senescence more slowly than wild type but at a rate similar to that of the *pif4/5* double mutant. This indicates that PIF4 and PIF5 are the major PIFs regulating senescence in our experimental conditions. The *pif4/5* mutant also expresses lower levels of *ORE1* and *NYE1* than wild type both at 28°C and 20°C (Fig. 2C). These results indicate PIF4/5 accelerate leaf senescence at high ambient temperature. It should be noted; however, that PIF4/5 are not the sole factors promoting senescence at high ambient temperature; both *pif4/5* mutant seedlings still senesce faster at 28°C than 20°C (Fig. 2B).

PIF4 accelerates senescence both by activating the expression of *ORE1* mRNA and also by activating ABA and ethylene

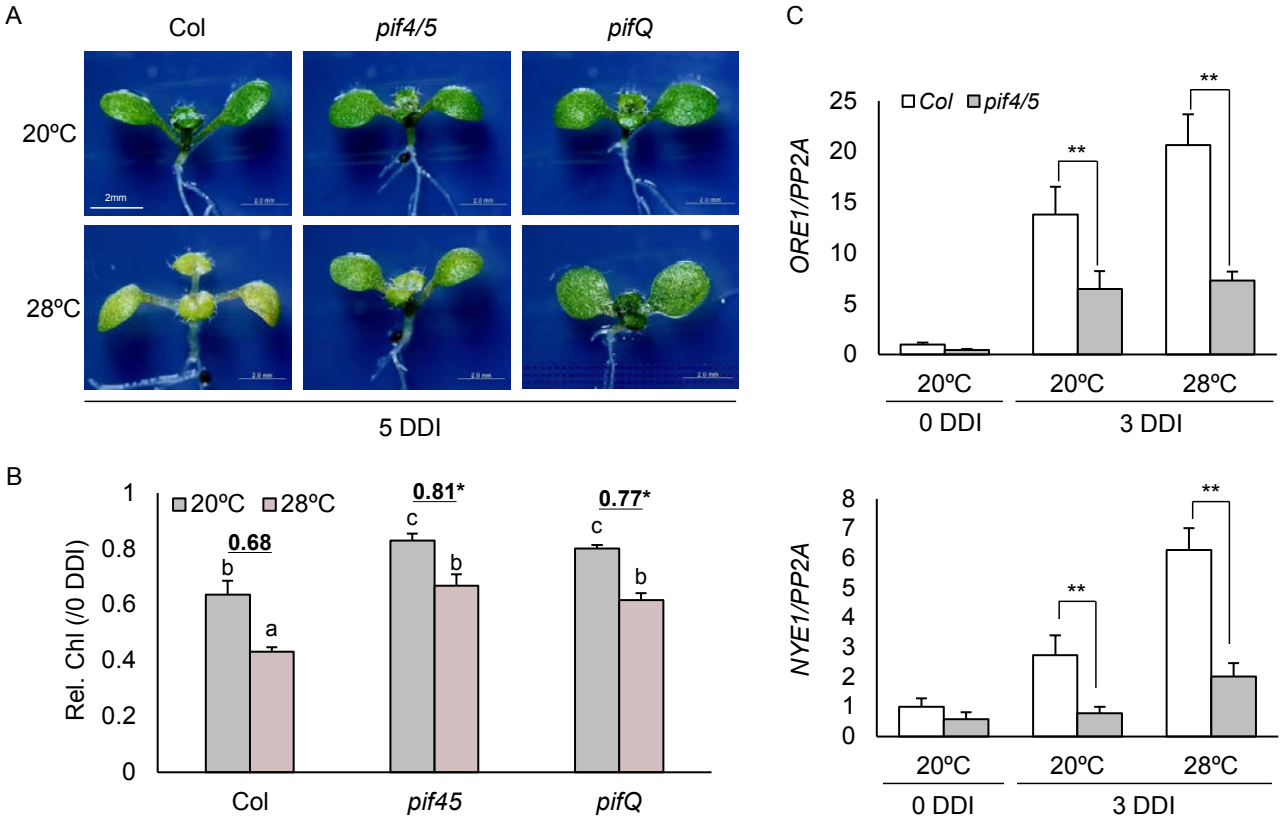

Fig. 2. High ambient temperature accelerates leaf senescence via PIF4 and PIF5. (A) Delayed leaf senescence of *pif4 pif5* (*pif4/5*) and *pifQ* mutant seedlings at high ambient temperature. Images taken at 5 DDI (days of dark incubation). Col, Col-0 ecotype. Scale bar = 2 mm. (B) Higher chlorophyll levels in *pif4/5* and *pifQ* mutant seedlings at high ambient temperature after 5 DDI. Chlorophyll levels were normalized by fresh weight and then by the chlorophyll level of each line at 0 DDI (Rel. Chl). Ratios of the chlorophyll level from samples grown at 28°C vs 20°C are indicated above the graph bars. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences between the corresponding values of wild type and mutants. $*P < 0.05$ (Student's *t*-test). Different letters indicate statistically significant differences (two-way ANOVA, Tukey's HSD, $P < 0.05$). (C) Lower expression of *ORE1* and *NYE1* mRNAs in *pif4/5* seedlings at high ambient temperature after 3 DDI. mRNA levels were normalized by *PP2AA3* mRNA levels. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences between corresponding values of wild type and mutants. $**P < 0.01$ (Student's *t*-test).

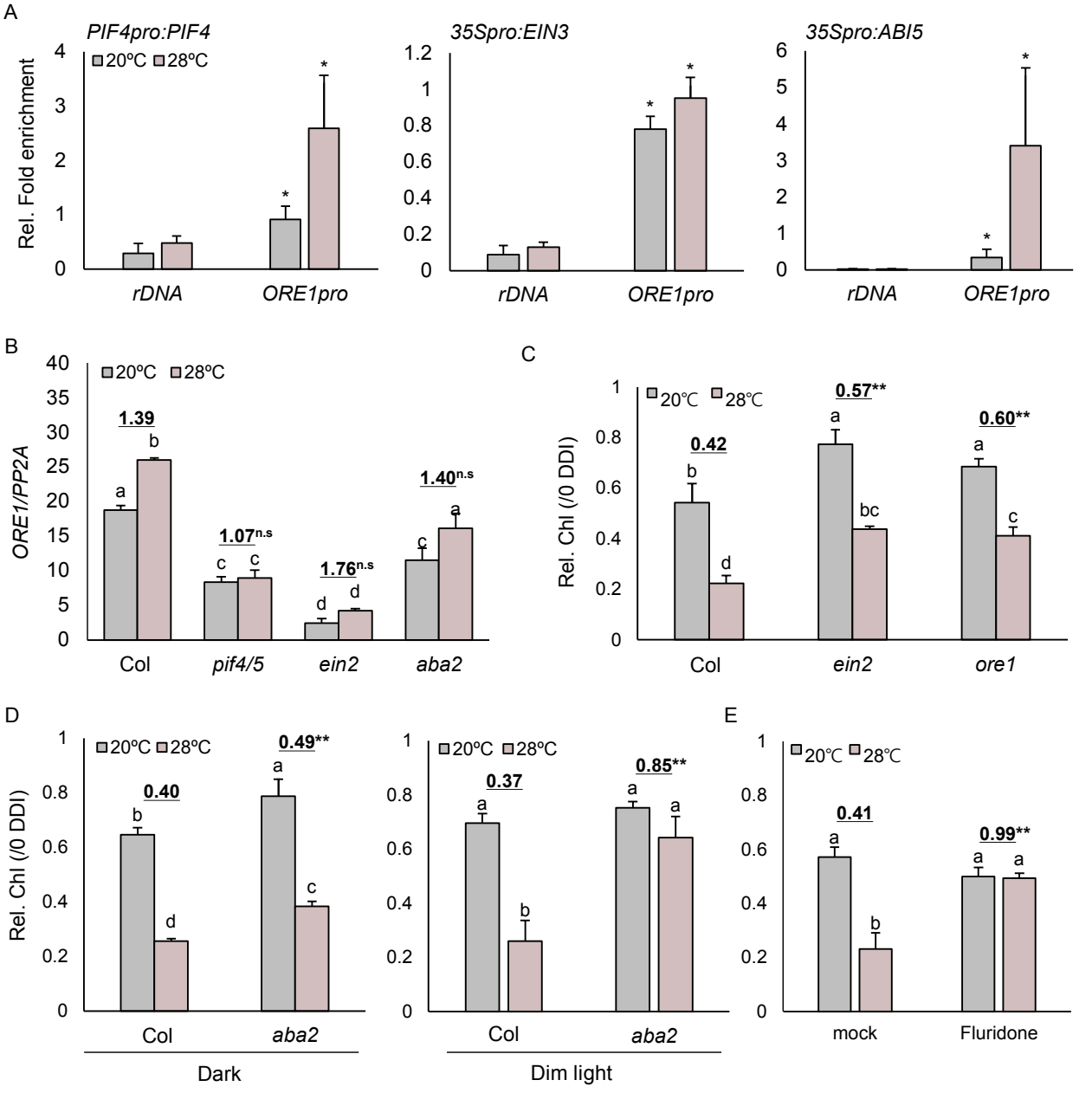

Fig. 3. PIF4 accelerates leaf senescence by increasing *ORE1* mRNA together with ABA and ethylene signaling at high ambient temperature. (A) ChIP assay showing binding of PIF4, ABI5, and EIN3 to the *ORE1* promoter. Chromatin cross-linked PIF4, EIN3, and ABI5 were immunoprecipitated using a FLAG antibody from *PIF4pro:PIF4-FLAG/pif4*, *35Spro:EIN3-FLAG*, and *35Spro:ABI5-FLAG* transgenic seedlings at 1 DDI (days of dark incubation). Relative enrichment expressed as the percentage of co-immunoprecipitated DNA fragments relative to *ORE1pro* at 20°C at 0 day and in the input. *ORE1pro* indicates *ORE1* promoter fragments containing G-box elements and *rDNA* indicates a DNA fragment 863 bp upstream of the 5.8S *rRNA* gene. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences in the corresponding rDNA and ORE1pro values. $*P < 0.05$, (Student's *t*-test). (B) Lower expression levels of *ORE1* mRNA in *pif4/5*, *aba2*, and *ein2* mutants at high ambient temperature after 3 DDI. mRNA levels were normalized by *PP2AA3* mRNA levels. Col, Col-0 ecotype. Error bars indicate SD (n = 3, biological replicates). (C) Higher chlorophyll levels in *ein2* and *ore1* mutants at high ambient temperature after 5 DDI. (D) Higher chlorophyll levels in *aba2* mutant at high ambient temperature after 6 DDI or after 9 days of cotyledon detachment under dim white light. (E) Higher chlorophyll levels in the presence of fluridone after 7 DDI. Detached cotyledons were floated on 100 μM fluridone in the dark. Chlorophyll levels were normalized by fresh weight and then by the chlorophyll level of each line at 0 DDI (Rel. Chl). For (B) to (E), the ratio of either gene expression (B) or chlorophyll levels (C to E) between 28°C and 20°C is indicated above the graph bars. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences between corresponding values of wild type and mutants. $**P < 0.01$, n.s, non-significant (Student's *t*-test). Different letters indicate statistically significant differences (two-way ANOVA, Tukey's HSD, $P < 0.05$).

signaling (Sakuraba et al., 2014). We asked whether the same pathways also promote senescence at high ambient temperature. Consistent with previous reports, we found via a ChIP assay that three transcription factors—PIF4, ABI5, and EIN3—bind to the *ORE1* promoter at high ambient temperature (Fig. 3A). To determine whether PIF4, ABA, and ethylene signaling activate the expression of *ORE1* mRNA at high ambient temperature, we measured *ORE1* mRNA levels in the *aba2* and *ein2* mutants. We used the *aba2* and *ein2* mutants to minimize the genetic redundancy of the ABA and ethylene signaling pathways as *ABA2* encodes a non-redundant ABA biosynthetic enzyme and *EIN2* encodes a non-redundant ethylene signaling component necessary for ethylene responses (Cutler et al., 2010; Dubois et al., 2018). We found that *ORE1* mRNA levels do not increase in the *pif4/5*, *aba2*, and *ein2* mutants as much as in wild type after the transfer to darkness regardless of ambient temperature (Fig. 3B). Consistent with this, the *ore1* and *ein2* mutants senesce more slowly than wild type (Fig. 3C). When we compared the relative decrease of chlorophyll levels at two different temperatures, chlorophyll levels decrease more slowly at 28°C in the *ore1* and *ein2* mutants than in wild type. For the *aba2* mutant, the relative decrease of chlorophyll levels is also lower than that of wild type at 28°C both in the dark and dim light (Fig. 3D). We further measured senescence while treating with the ABA synthetic inhibitor, fluridone. Similar to the *aba2* mutant, we found chlorophyll levels decrease more slowly at 28°C in the presence of fluridone (Fig. 3E). Taken together, our results indicate PIF4 accelerates leaf senescence at high ambient temperature by increasing *ORE1* mRNA levels with ethylene and ABA signaling.

High ambient temperature increases *PIF4* mRNA and PIF4 protein both phy-dependently and phy-independently after transfer to darkness

We next asked how high ambient temperature regulates PIF4 by measuring *PIF4* mRNA and PIF4 protein levels during senescence using a *PIF4pro:PIF4-FLAG/pif4* line that rescues the *pif4* mutant phenotypes for hypocotyl elongation (Fig. 4A). We found *PIF4* mRNA levels increase faster when seedlings are transferred to darkness at 28°C than 20°C (Fig. 4B), which is consistent with previous reports showing high ambient temperature induces *PIF4* mRNA expression. We found that *PIF4* mRNA levels reach similar plateaus; however, 16 h after the transfer to darkness at both temperatures. This indicates high ambient temperature increases *PIF4* mRNA levels only briefly right after the transfer. We next asked whether PIF4 protein levels follow *PIF4* mRNA levels. Interestingly, we found higher PIF4 protein levels at 28°C than 20°C, not only right after the transfer, but throughout the transfer for at least 4 days (Fig. 4C). Consistent with higher PIF4 protein levels after the transfer to darkness, *PIL1* and *HFR1*, two PIF4 target genes, also show higher expression levels at 28°C than 20°C throughout the period of darkness (Fig. 4B). These results suggest high ambient temperature also stabilizes PIF4 protein. To confirm this, we measured PIF4 protein levels using a transgenic line expressing *PIF4-YFP* under the control of the *UBQ10* promoter (*UBQ10pro:PIF4-YFP/Col*) (Fig. 4D). Again, we found higher PIF4-YFP protein levels at 28°C than 20°C after the transfer to darkness. These higher PIF4-YFP protein levels were not caused by higher *PIF4-YFP* mRNA levels at 28°C (Fig. 4D). Together, our results indicate high ambient temperature increases not only *PIF4* mRNA levels but also PIF4 protein levels when light-grown seedlings are transferred to darkness.

Since phyB suppresses senescence by inhibiting PIF4 (Sakuraba et al., 2014), we asked which step of *PIF4* expression is regulated by phyB at high ambient temperature. To clarify the role of phyB, we inactivated pre-existing Pfr with a far-red pulse (FRp) before the transfer to darkness (pre-FRp) and asked whether high ambient temperature still increases *PIF4* mRNA and protein levels. We mainly used a pre-FRp to avoid any complications associated with pre-existing growth or physiological differences between wild type and *phyB* mutant before the transfer to darkness. Using a pre-FRp, however, inactivates not only phyB but the other minor phytochromes (i.e., phyC, phyD, and phyE).

We found *PIF4* mRNA levels are higher at 28°C than 20°C 4 h after the transfer to darkness. A pre-FRp does not increase *PIF4* mRNA levels at 20°C nor does it decrease *PIF4* mRNA levels at 28°C (Fig. 5A). *PIF4* mRNA levels were also higher in *phyB* mutant at 28°C than 20°C. These results indicate high ambient temperature increases *PIF4* mRNA levels independent of phyB. PIF4 protein levels are also higher at 28°C than 20°C 1 day after the transfer to darkness. Unlike for *PIF4* mRNA, however, a pre-FRp increases PIF4 protein levels. This is especially apparent in Light-Response Bric-a-Brack/Tramtrack/Broad (BTB) E3 ubiquitin ligase 1 and 2 (*lrb1/2*) double mutant and *PHYB-OX* seedlings (Fig. 5B), which produce higher levels of phyB than wild type seedlings (Ni et al., 2014). This indicates that Pfr generated in the light period remains active to destabilize PIF4 protein even after the transfer to darkness. PIF4 protein levels, however, are still higher at 28°C than 20°C even when phyB is inactivated by a pre-FRp (Fig. 5B). This further suggests high ambient temperature increases PIF4 protein levels not only phy-dependently but also phy-independently. Together, our results indicate high ambient temperature increases *PIF4* mRNA levels phy-independently and PIF4 protein levels both phy-dependently and phy-independently.

Since COP1 and ELF3 regulate *PIF4* mRNA expression (Delker et al., 2014; Ezer et al., 2017; Gangappa and Kumar, 2017; Nusinow et al., 2011; Park et al., 2017), we asked whether COP1 and ELF3 regulate the phy-independent increase of *PIF4* mRNA expression induced by transfer to darkness at high ambient temperature. High ambient temperature induces *PIF4* mRNA in *cop1* mutant seedlings after they are transferred to darkness, but overall *PIF4* mRNA levels are far lower in *cop1* mutant seedlings than wild type at both 20°C and 28°C (Fig. 5C). Unlike in *cop1* mutant seedlings, however, *PIF4* mRNA levels are high in *elf3* mutant seedlings after they are transferred to darkness at 20°C. High ambient temperature does not further increase *PIF4* mRNA levels in *elf3* mutant seedlings. Consistent with the pattern we observed for overall *PIF4* mRNA expression levels, *cop1* mutant seedlings senesce more slowly than wild type at both temperatures, whereas *elf3* mutants senesce faster than wild type at both temperatures (Fig. 5D). Nevertheless, both *cop1*

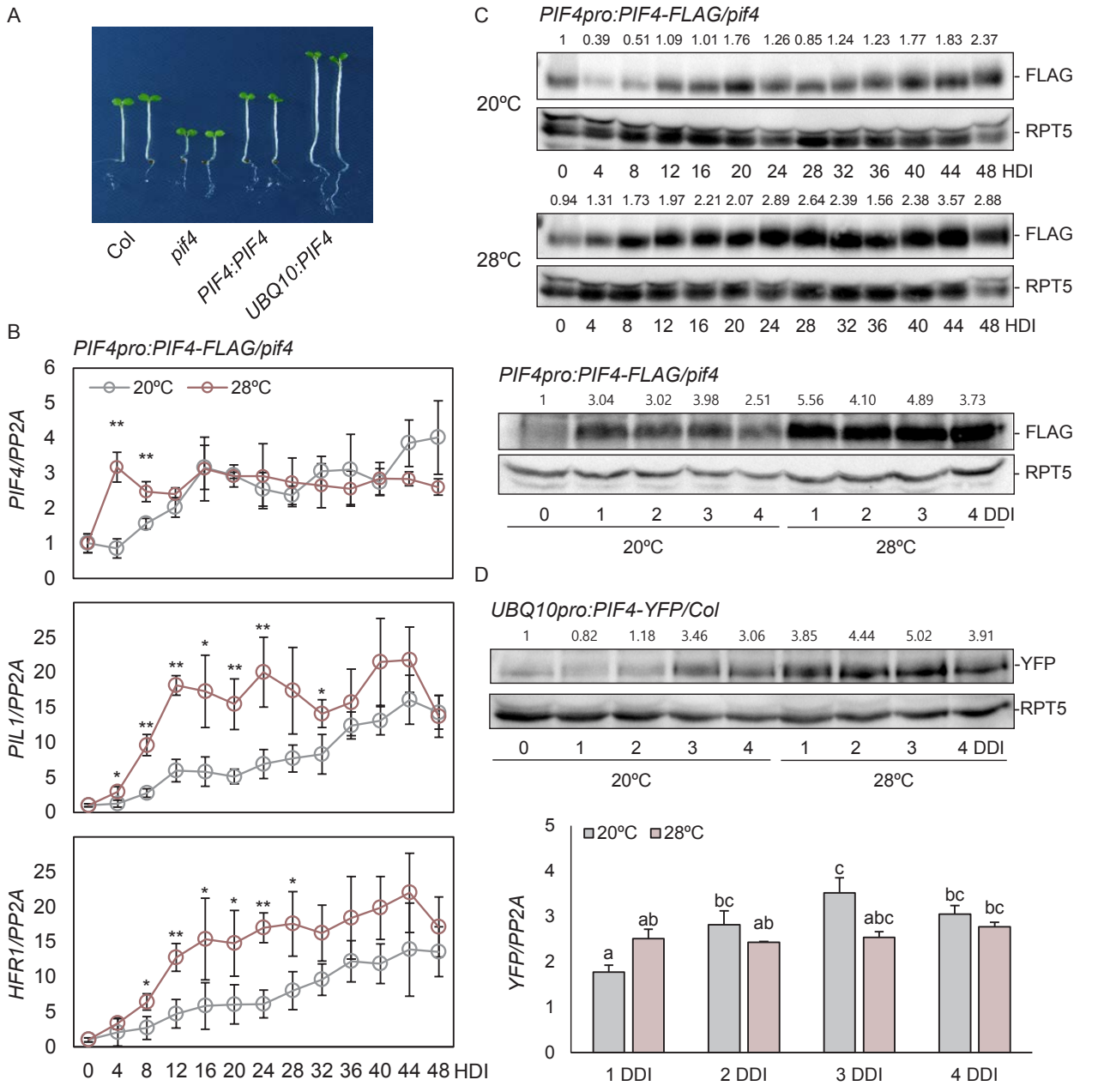

Fig. 4. High ambient temperature accelerates leaf senescence by increasing both *PIF4* mRNA and PIF4 protein. (A) Hypocotyl lengths of Col, *pif4, PIF4pro:PIF4-FLAG/pif4 (PIF4:PIF4)*, and *UBQ10pro:YFP-PIF4/Col (UBQ10:PIF4)*. Seedlings were grown in continuous red light (15 μmol $m^{-2}s^{-1}$) for 4 days. Col, Col-0 ecotype. (B) Increased *PIF4*, *PIL1*, and *HFR1* mRNA at high ambient temperature after transfer to darkness for the indicated number of hours (HDI, hours of dark incubation). Seven-day-old light-grown seedlings (*PIF4pro:PIF4-FLAG/pif4*) were transferred to darkness at either 20°C or 28°C and sampled every 4 h for mRNA expression analysis. mRNA levels were normalized by *PP2AA3* mRNA levels. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences between the corresponding values at 20°C and 28°C. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (C) Higher PIF4 protein levels in *PIF4pro:PIF4-FLAG/pif4* seedlings at high ambient temperature after transfer to darkness for the indicated number of hours (HDI) and days (DDI, days of dark incubation). PIF4 protein was detected with an anti-FLAG antibody and RPT5 protein was detected with an anti-RPT5 antibody. (D) Higher PIF4-YFP protein levels in *UBQ10pro:PIF4-YFP/Col* seedlings at high ambient temperature after 1 to 4 DDI. PIF4-YFP protein was detected with an anti-YFP antibody. *PIF4-YFP* mRNA levels were determined by real-time PCR. For (C) and (D), PIF4 protein levels relative to RPT5 protein levels are normalized by those before the transfer to darkness and indicated above the immunoblot data. Error bars indicate SD (n = 3, biological replicates). Different letters indicate statistically significant differences (two-way ANOVA, Tukey's HSD, $P < 0.05$).

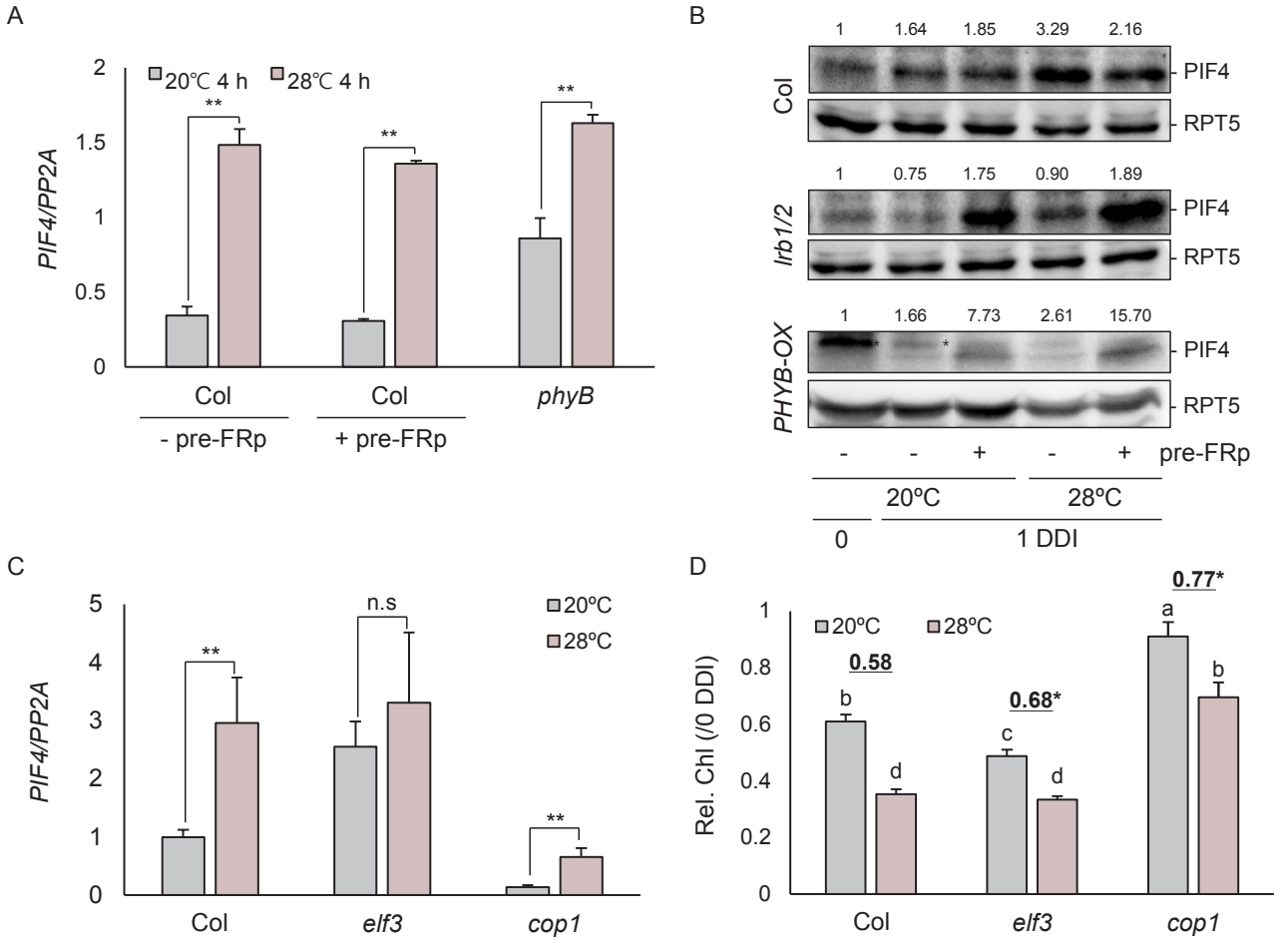

Fig. 5. High ambient temperature increases PIF4 both phy-dependently and phy-independently. (A) No effect of pre-FRp on the induction of *PIF4* mRNA by high ambient temperature. Wild type (Col) seedlings were transferred to darkness for 4 h at ambient or high ambient temperatures with or without a pre-FRp (2.5 μmol m^{-2} s^{-1}, 10 min) and sampled for mRNA expression analysis. *phyB* mutant seedlings were transferred to darkness for 4 h at ambient or high ambient temperatures without pre-FRp and sampled for mRNA expression analysis. mRNA levels were normalized by *PP2AA3* mRNA levels. Col, Col-0 ecotype. (B) The increase of PIF4 protein levels by a pre-FRp in Col, *lrb1 lrb2* (*lrb1/2*), and *PHYB-OX* seedlings after transfer to darkness for 1 DDI (days of dark incubation). PIF4 protein was detected with an endogenous PIF4 antibody and RPT5 protein was detected with an anti-RPT5 antibody. PIF4 protein levels relative to RPT5 protein levels are normalized before the transfer to darkness and indicated above the immunoblot data. (C) *PIF4* mRNA levels in *elf3* and *cop1* mutants after transfer to darkness for 4 h. Both wild type and mutant seedlings were transferred to darkness for 4 h at either ambient or high ambient temperature and sampled for mRNA expression analysis. mRNA levels were normalized by *PP2AA3* mRNA levels. For (A) and (C), error bars indicate SD (n = 3, biological replicates). Asterisks indicate the statistically significant differences. $**P < 0.01$, n.s, non-significant (Student's *t*-test). (D) Chlorophyll levels of *elf3* and *cop1* mutants after 5 DDI. Chlorophyll levels were normalized by fresh weight and then by the chlorophyll level of each line at 0 DDI (Rel. Chl). The ratio of chlorophyll levels between 28°C and 20°C after 5 DDI were indicated above graph bars. Error bars indicate SD (n = 3, biological replicates). Asterisks indicate the statistically significant differences between the corresponding values of wild type and mutants. $*P < 0.05$, (Student's *t*-test). Different letters indicate statistically significant differences (two-way ANOVA, Tukey's HSD, $P < 0.05$).

and *elf3* mutant seedlings senesce faster at 28°C than 20°C, which is consistent with high ambient temperature-induced PIF4 protein stabilization (Figs. 4C and 4D). Together, our results indicate COP1 is necessary for the increased expression of *PIF4* mRNA at both temperatures after the transfer to darkness, whereas ELF3 is necessary to repress *PIF4* mRNA expression at 20°C. The *PIF4* mRNA expression patterns we observed in these mutants indicate high ambient temperature increases *PIF4* mRNA levels phy-independently by inactivating ELF3.

Pfr generated before the transfer to darkness suppresses senescence in a prolonged dark condition

Since leaf senescence occurs relatively slowly after the transfer to darkness, we were curious to see whether Pfr generated before the transfer to darkness remains active to suppress senescence after the transfer to darkness. We therefore investigated the role of preexisting Pfr by inactivating Pfr with a

pre-FRp.

Wild type seedlings senesce faster at 20°C after exposure to a pre-FRp (Fig. 6A). Previous studies have shown PIF4/5 promote senescence downstream of phyB (Sakuraba et al., 2014; Song et al., 2014). Consistent with these results, not only did we find *pif4/5* double and *pifQ* mutant seedlings senesce more slowly than wild type, we also found pre-FRp exposure does not accelerate senescence in *pif4/5* and *pifQ* mutant seedlings at either 20°C or 28°C (Fig. 6A). This suggests the Pfr present in seedlings before the transfer to darkness suppresses senescence by inhibiting PIF4/5 after the transfer to darkness at ambient temperature. We next asked whether a pre-FRp can also accelerate senescence at high ambient temperature. Both wild type, *pif4/5*, and *pifQ* mutant seedlings senesce faster at 28°C than 20°C. Unlike what we observed at 20°C; however, we found pre-FRp exposure does not accelerate senescence in wild type seedlings at 28°C (Fig. 6A). This lack of any pre-FRp effect is not due to insufficient FR fluence as longer FR irradiation times also fail to accelerate senescence at 28°C (Fig. 6B). These results suggest Pfr is thermally reverted to its Pr form faster at 28°C than 20°C, with insufficient Pfr remaining at 28°C to suppress senescence.

Alternatively, it is possible pre-FRp exposure does not

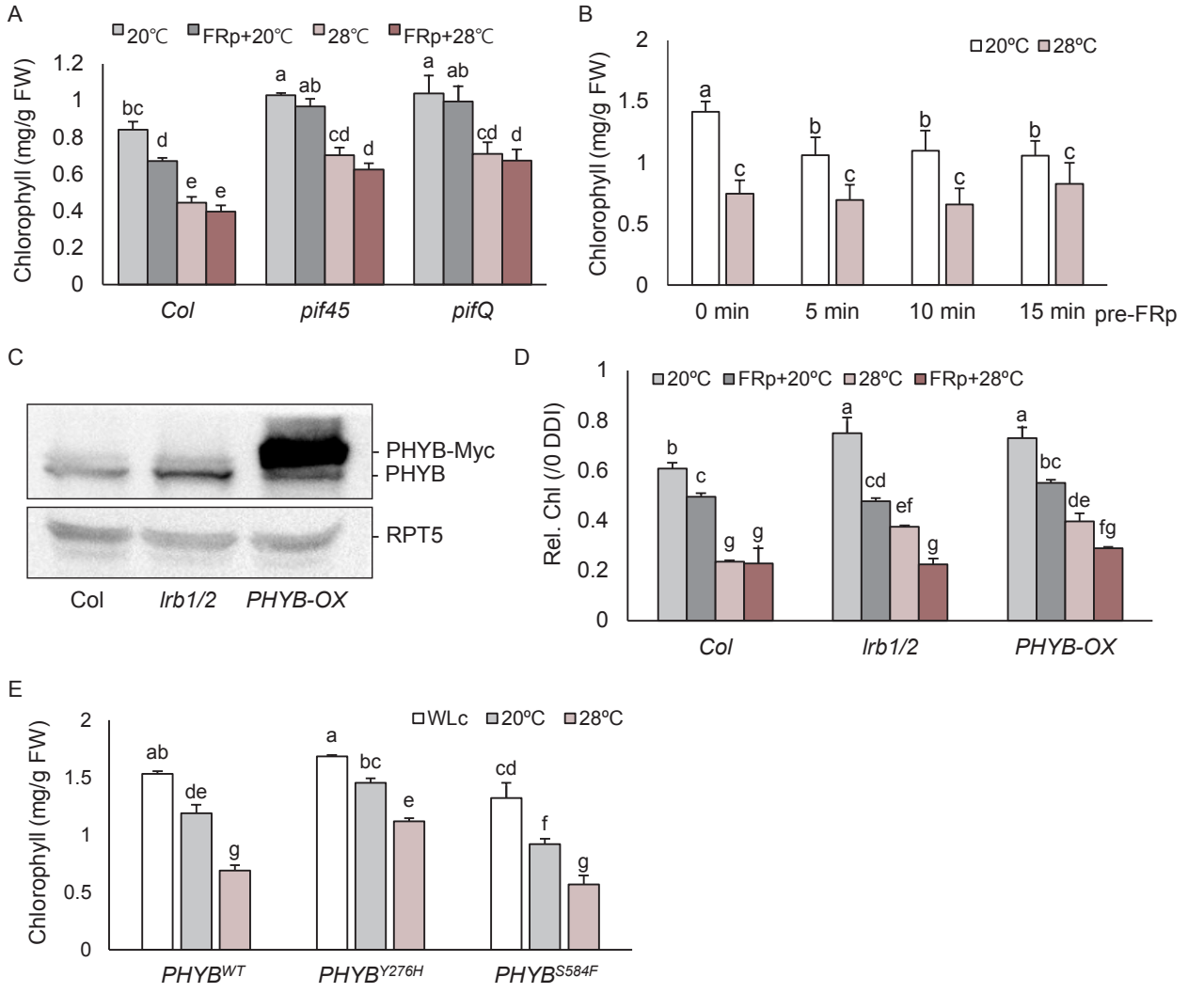

Fig. 6. Pfr generated in the light inhibits leaf senescence after the transfer to darkness. (A) Faster reduction of chlorophyll induced by a pre-FRp in wild type but not in *pif4/5* and *pifQ* mutant seedlings at ambient temperature after 5 DDI (days of dark incubation). Col, Col-0 ecotype. (B) Longer pre-FRp exposures do not increase the effect on chlorophyll levels at high ambient temperature after 5 DDI. (C) PhyB protein levels in *lrb1/2* and *PHYB-OX* seedlings. PhyB protein was detected in 7-day-old light-grown seedlings using an anti-phyB antibody and RPT5 protein was detected using an anti-RPT5 antibody. (D) Faster reduction in chlorophyll induced by a pre-FRp in *lrb1/2* mutant and *PHYB-OX* seedlings at both ambient and high ambient temperature after 6 DDI. (E) Chlorophyll levels of $PHYB^{WT}$, $PHYB^{Y276H}$, and $PHYB^{S584F}$ overexpression seedlings at 20°C and 28°C. For (A) to (E), error bars indicate SD (n = 3, biological replicates). Different letters indicate statistically significant differences (two-way ANOVA, Tukey's HSD, $P < 0.05$).

accelerate senescence at 28°C, not because high ambient temperature accelerates the thermal reversion of Pfr to Pr to levels below an activity threshold, but because Pfr cannot suppress senescence at high ambient temperature. If this is the case, pre-FRp exposure should not accelerate senescence even in seedlings with high levels of Pfr. We therefore examined senescence in *lrb1/2* double mutants and *PHYB-OX*, which produce two-fold and ten-fold more phyB protein than wild type, respectively (Fig. 6C). We found both *lrb1/2* and *PHYB-OX* mutants senesce more slowly than wild type at 20°C and 28°C (Fig. 6D). Pre-FRp exposures further accelerate the senescence of both *lrb1/2* mutant and *PHYB-OX* seedlings at 20°C and 28°C. Together, these results indicate Pfr is capable of suppressing senescence even at 28°C. This implicates a mechanism in which high ambient temperature accelerates senescence after the transfer to darkness by accelerating the thermal reversion of Pfr to Pr to levels below the threshold required for activity. Consistent with this result, constitutively active PHYB^{Y276H} suppresses senescence more strongly than wild type phyB, whereas the fast reverting PHYB^{S584F} suppresses senescence more weakly at both 20°C and 28°C (Fig. 6E).

High ambient temperature inactivates Pfr faster than ambient temperature in a prolonged dark condition

The suppression of senescence by pre-existing Pfr raises the question of how long Pfr remains active after the transfer to darkness. Since the number and size of phyB photobodies —distinct fluorescent dots in the nucleus—is correlated with phyB activity (Chen et al., 2003; Hahm et al., 2020), we examined how long phyB photobodies last after the transfer to darkness. As reported before, PhyB-GFP forms bright photobodies in the light (Fig. 7A). In the epidermis, the nuclear fluorescent signals grow more diffuse with fewer and weaker photobodies already at 12 h after the transfer to darkness. At 28°C, the effect is stronger, with more diffuse nuclear fluorescent signal and even fewer photobodies (Fig. 7B). One day after the transfer to darkness, nuclear fluorescent signals are even further reduced with virtually no discernible photobodies at either 20°C or 28°C. These results indicate phyB-GFP photobodies disappear quickly after the transfer to darkness at both 20°C than at 28°C, but lasting slightly longer at 20°C.

Although photobodies disappear quickly, diffuse GFP signal is still present in the nucleus 24 h after the transfer to darkness at both 20°C and 28°C (Fig. 7A). This suggests that a small portion of phyB may still be active. To more sensitively gauge the presence of active Pfr, we exposed seedlings to an FRp in the middle of the dark period (mid-FRp) at various times after the transfer to darkness and measured the expression of PIF target gene mRNAs (Fig. 7C). If Pfr is present at the time of the mid-FRp, then the mid-FRp should convert the remaining Pfr to Pr, releasing PIFs to activate the expression of PIF target gene mRNAs. We performed these experiments with *phyA* mutant seedlings to avoid FRp-induced phyA responses. At 20°C, we observed increased *PIL1* and *HFR1* expression upon mid-FRp exposure for up to 4 days after the transfer to darkness. At 28°C, in contrast, a mid-FRp did not induce any significant change in *PIL1* or *HFR1* levels 3 or 4 days after the transfer to darkness (Fig. 7D). The mid-FRp-induced increase in *PIL1* and *HFR1* mRNA expression can be attributed to the reversion of any remaining Pfr to Pr because the increase is prevented by exposure to a pre-FRp (Fig. 7E). Together, these results indicate that Pfr remains active for at least 4 days after the transfer to darkness at 20°C but for only 2 to 3 days at 28°C. This shorter lifespan of active Pfr at 28°C than 20°C in the darkness further suggests high ambient temperature accelerates senescence by accelerating the thermal reversion of Pfr to Pr.

DISCUSSION

PIF4 promotes leaf senescence in a prolonged dark condition, whereas phyB suppresses senescence by inhibiting PIF4 (Sakuraba et al., 2014; Song et al., 2014). In this study, we show high ambient temperature accelerates leaf senescence in the dark by increasing *PIF4* mRNA and PIF4 protein levels phy-dependently and phy-independently. In a phy-dependent pathway, high ambient temperatures accelerate the inactivation of Pfr, thus speeding the accumulation of PIF4 proteins after the transfer to darkness (Figs. 4C and 4D). In addition to this phy-dependent pathway, high ambient temperature also increases *PIF4* mRNA levels phy-independently through ELF3 and PIF4 protein levels and through other unknown components (Fig. 5). Increased PIF4 protein then activates the expression of *ORE1* while also activating ABA and ethylene signaling (Fig. 3), accelerating senescence at high ambient temperature. Our results support a role for the phy-PIF4 signaling module in integrating not only light signaling but also temperature signaling to regulate leaf senescence (Fig. 8).

Our data indicate some Pfr remains active relatively long after the transfer to darkness. We demonstrated this by measuring the induction of PIF4 target gene expression by exposure to a mid-FRp even 4 days after the transfer to darkness at 20°C (Fig. 7D). Direct measurements of different absorption spectra in vitro, in yeast, and in plants indicate that recombinant phytochromes undergo an initial rapid thermal reversion of Pfr-Pr heterodimers followed by a slower thermal reversion of Pfr-Pfr homodimers (Ádám et al., 2011; Eichenberg et al., 1999; 2000; Jung et al., 2016; Klose et al., 2015; Kunkel et al., 1995; Legris et al., 2016; Remberg et al., 1998; Sweere et al., 2001). In plants, the Pfr form of phyB also undergoes thermal reversion with a half-life of about 60 min at 25°C, but some Pfr remains even after 4 h in darkness. In fluorescent image analyses of phyB-GFP, although phyB-GFP photobodies disappear within 16 to 18 h in darkness, there is residual diffuse nuclear phyB-GFP signal even 24 h after the transfer to darkness (Huang et al., 2016; Van Buskirk et al., 2014). This suggests a portion of the phyB-GFP remains in the Pfr form even after 24 h of darkness. As we further probed biologically active Pfr by measuring the expression of FRp-inducible PIF target genes, we confirmed that Pfr remains active even 4 days after the transfer to darkness at ambient temperature (Fig. 7D). This relatively long survival of Pfr after the transfer to darkness is also evidenced by the destabilization of PIF4 protein 1 day after the transfer of *lrb1/2* mutants and *PHYB-OX* to darkness (Fig. 5B). Together, our results indicate that at ambient temperature, some active Pfr remains to suppress leaf senescence by inhibiting PIF4 protein

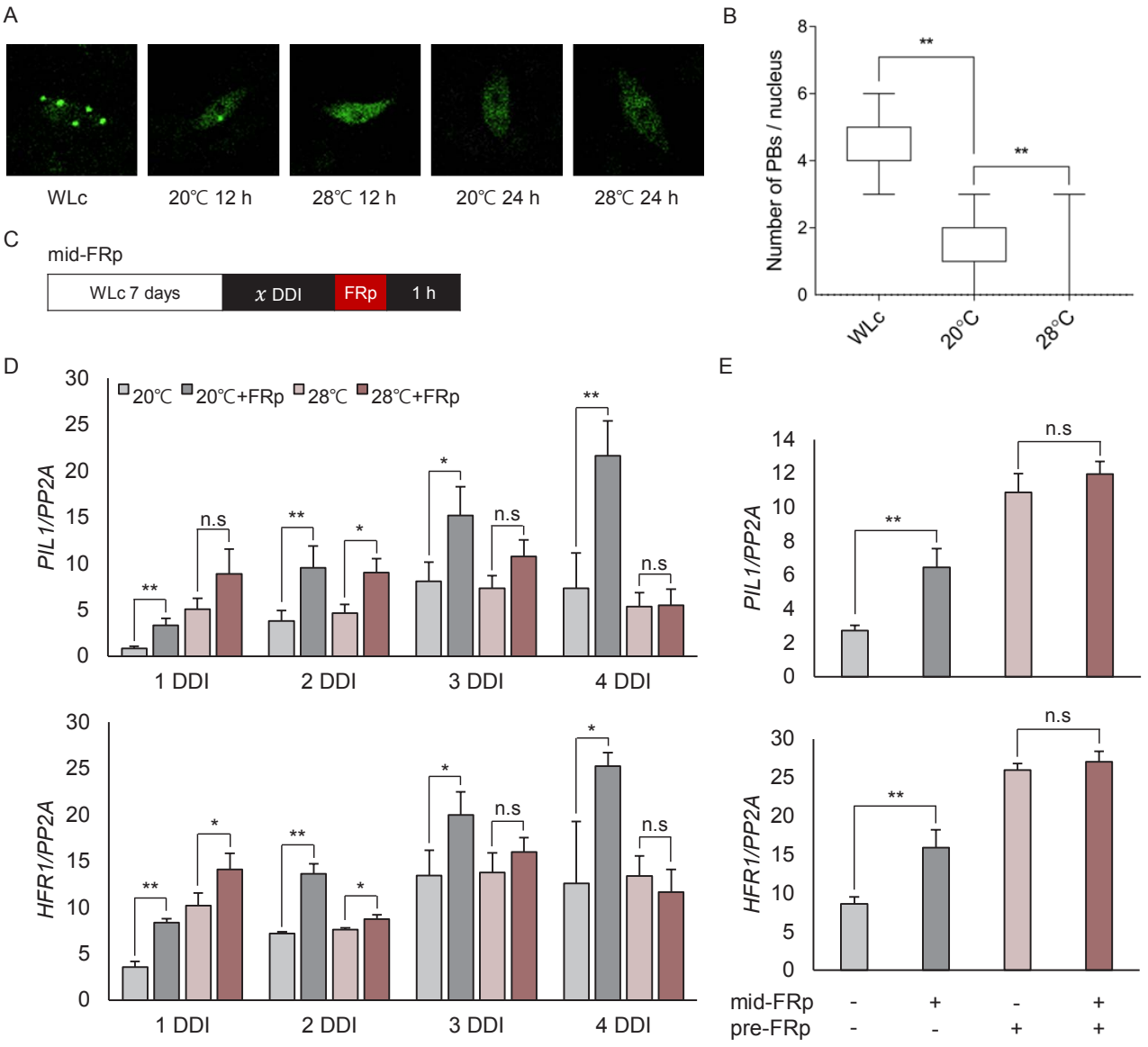

Fig. 7. High ambient temperature inactivates Pfr faster than ambient temperature after the transfer to darkness. (A) High ambient temperature accelerates the disappearance of phyB-GFP photobodies after the transfer to darkness. *35Spro:PHYB-GFP* seedlings were imaged after the transfer to darkness for 0, 12 and 24 h using a confocal microscope. WLc, continuous white light. (B) Quantification of the number of photobodies (PBs) per nucleus. Nuclei (50-130) were used for the quantification. Asterisks indicate statistically significant differences. $**P < 0.01$, (Student's *t*-test). (C) A diagram showing the far-red light pulse (FRp) treatment scheme given after the transfer to darkness (mid-FRp). Seedlings were sampled 1 h after mid-FRp exposure for RNA expression analysis. (D) Increased expression of *PIL1* and *HFR1* mRNAs induced by a mid-FRp on various days after the transfer to darkness. Seven-day-old light-grown *phyA* seedlings were transferred to darkness at two different temperatures and exposed to a mid-FRp at the indicated times after the transfer. mRNA levels were normalized to *PP2AA3* mRNA levels. (E) The abolishment of the mid-FRp effect on *PIL1* and *HFR1* mRNA expression by exposure to a pre-FRp. Seven-day-old light-grown *phyA* seedlings were transferred to darkness at ambient temperature with or without a pre-FRp before a mid-FRp was given 1 day after the transfer. mRNA levels were normalized to *PP2AA3* mRNA levels. For (D) to (E), error bars indicate SD (n = 3, biological replicates). Asterisks indicate statistically significant differences. $*P < 0.05$, $**P < 0.01$, n.s, non-significant (Student's *t*-test).

for at least 4 days after the transfer to darkness.

Our data show phyB acts not only as a photoreceptor but also as a thermosensor that regulates leaf senescence in prolonged darkness. PhyB's ability to sense temperature is likely due to a temperature-dependent increase in the rate of thermal reversion of the Pfr form to the Pr form. This was manifested in the reduced size of phyB-GFP photobodies and in direct absorbance measurements of Pfr level at high ambient

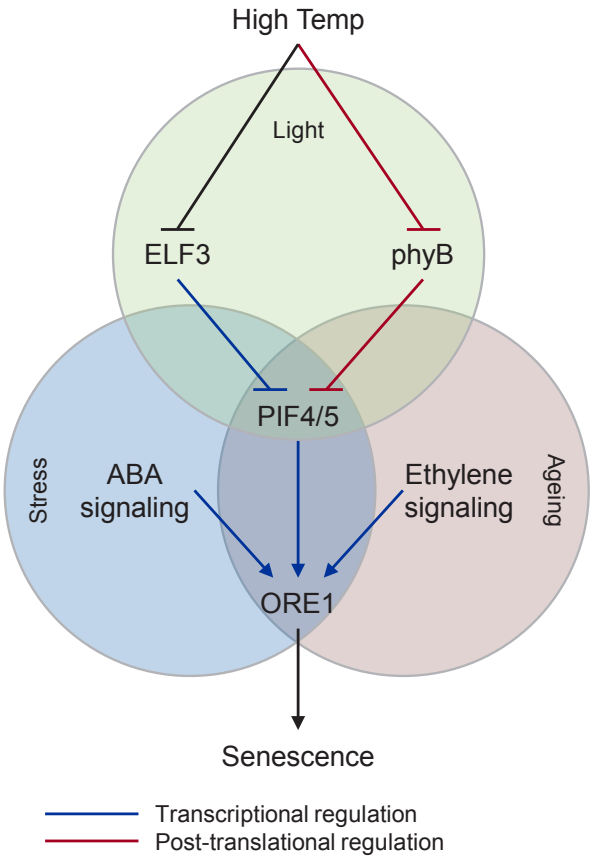

Fig. 8. A model for high ambient temperature-induced acceleration of leaf senescence. High ambient temperature accelerates leaf senescence via PIF4 and PIF5 when light-grown seedlings are transferred to darkness at different temperatures. High ambient temperature increases *PIF4* mRNA levels by inhibiting ELF3; it also increases PIF4 protein levels, in part by accelerating the thermal reversion of Pfr to Pr in the darkness. Increased PIF4 protein binds the *ORE1* promoter and activates *ORE1* mRNA expression while also activating ethylene signaling. Then, ORE1, a master regulator of leaf senescence, accelerates senescence. PhyB-independent stabilization of PIF4 protein by high ambient temperature is not included in the diagram for the sake of simplicity. Blue lines indicate transcriptional regulation, red lines indicate post-translational regulation, and black lines indicate unknown regulatory processes.

temperature in light-grown seedlings (Jung et al., 2016; Legris et al., 2016). We found, however, that phyB photobodies disassemble quickly after the transfer to darkness, making it difficult in our experimental conditions to determine whether Pfr lasts long enough to suppress senescence after the transfer to darkness (Fig. 7A). To detect the presence of biologically active Pfr, we measured the induction of PIF4 target gene mRNAs by an FRp given in the middle of the period of darkness (mid-FRp) (Fig. 7C). Such a mid-FRp should revert all the Pfr present at the time of irradiation to Pr, releasing PIF4 to activate the expression of its target genes. We found a mid-FRp increases PIF4 target gene mRNAs even if given 4 days after the transfer to darkness at 20°C. At 28°C, however, a mid-FRp given 3-4 days after the transfer to darkness does not affect PIF4 target gene mRNAs (Fig. 7D). These results indicate high ambient temperature accelerates the inactivation of Pfr, making phytochromes less active and therefore less able to suppress senescence.

The phytochromes, however, are not the only thermosensors regulating leaf senescence via PIF4 as high ambient temperature increases *PIF4* mRNA levels phy-independently after the transfer to darkness (Fig. 5). The increase of *PIF4* mRNA induced by high ambient temperature is largely restricted shortly after the transfer to darkness (Fig. 4B). Consistent with our data, previous reports showed high ambient temperature transiently increases *PIF4* mRNA levels in light-grown seedlings in a continuous white light condition (Koini et al., 2009; Sun et al., 2012). This faster increase in *PIF4* mRNAs at high ambient temperature after the transfer to darkness is not caused by a faster inactivation of Pfr because FRp exposure before the transfer to darkness (pre-FRp) does not abolish the transient induction of *PIF4* mRNA (Fig. 5A). Similarly, in *phyB* mutant seedlings under LD conditions during the dark period, *PIF4* mRNAs increase faster at 28°C than at 22°C (Nomoto et al., 2012). *PIF4* mRNAs also increase faster at 27°C than 22°C even in *phyABCDE* mutants and in a constitutively active *phyB*^{Y276H} line under an SD condition (Jung et al., 2016). Curiously, *PIF4* mRNAs are also induced faster and fall faster in cotyledons than in hypocotyls (Stavang et al., 2009), suggesting different tissues regulate *PIF4* mRNA expression differently in response to high ambient temperature.

High ambient temperature increases PIF4 protein levels both phy-dependently and phy-independently. Since *PIF4* mRNA levels reach similar plateaus at 28°C than 20°C, the higher PIF4 protein levels at high ambient temperature cannot be attributed to the increase in *PIF4* mRNA levels (Figs. 4B and 4C). Consistent with this, PIF4 protein levels are also higher at high ambient temperature in the *UBQ10pro:PIF4-YFP/Col* line, which produces similar levels of *PIF4* mRNA at both temperatures (Fig. 4D). PIF4 protein also accumulates less in the *hemera* (*hmr*) mutant when transferred from 21°C to 27°C, though wild type and the *hmr* mutant express similar levels of *PIF4* mRNA (Qiu et al., 2019). Also consistent with our observations, previous reports observed higher PIF4 protein levels in the *35Spro:PIF4* line at 25°C than at 15°C when the seedlings were grown in 12L:12D red or blue photocycles (Foreman et al., 2011). This was also the case when seedlings were transferred to 27°C rather than 22°C in white light (Kumar et al., 2012). The increase in PIF4 protein levels at high ambient temperature is partly phy-independent as PIF4 protein levels are still higher at 28°C than 20°C even after a pre-FRp (Fig. 5B). Consistent with our data, a pre-FRp was shown to slightly increase PIF4 protein levels at 15°C but not to levels high enough to match PIF4 protein levels at 25°C (Foreman et al., 2011). Dark-grown *35Spro:PIF4* seedlings also have higher PIF4 protein levels at 27°C than at 17°C (Johansson et al., 2014), further supporting a phy-independent mechanism. Together, our results indicate the presence of a thermosensor other than the phytochromes that regulates the expression of *PIF4* mRNA and the stability of PIF4 protein.

Currently, no other thermosensors are known to regulate

senescence via PIF4. Previous studies have shown that other plant photoreceptors, including phototropin (phot), cryptochrome (cry: cry1 and cry2), and UVR8, also undergo thermal reversion from an active state to an inactive state, making them good thermosensor candidates (Findlay and Jenkins, 2016; Fujii et al., 2017; Herbel et al., 2013). However, phot, cry, and UVR8 are unlikely to be the thermosensor that regulates senescence via PIF4 in prolonged darkness. First, phot has a short half-life (~30 s at 22°C), making it difficult to function in a prolonged darkness (Christie, 2007). Phot also does not regulate *PIF4* mRNA (Ohgishi et al., 2004). Second, cry1 binds more strongly under blue light to the promoters of PIF-target auxin-related genes such as YUC8, inhibiting their expression more strongly at high ambient temperature (Ma et al., 2016). This acts in opposition to the increase in PIF-target expression induced by high temperature via the thermal reversion of cry1. The cry1 cry2 double mutants also show wild type-like senescence in prolonged darkness (Sakuraba et al., 2014). Third, because we use only LED illumination lacking UV-B, we can exclude UVR8 as the other thermosensor that regulates senescence via PIF4 in prolonged darkness in our experiments. Beyond photoreceptors, one study showed that high ambient temperature evicts H2A.Z +1 nucleosomes from various genes (e.g., HSP70), thereby increasing their mRNA expression. At the same time, ARP6, a subunit of the SWR complex, can deposit H2A.Z +1 nucleosomes at 17°C (Kumar and Wigge, 2010). The same study, however, found that *PIF4* mRNA levels in *arp6* mutant seedlings are not higher than those of wild type seedlings at 17°C (Kumar and Wigge, 2010). This suggests H2A.Z +1 nucleosome eviction is not to blame for the induction of *PIF4* mRNAs by high ambient temperature.

ELF3 and COP1 could be closely associated with a thermosensor regulating high temperature-induced *PIF4* mRNA expression during senescence. Previous studies have shown high ambient temperature reduces the binding of the evening complex to the *PIF4* promoter, releasing the evening complex-mediated repression of *PIF4* mRNA expression (Box et al., 2015; Ezer et al., 2017; Nusinow et al., 2011; Silva et al., 2020). Consistent with this, the expression of *PIF4* mRNA is higher in *elf3* mutant seedlings than in wild type transferred to darkness at 20°C, with higher ambient temperature producing no further increase (Fig. 5C). This suggests ELF3 acts as either a thermosensor itself or as a key component mediating thermosensing signaling to increase the expression of *PIF4* mRNA in response to high ambient temperature. Previous studies also have shown high ambient temperature enhances nuclear localization of COP1 and that COP1 is required for the high ambient temperature-induced expression of *PIF4* mRNA either via its role in the degradation of HY5 or independently of HY5 (Delker et al., 2014; Gangappa and Kumar, 2017; Park et al., 2017). Under our experimental conditions, *cop1* mutant seedlings express *PIF4* mRNA at very low levels after the transfer to darkness, entering senescence slower than wild type seedlings regardless of temperature (Figs. 5C and 5D). Interestingly, however, the expression of *PIF4* mRNA in *cop1* mutants, although low overall, is still induced by high ambient temperature. This suggests COP1 is a general activator of *PIF4* mRNA expression regardless of temperature rather than a thermosensor inducing *PIF4* mRNA specifically at high ambient temperature. Together, our results indicate that ELF3 is required for the induction of *PIF4* mRNA at high ambient temperature during senescence, while COP1 is required for high expression of *PIF4* mRNA regardless of temperature. Further research is necessary to determine whether ELF3 or the evening complex are thermosensors.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

C.K., P.O.L., Y.I.P., and G.C. designed the experiments. C.K., S.J.K., J.J., E.P., and E.O. performed the experiments. C.K., E.O., P.O.L., and G.C. wrote the manuscript. All authors discussed the results and reviewed the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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