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Prefoldins Negatively Regulate Cold Acclimation in *Arabidopsis thaliana* by Promoting Nuclear Proteasome-Mediated HY5 Degradation

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ABSTRACT

The process of cold acclimation is an important adaptive response whereby many plants from temperate regions increase their freezing tolerance after being exposed to low non-freezing temperatures. The correct development of this response relies on proper accumulation of a number of transcription factors that regulate expression patterns of cold-responsive genes. Multiple studies have revealed a variety of molecular mechanisms involved in promoting the accumulation of these transcription factors. Interestingly, however, the mechanisms implicated in controlling such accumulation to ensure their adequate levels remain largely unknown. In this work, we demonstrate that prefoldins (PFDs) control the levels of HY5, an *Arabidopsis* transcription factor with a key role in cold acclimation by activating anthocyanin biosynthesis, in response to low temperature. Our results show that, under cold conditions, PFDs accumulate into the nucleus through a DELLA-dependent mechanism, where they interact with HY5, triggering its ubiquitination and subsequent degradation. The degradation of HY5 would result, in turn, in anthocyanin biosynthesis attenuation, ensuring the accurate development of cold acclimation. These findings uncover an unanticipated nuclear function for PFDs in plant responses to abiotic stresses.

Key words: prefoldins, cold acclimation, *Arabidopsis*, HY5, anthocyanin, DELLAs

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INTRODUCTION

Plants are sessile organisms and, as a result, they are continuously exposed to a changing environment. Adverse conditions originating from abiotic stresses limit their growth and geographical distribution in nature and reduce crop productivity, causing significant economic losses. Low temperature is one of the most important unfavorable environmental factors that restrict plant ranges and development. To overcome this constraint, many plants from temperate regions have evolved a sophisticated adaptive response, named cold acclimation, whereby they increase their freezing tolerance after being exposed for some days to low non-freezing temperatures (Levitt, 1980). Cold acclimation is a very complex process that includes many physiological and biochemical changes, most of them regulated at the transcriptional level (Chinnusamy et al., 2003; Gujjar et al., 2014; Nakashima et al., 2014). During the last years, however, protein modifications have been shown to play a crucial role in the correct development of this adaptive process, unveiling that it is also regulated at the post-translational level (Barrero-Gil and Salinas, 2013). Indeed, several components of the signal transduction pathways mediating cold response have been described to be post-translationally modified. These modifications, including phosphorylation and dephosphorylation, ubiquitination, SUMOylation, *N*-glycosylation, and lipid modification, determine key aspects of protein function, such as subcellular localization, stability, activity or ability to interact with other factors, and overlap with

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transcriptional regulation of gene expression to fine-tune the development of cold acclimation response (Barrero-Gil and Salinas, 2013).

HY5 is a bZIP transcription factor from *Arabidopsis* with a central role in photomorphogenesis (Oyama et al., 1997; Lau and Deng, 2010). In addition, it has been described to participate in plant response to several hormones, including abscisic acid (ABA) and auxins (Lau and Deng, 2010). Remarkably, moreover, HY5 also acts as a positive regulator of cold acclimation. Indeed, it activates the expression of about 10% of all cold-induced transcripts in *Arabidopsis*, including those implicated in anthocyanin biosynthesis that is required for the complete development of cold acclimation response (Catalá et al., 2011; Ahmed et al., 2015; Schulz et al., 2015). HY5 accumulates under low-temperature conditions, this accumulation being regulated at both transcriptional and post-translational levels (Catalá et al., 2011). Transcriptionally, *HY5* expression is transiently induced during cold acclimation through a C-repeat binding factor (CBF)- and ABA-independent pathway (Catalá et al., 2011). Post-translationally, the levels of HY5 increase via protein stabilization through the cold-induced nuclear depletion of COP1 (Catalá et al., 2011), a RING-finger E3 ubiquitin ligase that promotes the polyubiquitination and subsequent degradation of HY5 (Osterlund et al., 2000). All these data evidence the existence of different layers of regulation to promote HY5 accumulation in response to low temperature. Currently, however, the existence of mechanisms involved in attenuating the accumulation of HY5 to ensure its adequate levels during cold acclimation has not yet been reported.

Prefoldins (PFDs) are small proteins present in all eukaryotic cells, where they are organized in jellyfish-like heterohexameric complexes comprising two distinct α and four different β subunits (Leroux et al., 1999; Martin-Benito et al., 2002). PFDs participate in the folding of nascent actin and tubulin monomers at the cytoplasm during cytoskeleton assembly (Geissler et al., 1998; Vainberg et al., 1998). Interestingly, both α and β PFDs have also been detected in the nucleus, interacting with and modulating different aspects of proteins involved in the regulation of gene expression, including their degradation by promoting ubiquitination. Thus, the human PFD3 binds the INTEGRASE protein, facilitating its interaction with the VHL E3 ubiquitin ligase that mediates its polyubiquitination and decay (Mousnier et al., 2007). Analogously, the PFD5 from humans participates in the turnover of the E-box transcription factor c-Myc via the 26S proteasome by recruiting the ubiquitin ligase Skp2-ElonginC-ElonginB-Cullin2 complex (Kimura et al., 2007; Narita et al., 2012). *Arabidopsis* PFDs complement yeast *pfd*/*gim* mutants, revealing the functional conservation of this protein family in plants (Rodríguez-Milla and Salinas, 2009). Accordingly, *Arabidopsis* PFD3, PFD5, and PFD6 are required for correct cytoskeleton formation and activity through the stabilization of tubulin monomers in the cytoplasm (Gu et al., 2008; Rodríguez-Milla and Salinas, 2009). In addition, *Arabidopsis* PFDs function as positive regulators of plant tolerance to salt stress (Rodríguez-Milla and Salinas, 2009). More recently, the accumulation of the *Arabidopsis* PFD complex in the nucleus after the direct binding of α-PFDs (PFD3 and PFD5) to DELLA proteins has been described (Locascio et al., 2013). The nuclear localization would prevent the role of the cytoplasmic PFD complex in microtubule (MT) stabilization thus limiting, consequently, plant growth and development (Locascio et al., 2013). The function that plant PFDs may have evolved in the nucleus remains to be uncovered.

In this study, we report the functional characterization of PFDs in *Arabidopsis* response to low temperature. We provide genetic and molecular evidence that PFD4 negatively regulates cold acclimation by controlling the expression of genes involved in cold-induced anthocyanin biosynthesis. Our results suggest that this role is mediated through the post-translational control of HY5 stability in the nucleus, where PFD4 accumulates in response to low temperature by a DELLA-dependent mechanism. We demonstrate that, under cold conditions, PFD4 interacts with HY5 in the nucleus, promoting its polyubiquitination and subsequent proteasome-mediated degradation via, in all likelihood, a COP1-independent pathway. We further show that PFD3 and PFD5 play redundant roles with PFD4 in modulating the cold acclimation, strongly suggesting that PFDs act in this process as a complex. Collectively, the data reported here unveil an unexpected nuclear function for *Arabidopsis* PFDs to attenuate the accumulation of HY5 in response to low temperature, thus ensuring plant adaptation to this environmental constraint.

RESULTS

Prefoldins Negatively Regulate Cold Acclimation in *Arabidopsis*

Results from the eFP Browser database (bar.toronto.ca) indicated that *PFD4* was the only *PFD* gene from *Arabidopsis* whose expression levels significantly increased in response to low temperature (Winter et al., 2007). Accordingly, quantitative PCR (qPCR) experiments showed that *PFD4* mRNA accumulated in 2-week-old Columbia (Col-0) (wild-type [WT]) plants subjected to 4°C, reaching a peak after 2 days of treatment (Figure 1A). This accumulation was detected in all organs of adult *Arabidopsis* (Figure 1B). Western blot (WB) experiments using WT plants containing a PRO_{PFD4}-*PFD4-GFP* fusion revealed that the levels of PFD4 also increased under cold conditions, paralleling those of *PFD4* transcripts (Figure 1C).

The results described above suggested that PFD4 could be involved in *Arabidopsis* response to low temperature. To test this assumption, we first searched for *PFD4* T-DNA mutants. Plants containing a single T-DNA insertion located in the second exon of *PFD4* were identified (Figure 2A). *PFD4* mRNA was undetectable in T-DNA homozygous plants under both control and cold conditions, indicating that this new *PFD4* allele (*pfd4*) was null or highly hypomorphic (Figure 2B). *pfd4* plants, as did other *Arabidopsis pfd* mutants (Gu et al., 2008; Rodríguez-Milla and Salinas, 2009), showed reduced size compared with WT plants along all *Arabidopsis* development (Figure 2C–2E). Scanning electron microscopy analysis revealed that, consistent with the role of PFDs in cytoskeleton formation (Geissler et al., 1998; Vainberg et al., 1998), this phenotype was due to the small size of the *pfd4* cells (Figure 2F). Indeed, *pfd4* mutants contained reduced levels of both α and β tubulin subunits, and highly disorganized cortical MTs compared with WT plants (Figure 2G and 2H). The transformation of *pfd4* plants with the PRO_{PFD4}-*PFD4-GFP* fusion (*c-pfd4*) rescued all

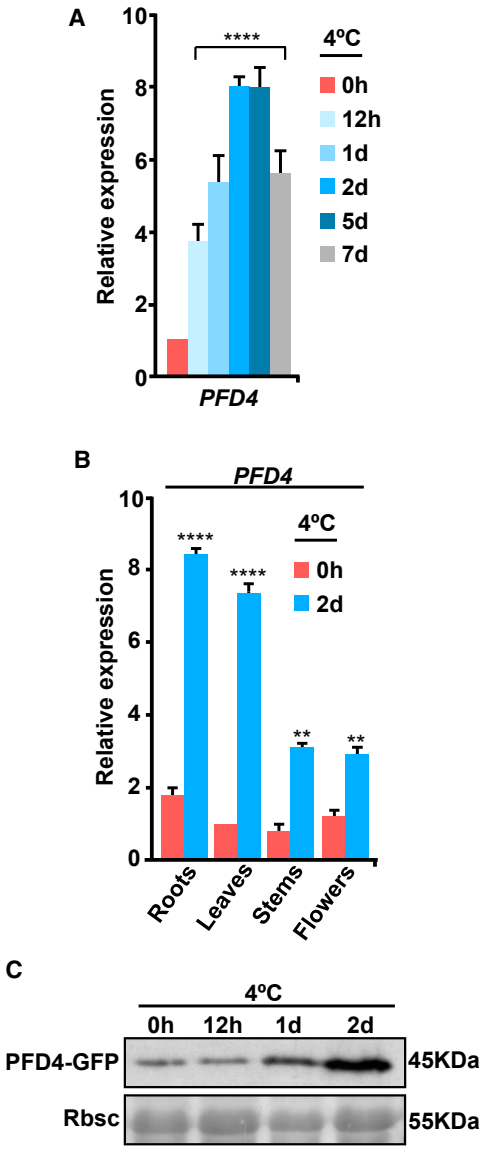

Figure 1. *Arabidopsis PFD4* Transcripts Accumulate in Response to Low Temperature.

(A and B) Levels of *PFD4* transcripts in leaves from 2-week-old WT plants **(A)**, and in roots and leaves from 2-week-old WT plants or stems and flowers from 6-week-old WT plants **(B)** exposed to 4°C for the indicated times. Error bars show the SD of three independent replicates. Asterisks represent significant differences (**$P \leq 0.01$, ****$P \leq 0.0001$) between cold-treated and control (0 h) plants, as determined by ANOVA (Dunnett's post hoc test) **(A)** or *t*-test **(B)**.

(C) Levels of PFD4-GFP fusion protein in 2-week-old WT plants exposed to 4°C for the indicated times. Coomassie staining of the large subunit of Rubisco (Rbsc) was used as a loading control.

WT phenotypes (Figure 2C–2H), validating the fusion and indicating that all alterations displayed by mutant plants originated from the absence of *PFD4* expression.

The involvement of PFD4 in cold response was studied by analyzing the capacity of *pfd4* mutants to tolerate freezing temperatures before and after cold acclimation. Non-acclimated mutants presented similar freezing tolerance as WT plants, both showing an LT_{50} (temperature that causes 50% of lethality) of about −4.2°C, indicating that PFD4 was not involved in the constitutive freezing tolerance of *Arabidopsis* (Figure 3A and 3B). Cold-acclimated (7 days, 4°C) *pfd4* mutants, however, exhibited higher capacity to tolerate freezing temperatures than cold-acclimated WT plants, the LT_{50} being in this case −10.5°C and −9°C, respectively (Figure 3C and 3D). The freezing tolerance of cold-acclimated *c-pfd4* plants was indistinguishable from that of WTs, evidencing that the increased capacity of *pfd4* mutants to cold acclimate was due to the lack of PFD4 (Figure 3C and 3D), therefore unveiling a negative role for this protein in *Arabidopsis* cold acclimation. As mentioned above, PFDs typically associate and function as heterohexameric complexes composed of different α and β subunits (Leroux et al., 1999; Martin-Benito et al., 2002). Hence, we evaluated whether *Arabidopsis* PFD subunits other than the β PFD4 could also be involved in cold acclimation. We analyzed the freezing tolerance of cold-acclimated T-DNA null mutants for the α subunits PFD3 and PFD5 (Rodríguez-Milla and Salinas, 2009). Interestingly, *pfd3* and *pfd5* mutants also showed an increased capacity to cold acclimate compared with WT plants (Supplemental Figure 1A and 1B), indicating that different PFD subunits negatively regulate cold acclimation in *Arabidopsis* and that, in all likelihood, the PFDs function as a complex in this adaptive response. Consistent with this function, though in contrast with the data reported at the eFP Browser database (see above) due in all likelihood to differences in the experimental conditions, transcripts corresponding to PFD1, 2, 3, 5, and 6 proteins exhibited cold-induced accumulation similar to that of *PFD4* mRNAs (Supplemental Figure 1C).

Prefoldins Negatively Regulate Cold Acclimation by Attenuating Anthocyanin Biosynthesis

Since cold acclimation involves an extensive transcriptome reprogramming (Chinnusamy et al., 2003) and PFDs have been implicated in regulating gene expression (Mori et al., 1998; Watanabe et al., 2002; Kim et al., 2008; Yoshida et al., 2008), we considered the possibility that these proteins could control the adaptive response by attenuating cold-induced gene expression. Transcriptomic analyses of 2-week-old WT and *pfd4* mutant plants exposed for 24 h to 4°C were performed using Agilent *Arabidopsis* Oligo Microarrays v4. Results unveiled 33 genes whose expression levels in *pfd4* mutants were increased at least two-fold (false discovery rate [FDR] ≤ 0.1) compared with WT plants (Supplemental Table 1). Intriguingly, 25 of these genes (i.e., 76%) had been previously reported to be induced (>2-fold) in response to low temperature (Vogel et al., 2005; Hannah et al., 2006; Oono et al., 2006; Kilian et al., 2007) (Supplemental Table 2). More importantly, 36% of them (i.e., 9) encoded proteins involved in anthocyanin biosynthesis (Jaakola, 2013; Shi and Xie, 2014) (Supplemental Table 2), a cold-induced pigment that is essential to prevent high levels of reactive oxygen species and to ensure the complete development of cold acclimation response (Catalá et al., 2011; Ahmed et al., 2015; Schulz et al., 2015). These results were validated by analyzing the expression of the nine anthocyanin biosynthesis-related genes in WT and *pfd4* mutant plants grown at 20°C or subjected for 24 h to 4°C by means of qPCR assays. In all cases, the expression patterns were coincident with those inferred from the microarrays

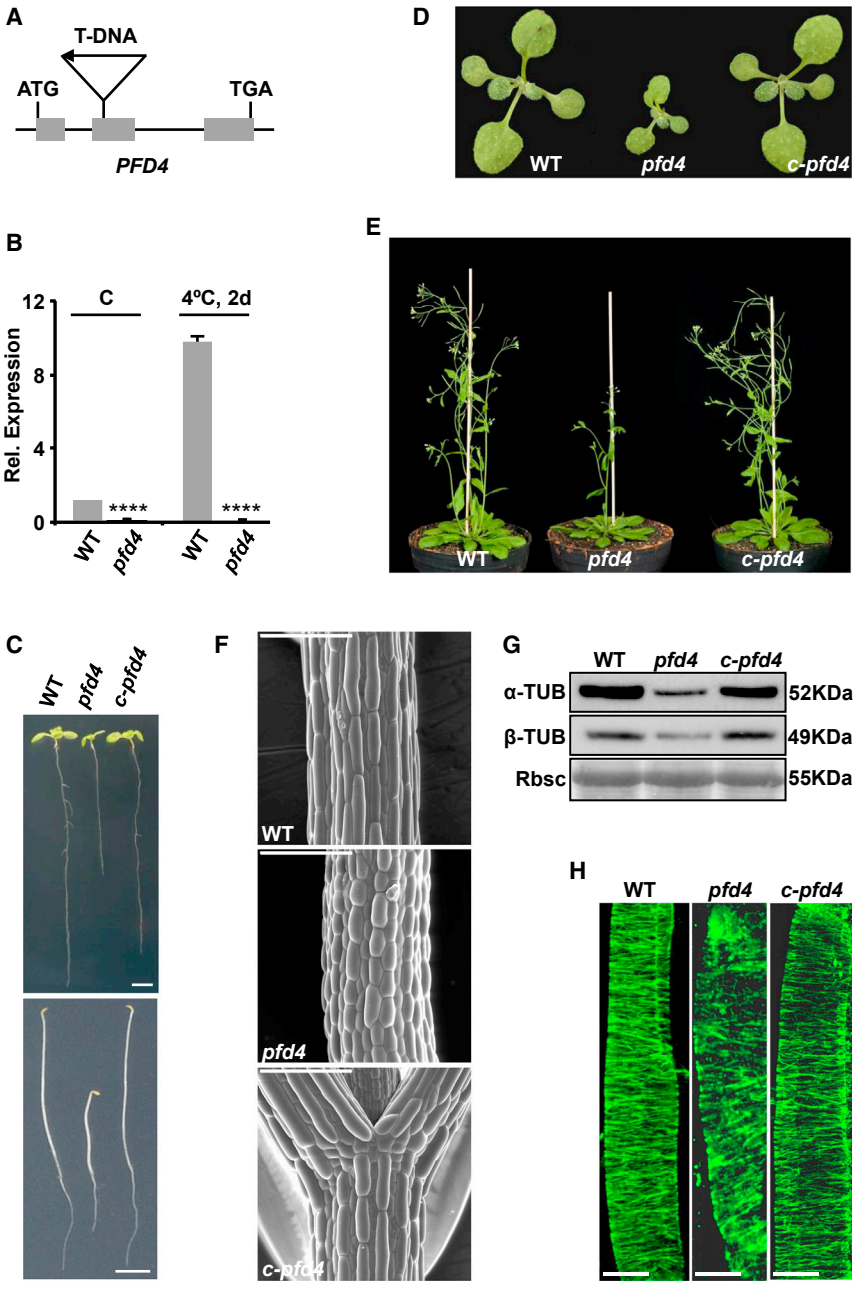

Figure 2. PFD4-Deficient *Arabidopsis* Plants Show Developmental Alterations Coupled to Cytoskeleton Defects.

(A) Schematic representation of *PFD4* showing a T-DNA insertion localized at the second exon.

(B) Expression of *PFD4* in 2-week-old WT and homozygous plants containing the T-DNA insertion (*pfd4*). Error bars indicate the SD of three independent replicates. Asterisks indicate significant differences (****$P \leq 0.0001$) between WT and *pfd4* plants, as determined by *t*-test.

(C) Six-day-old WT, *pfd4*, and *c-pfd4* seedlings grown under control (top) or etiolated (bottom) conditions. Scale bars, 50 mm.

(D and E) Three-week-old **(D)** and 6-week-old **(E)** WT, *pfd4*, and *c-pfd4* plants.

(F) Scanning electron microscopy images of hypocotyl cells from 6-day-old WT, *pfd4*, and *c-pfd4* seedlings. Scale bars, 200 mm.

(G) Western blots showing α- and β-tubulin levels in 6-day-old WT, *pfd4*, and *c-pfd4* seedlings. Coomassie staining of the large subunit of Rubisco (Rbsc) was used as a loading control.

(H) Microtubules in root-elongating WT, *pfd4*, and *c-pfd4* cells from 6-day-old seedlings revealed by immunolocalization using anti-α-tubulin and anti-Alexa Fluor 488 as primary and secondary antibodies, respectively. Scale bars, 10 mm.

(Figure 3E). Although *PAP1*, which encodes another protein directly involved in anthocyanin biosynthesis, was not among the 33 genes whose expression levels were increased at least two-fold in *pfd4* mutants because of the selection conditions (its FDR value was 0.102), qPCR analysis showed that, indeed, it was also significantly upregulated in cold-treated mutants (Figure 3E). Furthermore, the qPCR assays revealed that the expression of the 10 anthocyanin biosynthesis-related genes was not upregulated in mutant plants under control conditions (Figure 3E). Consistent with these data, mutants showed significantly higher anthocyanin levels than WT plants after exposure to 4°C for 7 days (Figure 3F), and accumulated the pigment in their leaf adaxial faces (Figure 3G). *c-pfd4* plants exhibited WT expression patterns for all validated genes and WT levels of anthocyanin (Figure 3E–3G), demonstrating that the *pfd4* mutant phenotypes were due to the absence of PFD4. *pfd3* and *pfd5* mutants also exhibited an increased content of anthocyanin in response to low temperature (Supplemental Figure 2A), easily detectable in the adaxial face of their leaves (Supplemental Figure 2B). Furthermore, as expected from these observations, the cold induction of genes involved in anthocyanin biosynthesis, including *PAP1*, *DFR*, *LDOX*, and *UF3GT*, was higher in *pfd3* and *pfd5* mutants than in WT plants (Supplemental Figure 2C). Altogether, these findings indicated that PFDs negatively regulate cold acclimation in *Arabidopsis*, at least in part by controlling the cold-induced expression of genes implicated in anthocyanin biosynthesis and, therefore, by attenuating the anthocyanin levels in response to low temperature.

PFDs Accumulate in the Nucleus in Response to Low Temperature through a DELLA-Dependent Mechanism

Animal PFDs have been described to modulate gene expression into the nucleus (Mori et al., 1998; Watanabe et al., 2002; Kimura et al., 2007; Kim et al., 2008; Yoshida et al., 2008; Narita et al., 2012). To examine whether *Arabidopsis* PFDs controlled cold-induced gene expression related to anthocyanin biosynthesis into the nucleus, we studied the subcellular distribution of PFD4 in *Arabidopsis* plants exposed to control or low-temperature

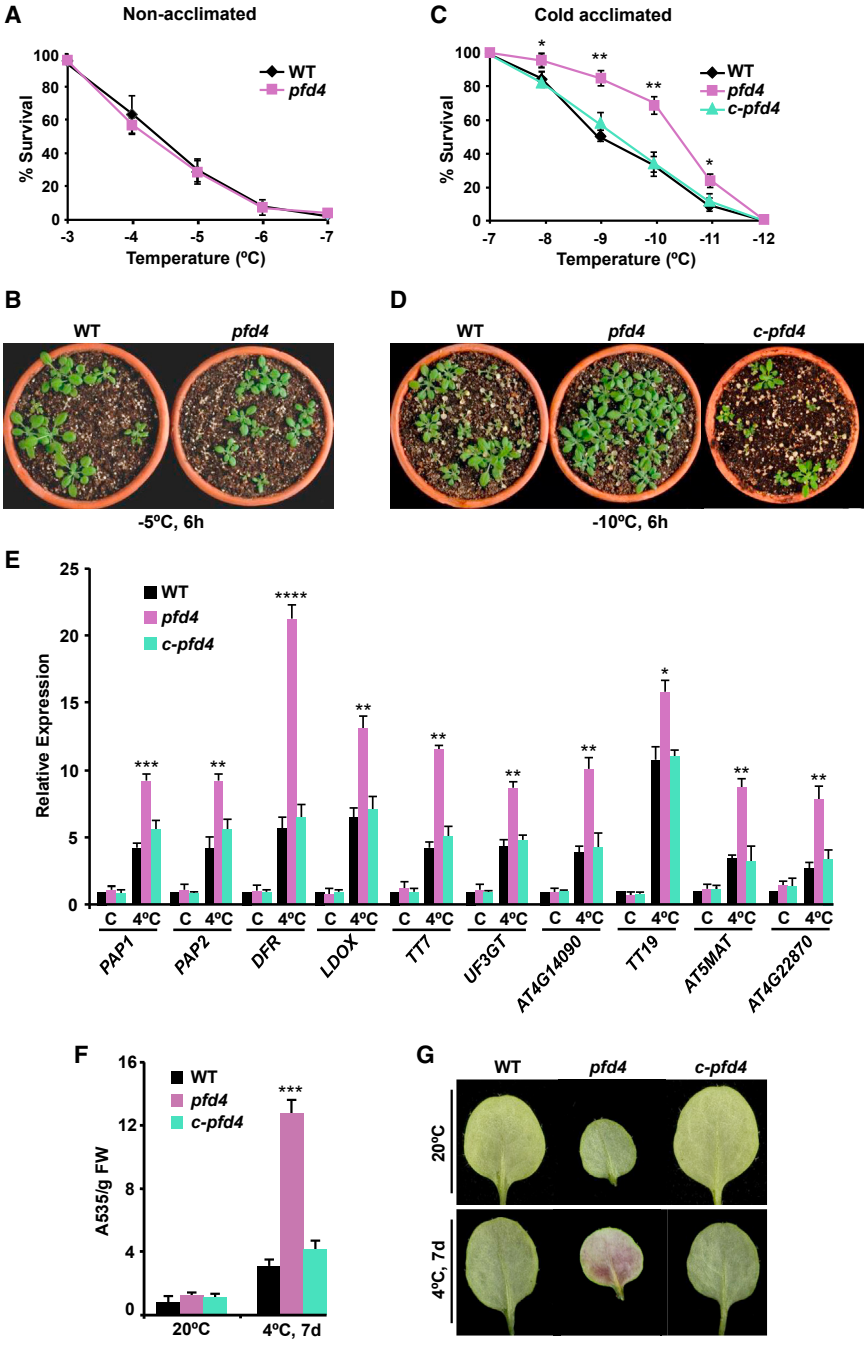

Figure 3. PFD4 Negatively Regulates Cold Acclimation Response Attenuating Gene Expression Involved in Anthocyanin Biosynthesis.

(A and B) Freezing tolerance assays of non-acclimated plants. Two-week-old WT and *pfd4* plants were exposed to the indicated freezing temperatures for 6 h. Survival rates were determined after 1 week of recovering at 20°C **(A)**. Representative recovered plants after being exposed for 6 h at −5°C **(B)**.

(C and D) Freezing tolerance assays of cold-acclimated plants. Two-week-old WT, *pfd4*, and *c-pfd4* plants grown under control conditions were transferred to 4°C for 1 week and subsequently exposed to the indicated freezing temperatures for 6 h. Survival rates were determined after 1 week of recovering at 20°C **(C)**. Representative recovered plants after being exposed for 6 h at −10°C **(D)**.

(E) Levels of transcripts corresponding to genes involved in anthocyanin biosynthesis in 2-week-old WT, *pfd4*, and *c-pfd4* plants grown under control conditions (C) or exposed to 4°C for 1 day.

(F) Anthocyanin levels in 2-week-old WT, *pfd4*, and *c-pfd4* plants grown under control conditions (20°C) or subjected to 4°C for 7 days.

(G) Leaf adaxial faces of 2-week-old WT, *pfd4*, and *c-pfd4* plants grown under control conditions (20°C) or exposed to 4°C for 7 days.

In **(A)**, **(C)**, **(E)**, and **(F)**, data represent the mean of three independent experiments and error bars indicate the SD. Asterisks indicate significant differences (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$) between *pfd4* and the other genotypes, as determined by ANOVA (Dunnett's post hoc test). No significant differences between WT and *c-pfd4* plants were observed in any case.

conditions. Confocal images of root tip cells from *c-pfd4* seedlings grown at 20°C showed that the PFD4-GFP fusion protein mostly localized in the cytoplasm (Figure 4A). The PFD4-GFP protein, however, showed a mainly nuclear accumulation when *c-pfd4* seedlings were exposed to 4°C for 2 days (Figure 4A). This huge increase in nuclear localization of PFD4 in response to low temperatures was confirmed by WB experiments using cytoplasmic and nuclear protein extracts obtained from control and cold-treated *c-pdf4* seedlings (Figure 4B).

Arabidopsis PFDs were found to accumulate in the nucleus under normal growth conditions through the direct interaction with DELLA proteins (Locascio et al., 2013). We then assessed whether DELLAs could also participate in the cold-induced nuclear accumulation of PFDs. To this end, we first analyzed the subcellular distribution of DELLA proteins under low-temperature conditions. According to Achard et al. (2008), confocal analysis indicated that DELLAs essentially localized to the nucleus in response to cold. Indeed, RGA-GFP and RGL3-GFP fusion proteins showed nuclear accumulation in root tip cells from *Arabidopsis* WT seedlings containing PRO_{RGA}-*RGA-GFP* and PRO_{35S}-*RGL3-GFP* constructs, respectively, exposed to 4°C for 2 days (Supplemental Figure 3A). This cold-induced nuclear accumulation of DELLAs was not PFD dependent, as shown by the nuclear localization of RGA-GFP in *pfd3*, *pfd4*, and *pfd5* mutants subjected to low temperature (Supplemental Figure 3A). Next, we introduced the PRO_{PFD4}-*PFD4-GFP* fusion into a *penta* DELLA-deficient mutant (*della*) (Feng et al., 2008) and determined the subcellular localization of the PFD4-GFP protein, at both 20°C and 4°C, by confocal imaging and WB experiments. Results revealed that the cold-induced nuclear localization of PFD4 was

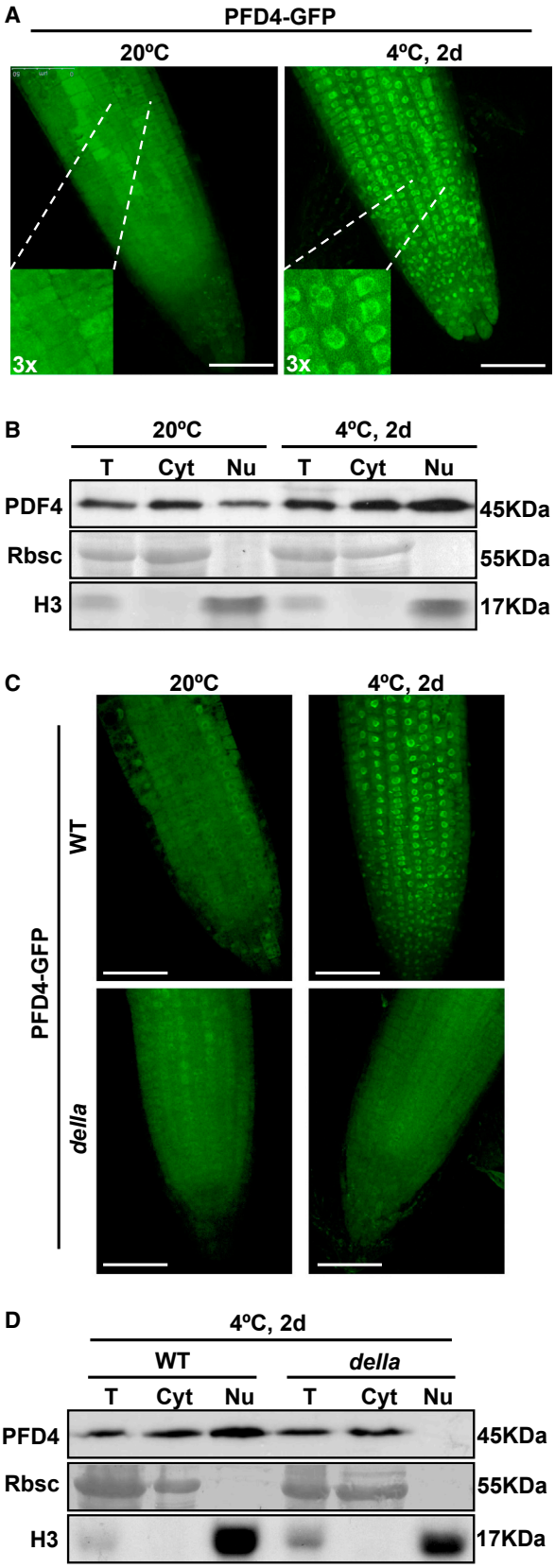

Figure 4. *Arabidopsis* PFD4 Accumulates in the Nucleus in Response to Low Temperature by a DELLA-Dependent Mechanism.

(A) Subcellular localization of PFD4-GFP fusion protein in root tip cells from 6-day-old *c-pfd4* seedlings grown under control conditions (20°C) or exposed to 4°C for 2 days. A 3× zoom is detailed. Scale bars, 50 μm.

(B) Levels of PFD4-GFP fusion protein in 2-week-old *c-pfd4* plants. Total (T), cytoplasmic (Cyt), and nuclear (Nu) fractions are shown from plants grown under control conditions (20°C) or exposed to 4°C for 2 days.

(C) Subcellular localization of PFD4-GFP fusion protein in 6-day-old seedlings from WT and *della* mutant grown under control conditions (20°C) or exposed to 4°C for 2 days. Scale bars, 50 μm.

(D) Levels of PFD4-GFP fusion protein in 2-week-old WT and *della* mutant plants exposed to 4°C for 2 days. Protein levels from total (T), cytoplasmic (Cyt), and nuclear (Nu) fractions are shown.

In **(B)** and **(D)**, membranes were reprobed with an anti-H3 antibody and the large subunit of Rubisco (Rbsc) was stained with Coomassie for control of fractionation.

abolished in root tip cells from *della* seedlings (Figure 4C and 4D), indicating that PFDs accumulate in the nucleus in response to low temperature through a DELLA-dependent mechanism.

PFDs Control HY5 Stability during Cold Acclimation

Nuclear-localized PFD subunits modulate gene expression in animals by promoting the degradation of transcription factors (Kimura et al., 2007; Narita et al., 2012). Since the *Arabidopsis* HY5 transcription factor accumulates in response to low temperature and activates anthocyanin accumulation by inducing the expression of several genes involved in anthocyanin biosynthesis (Catalá et al., 2011), we hypothesized that PFDs could attenuate anthocyanin biosynthesis during cold acclimation by controlling the turnover of HY5. To test this possibility, we determined the levels of HY5 in WT and *pfd4* plants under control and cold conditions. Confocal analysis of root tip cells from *hy5-1* and *pfd4* seedlings expressing the PRO_{HY5}-*HY5-YFP* fusion (Oravecz et al., 2006) revealed that, consistent with our hypothesis, the levels of fluorescence were in all cases higher in the nuclei of *pfd4* than in those of *hy5-1* plants (Figure 5A). WB experiments using an anti-HY5 antibody confirmed that, in fact, *pfd4* mutants contained higher levels of HY5 than WT plants under both standard and low-temperature conditions (Figure 5B). Moreover they uncovered that, while in WT plants HY5 accumulated transiently in response to cold reaching a peak after 2 days of treatment, in *pfd4* mutants the accumulation of HY5 was not transient but continued increasing during at least 5 days of cold exposure (Figure 5B). As expected, *c-pfd4* plants exposed to 4°C for different times presented a WT pattern of HY5 accumulation (Supplemental Figure 4A). qPCR assays showed that *HY5* expression was similar in control and cold-induced WT and *pfd4* plants (Figure 5C), confirming the microarray results (Supplemental Table 1) and evidencing that the high levels of HY5 noticed in *pfd4* mutants (Figure 5A and 5B) were not due to an increase in *HY5* transcripts.

To establish definitively whether PFDs control the stability of HY5 during cold acclimation, we investigated the HY5 levels in WT and *pfd4* plants simultaneously exposed to low temperature and cycloheximide (CHX), a potent inhibitor of translation. According to previous results (Catalá et al., 2011) and the results described above (Figure 5B), WT plants subjected for 1 day to 4°C exhibited

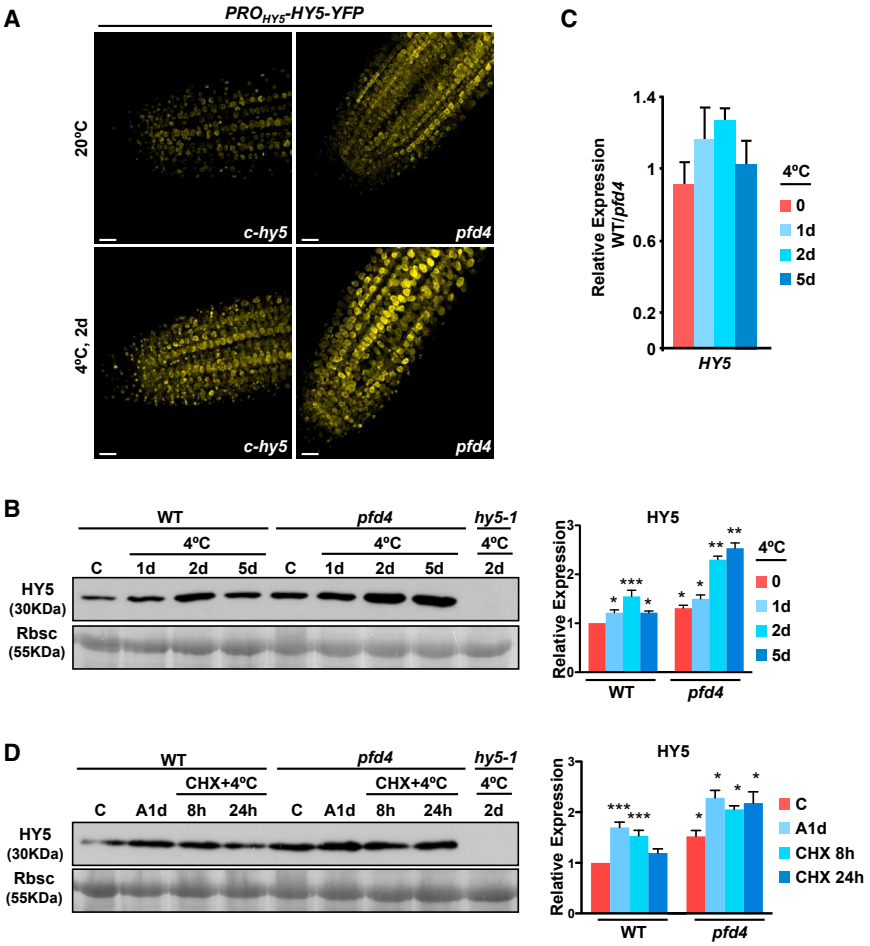

Figure 5. *Arabidopsis* PFD4 Controls HY5 Levels at the Post-translational Level.

(A) Subcellular localization of HY5-YFP protein fusion in root tip cells of 6-day-old *hy5-1* and *pfd4* seedlings grown under control conditions (20°C) or exposed to 4°C for 2 days. Scale bars, 25 μm.

(B) Levels of HY5 protein in 2-week-old WT and *pfd4* plants grown under control conditions (C) or exposed to 4°C for the indicated times.

(C) Levels of *HY5* transcripts in 2-week-old WT and *pfd4* plants exposed to 4°C for the indicated times. Error bars represent the SD of three independent replicates.

(D) levels of HY5 protein in 2-week-old WT and *pfd4* plants grown under control conditions (C), exposed for 1 day at 4°C (A1d), or subjected to 4°C for 8 h and 24 h in the presence of cycloheximide (CHX).

In **(B)** and **(D)**, protein samples from *hy5-1* mutant plants were included as a negative control in the western blots, and Coomassie staining of the large subunit of Rubisco (Rbsc) was used as a loading control. Charts representing blot signal quantifications are shown on the right. Data represent the mean of three independent experiments and error bars the SD. Asterisks indicate significant differences ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$) between control and low-temperature conditions in WT plants calculated by ANOVA (Dunnett's post hoc test). In *pfd4* mutants, asterisks indicate significant differences relative to WT plants for each time point analyzed, as determined by *t*-test.

higher levels of HY5 than plants grown under standard conditions (Figure 5D). These levels subsequently decreased following CHX treatment until reaching those displayed by unstressed plants (Figure 5D). In *pfd4* mutants, the levels of HY5 also increased after 1 day of cold exposure but, in contrast to WT plants, they did not decrease in the presence of CHX (Figure 5D), strongly suggesting that PFD4 controlled HY5 stabilization in response to low temperature. *c-pfd4* plants exposed for different times to 4°C displayed patterns of HY5 accumulation similar to those of WT plants (Supplemental Figure 4B). Importantly, *pfd3* and *pfd5* mutants, just as the *pfd4*, also showed increased levels of HY5 compared with WT plants under control and cold conditions (Supplemental Figure 5A). Moreover, these levels did not diminish when *pfd3* and *pfd5* mutants were subjected to 4°C and CHX treatments simultaneously (Supplemental Figure 5B). All these findings indicated that *Arabidopsis* PFDs control HY5 stability to attenuate anthocyanin biosynthesis in response to low temperature.

PFDs Interact with HY5 during Cold Acclimation and Promote Its Proteasome-Mediated Degradation Independently of COP1

Human PFDs have been reported to interact with transcription factors in the nucleus, fostering its ubiquitination and further decay (Kimura et al., 2007; Narita et al., 2012). Considering that the levels of HY5 are tightly regulated by ubiquitination and subsequent degradation through the proteasome (Osterlund et al., 2000), it was conceivable that *Arabidopsis* PFDs could control HY5 stability during cold acclimation by promoting its ubiquitination. To test this assumption, we first assayed the interaction between PFD4 and HY5 by means of bimolecular fluorescence complementation (BiFC) analysis in *Nicotiana benthamiana* leaves subjected to low temperature. Results revealed that a significant proportion of cells transformed with PFD4-nYFP and HY5-cYFP displayed intense yellow fluorescence (Figure 6A), indicating a positive interaction between these proteins. Consistent with the subcellular localization of HY5 and PFD4 at 4°C (Figures 4A, 4B, and 5A), PFD4–HY5 interaction was observed specifically in the nuclei of *N. benthamiana* cells (Figure 6A). This interaction was also detected under control conditions, but was much less evident than that observed in the cold (Supplemental Figure 6A). However, no interaction was noted between PFD4 or HY5 and PAP1, a nuclear protein used as a negative control (Figure 6A and Supplemental Figure 6A). Next, we determined the levels of ubiquitinated HY5 in WT and *pfd4* plants exposed for 2 days at 4°C. Plant extracts were incubated with p62 resin, which allows the isolation of total ubiquitinated proteins (Irigoyen et al., 2014), and HY5 was subsequently detected through WB experiments with an anti-HY5 antibody. Although total HY5 levels were higher in the mutant input, the amount of ubiquitinated HY5

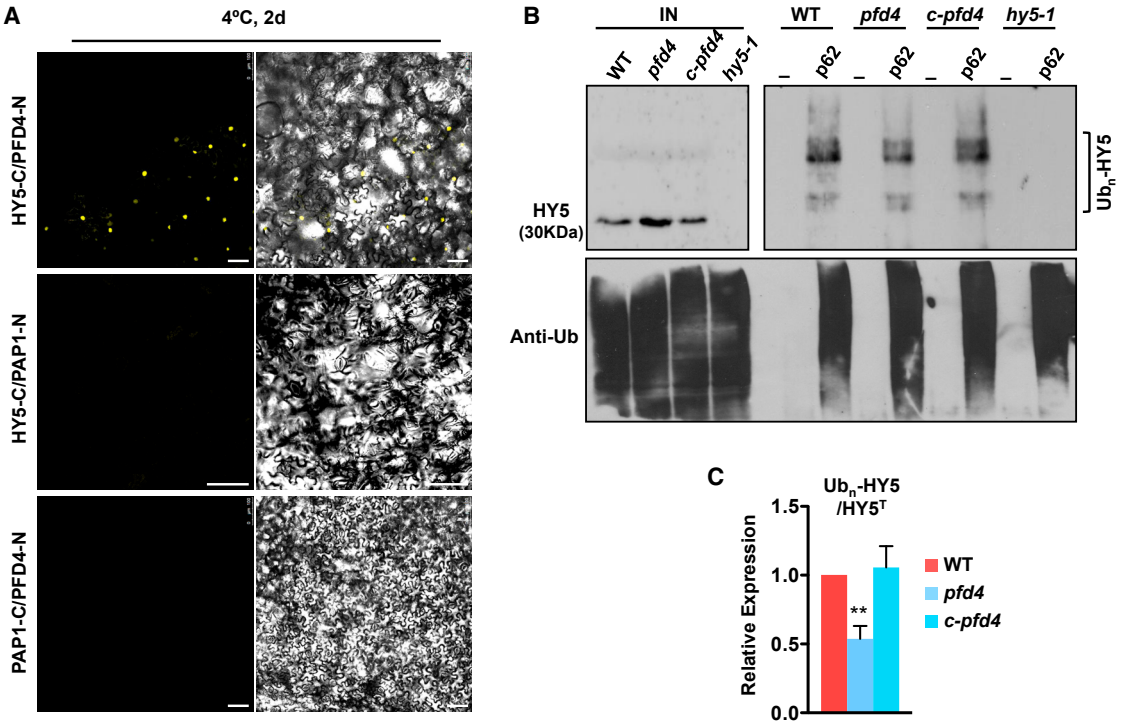

Figure 6. *Arabidopsis* PFD4 Interacts with HY5, Promoting its Polyubiquitination in Response to Low Temperature.

(A) *In vivo* interaction between *Arabidopsis* PFD4 and HY5 proteins by BiFC assays in *N. benthamiana* leaf cells under cold conditions (4°C, 2 days). Reconstitution of YFP (left) and overlay with transmission bright-field images (right) are shown. The interaction of PFD4 with PAP1 was also assayed as a negative control. Scale bars, 75 μm.

(B) Levels of HY5 from input (IN) total protein extracts (top left) and of polyubiquitinated HY5 (top right) in 2-week-old WT, *pfd4*, *c-pfd4*, and *hy5-1* plants exposed to 4°C for 2 days. The bracket indicates the positions of polyubiquitinated HY5 bands. Ubiquitinated proteins were affinity purified through binding to a p62 resin. An empty agarose resin was also used as a negative control (–). Immunoblots with an anti-Ub antibody were performed to detect total ubiquitinated proteins as a control of the experiment (bottom).

(C) Representation of polyubiquitinated/total HY5 ratios in 2-week-old WT, *pfd4*, and *c-pfd4* plants exposed to 4°C for 2 days. Error bars represent the SD of three independent replicates, and asterisks indicate significant difference (**$P \leq 0.01$) between *pfd4* and WT or *c-pfd4* plants, as determined by ANOVA (Dunnett's post hoc test).

detected was notably lower in mutant than in WT plants (Figure 6B and 6C). The ubiquitination levels of HY5 were also reduced in *pfd4* mutants grown at 20°C, but significantly much less than in response to low temperature (Supplemental Figure 6B and 6C). In all cases *c-pfd4* plants exhibited, as expected, analogous levels of ubiquitinated HY5 as the WT (Figure 6B and 6C; Supplemental Figure 6B and 6C). These results revealed the capacity of PFDs to interact with HY5 and control its levels by promoting its ubiquitination and subsequent proteasome-mediated decay, predominantly in response to low temperature.

It has been described that the E3 ubiquitin ligase COP1 targets HY5 for degradation through the proteasome (Osterlund et al., 2000). We therefore examined the capacity of COP1 to ubiquitinate HY5 in the presence or absence of PFDs under cold conditions by means of *in vitro* ubiquitination assays using yeast E1, rice His-Rad6 E2, HA-labeled plus unlabeled ubiquitin, and recombinant GST-HY5 and MBP-COP1 proteins. PFD4-GFP protein was immunopurified from 2-week-old *c-pfd4* plants exposed to 4°C for 2 days. GFP alone, isolated from WT *Arabidopsis* containing a *35S-GFP* fusion subjected to the same cold conditions, was used as a negative control. Ubiquitinated GST-HY5 proteins were detected in WB experiments with an anti-HY5 antibody. Results evidenced identical slow migrating bands corresponding to ubiquitinated HY5 proteins when PFD4-GFP or GFP purified proteins were included in the reactions (Figure 7A). Moreover, the levels of monoubiquitinated MBP-COP1, which were disclosed with an anti-HA antibody and used as a control of the COP1 ubiquitination activity, were also comparable in the presence of PFD4-GFP or GFP (Figure 7A), strongly suggesting that PFD4 has no effect on the ubiquitination of HY5 mediated by COP1. Next, even if our transcriptomic results showed that the expression levels of *COP1* were not affected in *pfd4* mutants at 4°C (Supplemental Table 1), we tested the possibility that PFDs could induce HY5 degradation by promoting the accumulation of COP1 in the nuclei during cold acclimation. Confocal analysis of root tip cells from *pfd4* mutant seedlings expressing a *PRO$_{35S}$-YFP-COP1* fusion exposed to low temperature unveiled that, in the absence of PFDs, COP1 accumulated as in cold-treated WT seedlings, i.e., *cop1-4* seedlings complemented with the *PRO$_{35S}$-YFP-COP1* fusion (Oravecz et al., 2006). As previously reported (Catalá et al., 2011), COP1 was rapidly depleted from the nuclei and later on, after 2 days at 4°C, its levels increased until reaching those of plants grown under control conditions (Figure 7B). WB experiments using an anti-COP1 antibody

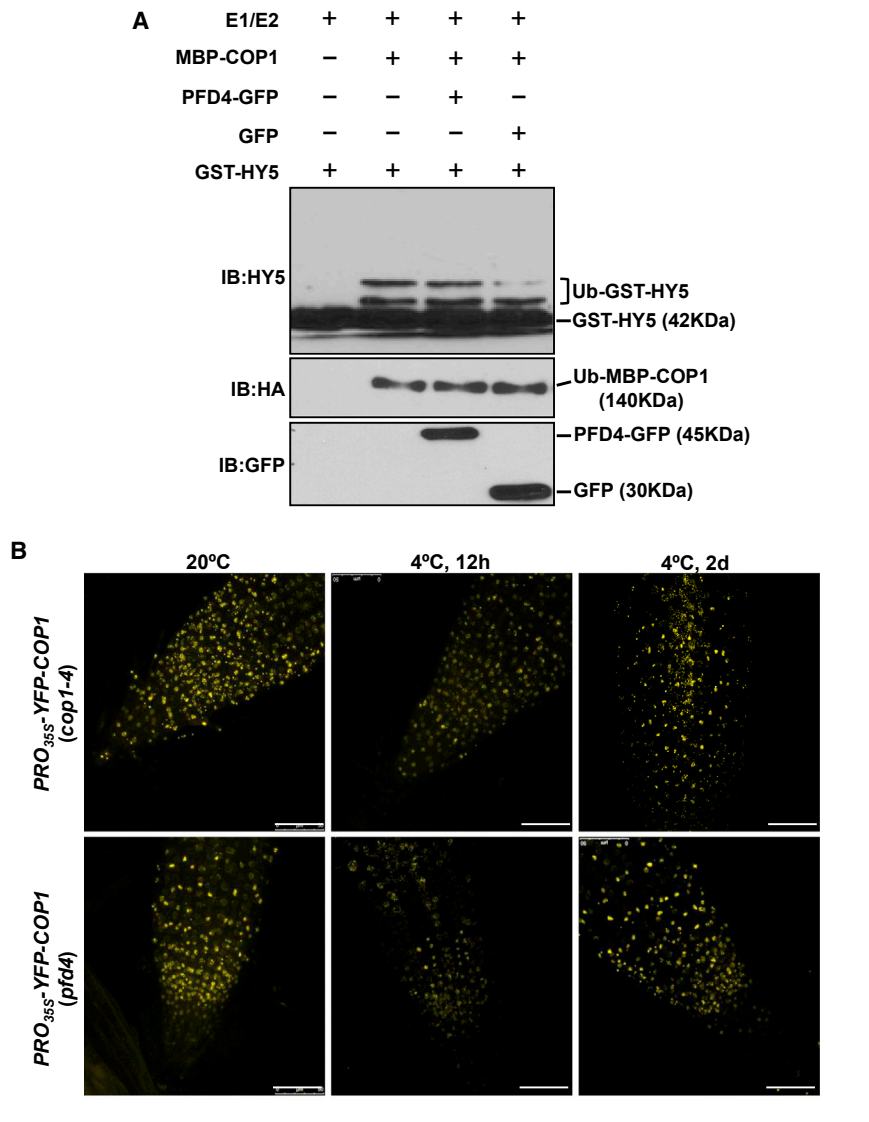

Figure 7. *In Vitro* Ubiquitination Assays and Subcellular Localization of COP1 in *pfd4* Mutants Indicate that PFD-Mediated HY5 Degradation in Response to Low Temperature Is Independent of COP1.

(A) GST-HY5 ubiquitination assays were performed using yeast E1, rice Rad6 (E2), and recombinant MBP-COP1 in the presence or absence of PFD4-GFP or GFP alone. The bracket indicates the positions of ubiquitinated GST-HY5 bands. Autoubiquitinated COP1 protein levels (Ub-MBP-COP1) were also included as a control of the COP1 ubiquitination activity. GST-HY5 and ubiquitinated MBP-COP1 were detected using anti-HY5 (IB:HY5) and anti-HA (IB:HA) antibodies, respectively. PFD4-GFP and GFP recombinant proteins were detected employing an anti-GFP antibody (IB:GFP).

(B) Subcellular localization of the YFP-COP1 fusion protein in main root tip cells from 6-day-old *cop1-4* and *pfd4* seedlings grown under control conditions (20°C) or exposed to 4°C for the indicated times. Scale bars, 50 μm.

(C) Levels of the YFP-COP1 fusion protein in 2-week-old WT and *pfd4* plants grown under control conditions (C) or exposed to 4°C for the indicated times. Protein samples from *cop1-4* were included as a negative control in the western blot, and Coomassie staining of the large subunit of Rubisco (Rbsc) was used as a loading control. A chart representing blot signal quantifications is shown on the right. Data represent the mean of three independent experiments and error bars the SD. Asterisks indicate significant difference (**$P \leq 0.01$) between control and low-temperature conditions in WT plants calculated by ANOVA (Dunnett's post hoc test). In *pfd4* mutants, no significant differences with respect to the WT plants were found at any time point analyzed, as determined by *t*-test.

confirmed that, in fact, COP1 accumulated similarly in WT and *pfd4* mutant plants subjected to 4°C (Figure 7C), demonstrating that PFDs are not involved in regulating the levels and the subcellular distribution of COP1 in response to low temperature. All these data indicated that PFDs interact with HY5 during cold acclimation to trigger its proteasome-mediated degradation, in all probability independently of COP1.

DISCUSSION

The control of protein stability represents a key step in the regulation of many plant biological processes. However, the mechanisms controlling protein turnover and their implications in plant responses to abiotic stresses are poorly understood. Here, we provide genetic and molecular evidence that PFDs negatively regulate the cold acclimation process in *Arabidopsis* by controlling the levels of HY5, a master activator of anthocyanin biosynthesis, in response to low temperature. We show that, under cold conditions, PFDs accumulate in the nucleus by a DELLA-mediated mechanism whereby they interact with HY5 promoting its ubiquitination and subsequent decay, which attenuates the levels of anthocyanins to ensure the correct development of cold acclimation. These findings unveil an unanticipated nuclear role for PFDs in plant adaptation to abiotic stresses.

Our expression analyses reveal that transcripts corresponding to *Arabidopsis PFD* genes increase in response to low temperature. Remarkably, the expression of *PFD4* has been shown to be down-regulated in *hy5* mutants when exposed to 4°C (Catalá et al., 2011). It is therefore tempting to hypothesize that the cold induction of

PFD genes could be positively regulated by HY5 at the transcriptional level. As expected assuming this possibility, HY5 protein and *PFD* transcripts transiently accumulate during low-temperature treatment, reaching the maximum of accumulation in both cases after about 48 h. Considering the negative function we have uncovered for PFDs on HY5 stability, the regulation of *PFD* expression by HY5 would constitute a novel feedback mechanism to control precisely the levels of HY5 during cold acclimation in *Arabidopsis*. Moreover, concomitantly with the increase of *PFD* transcripts, the content of PFD proteins also accumulates along the adaptive response, which is consistent with the uncovered function. Unexpectedly, this accumulation takes place mainly in the nucleus, which should engender, early in the response, a decrease in the levels of cytoplasmic PFDs that would account, at least in part, for the rapid and transient destabilization of MTs observed in plant cells subjected to cold (Bartolo and Carter, 1991; Wallin and Stromberg, 1995). The increase of PFD4 levels following cold exposure would contribute to restoring the adequate amounts of cytoplasmic PFDs, finally ensuring some MT repolymerization and a moderate cell growth rate under this unfavorable condition. According to this interpretation, we observed that the MTs of *pfd4* cells are unable to fully repolymerize after being depolymerized early in response to low temperature (Supplemental Figure 7).

The absence of PFD4 results in reduced plant size, which is associated with a lower content of α and β tubulin subunits, and, consequently, with impaired MT organization and function. Similar morphological and cellular defects have been observed in *pfd3*, *pfd5*, and *pfd6* mutants (Gu et al., 2008; Rodríguez-Milla and Salinas, 2009), indicating that PFDs are essential for the correct development of *Arabidopsis*. Prefoldins also seem to be required for the adequate relationship of plants with hostile environments. *pfd3*, *pfd4*, and *pfd5* mutants display enhanced capacity to tolerate freezing temperatures compared with WT plants, uncovering a negative role for the corresponding proteins in *Arabidopsis* cold acclimation, which should be accomplished, in all likelihood, in the context of the whole PFD complex. We previously showed that PFDs function as positive regulators of *Arabidopsis* tolerance to salt stress (Rodríguez-Milla and Salinas, 2009). All these data, therefore, demonstrate that PFDs play different functions in plant response to abiotic stresses, depending on the nature of the stress, and illustrate their plasticity in regulating plant adaptation to adverse environmental circumstances. The global transcriptome profiles indicate that PFDs negatively regulate cold acclimation in *Arabidopsis*, at least in part through a tight control of anthocyanin biosynthesis, a pigment that accumulates in response to low temperature to protect photosystems from reactive oxygen species (Catalá et al., 2011; Ahmed et al., 2015; Schulz et al., 2015). Under cold conditions, PFDs attenuate the expression of a number of genes encoding transcription factors and enzymes implicated in anthocyanin biosynthesis, such as *PAP1*, *PAP2*, *DFR*, *LDOX*, *TT7*, or *TT19*. Yet the expression of these genes is not upregulated in *pfd3*, *pfd4*, and *pfd5* mutants grown at 20°C, which is consistent with the fact that PFDs are not involved in controlling the constitutive freezing tolerance of *Arabidopsis*.

As discussed above, the results presented show that *Arabidopsis* PFDs restrain the accumulation of HY5, a central transcription factor for anthocyanin biosynthesis (Catalá et al., 2011). Although this restriction takes place to a certain extent under control conditions, it plays a pivotal role during cold acclimation. Among the anthocyanin biosynthesis-related genes whose expression is negatively regulated by PFDs at 4°C we did not find *HY5*, suggesting that the levels of HY5 are not controlled by these proteins transcriptionally. Indeed, the data presented here demonstrate that *Arabidopsis* PFDs control the accumulation of HY5 post-translationally to ensure its appropriate content all along the cold acclimation response. Moreover, we provide evidence that the PFD4 subunit binds to HY5 in the nucleus, predominantly in response to low temperature, promoting its ubiquitination and further decay. Prefoldins have been conventionally considered as highly specialized co-chaperones for actin and tubulin folding (Geissler et al., 1998; Vainberg et al., 1998). Nevertheless, in addition to this role directly linked to the cytoskeleton, PFDs have also been connected to phenomena that are not cytoplasmic. Several studies have reported the presence of PFDs in the nucleus and the existence of different mechanisms regulating their cytoplasmic–nuclear trafficking. Thus, human PFD2 and PFD6 seem to shuttle between nucleus and cytoplasm together with RNA polymerase II (Mita et al., 2013). In yeast, PFDs concentrate in the nucleus when nuclear export systems become genetically inactivated (Delgermaa et al., 2004), suggesting the existence of an active mechanism mediating their subcellular shift. Recently, plant PFDs have also been described to accumulate in the nucleus after a physical interaction with DELLA proteins, severely compromising plant growth (Locascio et al., 2013). Consistent with these results and with those from Achard et al. (2008) showing the nuclear accumulation of DELLAs in response to low temperature, we found that PFD4 concentrates in the nucleus of *Arabidopsis* cells exposed to 4°C by a DELLA-dependent mechanism. The cold-induced nuclear accumulation of DELLAs, however, is not dependent on the PFDs. Our findings, therefore, unveil a cooperative function between PFD and DELLA proteins in cold acclimation. The increase of DELLA levels under low-temperature conditions would limit *Arabidopsis* growth by transferring PFDs to the nucleus where they regulate HY5 turnover. Intriguingly, in contrast to PFDs, DELLAs have been described to act as positive regulators of cold acclimation as components of the CBF1-mediated cold stress response (Achard et al., 2008). Whether they may also be implicated in inducing anthocyanin biosynthesis remains to be determined.

Eukaryotic PFDs also have a prominent nuclear role in regulating gene expression through chromatin remodeling or by modulating the activity of transcription factors (Millán-Zambrano and Chávez, 2014). In particular, human PFDs have been shown to regulate gene expression through their interaction with transcription factors in the nucleus to control their stability by triggering its ubiquitination and subsequent proteasome-mediated degradation (Kimura et al., 2007; Narita et al., 2012). Our data reveal that this nuclear role of PFDs is also functionally conserved in plants. Actually we demonstrate that *Arabidopsis* PFD4 binds to HY5 in the nucleus under low-temperature conditions, activating its ubiquitination and further decay. To date, only the COP1 E3 ubiquitin ligase has been involved in HY5 degradation during the cold acclimation process (Catalá et al., 2011). It was, therefore, reasonable to investigate the possibility that the accumulation of PFDs in the

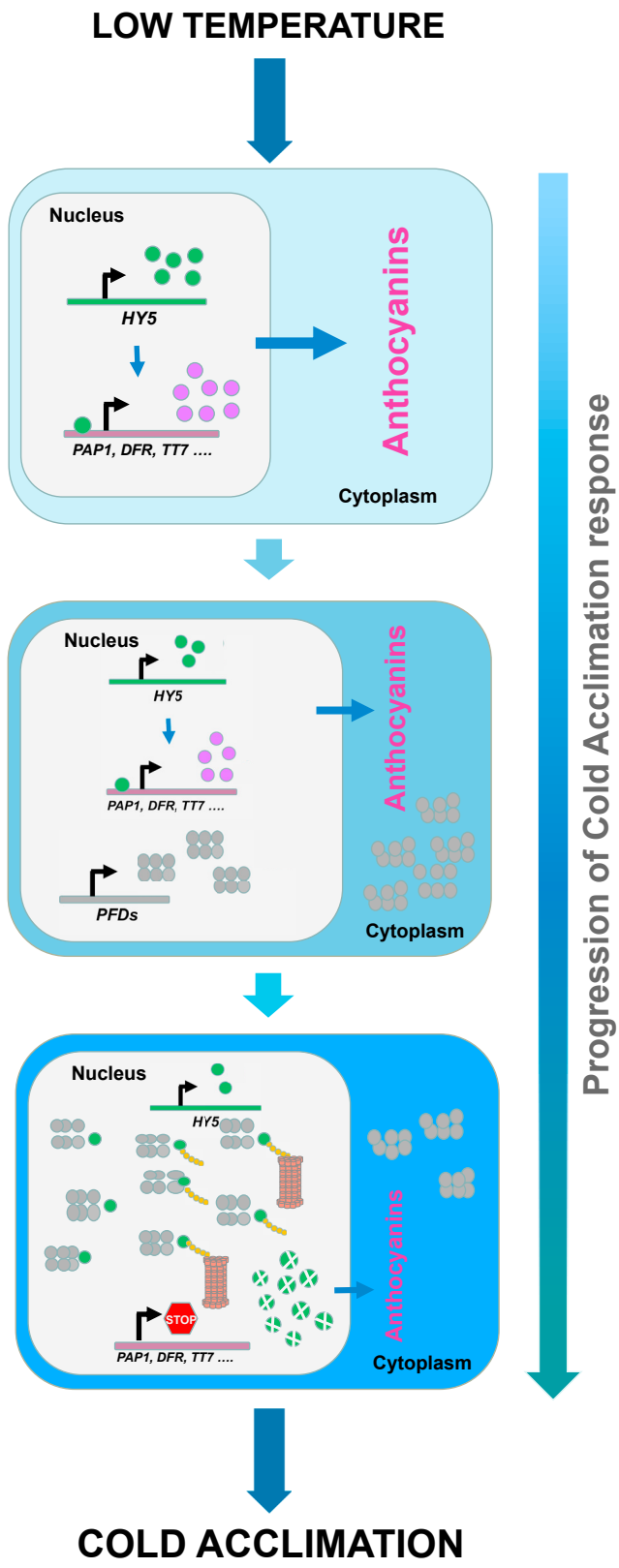

Figure 8. Hypothetical Model for the Function of the *Arabidopsis* PFDs in Cold Acclimation Response.

nucleus might affect the levels, subcellular localization, or activity of COP1 in response to low temperature, which in turn would generate a reduction in the HY5 levels. The results displayed here indicate, however, that PFD4 has no effect on the characteristics of COP1 we have analyzed, including its activity *in vitro*. Although it cannot be excluded that PFD4 may regulate some other aspect of COP1, our findings suggest that PFDs foster the turnover of HY5 in the nucleus during cold acclimation independently of COP1, through another E3 ubiquitin ligase still to be uncovered.

Based on the data described in this work, a hypothetical model for the function of the *Arabidopsis* PFDs in cold acclimation is presented in Figure 8. In response to low temperature, once the HY5 levels have increased triggering the induction of genes involved in anthocyanin biosynthesis, the expression of *PFD* genes would be induced and PFDs would accumulate in order to control HY5 levels and guarantee appropriate levels of anthocyanins during cold acclimation. To this end, PFDs would accumulate in the nucleus, in a DELLA-dependent way, where they would interact with HY5. This interaction, in turn, would promote the polyubiquitination of HY5 and its subsequent proteasome-mediated degradation, most likely in a COP1-independent manner. The attenuation of HY5 accumulation would ensure proper levels of anthocyanins and, finally, the correct development of cold acclimation response. In conclusion, our results uncover an unexpected nuclear function for PFDs in plant adaptation to adverse environmental conditions by modulating gene expression at the post-translational level. Identifying the E3-ubiquitin ligase involved in the cold-induced PFD-mediated decay of HY5 constitutes a remarkable goal for future studies.

METHODS

Plant Materials, Constructs, Growth Conditions, Treatments, and Tolerance Assays

Arabidopsis Col-0 ecotype was used in all experiments. The T-DNA insertion line for *PFD4* (*pfd4*) was obtained from SALK (*SALK_050049*). *pfd3* and *pfd5* T-DNA lines were previously described (Rodríguez-Milla and Salinas, 2009). *hy5-1* and *cop1-4* mutants were obtained from the Nottingham *Arabidopsis* Seeds Center. Mutants *hy5-1* and *cop1-4* complemented with PRO_{HY5}-*HY5-YFP* and PRO_{35S}-*YFP-COP1* fusions, respectively, were provided by R. Ulm (University of Geneva, Switzerland) and subsequently crossed with the *pfd4* mutant. Transgenic lines expressing PRO_{RGA}-*RGA-GFP* and PRO_{35S}-*RGL3-GFP* were kindly provided by Salome Prat (CNB, Spain), and subsequently the PRO_{RGA}-*RGA-GFP* line was crossed with the *pfd4* mutant. Transgenic lines expressing a PRO_{PFD4}-*PFD4-GFP* fusion were generated by cloning the genomic PFD4 region, including 1.5 kb of the corresponding promoter, with appropriate primers (see Supplemental Table 3), into the pGWB4 gateway binary vector (Nakagawa et al., 2007). The fusion was verified by sequencing and introduced in Col-0 via *Agrobacterium tumefaciens* C58C1 using the floral dip method (Clough and Bent, 1998). The PRO_{PFD4}-*PFD4-GFP* fusion was also introduced in *pfd4* (*c-pfd4*) and *pentaDELLA* mutants by *Agrobacterium* transformation. For BiFC assays, full-length cDNAs corresponding to *PFD4*, *PAP1*, and *HY5* genes were amplified with appropriate primers (see Supplemental Table 3) and fragments were subsequently cloned into pYFN43 and pYFC43 Gateway binary vectors (Belda-Palazón et al., 2012). After sequencing, plasmids were introduced into *A. tumefaciens* C58C1 for agroinfiltration of leaves from 3-week-old *Nicotiana benthamiana* plants, essentially as described by English et al. (1997). The reconstitution of YFP activity was assayed 3 days after agroinfiltration.

Plants were grown at 20°C under long-day photoperiod (16 h of cool-white fluorescent light, photon flux of 90 μmol m^{-2} s^{-1}) in pots containing a mixture of organic substrate and vermiculite (3:1 v/v) or in Petri dishes containing Murashige and Skoog medium supplemented with 1% sucrose (GM) and solidified with 0.8% (w/v) agar. Low-temperature treatment for gene expression, subcellular localization, and immunoblot analyses was performed by transferring plants growing on soil or in Petri dishes under control conditions to a growth chamber set to 4°C for different times under long-day photoperiods with a photon flux of 40 mmol m^{-2} s^{-1}. Tolerance to freezing temperatures was determined on 2-week-old plants grown on soil as described by Catalá et al. (2011). Data reported are expressed as means of three independent experiments with 50 plants each.

Anthocyanin Quantification

Anthocyanins were extracted from *Arabidopsis* leaves as described by Mancinelli (1990) with minor modifications. In brief, leaves from approximately 100 plants were ground in liquid nitrogen and 100 mg of pulverized tissue were further homogenized in 600 μL of methanol containing 1% HCl (v/v) and maintained for 2 h at 4°C. Suspension particles were removed by centrifugation and chlorophyll extracted with one volume of chloroform. The absorbance of the cleared supernatant was measured at 530 nm, and mean values were obtained from three independent experiments.

Microscopy Analysis

Subcellular localization of PFD4-GFP, RGA-GFP, RGL3-GFP, HY5-YFP, and COP1-YFP fusion proteins was performed by confocal microscopy in 6-day-old transgenic seedlings grown under control conditions or subjected to stress as described above. Reconstitution of YFP activity in BiFC experiments was also analyzed by confocal microscopy. Images were collected using a confocal laser spectral microscope TCS SP5 (Leica Microsystems, Wetzlar, Germany). The excitation line for imaging GFP and YFP fusions were 488 nm and 514 nm, respectively.

For cell visualization by scanning electron microscopy (SEM), 6-day-old seedlings were fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) overnight at 4°C and then dehydrated in ethanol series. Tissues were critical-point-dried in liquid CO_2, sputter-coated with gold, and analyzed with a Philips XL 30 FEG scanning electron microscope.

Visualization of Microtubules

Cortical MTs in root-elongating cells from 6-day-old WT, *pfd4*, and *c-pfd4* seedlings grown under control conditions or exposed to 4°C were visualized by whole-mount immunolocalization, essentially as described by Baskin et al. (1999). Seedlings were fixed in 4% paraformaldehyde and 1% glutaraldehyde in PME buffer (50 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$ [pH 6.9]) for 1 h at 20°C or 4°C, depending on the treatment before fixation. After fixation, seedlings were rinsed 3 × 10 min in PME buffer, digested with 0.5% pectolyase in PBS for 20 min, and rinsed 3 × 5 min in 10% glycerol and 0.2% Triton X-100 in PME. Cells were extracted with methanol at −20°C for 15 min, rehydrated by rinsing 3 × 5 min in PBS, incubated for 20 min with $NaBH_4$ to eliminate autofluorescence from glutaraldehyde, and rinsed in PBS for 30 min. Root segments were incubated overnight at room temperature with anti α-tubulin DM1A mouse monoclonal antibody (BioNova, 1:200 dilution in PBS), washed 3 × 5 min with PBS, and incubated for 2 h at 30°C with the secondary antibody Alexa Fluor 488 goat anti-mouse immunoglobulin G (A-11017; Molecular probes) used at a 1:200 dilution in PBS. Samples were washed 5 × 5 min with PBS, mounted in 50% glycerol in PBS, and analyzed by confocal microscopy.

Immunoblot Analysis and Nuclear/Cytoplasmic Fractionation

Total proteins were extracted from 2-week-old plants grown under control conditions or exposed to 4°C for the indicated times and quantified by Bradford assay. Ten micrograms was then loaded and fractionated by 15% SDS–PAGE for immunoblotting analysis (Catalá et al., 2011). For PFD4-GFP fusion detection, monoclonal anti-GFP (ab290, Abcam, 1:5000) was employed. A polyclonal anti-HY5 (Ad-16, Santa Cruz Biotechnology, 1:1000) was used for HY5 measurements. Anti-COP1 was kindly provided by Xing Wang-Deng (Yale, USA, 1:500). Monoclonal anti α-tubulin DM1A (NeoMarkers, 1:5000) and anti β-tubulin TUB2.1 (Sigma, 1:5000) were used for analysis of tubulin proteins.

Subcellular fractionation was performed as reported previously (Locascio et al., 2013) using extracts from 2-week-old plants growth under control conditions or subsequently exposed to 4°C for 2 days. Isolated proteins were separated by 15% SDS–PAGE and analyzed by immunoblotting (Catalá et al., 2011) using anti-GFP for PFD4-GFP detection (see above) or polyclonal anti-H3 (sc-10809, Santa Cruz, 1:2000) for control of the nuclear fraction.

In all cases, horseradish peroxidase-conjugated secondary antibodies (1:10 000) were employed for primary antibody detection. Coomassie brilliant blue staining of the large subunit of Rubisco was employed as a loading control. All assays were realized in triplicate employing three independent protein samples.

Protein Stability, Affinity Purification of Ubiquitinated Proteins, and *In Vitro* HY5 Ubiquitination Assay

For protein stability experiments, total proteins were extracted from cold-treated 2-week-old plants incubated in GM liquid medium supplemented with 200 μg/mL cycloheximide at 4°C for different times after application of vacuum for 10 min.

Isolation of ubiquitinated proteins was performed as described by Irigoyen et al. (2014), employing extracts from plants exposed for 2 days at 4°C and subsequently incubated for 8 h in GM liquid medium supplemented with 50 μM MG132 at 4°C. Isolated proteins were separated in parallel by 10% and 6% SDS–PAGE and analyzed by immunoblotting using anti-HY5 (see above) or anti-Ub (Boston Biochem, 1:1000) to detect ubiquitinated proteins as a control of the experiments, respectively.

In vitro ubiquitination assays were performed as previously reported (Yu et al., 2008) with minor modifications. Ubiquitination reaction mixtures contained 50 ng of yeast E1 (Boston Biochem), 50 ng of rice His-Rad6 E2 (Yamamoto et al., 2004), 5 μg of HA-labeled plus 5 μg of unlabeled ubiquitin (Boston Biochem), 150 ng of GST-HY5, and 2 μg of MBP-COP1 (previously incubated with 20 μM $ZnCl_2$) in 30 μL of reaction buffer (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT). Equal amounts of immunopurified PFD4-GFP or GFP were added to the reactions. After 2 h of incubation at 30°C, reaction mixtures were stopped by adding 30 μL of SDS–PAGE sample buffer (80 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol), and a half of the mixtures (30 μL) were boiled for 5 min and separated by 7.5% SDS–PAGE. GST-HY5 and ubiquitinated MBP-COP1 were detected using anti-HY5 (see above) and anti-HA (Roche) antibodies, respectively. Similarly, 10 μL of the mixtures was used to detect PFD4-GFP and GFP recombinant proteins with an anti-GFP antibody (see above).

All assays were performed in triplicate employing three independent protein samples.

Western Blot Quantifications

ImageJ software (https://imagej.nih.gov/ij/download.html) was used to quantify blot signals prior to statistical analysis (see below). In all cases, three independent replicates were quantified.

Gene Expression Analysis and Array Experiments

For gene expression, qPCR experiments were performed as described by Catalá et al. (2011). Primers used are listed in Supplemental Table 3. All

reactions were realized in triplicate employing three independent RNA samples.

For microarray experiments, total RNA was extracted from 2-week-old WT and *pfd4* plants exposed to 4°C for 1 day using TRIzol reagent (Life Technologies) and cleaned with an RNeasy Plant Mini Kit (Qiagen). Three biological replicates were independently hybridized per transcriptome comparison. RNA amplification and labeling were carried out as reported by Goda et al. (2008). Hybridizations were performed on Agilent *Arabidopsis* Oligo Microarrays v4 (catalog no. G2519F-V4021169) in accordance with the manufacturer's specifications. The statistical significance of the results was evaluated with FIESTA software (http://bioinfogp.cnb.csic.es). Genes with an FDR value of ≤ 0.1 and a fold change of ≥ 2 were selected for further consideration. Data from these microarray experiments have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GEO: GSE90746.

Statistical Analysis

Datasets were analyzed using Prism 6 software (GraphPad Software, USA). Comparisons between two groups were made using Student's *t*-test. Comparisons between multiple groups were made using one-way ANOVA and Dunnett's post hoc test. The values of control conditions or WTs were considered as references.

ACCESSION NUMBERS

Sequence data from this article can be found in the GenBank data library under accession numbers AT2G07340 (PFD1), AT3G22480 (PFD2), AT5G49510 (PFD3), AT1G08780 (PFD4), AT5G23290 (PFD5), AT1G29990 (PFD6), AT1G56650 (PAP1), AT1G66390 (PAP2), AT5G42800 (DFR), AT4G22880 (LDOX), AT5G07990 (TT7), AT5G54060 (UF3GT), AT4G14090, AT5G17220 (TT19), AT3G29590 (AT5MAT), AT4G22870, AT5G11260 (HY5), AT2G32950 (COP1), AT2G01570 (RGA), and AT5G17490 (RGL3).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

C.P.-R., M.A.R.-M., E.I., V.R., and J.S. conceived the experiments. C.P.-R., M.A.R.-M., and E.I. performed the experiments. C.P.-R., M.A.R.-M., E.I., V.R., and J.S. analyzed the data. C.P.-R. and J.S. wrote the manuscript.

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