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Phytochrome Signaling in Green *Arabidopsis* Seedlings: Impact Assessment of a Mutually Negative phyB–PIF Feedback Loop

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ABSTRACT The reversibly red (R)/far-red (FR)-light-responsive phytochrome (phy) photosensory system initiates both the deetiolation process in dark-germinated seedlings upon first exposure to light, and the shade-avoidance process in fully deetiolated seedlings upon exposure to vegetational shade. The intracellular signaling pathway from the light-activated photoreceptor conformer (Pfr) to the transcriptional network that drives these responses involves direct, physical interaction of Pfr with a small subfamily of bHLH transcription factors, termed Phy-Interacting Factors (PIFs), which induces rapid PIF proteolytic degradation. In addition, there is evidence of further complexity in light-grown seedlings, whereby phyB–PIF interaction reciprocally induces phyB degradation, in a mutually-negative, feedback-loop configuration. Here, to assess the relative contributions of these antagonistic activities to the net phenotypic readout in light-grown seedlings, we have examined the magnitude of the light- and simulated-shade-induced responses of a pentuple *phyBpif1pif3pif4pif5* (*phyBpifq*) mutant and various multiple *pif*-mutant combinations. The data (1) reaffirm that phyB is the predominant, if not exclusive, photoreceptor imposing the inhibition of hypocotyl elongation in deetiolating seedlings in response to prolonged continuous R irradiation and (2) show that the PIF quartet (PIF1, PIF3, PIF4, and PIF5) retain and exert a dual capacity to modulate hypocotyl elongation under these conditions, by concomitantly promoting cell elongation through intrinsic transcriptional-regulatory activity, and reducing phyB-inhibitory capacity through feedback-loop-induced phyB degradation. In shade-exposed seedlings, immunoblot analysis shows that the shade-imposed reduction in Pfr levels induces increases in the abundance of PIF3, and mutant analysis indicates that PIF3 acts, in conjunction with PIF4 and PIF5, to promote the known shade-induced acceleration of hypocotyl elongation. Conversely, although the quadruple *pifq* mutant displays clearly reduced hypocotyl elongation compared to wild-type in response to prolonged shade, immunoblot analysis detects no elevation in phyB levels in the mutant seedlings compared to the wild-type during the majority of the shade-induced growth period, and phyB levels are not robustly correlated with the growth phenotype across the *pif*-mutant combinations compared. These results suggest that PIF feedback modulation of phyB abundance does not play a dominant role in modulating the magnitude of the PIF-promoted, shade-responsive phenotype under these conditions. In seedlings grown under diurnal light–dark cycles, the data show that FR-pulse-induced removal of Pfr at the beginning of the dark period (End-of-Day-FR (EOD-FR) treatment) results in longer hypocotyls relative to no EOD-FR treatment and that this effect is attenuated in the *pif*-mutant combinations tested. This result similarly indicates that the PIF quartet members are capable of intrinsically promoting hypocotyl cell elongation in light-grown plants, independently of the effects of PIF feedback modulation of photoactivated-phyB abundance.

Key words: Light regulation; light signaling; genetics; molecular biology; transcriptional control and transcription factors; photomorphogenesis.

INTRODUCTION

Plants monitor and respond to informational light signals from the environment using a set of sensory photoreceptors that include the phytochrome (phy) family (phyA to phyE in *Arabidopsis*) (Rockwell et al., 2006; Schafer and Nagy, 2006;

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Franklin and Quail, 2010; Quail, 2010). The phys track the relative levels of incident red (R) and far-red (FR) light by virtue of a capacity to switch reversibly between the biologically inactive Pr and active Pfr, conformers of the molecule, upon sequential absorption of R and FR photons. In dark-germinated seedlings, the inaugural conversion of Pr to Pfr upon initial exposure to light induces the familiar deetiolation process. In fully deetiolated, light-grown seedlings, exposure to vegetative shade imposes a variable reduction (but not abolition) of Pfr in the cell, because of the depletion of R, but not FR, photons (a reduction in R/FR ratio) from radiation filtered through, or reflected from, neighboring vegetation. This color-change-imposed reduction in Pfr levels induces the Shade-Avoidance Syndrome (SAS) in affected seedlings, displayed as accelerated extension-growth rates in hypocotyls, internodes, and petioles, retarded expansion rates in cotyledons, and retarded chloroplast development (Child and Smith, 1987; Smith and Whitelam, 1997; Franklin, 2008; Ballare, 2009; Ruberti et al., 2011). Under diurnal, light–dark cycles, the level of Pfr established at the end of the light period persists in the subsequent darkness, continuing to exert its regulatory activity for a defined period. Experimentally, a pulse of FR light, administered at the termination of the light period, a so-called End-of-Day-Far-Red (EOD-FR) treatment, that effectively removes this residual Pfr from the cell for the duration of the dark period is frequently used to examine this activity (Smith and Whitelam, 1997; Franklin, 2008; Franklin and Quail, 2010).

Current evidence indicates that the intracellular pathway by which the photoreceptor transduces these light signals involves translocation of the photoactivated phy molecule from the cytoplasm into the nucleus (Nagatani, 2004), where it induces changes in gene expression as a result of direct, physical interaction with members of a subfamily of basic helix-loop-helix (bHLH) transcription factors, called Phytochrome-Interacting-Factors (PIFs) (Castillon et al., 2007; Jiao et al., 2007; Bae and Choi, 2008; Leivar and Quail, 2011). The data indicate that the signal-transfer mechanism from the phy to the PIF molecule involves rapid phy-induced phosphorylation of the bHLH factor, which, in turn, triggers degradation of this factor via the ubiquitin–proteasome system (Bauer et al., 2004; Park et al., 2004; Shen et al., 2005; Al-Sady et al., 2006; Oh et al., 2006; Nozue et al., 2007; Shen et al., 2007; Al-Sady et al., 2008; Lorrain et al., 2008; Shen et al., 2008).

Genetic studies, using loss-of-function mutations in four of the PIFs (PIF1, PIF3, PIF4, and PIF5, designated here as the PIF quartet), have provided compelling evidence that these factors function with overlapping redundancy in dark-grown seedlings, to promote skotomorphogenesis, and that initial exposure to light induces deetiolation (the transition from skotomorphogenic to photomorphogenic development) as a consequence of the rapid, phy-triggered PIF degradation (Leivar et al., 2008a). Transcriptome analysis of the partially constitutively photomorphogenic *pif1pif3pif4pif5* quadruple (*pifq*) mutant has identified the genes genome-wide that are regulated by these PIFs under phy control (Leivar et al., 2009; Shin et al., 2009), and has defined a subset, enriched in transcription-factor-encoding loci, that respond rapidly (within 1 h) to the initial light signal (Leivar et al., 2009). These rapidly light-responsive genes are thus considered candidates for being components of the primary transcriptional network targeted through the phy–PIF system.

Definition of the biological function(s) of the PIFs in fully deetiolated, green plants has been somewhat more complicated. Monogenic and higher-order *pif* mutants display light-hypersensitive seedling-phenotypes (shorter hypocotyls and larger cotyledons than wild-type (WT)) when grown under constant, prolonged R, FR, or white light (WL) irradiation (Huq and Quail, 2002; Kim et al., 2003; Fujimori et al., 2004; Huq et al., 2004; Monte et al., 2004; Oh et al., 2004; Khanna et al., 2007; Leivar et al., 2008b; Lorrain et al., 2008, 2009). This observation has been taken to indicate that the PIFs function as negative regulators of photomorphogenesis (Duek and Fankhauser, 2005; Castillon et al., 2007; Bae and Choi, 2008). Conversely, *pif4*, *pif5*, and *pif4pif5*-double mutants have reduced responsiveness to simulated shade (reduced hypocotyl elongation and marker-gene responsiveness compared with WT), and PIF4- and PIF5-overexpressors have the opposite phenotype (approaching constitutively long hypocotyls and petioles, and high marker-gene expression) (Lorrain et al., 2008). The mechanism by which these PIF activities might be exerted, in both light and shade, could in principle simply be by partial retention of the intrinsic skotomorphogenic-promotive activity of these factors. Consistent with this possibility, the evidence indicates that the reduction in PIF levels induced in light-grown WT seedlings does not completely abolish these proteins from the cell, but rather establishes a new, lower steady-state level than was present in darkness (Monte et al., 2004). Similarly, the abundance of the PIF4 and PIF5 proteins increases rapidly in white-light-grown WT seedlings upon their transfer to simulated shade, consistent with a function in promoting hypocotyl elongation (Lorrain et al., 2008). An alternative mechanism might involve feedback inhibition of the photomorphogenic-promotive activity of the phy molecule. Consistent with this possibility, the genetically imposed absence of the PIFs has been found to result in higher levels of phyB in the light, thus enhancing the photosensory, and thereby the photomorphogenic-inducing, capacity of the photoreceptor in the *pif* mutants compared with the WT (Khanna et al., 2007; Monte et al., 2007; Al-Sady et al., 2008; Leivar et al., 2008b). These data have thus been interpreted to indicate the existence of a mutually negative-feedback loop between the phyB and PIF proteins (Leivar and Quail, 2011) and there is evidence that PIF-induced phyB degradation is mediated via the ubiquitin–proteasome system using COP1 as an E3 ligase (Jang et al., 2010). The elevated phyB levels observed in the *pif* mutants have the capacity both to impose the observed light-hypersensitivity and to attenuate the extent of the shade response in these mutants, as has been demonstrated for seedlings engineered to overexpress phyB