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PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) positively regulates dark-induced senescence and chlorophyll degradation in *Arabidopsis*

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ABSTRACT

Darkness is a known environmental factor that induces plant senescence. Here, PHYTOCHROME-INTERACTING FACTORS (PIFs), several bHLH transcription factors involved in plant skotomorphogenesis, were examined for their roles in the regulation of dark-induced senescence and chlorophyll breakdown in *Arabidopsis thaliana*. After light-grown seedlings were transferred to darkness, green leaves turned yellow, and chlorophyll contents decreased, but membrane lipid peroxidation and cell death increased in wild-type Col-0. These responses were enhanced in overexpression line *PIF5OX* but decreased in mutant *pif5-3*. Darkness significantly induced expression of several genes involved in chlorophyll breakdown, including *SGR*, *NYC1*, *NOL*, and *PAO*, as well as genes encoding for transcription factors that have been shown to be required for dark-induced senescence, including *WRKY22*, *NAP*, *EIN3*, *EIL1*, and *ORE1*. These effects on gene expression were also enhanced in *PIF5OX* but decreased in *pif5-3* relative to Col-0. Further analyses using ChIP-qPCR, EMSA, and protoplast transient assays indicated that PIF5 binds to the G-box motifs in the promoters of *SGR*, *NYC1*, and *ORE1* genes and stimulate their expression. Collectively, our data indicate that PIF5 is a key factor that positively regulates dark-induced senescence upstream of ORE1 and regulates chlorophyll breakdown upstream of SGR and NYC1.

1. Introduction

Leaf senescence is the final stage of leaf growth and development, characterized by loss of chlorophylls, degradation of proteins, nucleic acids, and nutrient remobilization [1]. It generally occurs in an age-dependent manner, controlled by endogenous factors, such as ethylene and nitric oxide, but is also induced by various environmental stresses, including darkness, drought, high temperature, and high salinity [1,2].

To understand the molecular mechanisms of leaf senescence, different sets of genes, designated senescence-associated genes (*SAGs*) were found to be up-regulated during senescence and encode proteins involved in the breakdown of cellular components, including nucleases, proteases, and cell wall hydrolases [1], which were often used as senescence marker genes and their gene expression will quickly increase upon senescence onset [3–6]. In addition, various transcription factors (TFs) have been found to play important roles in modulating senescence. ORESARA1 (ORE1, also known as NAC092), a TF belongs to the NAC family, has been identified as a positive regulator of senescence [4,7]. Overexpression of *ORE1* in transgenic *Arabidopsis* triggers early senescence, while its down-regulation retards senescence [4,7]. ORE1 promotes senescence by regulating the expression of hundreds of *SAGs* that function in the breakdown of nucleic acids and proteins [7]. NAC-LIKE, ACTIVATED BY AP3/PI (NAP, also known as NAC029), another NAC TF, also promotes senescence, as demonstrated by the delayed senescence phenotype of *nap* mutant [8]. ETHYLENE-INSENSITIVE3 (EIN3), a key positive TF involved in ethylene signaling, was recently found to be an inducer of senescence [5]. Overexpression of *EIN3*

Abbreviations: ChIP, chromatin immunoprecipitation; CLH, chlorophyllase; CV, chloroplast vesiculation; EMSA, electrophoretic mobility shift assay; fLUC, firefly luciferase; NOL, NYC1-like; NYC1, non-yellow coloring 1; ORE1, oresara 1; PAO, pheide a oxygenase; PIF, phytochrome-interacting factor; qRT-PCR, real-time quantitative reverse transcription PCR; REN LUC, renilla luciferase; SGR, stay green; SAG, senescence-associated gene; TBARS, thiobarbituric acid reactive substance.

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is sufficient to accelerate leaf senescence, but loss of function of *EIN3* and its close homolog EIN3-LIKE1 (EIL1) conversely leads to delay in age-dependent and dark-induced leaf senescence, and further studies indicated that EIN3 could negatively regulate the expression of *microRNA164* through directly binding to its promoter, whereas microRNA164 negatively regulates *ORE1* [4,5,9]. In addition, three WRKY TFs have been studied for their involvement in senescence: *WRKY6* positively elevates the transcript level of *SIRK*, whose expression is strongly induced specifically during leaf senescence [10]; *WRKY53* mainly expresses at the early stage of leaf senescence, and its overexpression and knockout plants showed accelerated and delayed senescence phenotypes, respectively [11,12]; *WRKY22* transcription was promoted by darkness, and its overexpression and knockout lines also exhibited accelerated and delayed senescence phenotypes after darkness treatment, respectively, implying its role in dark-induced senescence [6].

Loss of green color is a dramatically visualized sign of leaf senescence, which is mainly resulted from chlorophyll breakdown [13]. The initial reaction during chlorophyll degradation is the removal of the phytol residue and the central Mg by chlorophyllase (CLH) and metal chelating substance (MCS), respectively. *CHLs* were the first cloned genes involved in chlorophyll degradation. Subsequently, Pheophyinase (PPH) was identified to function in porphyrin-phytol hydrolysis in senescence-related chlorophyll degradation, and the resulting pheophorbide (pheide) *a* is converted into a primary fluorescent chlorophyll catabolite (*p*FCC), which is catalyzed by pheide *a* oxygenase (PAO) and red chlorophyll catabolite reductase (RCCR) sequentially [14]. More importantly, two other genes, namely *NON-YELLOW COLORING1* (*NYC1*) and *STAY GREEN* (*SGR*), were identified in *Arabidopsis*. *NYC1* and its homolog *NYC1-LIKE* (*NOL*) encode two subunits of chlorophyll *b* reductase, catalyzing the chlorophyll *b* to chlorophyll *a* reduction [14,15]. SGR could directly interacts with a subset of the proteins in the light-harvesting chlorophyll *a*/*b*-protein complex II (LHCII), implying that SGR may be involved in destabilizing pigment–protein complexes as a prerequisite for chlorophyll degradation enzymes to access their substrates during leaf senescence [16]. More recently, another novel gene, *CHLOROPLAST VESICULATION* (*CV*), encoding a protein that could destabilize the chloroplast, was identified to be an important factor positively regulating plant senescence [17].

Light is one of the most important environmental factors for plant growth, which not only provides the source of energy for plant life, but also acts as a signal affecting plant growth and development throughout the life cycle from germination to flowering [18]. PHYTOCHROME-INTERACTING FACTORS (PIFs), a small subfamily of basic helix-loop-helix (bHLH) TFs, have been indicated to be centrally critical to the promotion of skotomorphogenic development in dark-grown seedlings [19,20]. At the molecular level, when plants were transferred to light, active phytochromes directly interact with PIFs to induce their detachment from DNA and promote their degradation by the 26S proteasome [20]. Primary characterization of the PIFs indicated that these factors regulate various light responses and act as important hubs of light, auxin, gibberellin, and brassinosteroid pathways through regulating the expression of many genes by directly binding to their promoters at the G-box (CACGTG) or other E-box variants [19,21]. Different PIFs play redundant and distinct roles in *Arabidopsis* [21]. For example, PIF1 negatively regulates seed germination and chlorophyll biosynthesis [22,23]; PIF3 negatively regulates chloroplast development and chlorophyll biosynthesis [24,25], whereas it positively regulates anthocyanin accumulation [26]; PIF4 and PIF5 negatively regulate phytochrome-mediated inhibition of shade avoidance [27], and several recent association studies implicated involvement of PIF4 in high-temperature response and flowering time regulation [28,29]. Additionally, a quadruple *pif* mutant (*pifq*), which lacks PIF1, PIF3, PIF4 and PIF5, displays morphogenic development in full darkness that resembles the normal light-grown seedlings [24,30], indicating that PIF1, PIF3, PIF4, and PIF5 function redundantly in the regulation of hypocotyl elongation.

The role of light in the regulation of leaf senescence has been widely studied. Red light is considered a negative signal for this process, because pulses of red light substantially delay dark-induced senescence [31]. Plants that overexpress the light receptors phytochrome A or B show delayed leaf yellowing [32,33], whereas the loss-of-function mutant *phyB* is hyposensitive to dark treatment [34], suggesting a role of phytochromes in the regulation of leaf senescence. Recently, two studies found that phytochrome-interacting transcription factors PIF4 and PIF5 are both positive factors of dark-induced senescence in *Arabidopsis* [3,35]. Here, we found that PIF5 is a key player for regulation of dark-induced chlorophyll degradation and senescence but PIF1, PIF3 and PIF4 are less important in these processes. Using ChIP-qPCR, EMSA, and protoplast transient assays, it was revealed that PIF5 directly binds to the G-box motifs of *SGR*, *NYC1*, and *ORE1* gene promoters to activate their expression.

2. Materials and methods

2.1. Plant materials and growth conditions

The *pif1-2* (*pif1*), *pif3-3* (*pif3*), *pif4-2* (*pif4*), *pif5-3* (*pif5*), *pif4pif5*, *pifq*, and *PIF5OX* were kindly provided by Prof. Peter H. Quail at University of California, and the transgenic line *35S:PIF5-HA* was a gift from Prof. Julin N. Maloof also at University of California. Homozygous lines of *sgr-2* (Salk_070891) and *nyc1-1* (Salk_017680) were obtained from the Nottingham Arabidopsis Stock Center (NASC). All the materials are in *Arabidopsis thaliana* Columbia (Col-0) ecotype background. The double mutants *PIF5OX/sgr-2* and *PIF5OX/nyc1-1* were generated by crossing *PIF5OX* to *sgr-2* and *nyc1-1*, respectively, and homozygous lines were selected through PCR genotyping (primers used are listed in Table S1). Seeds were surface-sterilized with 20% (v/v) bleach solution for 10 min, rinsed with sterile water for three times, and then sown on media containing 1/2 Murashige and Skoog (MS) salt, and 0.68% phytoblend agar. Seeds on agar plates were cold-treated at 4 °C for 3 days in the dark and then transferred to a chamber at 22 °C with a continuous white light condition (80 μmol m^{-2} s^{-1}) for growth. After 3 weeks, the plants were transferred to darkness for treatment.

2.2. Measurement of chlorophyll pigments, ion leakage, TBARS, and cell death

Total chlorophyll content was measured according to Zhou et al. [6]. Chlorophyll pigments were extracted with 80% ice-cold ethanol from leaf tissues of plants transferred to darkness for indicated time. Extracts were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the absorbance at 645 and 663 nm was determined using an UV–vis spectrophotometer. The chlorophyll content was calculated according to Lichtenthaler [36].

Ion leakage caused by dark-induced senescence was determined according to Sakuraba et al. [3] with minor modifications. In brief, five pieces of the sixth leaves were placed in a tube with 10 mL of deionized water at room temperature for 2 h, and the initial conductivity (C_1) in the bathing solution was determined, and then the samples were heated in boiling water for 10 min to induce 100% leakage, followed by cooling at room temperature and measurement of final electrolyte conductivity (C_2). The relative ion leakage was expressed as a percentage of the total ion leakage and calculated as relative ion leakage (%) = $C_1/C_2 \times 100$.

Thiobarbituric acid reactive substance (TBARS) was measured as the amount of MDA (malonyldialdehyde) according to Liu et al.