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SHORT COMMUNICATION

PHYTOCHROME INTERACTING FACTOR1 interactions leading to the completion or prolongation of seed germination

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ABSTRACT

In *Arabidopsis thaliana*, the basic Helix Loop Helix transcription factor, PHYTOCHROME INTERACTING FACTOR1 (PIF1) is known to orchestrate the seed transcriptome such that, ultimately, proteins repressing the completion of germination are produced in darkness. While PIF1-mediated control of abscisic acid (ABA) and gibberellic acid (GA) anabolism/catabolism is indirect, PIF1 action favors ABA while discriminating against GA, firmly establishing ABA's repressive influence on the completion of germination. The result is tissue that is more sensitive to and producing more ABA; and is less responsive to and deficient in GA. Illumination of the appropriate wavelength activates phytochrome which enters the nucleus, and binds to PIF1, initiating PIF1's phosphorylation by diverse kinases, subsequent polyubiquitination, and hydrolysis. One mechanism by which phosphorylated PIF1 is eliminated from the cells of the seed upon illumination involves an F-BOX protein, COLD TEMPERATURE GERMINATING10 (CTG10). Discovered in an unbiased screen of activation tagged lines hastening the completion of seed germination at 10°C, one indirect consequence of CTG10 action in reducing PIF1 titer, should be to enhance the transcription of genes whose products work to increase bioactive GA titer, shifting the intracellular milieu from one that is repressive to, toward one conducive to, the completion of seed germination. We have tested this hypothesis using a variety of Arabidopsis lines altered in CTG10 amounts. Here we demonstrate using bimolecular fluorescence complementation that PIF1 interacts with CTG10 and show that, in light exposed seeds, PIF1 is more persistent in *ctg10* relative to WT seeds while it is less stable in seeds over-expressing CTG10. These results are congruent with the relative transcript abundance from three genes whose products are involved in bioactive GA accumulation. We put forth a model of how PIF1 interactions in imbibed seeds change during germination and how a permissive light signal influences these changes, leading to the completion of germination of these positively photoblastic propagules.

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Introduction

The capacity of the hydrated seed to monitor its external environment is largely dedicated to evaluating conditions favorable for seedling establishment. One such environmental cue for either positively- or negatively-photoblastic seeds is light[1], exerting its influence, depending on the seed, through the phytochrome and/or cryptochrome photosensory systems[2,3]. Once an orthodox seed[4] takes up water (imbibes) it continues germinating until some portion of the embryo protrudes visibly from the covers, at which point germination is complete and seedling establishment commences[5]. Upon the perception of light quality and quantity through phytochrome[6,7], the signal transduction pathway orchestrating the completion, or prolongation, of germination is elaborate, ultimately impinging both on the relative abundance of, and sensitivity to, abscisic- and gibberellic-acid (ABA and GA[8]), the final arbiters of whether and when the seed completes germination[9]. PHYTOCHROME INTERACTING FACTOR1 (PIF1; At2g20180) is a light labile, basic Helix-Loop-Helix (bHLH) transcription factor[10] responsible for deploying a transcriptome whose products are antagonistic to the completion of seed germination[11]. Although PIF1 does not interact with the genes encoding the enzymes for active ABA- or GA-production or degradation directly[11], it still influences their expression[12]. Consequently, alterations influencing PIF1 bHLH dimerization[13], DNA occupancy[14], and/or stability[6,15] all impact transcript abundance of genes encoding enzymes that produce, or metabolize, bioactive GA and/or ABA.

One of two known pathways leading to PIF1 degradation involves an E3 ligase complex employing the COLD TEMPERATURE GERMINATING10 F-Box protein (At4G19330; DQ666277) for phospho-PIF1 recruitment, a process enhancing the completion of Arabidopsis seed germination[6,16,17]. We tested the PIF1-CTG10 interaction *in planta* with bimolecular fluorescence complementation (BiFC) and the consequences of such an interaction on PIF1 abundance using western blots of genotypes with reduced or enhanced expression of *CTG10*. The seed from those same plants were used to examine the influence such alterations had on transcript abundance, as measured relative to the 18S

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ribosomal RNAs (AT2G01010 and AT3G41768), of two genes encoding enzymes responsible for active GA production (GA3OX1, AT1G15550; GA3OX2, AT1G80340) and one gene whose product reduces active GA in the seed (GA2OX2, AT1G30040). We present a model to elucidate the multiplicity of molecular mechanisms involved in altering the titer of PIF1 during seed germination and its DNA occupancy.

Results

Initial isolation of CTG10, involved in the completion of germination, was the result of an unbiased screen of an activation-tagged population of seeds for the early completion of germination at 10°C (Supplemental data 1 movie;[16]). The screen was designed to eliminate seed dormancy as an influencing factor on the time to completion of germination as Arabidopsis seeds can alleviate dormancy at 10°C. From the sensitivities of *CTG10-OE* seeds to exogenous GA after paclobutrazol application and, of the *ctg10* knockdown mutant to paclobutrazol treatment[17], the transcription factor, PIF1, was tested as a potential interacting partner given its overarching effect on the completion of germination and known, though indirect, effects on hormonal balance in the seed.

The Sonchus Yellow Net virus (SYNV) nucleocapsid- (N) and phosphor- (P) proteins are known to interact in the plant nucleus[18] and acted as positive controls for *in planta*, split YFP interactions between CTG10 and PIF1 (Figure 1A). Cells bombarded with nano-gold particles bearing plasmid DNA encoding the various protein pairs were identified through fluorescence caused by a third, co-bombarded plasmid encoding a nuclear localized Ds-RED (NLS-Ds-Red) or a Microtubule Associated Protein 65 also fused to Ds-Red (MAP65-Ds-Red). Co-bombardment of N- and P-proteins fused with the carboxy- or amino-terminal halves of YFP, respectively, re-associated a functional fluorescent protein, resident in the nucleus (Figure 1A, i). When CTG10, fused to the amino terminal half of YFP, was introduced into tobacco cells along with a non-binding, SYNV-N partner protein containing the carboxy-terminal half of YFP, no detectable YFP signal was observed (Figure 1A, ii). However, upon co-bombardment of CTG10-Y and PIF1-FP plasmids, functional fluorescent protein was again established in the nucleus (Figure 1A, iii).

An *in vivo* association of the F-Box protein, CTG10 with PIF1 in the nucleus does not necessarily dictate that PIF1 steady state amounts will subsequently be reduced. To demonstrate a negative correlation between CTG10 and PIF1 titers, western blot analysis of seeds from a variety of genotypes, imbibed in the light for 30 min (which is required for germination of Arabidopsis seeds to progress to its completion in subsequent darkness), followed by 24 h darkness before brief illumination for 10 min, determined that PIF1 was not present in *pif1* seeds, was rapidly eliminated in *CTG10-OE* seeds, and was in greater abundance in *PIF1-OE* seeds. PIF1 protein was stabilized in seeds of the *ctg10* mutant relative to the WT (Figure 1B, C).

Alterations of PIF1 titer through direct modification of *PIF1* gene integrity (*pif1*) or ectopic over-expression (*PIF1-*

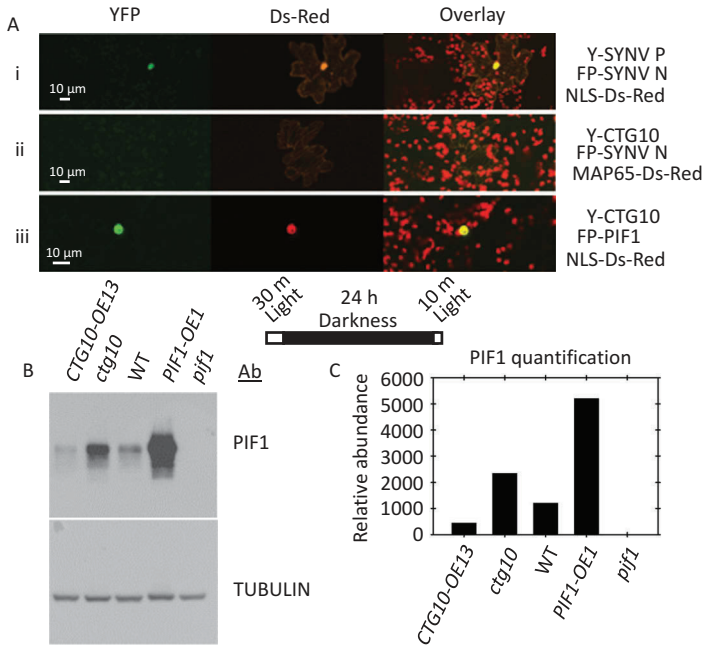

Figure 1. Bi-molecular fluorescence complementation assays determined that PIF1 and CTG10 interact in the plant nucleus with consequences for the stability of PIF1. A) The Sonchus Yellow Net virus (SYNV) nucleocapsid- (N) and phosphor- (P) proteins are known to interact in the plant nucleus and were used as positive controls for *in planta*, split YFP interactions between Y-CTG10 and FP-PIF1 fusion proteins. Co-bombarded Y-SYNV-P and FP-SYNV-N plasmids in tobacco leaf disks (*Nicotiana tabacum* cv KY160) reformed an active YFP localized to the nucleus (i) but Y-CTG10, FP-SYNV-N plasmids did not (ii). Co-bombarded Y-CTG10 and FP-PIF1 also reformed an active fluorescent protein in the plant nucleus (iii). YFP: YFP confocal channel; Ds-RED: Channel for Ds-RED; overlay: overlay of signal from both channels. The scale bars are 10 µm. B) PIF1 protein accumulation. Seeds from a variety of genotypes were imbibed in light for 30 min before being subjected to darkness for 24 h. At the end of this time, seeds were exposed for 10 min to light prior to extracting total protein from them. Protein was quantified, and equal amounts run on SDS-PAGE gels, transferred to membrane, and assayed using PIF1 antibody followed by TUBULIN Ab. C) Signal intensities (from panel 1B) were quantified and are presented relative to TUBULIN amounts. WT: wild type; *CTG10-OE13*: line 13 CTG10 over-expressing line; *ctg10*: CTG10 insertional mutant; *PIF1-OE1*: PIF1 over-expressing line; *pif1*: PIF1 insertional mutant. Ab: antibody.

OE) result in changes in the transcript abundance from genes encoding proteins involved in metabolizing bioactive GA (Figure 2A). Similarly, PIF1 titer alterations through manipulation of CTG10 amounts through mutation (*ctg10*) or *CTG10* over-expression also resulted in adjustments in transcript abundance for genes encoding proteins involved in GA metabolism. Reverse transcribed mRNA from seeds of a variety of genotypes was subjected to PCR. The abundance of the resulting amplicons, relative to the 18S ribosomal RNAs, showed that transcript encoding enzymes involved in bioactive GA production (GA3OX1 and GA3OX2) were up-regulated, relative to WT and VC, in *CTG10-OE* lines, and the *pif1* mutant. The *GA2OX2* transcript, encoding an enzyme responsible for catabolizing bioactive GA to inactive GA, was down-regulated, relative to WT in these same lines. Conversely, the *PIF1-OE* line and the *ctg10* mutant, relative to WT and the empty vector control seeds (VC), had transcript abundances encoding enzymes synthesizing bioactive GA that were less than WT and VC, while the opposite was true of the transcript encoding the GA catabolizing enzyme.

A prior report has investigated the repression of *CTG10* transcription by PIF1, presumably in heterologous association with another bHLH transcription factor which would allow PIF1 to bind any of 10 E-boxes found in the *CTG10* promoter[6]. The repressive effects of PIF1 on *CTG10* transcription are predicated on both PIF1 and its associating bHLH protein partner presence in the nucleus. We have presented a model of the various mechanisms known to control PIF1 titer and DNA occupancy in the germinating seed (Figure 3).

Discussion

The results from both BiFC assays and western blot of proteins from various genotypes of afterripened Arabidopsis seed after 24 HAI in darkness exposed to 10 min white light, are consistent with the proposition that, following illumination, the CTG10 F-BOX interacts with PIF1 to destabilize PIF1 leading to its decline in the seed (Figure 1). The consequences of this reduction in PIF1 titer includes an up-regulation of *GA3OX1* and *GA3OX2* transcript amounts, the products from which increase bioactive

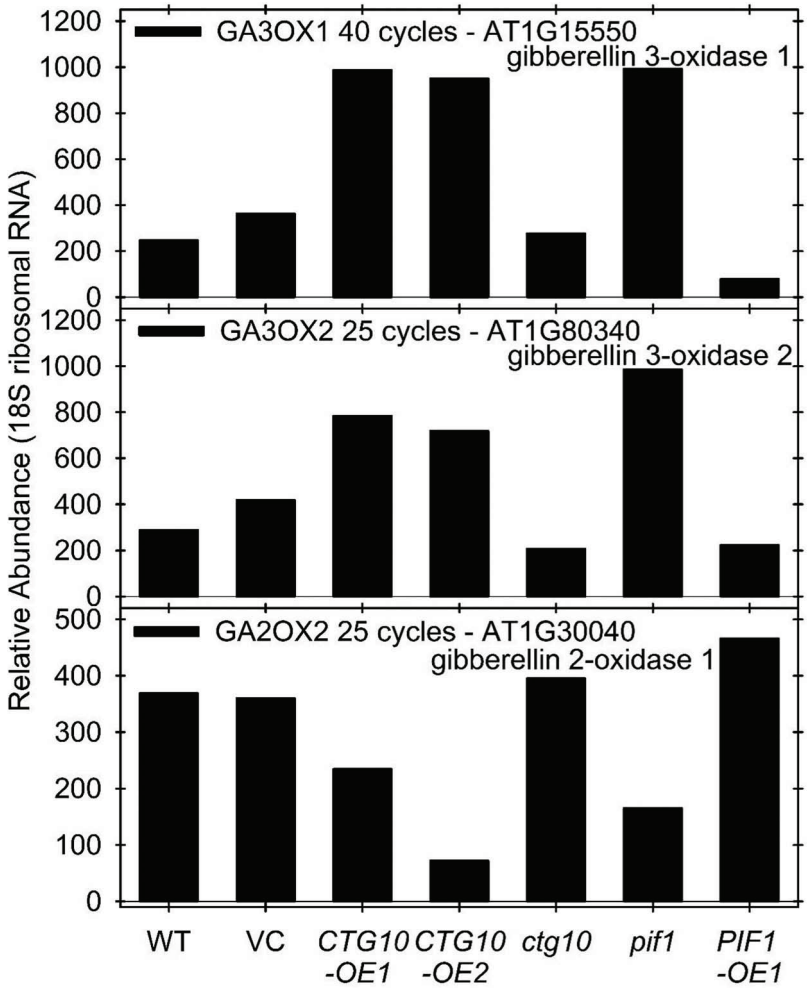

Figure 2. Alterations of PIF1 titer and CTG10 steady amounts (which affects PIF1 titer) result in changes in transcript abundance from genes encoding proteins involved in metabolizing GA. Reverse transcribed mRNA from seeds of a variety of genotypes were subjected to PCR. The abundance of the resulting amplicons, relative to the 18S ribosomal RNA, shows that transcript encoding enzymes involved in bioactive GA production (*GA3OX1* and *GA3OX2*) are up-regulated, relative to WT and VC, in *CTG10-OE* lines, and the *pif1* mutant. The *GA2OX2* transcript encoding an enzyme responsible for catabolizing bioactive GA to inactive GA was down-regulated, relative to WT in these same lines. Conversely, the *PIF1-OE* line and the *ctg10* mutant, relative to WT and VC, had transcript abundances encoding enzymes synthesizing bioactive GA that were less than WT and VC, while the opposite was true of the transcript encoding the GA catabolizing enzyme. WT: wild type; VC: empty vector control; otherwise as in Figure 1C.

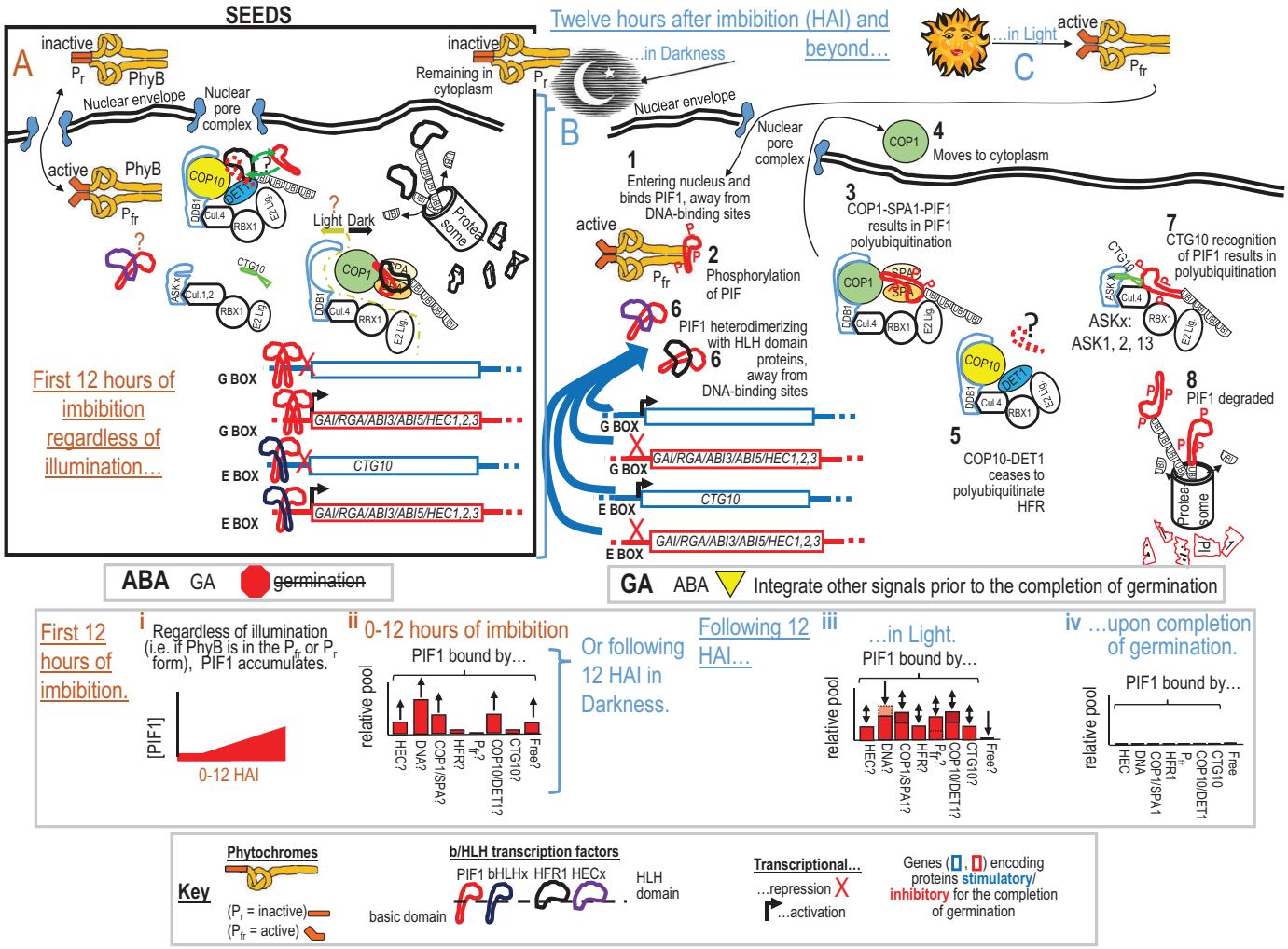

Figure 3. A model of the light-determined imposition and removal of PIF1 control of the completion of seed germination. A) During the first 12 hours after imbibition (HAI; brown text), PIF1 titer increases, regardless of illumination (left side of figure and i[6]). This is despite the presence of PhyB at least, and its capacity to activate and enter the nucleus in illuminated seeds[7]. ii.) The relative amounts of total PIF1 titer in the nucleus has been partitioned based on its association with other nuclear-localized molecules. The graphs (i, ii, iii and iv) are not suggesting actual ratios of PIF1 but hypothesizing relative pools based on published accounts (question marks indicate this uncertainty).

Rising PIF1 titer may saturate DNA binding requirements as well as requirements for E3 ubiquitin ligase complexes (see below) potentially resulting in some free PIF1 (upward arrows in "ii"). Following 12 HAI (blue text), in darkness (B), inactive phytochrome remains localized to the cytoplasm. PIF1 occupies G-boxes in promoters as homodimers or E-boxes as heterodimers with other bHLH proteins (bHLHx). PIF1, unbound to DNA cognate sites, is also present, some of which, in 4 d dark-grown seedlings, is in association with the COP1/SPA1 complex[15], some with the COP10/DET1 complex[34], and some of which is free. Based on PIF1 stability in seeds and the concomitant seed germination phenotypes reported for mutants of *cop1, spaQ*[15], and the PIF1 destabilization and seed germination phenotypes of *cop10* and *det1*[34], it is logical to assume that PIF1 is also bound by these ubiquitination complexes in germinating seeds. HFR1 is recognized and polyubiquitinated by the PIF1-associated COP1/SPA1 complex[15,42]. Thus, HFR1, with an affinity for PIF1, binds little PIF1 at this time ("ii") due to HFR1's 26S proteasomal turnover. In darkness, the resulting transcriptome encodes proteins inhibitory for the completion of germination and requires an environmental signal (light) to proceed to the completion of germination.

Upon illumination (C; right side of the figure), phytochrome is activated and 1) enters the nucleus, recruiting PIF1 away from its cognate DNA binding sites (blue arrows). This results in the 2) phosphorylation of Phy-bound PIF1. 3) The COP1-SPA-PIF1 complex now docks with the larger CUL4 ubiquitin ligase assemblage and PIF1 is polyubiquitinated. 4) Nuclear localized COP1 participating in the polyubiquitination of PIF1 commences leaving the nucleus due to the light signal, potentially curtailing its influence on PIF1 polyubiquitination. 5) The COP10-DET1-PIF1 complex, hitherto polyubiquitinating HFR1, somehow ceases this activity, potentially through loss of PIF1 to $P_{(fr)}$, phosphorylation of PIF1, or disruption of the COP10-DET1 complex. This uncertainty is emphasized with a question mark over a dotted silhouette of a PIF1 protein removed from the complex. 6) Illumination increases the nuclear titer of HFR1 (through its stabilization) and that of the HEC proteins, which, along with activated Phy, also sequester PIF1 away from its DNA binding sites (blue arrows). Whether this sequestration to HFR1 and HECs occurs after PIF1 phosphorylation is currently unknown. 7) PIF1 is bound by the CTG10 F-BOX protein and, in association with an SCF-complex, is polyubiquitinated. 8) Polyubiquitinated PIF1 is degraded through the 26S proteasome. The hypothetical amounts of PIF1 bound within the illuminated cells of the seed iii) dynamically switch from free, DNA-, and E3 complex-bound; to $Phy_{(fr)}$, HEC, HFR1 sequestered; potentially to $Phy_{(fr)}$ from HEC and HFR1 if they sequester un-phosphorylated PIF1; to E3 complexes; and finally to the 26S proteasome. This dynamism is reflected in the decline in DNA-bound and free PIF1; double headed arrows showing transient increases in HEC-, HFR1-bound PIF1 and differentially shaded bars as PIF1 shuttles to/from P_{fr} to E3 ligase complexes. Ultimately, the capacity of activated Phy to acquire and phosphorylate PIF1 and the affinity of the E3 ligases for phosphorylated PIF1 leads to the polyubiquitination of the entire PIF1 pool which is now 8) degraded by the 26S proteasome to the point where PIF1 is no longer detectable (iv) upon the completion of germination. The "stop sign" plus the text "~~germination~~" and the "yield sign" plus the text "Integrate other signals prior to the completion of germination" are in place together with the relative abundance and sensitivities (as the size of the font and boldness) of the seed to ABA and GA to portray the overall effect of the hormones and signaling that results from the changes shown in PIF1 abundance.

GA, and a down-regulation of the transcript amounts for the GA-metabolizing enzyme encoded by *GA2OX2* (Figure 2). PIF1 amounts in the seed have been shown to be correlated with the amount of transcript produced from these genes in previous reports[19]. There is a parity of phenotypes (transcript abundance among *GA3OX1, GA3OX2*, and *GA2OX2*), between lines hyper- and hypo-accumulating PIF1, relative to WT and empty vector control seeds. Hence, *CTG10* over-expressing lines exhibit transcript amounts encoding GA synthesizing enzymes (*GA3OX1, GA3OX2*) similar to that of the *pif1* mutant while, conversely, the *ctg10* mutant most closely resembles the *PIF1* over-expressing line regarding the amount of these transcripts (Figure 2). The same is true for the PIF1-indirectly-up-regulated transcript, *GA2OX2* (Figure 2). Although PIF1 influence over the transcription of these genes is indirect[11], the consequences of PIF1 reduction (*CTG10-OE, pif1*) or retention (*ctg10, PIF1-OE*) relative to WT (viz. alterations in the transcript abundance of *GA3OX1, GA3OX2* and *GA2OX2*) is entirely consistent with our hypothesis that CTG10 assists in the polyubiquitination of PIF1 leading to its removal by the 26S proteasome (Figure 2, 3).

A previously reported mechanism to degrade PIF1 in response to light[15], as well as multiple means to sequester PIF1 away from its target promoter sites following photoperception[13,14,20], is a testament to the critical nature of the light cues the seed must integrate to make the irrevocable commitment to complete germination. Such a redundancy would be, in hindsight, an expectation given that PIF1 amounts are seemingly at the crux of controlling light-mediated completion of germination (Figure 3).

To summarize our understanding of the control light exerts over the completion of germination in Arabidopsis upon rehydration, we present a model of factors influencing PIF1 abundance and associations during seed germination that impact its residence on its cognate gDNA binding sites (Figure 3).

First hours after imbibition (HAI): setting up a de-repressible system

The hormone balance theory of seed germination/dormancy[21,22] proposes that the abundance of, and sensitivity to, ABA and GA in the seed tissues ultimately controls whether the seed completes germination or not. The initial response of the seed upon taking up water (i.e. during imbibition; phase I of seed germination[5]) is to ensure the proteome is inhibitory to the completion of the process, in part through decreasing the amount of, and sensitivity to, bioactive GA[23] setting up a de-repressible system[24]. A second mechanism by which this is accomplished in Arabidopsis is through the continuous generation of ABA (by the endosperm transported to the embryo[25,26]) while dampening its conversion to inactive metabolites[27,28] which is sustained under conditions unfavorable for seedling establishment. With progression toward radicle protrusion blocked, the seed is now forced to integrate light conditions, along with the myriad of other signals, to determine if all indicators point to an environment conducive to establishing an autotrophic seedling. If so, then, and only then, does the seed begin to remove these impediments to the completion of germination overhauling the proteome to a state stimulatory to the completion of germination. This constitutes a system in which the progression from a seed (most impervious-) to a seedling (most susceptible-to detrimental conditions) is automatically prevented initially. This active and automatic imposition of hinders to the completion of germination includes an increase in abundance of PIF1 during the first 12 HAI, regardless of illumination (a default[6], Figure 3,"A"). PIF1 transcript and protein are present in the stored transcriptome[29] and proteome[6], respectively and PIF1 protein amounts are augmented over the first 12 HAI[6]. Despite the fact that Phy B, at least, is present, functional, and capable of nuclear import in the Arabidopsis seed during this time[7], it does not prevent the rise in PIF1 titer, even in constant light. This increase in PIF1 titer from 0–12 HAI is depicted as a rising PIF1 concentration (Figure 3 "i") and as red bars representing presumptive PIF1 distribution in the cell (Figure 3 "ii"). How PIF1 accumulation from 0–12 HAI in an illuminated seed might be orchestrated is unclear but may be due to PIF1 production outstripping the Ubiquitin-Proteasome System (UPS) re-activation following the early hours after imbibition[30]. The amount of ABA in the dry seed is high and requires some hours after imbibition to decline, even under the most favorable conditions for seedling establishment[31]. ABA dampens both protein ubiquitination and proteasome activity in the imbibed seed[30]. Due to the large assemblage of proteins required for UPS function, absences of a single component may render it ineffective. For example, although CTG10 F-BOX protein is abundant in the dry seed[6], the ASK13 protein, one to which CTG10 F-BOX binds to polyubiquitinate PIF1[17], increases in abundance during germination and, when over-expressed, can hasten the completion of germination[32] suggesting that the lack of components of the UPS may be a bottleneck early during germination.

There is sufficient PIF1 at the end of 12 HAI to exert a repressive influence on the completion of germination through DNA binding resulting in direct upregulation of some *DELLA* genes[11], the proteins of which repress GA sensitivity, and indirectly by down regulating transcription leading to enhanced bioactive GA amounts[23]. The DELLA genes *RGA* and *GAI* have a promoter containing both G- and E-boxes to which PIF1 binds[11] and so have been placed under the positive transcriptional influence of both PIF1 homo- and hetero-dimers (Figure 3). PIF1 can indirectly both increase ABA biosynthesis and inhibit its catabolism[11]. Simultaneously, PIF1 directly binds and upregulates transcription of both *ABI3* (AT3G24650) and *ABI5* (AT2G36270), the products from which boost the sensitivity of the seed to ABA[12,33]. Other than altering transcription, PIF1 is recruited into at least two forms of ubiquitination machinery[15,34] where, in the COP1-SPA assemblage at least, it assists recognition, binding, and destruction of LONG HYPOCOTYL IN FAR RED1 (HFR1 is stimulatory to the completion of germination[13]). Apparently, the ubiquitination of HFR1 by COP1-SPA-PIF1 in darkness occurs without recruitment of this assemblage into the larger CUL4 complex[15] an observation described for etiolated seedlings which may also function during the first 12 HAI in seeds. In Figure 3, there has been a gap introduced between COP1 and DDB1 to reflect this uncertainty and a question mark placed over "Light". The second E3 ligase complex recruiting PIF1 (COP10-DET1) stabilizes PIF1, potentially providing another means by which PIF1 can accumulate in seeds on water up to 12 HAI regardless of illumination. The same

complex also targets HFR1 for polyubiquitination[34]. Because PIF1 and HFR1 are known to interact, one intriguing question is the nature of PIF1/HFR1 recognition and binding by COP10-DET1 (Figure 3 question mark between free PIF1 and the COP10-DET1 complex). Do the PIF1/HFR1 proteins compete for binding (Figure 3 double headed green arrow at this complex) which appears likely as both are bound by the same region on DET1 at least[34]. Or can PIF1/HFR1 proteins interact with COP10-DET1 concurrently (possibly through COP10 or PIF1/HFR1 affinity) resulting in simultaneous PIF1 stabilization and the introduction of HFR1 to the E3 ligase for polyubiquitination (Figure 3 green arrow from free PIF1 to the silhouette of PIF1 bound by the complex)? Both PIF1 and HFR1 have been shown to physically interact with COP10 and DET1[34] and with each other[13].

A second small family of HLH proteins, HECATE (HEC) are somewhat stable in darkness and known to bind PIF1, sequestering PIF1 away from its DNA binding sites[20] (Figure 3. Whether any HECs are active during the first 12 HAI or not (question mark over this complex in Figure 3, at 0 −12 HAI) is currently unknown but the amount of PIF1 bound by this group has, nevertheless, been depicted as increasing from 0–12 HAI (Figure 3,"ii"). Additionally, PIF1 recruitment into the COP1-SPA complex also stimulates the destruction of HFR1 and other proteins stimulatory to the completion of germination (Figure 3). Now the imbibed seed is ready to sample the environment and is capable of orchestrating appropriate changes to its metabolism based on this information.

Positively photoblastic seed germination

In darkness, the amount of PIF1 in the seed cells is maintained at a concentration such that repression of the completion of germination, imposed upon initial imbibition, is maintained during the lag phase of germination, however long this may be,[5] (Figure 3,"B"). The blue brackets encompassing the seed cell and PIF1 distribution during the first 12 HAI (Figure 3,"ii") suggests that, 12 HAI in darkness the cellular PIF1 titer has probably plateaued and remains largely unchanged. Upon appropriate illumination Phy is activated (Figure 3,"C"), enters the nucleus, initiating a cascade of events that progressively removes the negative influence PIF1 exerts over the completion of germination. Depending on the quality of impinging light, cytoplasmically localized Phy is activated and enters the nucleus (Figure 3,"1"). PIF1, even those proteins associated with DNA at the time, can be sequestered away from their DNA targets by active Phy[14] and, while bound to Phy, undergo phosphorylation by a variety of kinases[35,36,](Figure 3,"2"). The transition from darkness to light also promotes the association of COP1-SPA-PIF1 (where PIF1 has been acting as a co-factor to recognize such proteins as HFR1) with a larger complex comprised of DDB1-CUL4-RBX-E2 whereupon PIF1 now becomes a polyubiquitination target 15,(Figure 3,"3"). PIF1 polyubiquitination by this complex may be progressively attenuated as COP1 exits the nucleus due to illumination although the relative time scales of PIF1 degradation in seeds and COP1 nuclear egress[37] in seeds are unknown (Figure 3 "4"). Substrate proteins, hitherto polyubiquitinated by the COP1-SPA-PIF1 or the DDB1-COP10-DET1 (Figure 3 "5") complexes, are now stabilized and one of these target proteins, HFR1, is a HLH protein that, along with members of the HEC family proteins (the stability of which also improves in light), are capable of interacting with PIF1 and sequestering it away from its DNA targets[13,20,](Figure 3,"6"). The F-BOX protein CTG10 also binds and targets PIF1 for polyubiquitination upon illumination (Figure 3,"7"). The dynamics of transfer from DNA-bound and free PIF1, to Phy- and HLH-bound PIF1, and finally to E3 ligase complexes is depicted (Figure 3,"iii"). The onslaught of these various E3 ligases on PIF1 under light regimes conducive to the completion of germination results in the reduction of PIF1 titer (Figure 3,"8"), although this occurs rather more slowly than is seen in seedling de-etiolation[6,15,34]. This decline continues to the point where the amount of PIF1 in the seed falls below the level of detection[6] (Figure 3,"iv") and the radicle protrudes, concluding germination and commencing seedling establishment[5].

Materials and methods

Plant material

Arabidopsis plants (*Arabidopsis thaliana*) (Col 0) were grown in a growth room with constant light (135 μmol•m^{-2}•sec^{-1}) at 25°C unless otherwise stated. In each experiment, seeds from plants grown under the same conditions, planted and harvested at the same time, were compared. Tobacco (*Nicotiana tabacum* cv KY160) plants for Bimolecular Fluorescence Complementation (BiFC) assays were grown aseptically on minimal MS salt[38], 3% (w/v) sucrose, 0.8% (w/v) phytagar plates under 16/8 h light/dark cycles (135 μmol•m^{-2}•sec^{-1}) at constant 25°C and leaves collected from 3- to 4-week-old plants for particle bombardment. The *pif1* (*pil5*) mutant and *PIF1-OE* line were obtained from Prof. Giltsu Choi (KAIST, South Korea) while the *ctg10* mutant line and *CTG10-OE* were acquired as previously described[17].

Bimolecular fluorescence complementation analysis

Bimolecular Fluorescence Complementation (BiFC) was accomplished using transient expression of CTG10 fused with the amino-terminal portion (174 amino acids) of the eYFP reporter (Y:CTG10) and PIF1 fused to the carboxy-terminal moiety (67 amino acids, FP:PIF1) orchestrated through GATEWAY technology into pSITE-BiFC vectors[39]. The Sonchus Yellow Net virus (SYNV) nucleocapsid- (N) and phosphor- (P) proteins are known to interact in the plant nucleus[18] and Y:SYNV-P co-bombarded with FP:SYNV-N acted as a positive, nuclear-localized, technical control. A negative control included Y:CTG10 co-bombarded with the inappropriate, nuclear-localized, FP:SYNV-N. Cells receiving nanogold and their transcriptional/translational capacity were confirmed by including a plasmid constitutively expressing nuclear-localized Ds-Red or the tubulin-targeting MICROTUBULE ASSOCIATED PROTEIN65–1 (MAP65-1:Ds-Red) on the particles for each experiment.

Leaf disk transformation was accomplished using a PDS1000 DuPont Bio/Rad Microprojectile delivery system (BioRad Laboratories, Hercules, CA, USA). Following bombardment, leaf disks were cultured for 36 h on media[40] in darkness. To visualize any eventual interactions, the disks were quickly mounted on slides under very dim light, and

Table 1. Primers used in this project.

Target gene	Primer direction	Primer nucleotide sequence presented 5′→3′	Locus
GA3OX1	F	5′-tggatccCACAAACATCTATCAAATTTAC-3′	AT1G15550
	R	5′-ctctagaCAAATCATATTGCTGAAAT-3′	
GA3OX2	F	5′-tggatccATGAGTTCAACGTTGAGCGATG-3′	AT1G80340
	R	5′-ctctagaAGTTAATTTCTAATAATGGAAAG-3′	
GA2OX2	F	5′-TGACTCGGTTAGAGCAGGAG-3′	AT1G30040
	R	5′-CTTGAACCTCCCGTTAGTCA-3′	
18S rRNA	F	5′-CTCTGCCCGTTGCTCTGATGATTC-3′	AT2G01010 AT3G41768
	R	5′-GAGCGTAGGCTTGCTTTGAGCACT-3′	

Lower case nucleotides are non-binding extraneous 5′ additions. Underlined nucleotides are engineered restriction enzyme sites used for cloning amplicons. F: forward primer; R: Reverse primer.

emission from the various reporter genes and, in some instances, chlorophyll autofluorescence, viewed using an Olympus FV1000 laser-scanning confocal microscope (Olympus America, Inc., Center Valley, PA, U.S.A.).

Protein extraction, western blot preparation and development

Seeds were exposed to white light (135 μmol•m^{-2}•sec^{-1}) for 30 min at 25°C while imbibing on germination blotter (Ahlstrom-Munksjö Filtration LLC, Madisonville, KY, USA) in a Petri dish before the dish was wrapped in 3 layers of aluminum foil for 24 h. The foil was removed under white light (135 μmol•m^{-2}•sec^{-1}) to which the seeds were exposed for 10 min at 25°C prior to protein extraction. Seeds were ground in liquid nitrogen and homogenized in 50 mM Tris-HCl pH 8, 10 mM EDTA, 50 mM NaCl, 0.5% (w/v) SDS and Millipore Sigma plant protease inhibitor cocktail (1:100; St. Louis, MO, USA), including MG132 at a final concentration of 200 μM. The homogenate was centrifuged at 13,000 x g for 10 min at 4°C and the supernatant collected and pipetted into microfuge tubes in 50 μL aliquots which were snap frozen in liquid nitrogen and stored at −20°C until use. Thawed protein extracts were mixed with sample loading buffer (pH 6.8), boiled for 5 min and size fractionated using 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Protran BA85, Whatman International, Ltd., Maidstone, UK) using a transblot buffer (25 mM Tris, 192 mM glycine, and 20% v/v methanol). The blot was then blocked using 5% (w/v) milk powder dissolved in TBST for 1 h with gentle agitation at room temperature before it was probed with PIF1 primary antibody (1:12,000) in blocking solution. The blot was incubated for 1 h with gentle agitation, washed 3 times with TBST for 10 min each time before applying a horse radish peroxidase-conjugated, goat-anti-rabbit secondary antibody (1:25,000, Cat No. 374–1506 KPL Gaithersburg, MD, USA) for 1 h. Following three washes with TBST for 10 min each, blots were exposed to luminol (KPL LumiGLO Reserve™) and imaged using a cooled CCD camera (Azure™ c300; Azure Biosystems, Inc., Dublin, CA, USA). As loading controls, the blots were re-probed using tubulin primary, monoclonal antibodies (1:5000, alpha-tubulin 4a (antibody clone B-5-1-2); Cat. No. T6074 Millipore Sigma) following the same procedure outlined above except using a goat-anti-mouse, HRP-conjugated secondary antibody (Cat No. 374–1806; KPL).

RNA isolation and RT-PCR analysis

Total RNA was extracted from 22 h dark-imbibed seeds according to Wan and Wilkins[41]. Total RNA was reverse transcribed using random hexamers and SuperScript™ RNase H$^-$ reverse transcriptase (Invitrogen part of ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primers for RT-PCR were made to *18S RIBOSOMAL RNA, GA3OX1, GA3OX2* and *GA2OX2* (Table 1) cDNA. The amount of amplicon generated for each transcript of interest after various numbers of cycles (transcript dependent) was normalized to the amount of *18S RIBOSOMAL RNA* obtained after 25 cycles.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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