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Differential Expression of P1F1-Targeted Genes in Various *pif1* and *ctg10* Mutants

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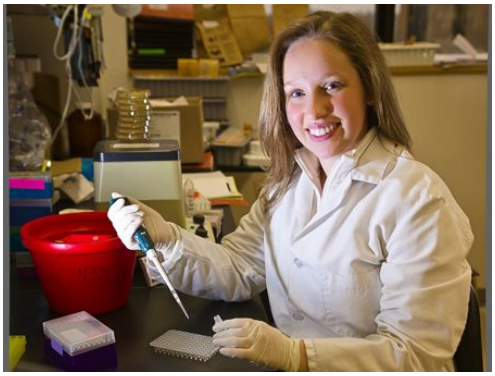

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Abstract:

Successful completion of germination of *Arabidopsis thaliana* seeds is contingent upon the actions of PHYTOCHROME (Phy) and PHYTOCHROME INTERACTING FACTOR 1 (PIF1). Previous studies have shown that PIF1, a basic helix-loop-helix (bHLH) transcription factor, regulates genes through preferential binding to G-box motifs in their regulatory regions. A poly-ubiquitin-mediated degradation pathway has been identified as a regulator of PIF1 amounts. One aspect of this pathway is hypothesized to be the binding of the kelch beta-propeller of the COLD TEMPERATURE GERMINATING 10 (CTG10) F-BOX protein to phosphorylated PIF1 following a PIF1 phosphorylation event caused by the movement of active Phy into the nucleus. The binding of CTG10 leads to the polyubiquitination of PIF1 and its subsequent degradation by way of the 26S proteasome pathway. This experiment investigated the interaction of PIF1 and CTG10 indirectly through a study of the relative expression of PIF1 direct-target genes using quantitative Real Time – Polymerase Chain Reaction (qRT-PCR). Over-expressing and knockdown mutants of *PIF1* along with over-expressing mutants and an RNAi line of *CTG10* were verified as affecting transcript abundance for *PIF1* and *CTG10*, respectively. Additionally, indirect evidence supporting, in some instances, the hypothesized interaction of PIF1 and CTG10 was acquired from two up- and two down-regulated PIF1 direct target gene transcripts using qRT-PCR. The preponderance of these results indirectly corroborate the interaction of the two proteins, PIF1 and CTG10, which can lead to the degradation of PIF1, thus allowing the completion of germination in the presence of light of the seeds from the positively photoblastic model plant.

Introduction:

It has been shown that the movement of PHYTOCHROME (phy) into the nucleus leads to the phosphorylation of the basic Helix-Loop-Helix (bHLH) transcription factor, PHYTOCHROME INTERACTING FACTOR 1. This phosphorylation event targets PIF1 for polyubiquitination and subsequent destruction via the 26S proteasome pathway.

F-BOX proteins have been identified as important components of the E3 Ubiquitin ligase machinery mediating the interaction of target proteins and Arabidopsis Skp1-like proteins (ASKs) proteins in the E3 complex. Unpublished data supports the contention that the kelch beta-propeller-containing F-BOX protein, COLD TEMPERATURE GERMINATING 10 (CTG10), mediates PIF1 polyubiquitination.

The impetus for this project was to further our knowledge of the interaction between PIF1 and CTG10 in the described degradation pathway. The mode of investigation was through the measurement of the differential expression of PIF1-targeted genes in various PIF1 and CTG10 mutants. The goal was to illustrate the effect of varying CTG10 concentration on PIF1 abundance through recording the expression of genes whose transcript abundance is directly regulated by PIF1.

Results and Discussion:

To best illustrate the effect varying concentrations of PIF1 and CTG10 have on direct PIF1 targets, two up-regulated and two down-regulated PIF1-targeted genes were selected from Table 1 in Oh et al. (2009). Transcripts from these genes, along with transcripts from the *PIF1* and *CTG10* genes, were evaluated with quantitative Real Time-PCR (qRT-PCR) in both knockouts and over-expressers of *PIF1* and *CTG10* compared to WT. The C_t values were evaluated using the $\Delta\Delta C_t$ method and analyzed for significance with a student T-test.

Over-expressing mutants inherently increase the transcription of the targeted gene, shown in Figures 1 and 2 with the *PIF1* and *CTG10* concentrations, and the assumption being, that this will, in turn increase the amounts of the protein the transcript encodes. The increase in the amount of protein would potentially provide feedback control from down-stream protein-protein interaction. The same premise in reverse applies to knockout mutants/RNAi lines. The transcript level of the knockout mutants/RNAi lines would be much lower and would potentially release the control of PIF1 on the targeted genes. The hypothesis of this experiment depends on the effect of an increased or decreased amount of PIF1 and CTG10 on the transcription level of *PIF1* regulated genes. *PIF1* and *CTG10* were evaluated to show that qRT-PCR was working and to validate the behavior of the selected mutants. Figures 1 and 2 demonstrate a confirmation of both the qRT and the mutants. Figures 4-7 illustrate the experimental results for the two up-regulated and two down-regulated PIF1-targeted genes.

It is essential to recognize that the measurements taken in qRT-PCR are strictly transcript levels and cannot be mistaken for translated protein (illustrated in Figure 3). The results shown can be logically linked to a feedback mechanism due to protein-protein interaction of PIF1 and CTG10 in some but not all instances.

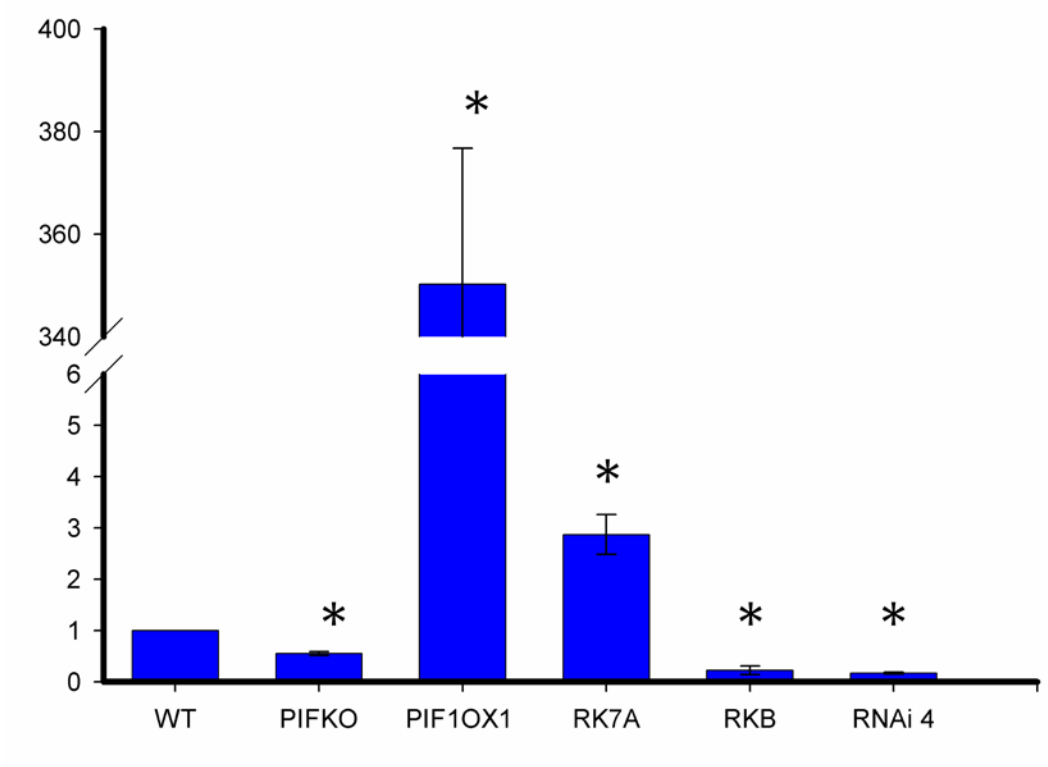

Figure 1: *PIF1* transcript levels
Measurements of *PIF1* transcript were taken to validate both the behavior of the mutants and the qRT-PCR results.

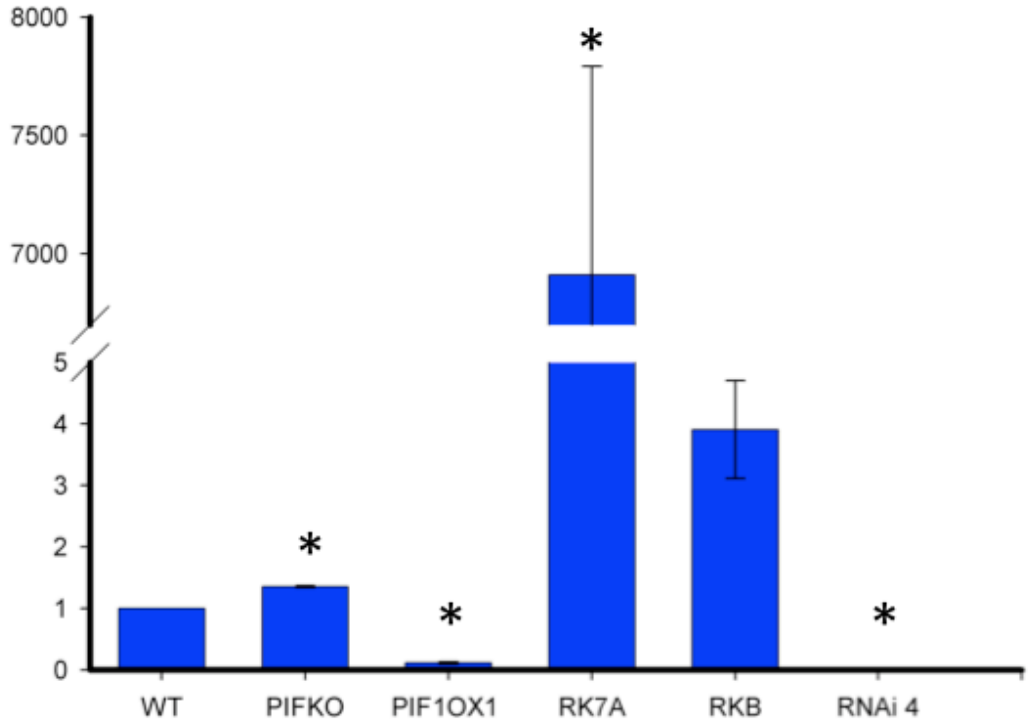

Figure 2A: *CTG10* transcript levels
CTG10 values were also found to validate the qRT-PCR results along with the behavior of the mutants.

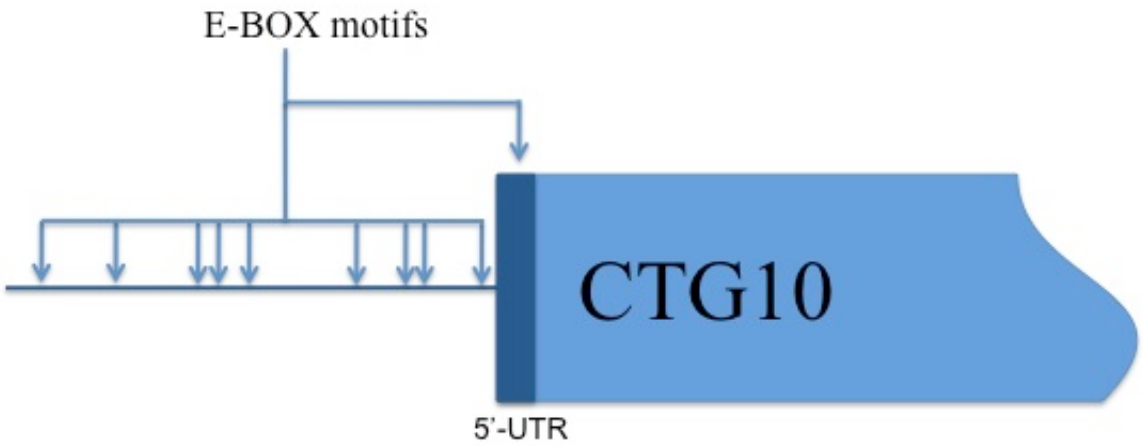

Figure 2B: *CTG10* promoter region
The inverse relationship between the *PIF1* and *CTG10* values for PIFKO and PIF1OX1 may suggest PIF1 regulation of *CTG10* transcription through heterodimerization and binding to any of the ten E-BOX sites in the *CTG10* promoter region[5]. As a homodimer, *PIF1* has been shown to only bind to a sub-class of the E-BOX motif, the G-BOX. Heterodimerization would potentially increase the robustness of the binding affinity such that the heterodimer could bind to the less specific E-BOX.

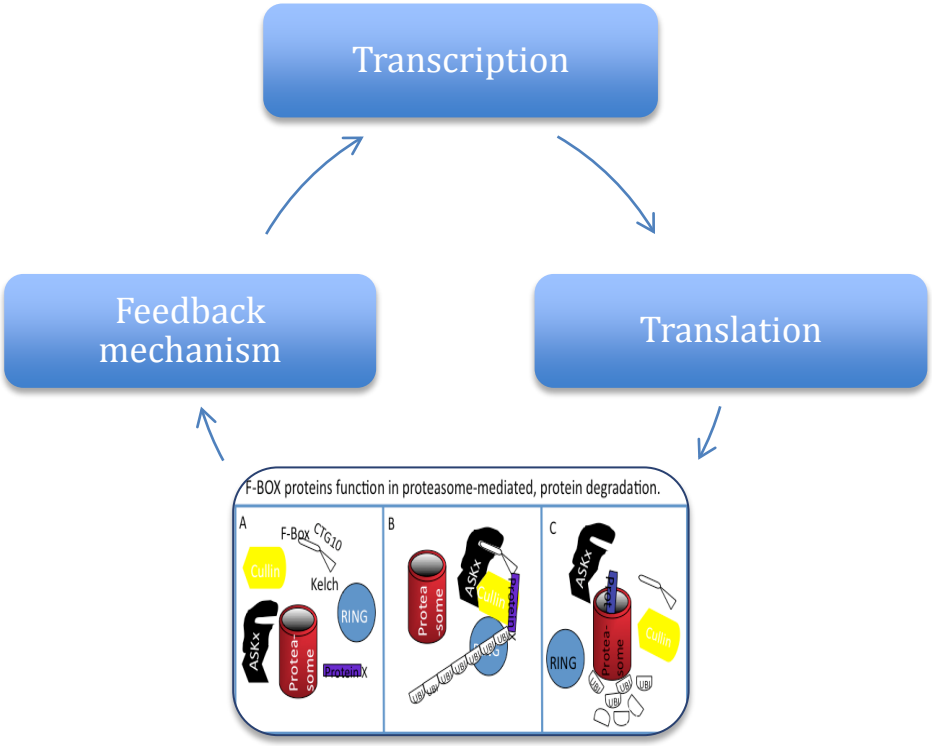

Figure 3: Diagram of cycle required for "visualizing" feedback in qRT-PCR

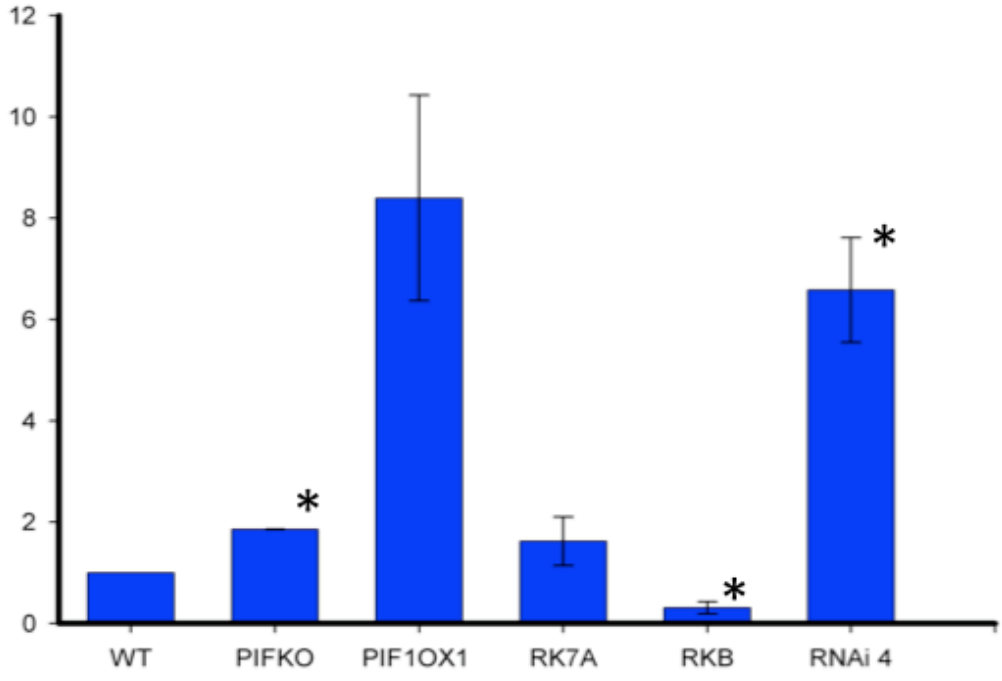

Figure 4: *CYTOKININ OXIDASE 5* (*AtCKX5*) (At1g75450)
Experimental results for the up-regulated gene, *AtCKX5*

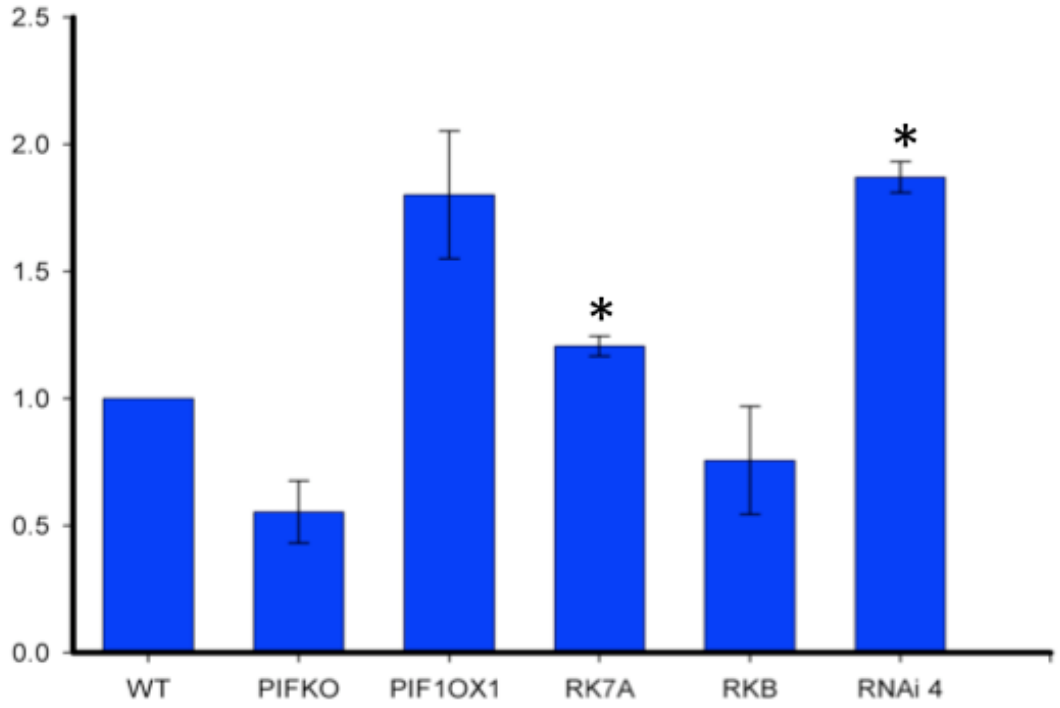

Figure 5: *REPRESSOR OF GA1-3 1* (*RGA*) (At2g01570)
Experimental results for up-regulated gene, *RGA*

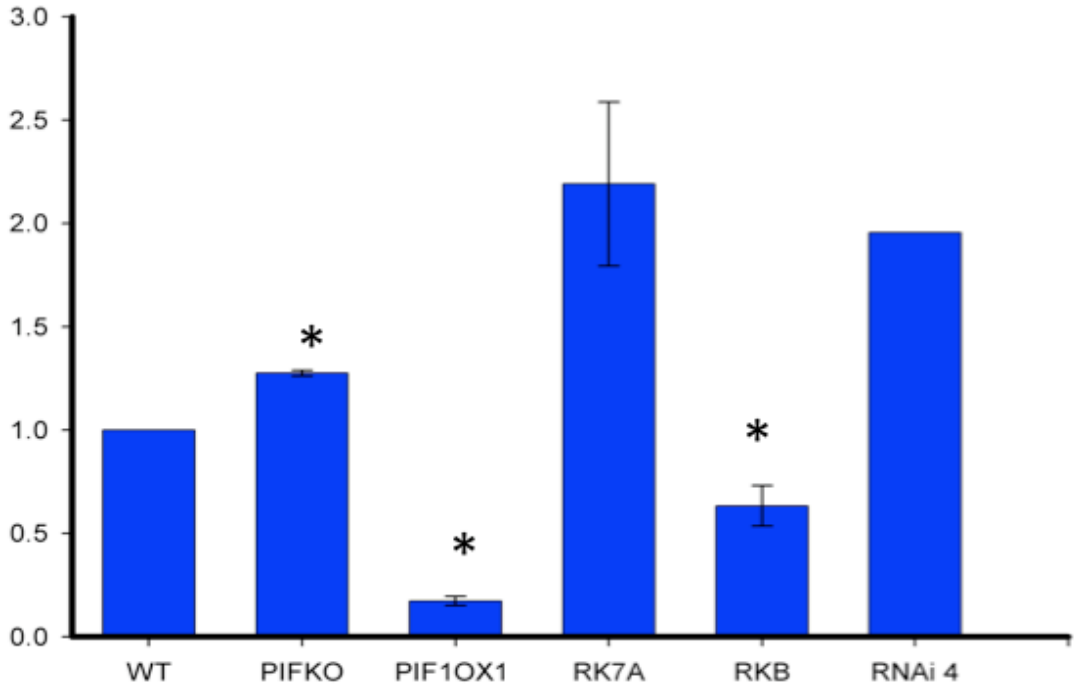

Figure 6: *EXPANSIN 10* (At1g26770)
Experimental results for down-regulated gene, *EXP10*

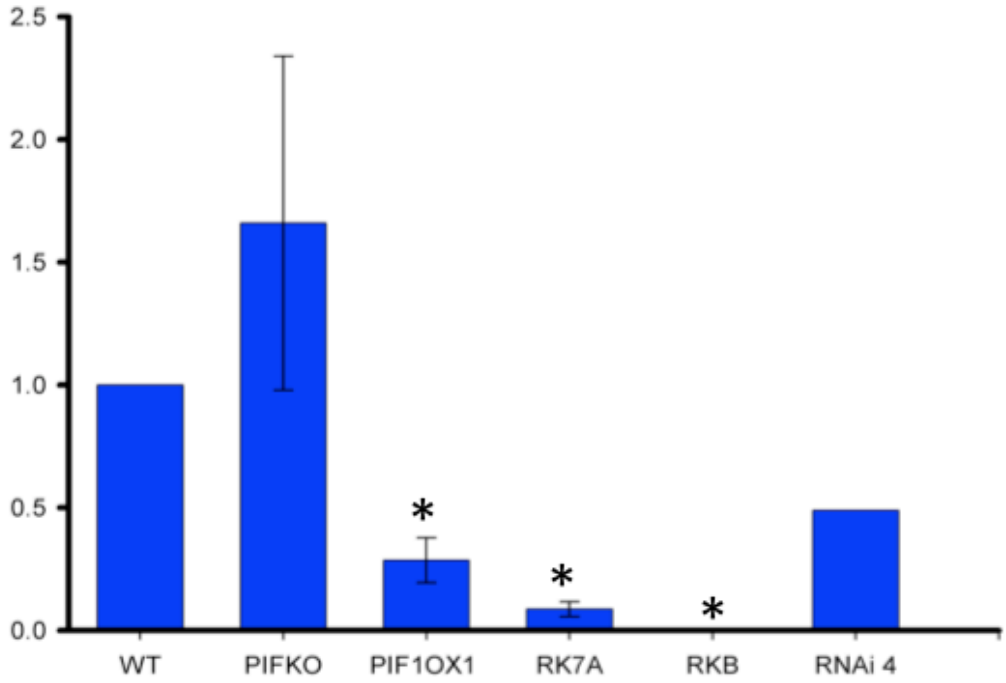

Figure 7: *L-ASPARAGINASE* (At3g16150)
Experimental results for the down-regulated gene, *L- ASPARAGINASE*

Conclusions:

The results of this experiment interpret the relative expression of direct PIF1-targeted genes in mutants of PIF1 and CTG10. Though a feedback mechanism can be inferred in some instances, the design of this experiment did not allow a measured amount of time for this reaction to occur. Therefore, the PIF1 mutants display the anticipated effects on transcription levels in the genes, while the CTG10 mutants provide a second degree of experimentation in evaluating the possible feedback mechanism from the protein-protein interaction of PIF1 and CTG10. A new set of data is currently being collected with the etiolated seedlings treated with a period of red-light exposure in order to better visualize the feedback mechanism suspected. Current results are encouraging but not complete and thus have not been included.

Overall, the results support the interaction of PIF1 and CTG10 proteins through transcription level analysis as well as the influence of presumptive PIF1 amounts on the transcription of PIF1-targeted genes.

The inverse relationship between *PIF1* transcript levels and *CTG10* may lead to discovery of a heterodimerization of PIF1 and another bHLH protein, regulating transcription of the F-BOX responsible for its destruction, through binding to any of 10 E-BOX motifs in the *CTG10* promoter region.

The implications of these results provide significant evidence in favor of CTG10 mediating the destruction of PIF1 through polyubiquitination and subsequent degradation via the 26S proteasome

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