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ORIGINAL PAPER

Pirin1 Is a Non-Circadian Regulated Transcript and Protein, but Highly Responsive to Light/Dark Periods in the Seed-to-Seedling Transition in *Arabidopsis thaliana*

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Abstract As non-motile organisms, plants must respond to constantly changing environmental conditions through dynamic means. The *Arabidopsis thaliana* Pirin1 (PRN1) is an enzyme that regulates the UV-screening compound quercetin and exhibits multiple light-dependent mutant phenotypes. However, regulation of the *PRN1* gene or protein is not well studied. Herein, we investigated the light regulation of expression of the *PRN1* transcript and protein early in development under multiple light conditions (constant darkness, constant white light, 12:12, 16:8, and circadian conditions). *PRN1* transcript was more highly expressed in continuous darkness than in continuous light or 16:8 conditions in 7-day seedlings. However, in these identical samples, protein detected by a specific antibody revealed quite different protein band patterns, with larger bands than that observed for in vitro-translated PRN1 alone. In entrainment studies, exploration of 12:12 expression indicated that transcript generally was reduced in "evening" of the photoperiod. PRN1 protein expression revealed multiband patterns, varying over time and light conditions. *PRN1* transcript did not display typical diurnal or circadian regulation; protein from the same samples indicated unique patterns in the free-running light versus free-running dark. Purified in vitro-translated PRN1 protein briefly treated with cell extract produced higher molecular mass bands, indicating posttranslational modification. *prn1* mutant data indicated that full transcript was not produced, and any protein resulting was likely to be targeted to the proteasome; data indicated that for wild-type (WT) PRN1, the lower molecular weight bands (~36–40 kD) were likely active protein. RNA-seq analysis of 6-day-old dark-grown WT versus *prn1* mutant seedlings indicated few significantly altered genes. High sequence conservation of PRN1 among plants indicates that Arabidopsis is a model system to understand its regulation.

Keywords Pirin · Light · Seedling · Development · Posttranslational · Transcript · Ubiquitin

Introduction

As sessile organisms, plants must be capable of responding to an array of changing environmental conditions, including daily oscillations of dark and light cues, in order to survive and reproduce. Responses to abiotic and biotic signals often include alterations in gene expression to produce gene products that help the plant adapt to its environment. Many plant genes are specifically responsive to light cues. In the plant *Arabidopsis thaliana* (Arabidopsis), for example, 20 % of the genome is estimated to be regulated by white light, as analyzed by microarray analysis (Jiao et al. 2005). In addition to inducing transformative developmental changes in early seedling development, light is also perceived diurnally by a plant throughout its life cycle to regulate important biological functions such as photosynthesis. Additionally, light provides signals to entrain the plant circadian clock, and many plant genes are directly regulated by endogenous circadian rhythms. By global transcriptome analysis, approximately one third of Arabidopsis transcripts are estimated to be circadian regulated (Covington et al. 2008).

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Pirin proteins are highly conserved members of the cupin superfamily (Dunwell et al. 2001), found in prokaryotes, fungi, plants, and expressed at low levels in all examined cell types in mammals (Wendler et al. 1997). The first identified Pirin homolog in plants was implicated in programmed cell death in tomato (Orzaez et al. 2001). In *A. thaliana*, the Pirin1 (PRN1) protein has several described environmental response functions, including regulation of ABA-induced germination (Lapik and Kaufman 2003), blue light (B) regulation of the light-harvesting chlorophyll-binding (*Lhcb*) transcript (Warpeha et al. 2007), and defense against *Cryptococcus* fungi (Warpeha et al. 2013). *PRN1* is also reported in high-throughput data derived from carbon status changes (Usadel et al. 2008), meta-analysis of microarrays of plant hormone regulation (Nemhauser et al. 2006), and in expression analysis where *PRN1* is induced by drought (Catala et al. 2007). Recently, we have identified that PRN1 also plays important roles in light-directed developmental responses, including responses to high-energy, apoptosis-inducing ultraviolet (UV) irradiation, white light-orientation of the seedling shoot, and overall size of the seedling (Orozco-Nunnelly et al. 2014). Interestingly, PRN1's developmental effects appear to be light-specific, as multiple *prn1* mutant phenotypes (shoot orientation, overall seedling size, and seedling survival) were observed in response to light (white light or UV), but no phenotypic differences were observed in complete darkness (Orozco-Nunnelly et al. 2014). We have also identified a number of hormone-, abiotic-stress-, and light-responsive motifs in the PRN1 promoter (Orozco-Nunnelly et al. 2014).

Pirin proteins are known to possess several activities. The first identified Pirin was reported to interact with the heterotrimeric nuclear factor I/CCAAT box transcription factor NFY (aka NFI/CTF1; HAP) to drive adenovirus DNA replication and polymerase II transcription (Wendler et al. 1997). In Arabidopsis, PRN1 is capable of performing a co-transcription factor activity. PRN1 can act as a light-responsive transcription co-factor, regulating *LhcB* transcript expression in response to blue light via G-protein alpha-subunit (GPA1) interaction, then subsequent interaction with specific NF-Y proteins (Warpeha et al. 2007). PRN1 also possesses an enzymatic activity as a quercetinase (Orozco-Nunnelly et al. 2014), breaking down quercetin, a UV-screening and antioxidant flavonoid compound reviewed by Agati et al. (2012) and Rozema et al. (2002). Interestingly, quercetin has been shown to have effects on transcription (Boege et al. 1996; Ruiz et al. 2007; Spencer et al. 2003; Ciolino et al. 1999; Xing et al. 2001), indicating that PRN1's quercetinase and transcriptional co-factor activities may actually be linked.

Although PRN1 can act as a B light-regulated co-transcription factor (Warpeha et al. 2007), has several light-specific mutant phenotypes (Orozco-Nunnelly et al. 2014), and has multiple frequently repeated light-responsive cis-regulatory motifs (Orozco-Nunnelly et al. 2014), there is no detailed transcriptional or translational light analysis of PRN1. Lapik and Kaufman reported that *PRN1* transcript levels are upregulated in 6-day-old etiolated seedlings in response to a brief pulse of low-fluence red light (Lapik and Kaufman 2003), but no other aspects of the regulation of PRN1 are known. Due to the light-specific *prn1* phenotypes (and lack of growth response phenotypes in darkness), and PRN1 activities (Orozco-Nunnelly et al. 2014), and due to the prevalence of diurnal or circadian regulation of transcript and proteins in plants in general, we hypothesized that PRN1 is a diurnally and/or circadian-regulated transcript and protein. Additionally, since PRN1 possesses a light-regulated transcription co-factor activity (Warpeha et al. 2007) as well as an enzymatic quercetinase activity (Orozco-Nunnelly et al. 2014), we also hypothesized that PRN1 directly or indirectly regulates genes involved in light and/or circadian responses, and in quercetin metabolism.

To explore the possibility that PRN1 (and therefore its activities) could directly be light-regulated, we undertook a detailed analysis of the PRN1 transcript and protein under multiple light conditions (continuous darkness [Dc], continuous white light [Wc], 12:12, and circadian). Second, we explored and addressed the mode of protein expression under these same light conditions. Last, we performed an RNA-Seq analysis to identify transcripts that were significantly altered in a *prn1* mutant background compared to a wild-type (WT) background. We report herein that *PRN1* is a low expressed transcript and does not display circadian regulation, yet appears responsive to specific light or dark conditions. Prolonged exposure to light appeared to reduce *PRN1* expression, but not PRN1 protein accumulation. Prolonged exposure to darkness appeared to increase transcript, but reduce protein accumulation; however, the type of light conditions were also important (Dc vs 12:12 for example). Once false discovery rate was applied to transcriptomic data of *prn1* mutants compared to WT, few transcripts, albeit involved in metabolism and self-regulation, showed significant change, indicating that PRN1 has very specific impacts on the young seedling. This study reveals an interesting regulatory contrast: a protein that has major impacts on light-directed responses of the cell and whole seedling (Orozco-Nunnelly et al. 2014), whose gene expression does not undergo major changes at the steady-state level under rigorous conditions but whose protein does change over specific light conditions. Such findings argue for a circumspect view of the many levels of regulation influencing light-directed early development.

Materials and Methods

Chemicals All chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

Plant Materials, Seed Stocks, and Accessions Seeds of WT Col-0 *A. thaliana* and mutants carrying a T-DNA insertion within the coding region of *PRN1* (SALK_006939 or SALK_063087) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH) (Alonso et al. 2003). The mutant lines have been reported homozygous null for the insertions. Plants intended for seed stocks were grown in Scott Metromix 200 (Scotts, Marysville, OH) in continuous white light (10^2 μmol m^{-2} s^{-1}) chambers (Lapik and Kaufman 2003). Sequence data from this article can be found in the EMBL/GeneBank data libraries under accession numbers At2g46830 (CCA1), At3g02780 (IPP2), At3g59220 (PRN1), and At4g05320 (UBQ10).

Plant Growth Conditions for Experiments Seedlings of *A. thaliana* WT or insertion mutants were grown on 50-mL 0.8 % agarose phytatrays containing only 0.5× Murashige and Skoog media as described (Lapik and Kaufman 2003). The growth medium contained no added sugars, hormones, vitamins, or other nutrients. For all experiments, seedlings were sterilized in a bleach solution, washed in sterile water, sown on phytatrays, and stratified for 48 h in complete darkness at 4 °C as described (Warpeha et al. 2006), without a light vernalization/treatment. All sterilization, planting, and manipulations were carried out under dim green light (Warpeha et al. 2006). Cold-vernalized seeds were then moved to appropriate dark and/or light conditions, detailed below, and grown between 5 and 8 days in growth chambers as described, depending on the experiment. All were grown at 20 °C.

Light Regulation and MG132 Treatments After a 48-h cold stratification, phytatrays were moved to 20 °C, then were grown in either Dc for 7 days, Wc for 7 days, 16:8 light/dark cycles (16–8) for 7 days, or Dc for 6 days then treated with red light (10 μmol m^{-2} s^{-1}) for 6 h, then returned to Dc for 24 h (Dc + RL). For diurnal experiments, phytatrays were placed in 12 h white light (8 a.m. to 8 p.m.), 12 h dark (8 p.m. to 8 a.m.) [12:12], and on day 6 of growth, aerial portions of seedlings were harvested every 4 h for 24 h. Circadian free-running light experiments were performed in a similar fashion to Hong et al. (2010). Seedlings were entrained in 12:12 conditions for 5 days. On day 6 (8 a.m.), phytatrays were moved to constant light (LL) and harvested (as described above) at 4-h intervals for 68 h in constant white light. For circadian free-running dark, seedlings were entrained in 12:12 for 5 days, and on day 6 (8 a.m.), phytatrays were moved to constant darkness (DD) and harvested (as described above) at 4-h intervals for 68 h in constant darkness. For MG132 experiments, experiments were carried out according to the methods of Jang et al. (2010). After a 48-h cold stratification, phytatrays were moved to Dc. On day 6 in dim green light, seedlings were then sprayed with 300 μL of 25 μM MG132 (Sigma) or DMSO (1:400). Seedlings were either treated with red light (RL) for 6 h (then returned to darkness) or returned to Dc and harvested 24 h later into liquid nitrogen at the indicated time points and used for RNA or protein analysis, as described by Warpeha et al. (2007) or at 10 a.m. for MG132 experiments.

Quantitative PCR Using the samples outlined above, complementary DNA (cDNA) was pre-amplified using TaqMan PreAmp Master Mix (Life Technologies) according to manufacturer's instructions. After the pre-amplification, gene expression quantitative PCR (qPCR) was performed according to manufacturer's instructions on a ViiA™ 7 Real-Time PCR System (Life Technologies), using TaqMan Fast Advanced Master Mix (Life Technologies) and standard pre-designed assays for *PRN1* (At02196797_gH, At02196799_g1, At02163341_gH, At02173357_g1). Steady-state transcript levels of *At3g01345* were also assessed (Life Technologies, TaqMan Assay At02329200_s1). Expression values are relative to the *IPP2* reference gene (Life Technologies, TaqMan Assay At02163341_gH). Data was analyzed by the comparative C_T method (Schmittgen and Livak 2008), which is an established method for analyzing Arabidopsis-derived qPCR data (Sun et al. 2008). IPP2 was used as an internal reference gene.

RNA Extraction, First-Strand Synthesis, and RT-PCR Similar to Hong et al. (2010), total RNA was extracted from liquid nitrogen-frozen, ground plant tissue samples according to the protocol of the RNeasy Plant Mini Kit (QIAGEN, Germantown, MD). Equal concentrations of RNA samples were then converted to cDNA, employing random primers, according to the manufacturer's instructions (Life Technologies, Grand Island, NY). Reverse transcription polymerase chain reactions (RT-PCR) were then run with equal volumes of each cDNA sample using Phusion High-Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA). Primers were designed to amplify the *PRN1* CDS, the *CCA1* CDS (control gene), or a region of the *UBQ10* CDS (normalization gene) as described by Scortecci et al. (2003). Primer sequences are listed in Supplementary Table 2. RT-PCR samples were separated by gel electrophoresis, stained with ethidium bromide, and imaged (AlphaImager® HP System). Bands were quantified using AlphaView™ Stand Alone Software.

Protein Extraction, Gel, Blotting, and Probing All standard protein materials/solutions were purchased from Bio-Rad (Hercules, CA), unless otherwise stated. Protein was extracted from frozen, ground plant tissue samples by adding 1:1 tissue: Protein extraction buffer (PEB) (containing 10 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA pH 8.0, 25 mM Tris–Cl pH 7.2, 0.1 % Sigma protease inhibitor cocktail for plant cell and tissue extract, 0.02 % dithiothreitol [DTT] and 0.06 % β-

mercaptoethanol). Samples were allowed to shake at 225 rpm for 1 h, then sonicated for 1 min at 12 % amplitude, with 10-s sonication then 10-s rest in an ice bath. The samples were then spun at 10,000 rpm, and the supernatant was saved. All samples were quantified using the Bradford assay. Equal concentrations of each protein sample (with 0.05 % BME) was denatured by heat, then separated by standard SDS gel electrophoresis methods. The samples were then transferred to a blotting membrane and blocked with 5 % non-fat dry milk. The samples were washed and probed with a polyclonal anti-PIRIN1 (*Arabidopsis*) antibody, (1:1000 dilution, 5 % non-fat dry milk, 45 rpm, overnight, 4 °C), designed against a unique peptide in the PRN1 protein (N'-PSTEK MTEPK YKELS SLDC) (custom antibody, Proteintech, Chicago, IL). The blots were washed and probed with a Proteintech anti-rabbit secondary antibody (1:20,000 dilution, 5 % non-fat dry milk, 45 rpm, 60 min). The blots were again washed and then incubated with Western Bright™ ECL Western Blotting Detection Kit (Advansta Inc., Menlo Park, CA). In safelight, blots were exposed to HyBlot CL® Autoradiography Film (Denville Scientific Inc., South Plainfield, NJ), and film was developed. The blots were stripped and re-probed with a Sigma monoclonal anti-ACTIN (Plant) antibody and/or an anti-PhyD (aN-17): sc-12710 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX). Semi-quantification of bands was performed using ImageJ software.

Protein Shift Assay PRN1 protein expression was induced by IPTG in C41(DE3) *E. coli* cells from a PET29b vector, containing a C-terminal His tag. PRN1 protein was then affinity purified according to the manufacturer's instructions, using a HisPur Cobalt Purification Kit (Thermo Fisher Scientific Inc.) and subsequently dialyzed in 1× PBS buffer, pH 6.8. Cell extract material was obtained from youngest leaves harvested from ~4-week-old light-grown WT Col-0 plants, ground into an assay buffer (containing 45 mM HEPES [pH 7.2], 150 mM NaCl, 5 mM $MgCl_2$, 0.025× MS, and 0.1 mM DTT), then the homogenate was subjected to a low-speed spin (5000 rpm) and the supernatant retained. Purified PRN1 protein was then treated with this cell extract for 15 min. PRN1 protein samples treated with the supernatant or with the buffer alone were then denatured and analyzed via Western blotting, as described above.

Transcriptome Sequencing and Analysis Total RNA extracted was processed for high-throughput next-generation RNA-seq. RNA was obtained from three independent biological replicates of WT and *prn1* aerial portions of seedlings, where each replicate was composed of ~1000–1200 seedlings. Polyadenylated messenger RNAs (mRNAs) were enriched from total RNA using the Dynabeads® mRNA Purification Kit (Life Technologies). Subsequently, mRNA was processed for sequencing on the Illumina platform using the Apollo 324 library preparation robotic system (IntegenX, Pleasanton, CA) with the PrepX RNA-Seq Library Preparation Kit. Barcoded adapters were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). After the library preparation, sequencing-adapter ligated fragments of 150–500 bp in length were selected using the PippinPrep automated electrophoresis system (Sage Scientific, Beverly, MA); most fragments were 200–300 bp. Libraries were quantitated using the KAPA library quantification kit for Illumina (KAPA Biosystems, Inc., Woburn, MA). Libraries were pooled in equimolar ratio, and the total library was diluted to a concentration of 10 nM. Sequencing was performed using a single lane of 1×100 base reads on an Illumina HiSeq2000 instrument at the High-Throughput Sequencing and Genotyping core facility at the University of Illinois at Urbana-Champaign.

Raw sequence data were processed using the software package CLC genomics workbench 6.0.1 (CLC bio, Denmark). Raw sequences from each sample were trimmed using a Q20 (0.01) threshold, and sequences shorter than 50 bases after trimming were removed from the dataset. Subsequently, trimmed data were analyzed using the RNA-seq algorithm implemented within the CLC genomics workbench, using default parameters for mapping and the annotated *A. thaliana* genome (Col-0 TAIR 10) as reference. Normalized expression values, reads per kilobase per million mapped reads (RPKM), were generated for each gene and each sample as described (Mortazavi et al. 2008). Expression (RPKM) data were further log-transformed and normalized using mean scaling and analyzed using the empirical analysis of differential gene expression (EDGE) implemented within CLC genomics workbench. False discovery rate (FDR) corrected *p* values were also calculated within CLC genomics workbench.

Results

PRN1 Appears to Be a Dark-Abundant, but Low-Expressed Transcript Past investigation of *PRN1* expression at the transcriptional level has been limited to response to a single short pulse of low fluence red or B light in 6- to 7-day-old dark-grown seedlings at one time point, 2 h after light treatments (Lapik and Kaufman 2003). Moreover, evaluation of direct PRN1 protein expression has not been explored. There are indications that PRN1 activities are regulated by light at nuclear, cellular, and whole seedling levels (Lapik and Kaufman 2003; Warpeha et al. 2007; Orozco-Nunnelly et al. 2014), and the *PRN1* promoter exhibits multiple frequently repeated light-responsive cis-regulatory motifs (Orozco-Nunnelly et al. 2014).

To explore *PRN1* expression in response to different lighting regimes of similar age seedlings, seedlings were grown for

7 days in Dc, Dc with a RL treatment on day 6 then harvested 24 h later, grown under 16:8 conditions or Wc. Steady-state level RNA or protein was then extracted from aerial portions of the seedlings on the morning of day 7 at the same time point. qPCR data indicated that *PRN1* transcript accumulated to higher levels in under Dc conditions compared to 16:8 conditions or Wc (Fig. 1a). RL treatment of Dc seedlings did increase detectable transcript, where it was observed that RL could induce transcription (Lapik and Kaufman 2003). The differences between Dc and Dc + RL were significant (two-tailed *t* test, $p=0.032$) but was only significant in a one-tailed test for the other light conditions (shown on figure), showing a trend toward increased transcript in a Dc environment, comparatively.

PRN1 Protein Expression Varies in Response to Specific Light Conditions Using a custom-made polyclonal antibody, PRN1 protein was detected in these same samples (compare Fig. 1a, b). In vitro-expressed PRN1 was used as a positive control and was determined to be ~33–34 kD, above the sequence-predicted PRN1 size of ~31.6 kD. In experimental samples, each light condition produced a protein pattern detected by the specific antibody. Bands corresponding to PRN1 in the 7-day Dc seedling sample ran higher than the in vitro PRN1 control, with the most intense band(s) appearing to run at ~50–52 kD, with two smaller bands estimated at 35 and ~37–38 kD. The Dc + RL sample indicated that the higher band was detected but with greater intensity of the band running at ~37–38 kD. Light conditions with more prolonged light exposure, 16:8 and Wc, also possessed stronger detection of the lower-sized band(s), with Wc exhibiting the highest signal intensity with the same protein load (the image shown is a lesser exposure of the same gel as the other sample lanes; lighter exposure chosen for that lane to enable banding pattern to be seen). The multiple bands pattern, variable with light regime, indicated possible posttranslational modifications. The ExPASy PROSITE database (http://prosite.expasy.org) for PRN1 sequence indicates that posttranslational changes, such as N-glycosylation, N-myristoylation, and multiple phosphorylation types (casein kinase II, tyrosine kinase, and protein kinase C) are possible, and known covalent modifications like ubiquitin or ubiquitin-like modifications are possible. Western blots of WT material with either a pre-blocked antibody or with only the secondary

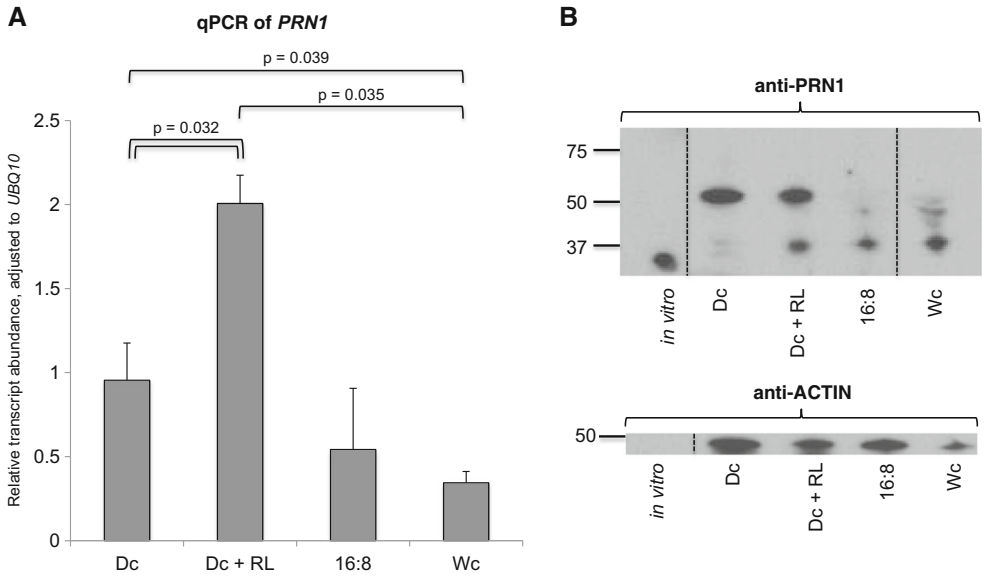

Fig. 1 Expression of *PRN1* transcript and protein is dependent on light regime. **a** Changes in steady-state *PRN1* transcript levels in different light regimes. Aerial portions of 7-day-old WT dark-grown (Dc), dark-grown with a red light treatment (Dc + RL), 16:8-grown (16:8), or white light-grown (Wc) seedlings were harvested into liquid nitrogen. RNA was extracted, converted to cDNA, and used in qPCR reactions (as described in the "Materials and Methods" section) to determine steady-state transcript levels of *PRN1* (Life Technologies, TaqMan Assay At02196797_gH). Expression values are relative to the *UBQ10* reference gene (Life Technologies, TaqMan Assay At02358313_s1). Mean values are displayed with SEM ($n=2$–4 independent samples, with 500–750 seedlings per sample). Significance was assessed by a two-tailed (*double brackets*) Student's *t* test, with exact *p* values displayed above brackets. 16:8 and Wc regime comparisons shown failed to reach significance under two-tailed test, but differences indicate the trend (one-tailed test; *single bracket*). **b** PRN1 protein band patterns in different light regimes. Aerial portions of 7-day-old dark-grown (Dc), dark-grown with a red light treatment (Dc + RL), 16:8-grown (16:8), or white-light-grown (Wc) WT seedlings were harvested into liquid nitrogen (at 10 a.m.). Protein was extracted, separated by gel electrophoresis, transferred to blotting membrane, and probed with either an anti-PRN1 antibody (*top panel*) or an anti-ACTIN antibody (*bottom panel*). In vitro-translated PRN1 (in vitro) was used here as a control. $n=2$ independent samples, with 500–750 seedlings per sample. One representative blot is shown with protein size listed on the left in kD. *Black dashed lines* are used to indicate representative blots that were pieced together for this composite image; however, the Wc lanes is the same gel/lane as shown just a lighter exposure as Wc induced much more protein, and it was not possible to get comparable exposure to Dc if same protein level was loaded (all loads equal protein concentration)

antibody indicated specificity of the antibody (Supplementary Fig. 1).

PRN1 Transcript Accumulation and Protein Accumulation Vary Distinctly in Different Photoperiods In order to better understand PRN1's light responsiveness, WT seedlings were entrained under short-day 12:12 (12-h light/12-h dark) conditions to 6 days old, then samples were collected every 4 h. The steady-state level of *PRN1* transcript was assessed. Corresponding levels of PRN1 protein were also assessed on day 6 to 7 at the same time points. qPCR analysis showed that *PRN1* transcript levels increased in day from 4 a.m. to 12 noon, decreasing by 4 p.m. and were decreased in the night time points (Fig. 2a). Data are shown compared to *CCA1*, an early-morning-expressed transcript (Wang and Tobin 1998), used hereafter as a control (Fig. 2b). *CCA1* expression was observed at much higher expression than *PRN1* (standardized to a different gene control due to the huge differences), confirming that *PRN1* appears to be a low-expressed transcript; the low expression also indicated elsewhere by microscopy (Orozco-Nunnelly et al. 2014). While transcript had an increased trend in accumulation in daylight hours (8 a.m. through transition to darkness at 8 p.m.), protein decreased by midnight and 4 a.m. points, where expression at all detected sizes was lower (Fig. 2c). Interestingly, at 8 a.m. through to 8 p.m., total band intensity increased, then a downward trend was observed after 8 p.m. (the time when lights go off) at 12 a.m. and 4 a.m. Quantitation on ImageJ of total bands for the replicates is indicated in Fig. 2d.

Potential circadian regulation of the *PRN1* transcript and protein was also explored using described methods (Hong et al. 2010). After entrainment, 5-day-old seedlings were transferred to either LL (free-running white light) or DD (free-running darkness). At time point 0 h (transfer) and every 4 h, from 0 to 68 h, steady-state level RNA and protein were extracted from seedling aerial portions. RT-PCR analysis showed that the *PRN1* transcript accumulation did not exhibit a circadian regulation in free-running light or free-running dark conditions compared to CCA1 control (Supplementary Figs. 2 and 3). The protein accumulation data indicated that in LL conditions, protein expression of the identified sizes were observed fairly consistently, with lower bands (34–40 kD) appearing at most time points, with protein expression at every time point. Conversely, in the free-running dark (DD), after the first 8 h in darkness, only once approximately every 24 h were any lower bands detected at all. Interestingly, the 50-kD band was always observed but still much less detectible after the first 24 h.

The PRN1 In Vitro-Translated Protein Can Be Modified by Cell Extract of WT Leaves, Resulting in Higher-Mass Migrating Bands Potential posttranslational modification was tested by treating in vitro-translated PRN1 (lane 3) with extract of live cells, the supernatant fraction obtained from WT leaves (cell debris removed; lane 1). After the purified PRN1 protein was incubated with cell extract for 15 min, it was denatured, then samples were separated by gel electrophoresis. The samples were transferred to membrane, then probed with anti-PRN1 antibody, shown in Fig. 3. This treatment (in vitro translated + Sup.) resulted in multiple bands detected by anti-PRN1, at very similar sizes to the bands running at higher mass for in vivo samples, as shown in Fig. 1. There were no bands observed in the *extract supernatant* only lane (Sup. Only; lane 1), indicating that these higher mass bands in lane 2 were indeed modified in vitro-translated PRN1.

prn1 Mutant Does Not Produce Full-Length Transcript, but Individual Exons Are Transcribed, Producing a Different Profile of Protein Detection by Anti-PRN1 *prn1* mutants were reported as null (Lapik and Kaufman 2003). Subsequent studies explored mutant phenotypes and complementation. Typically, investigators do the standard genotyping to look at full cds transcription (which had shown null results for this gene), but it was observed for *prn1* mutants that exons transcribed around the insertion was possible. We further investigated the nature of the mutation, and in retyped the *prn1* mutant based on ABRC/TAIR recommendations and SALK standard primers, shown in Fig. 4. Genomic and cDNA full-length sequence was not detected. Sequence was detected in WT and at higher levels in the overexpressor *35S*::PRN1 (in wt background) seed line, as could be expected (Fig. 4b). The insertion was detected using the recommended LBb1.3 primer and RP primer, and data indicate that the insertion is homozygous. The insertion is in exon 4, and from doing PCR with exon-junction primers, we detected that individual pieces of the coding sequence is upregulated in *prn1* mutants compared to WT (Supplementary Fig. 4). The 35S promoter based on its orientation would not read through the coding region *PRN1*.

Two different insertion lines of *prn1* were grown in Dc and treated in an identical manner to samples described for Fig. 1, and seedlings harvested on the morning of day 7, protein extracted, transferred to membrane, and probed with anti-PRN1, also in the same manner as described for data in Fig. 1. Mutant lines characteristically and reproducibly showed a heavy band at ~50 kD and very little to no visible bands at any lower size, even with maximum exposure of the immunoblot.

MG132 Experimental Data Indicate that PRN1 Is Targeted to the Proteasome MG132 is a well-known cell-permeable proteasome inhibitor and as such, reduces the degradation of proteins that have been ubiquitin-conjugated in cells. We explored effects of MG132 on WT and *prn1* mutant seedlings (aerial portions) of Dc seedlings, with or without a RL treatment following MG132 incubation or DMSO only (control) according to the methods of Jang et al. (2010). Samples shown

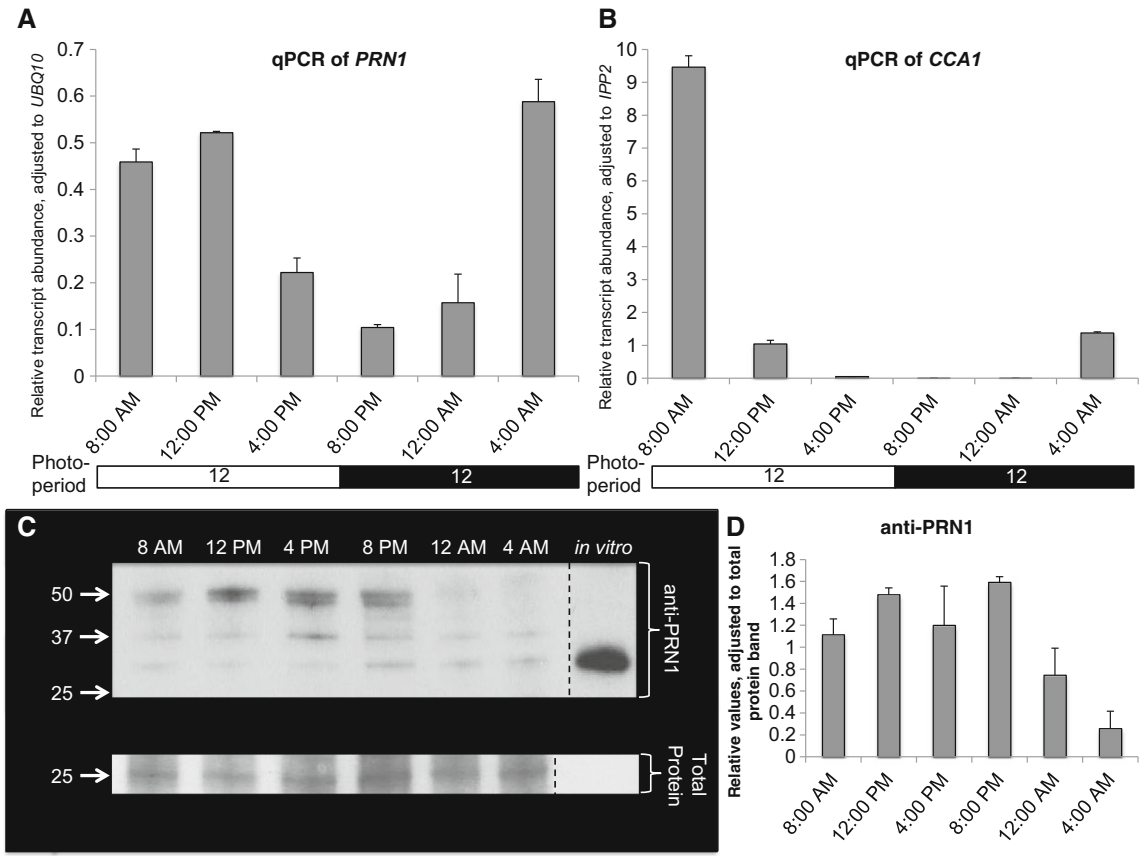

Fig. 2 Responses of PRN1 to 12:12 growth regime. **a** Changes in steady-state *PRN1* transcript levels in diurnal 12:12 conditions. Aerial portions of 5-day-entrained (12-h light [8 a.m. to 8 p.m.]/12-h dark [8 p.m. to 8 a.m.]) seedlings were harvested into liquid nitrogen at the indicated time points. RNA was extracted, converted to cDNA, and used in qPCR reactions (as described in the "Materials and Methods" section) to determine steady-state transcript levels of *PRN1* (Life Technologies, TaqMan Assay At02196797_gH). Expression values are relative to the *UBQ10* reference gene (Life Technologies, TaqMan Assay At02358313_s1). Mean values are displayed with SEM (n=2 independent samples, with 500–750 seedlings per sample). Periods of light and darkness (photoperiod) are represented, respectively, by the *white bar* and *black bar*. **b** Changes in steady-state *CCA1* transcript levels in diurnal 12:12 conditions. Using the same samples and conditions as in **a**, steady-state transcript levels of *CCA1* (Life Technologies, TaqMan Assay At02173357_g1) were determined. Expression values are relative to the *IPP2* reference gene (Life Technologies, TaqMan Assay At02163341_gH). Mean values are displayed with SEM (n=2 independent samples, with 500–750 seedlings per sample). Periods of light and darkness (photoperiod) are represented, respectively, by the *white bar* and *black bar*. **c** PRN1 protein levels in diurnal 12:12 conditions. Aerial portions of 5-day-entrained (12-h light [8 a.m. to 8 p.m.]/12-h dark [8 p.m. to 8 a.m.]) seedlings were harvested into liquid nitrogen at the indicated time points. Protein was extracted, separated by gel electrophoresis, transferred to blotting membrane, and probed with an anti-PRN1 antibody (*top panel*) or stained for total protein (*bottom panel*). In vitro-translated PRN1 (in vitro) was used here as a control. n=2 independent samples, with 500–750 seedlings per sample. One representative blot is shown with protein size indicated in kD. *Black dashed lines* are used to indicate representative blots that were pieced together for this composite image. **d** Quantification of total PRN1 in diurnal 12:12 conditions. Semi-quantification of total PRN1 protein was performed using ImageJ software on the immunoblot, where values are relative to a band. Mean values are displayed with SEM (n=2 independent samples, with 500–750 seedlings per sample)

(lanes 1–7) are identical samples in the three panels displayed (Fig. 5). WT seedlings show the characteristic multiband pattern in control conditions (lane 4), and with MG132, the upper band increases accumulation compared to lower bands (lane 5). RL treatment also shows this result, with considerably more intensity in the upper band (lanes 6 and 7), indicating that detected protein at ~50–52 kD is targeted to the proteasome. The band size ~45 kD could have some sort of ubiquitin modification, as it is also accumulating.

prn1 mutant seedlings actually exhibit the opposite responses of WT in these experiments, which if the protein were non-functional, we would expect this result. Normally (in control conditions), *prn1* mutants show a majority, if not all protein is localized to the ~50 kD size (lane 2), which based on MG132 treatment for WT, we propose this is the ubiquitinated protein destined for the proteasome. Treatment with MG132 results in less intensity of the upper band, and the appearance of lower bands perhaps reflecting newly translated material ubiquitinated right away due to defects (mutation) and accumulating or backing up (cannot be targeted or modified) (lane 3).

The pattern of data obtained are similar to the response detected for the PhyD control (middle panel), originally

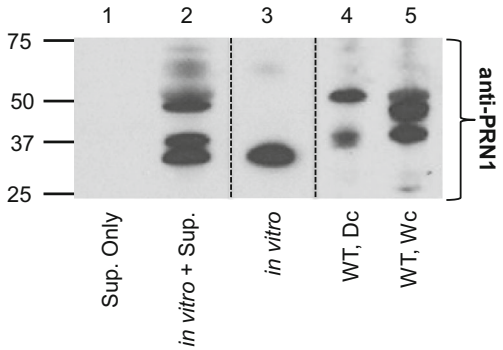

Fig. 3 PRN1 protein shift assay indicates that enzymes necessary are available in the cell extract to effect posttranslational modifications on PRN1 protein. In vitro-translated PRN1 was treated with a clarified cell extract (supernatant of a low speed spin) of 3-week-old Wc-grown WT leaves that were ground into buffer (described in the "Materials and Methods" section). After a low-speed spin, the supernatant only (Sup. Only), PRN1-treated with supernatant (in vitro + Sup.) or PRN1 protein alone (in vitro) were separated by gel electrophoresis, transferred to blotting membrane, and probed with an anti-PRN1 antibody. Seven-day-old dark-grown (Dc) or white-light-grown (Wc) WT seedlings were also used to show in vivo PRN1 banding patterns. $n=2$ independent samples. One representative blot is shown with *lanes 1–5* and protein size listed on the left in kD. *Black dashed lines* are used to indicate representative blots that were pieced together for this composite image

published by Jang et al. (2010). PhyD antisera indicated a similar intensity increase in the higher molecular weight band, for both WT and *prn1* mutants treated with MG132.

RNA-seq Analysis Reveals Multiple Differentially Regulated Transcripts in 6-Day-Old Seedlings (prn1 Compared WT), but Few Transcripts That Change Dramatically To understand more about PRN1 impact, we conducted an RNA-seq analysis on aerial portions of 6-day-old dark-grown WT seedlings to compare to values measured for *prn1* mutant seedlings. Stringent analysis indicates that 26 genes have differential expression of 1.5× or higher (in either direction) (Supplementary Table 1), but accounting for false discovery rate, only two transcripts are dramatically different in *prn1* mutants compared to WT. The top hit on the transcriptome was the *PRN1* gene itself. *prn1* (*prn1*/WT) indicated such high expression that we viewed the raw data—where the vast majority of the signal was the sixth (and most 3') exon. This increase in the sixth exon of *PRN1* transcript was confirmed by qPCR experiments (Supplementary Fig. 4). Differentially regulated genes identified in the RNA-seq are involved in responding to different biotic and abiotic stresses, some of which include responses to bacteria, hormones, metals, salt, and light (Supplementary Table 1). Several of these differentially regulated genes were assayed via qPCR analysis. Transcript *At3g01345* (unnamed hydrolase of O-glycosyl compounds) was confirmed to be significantly increased; $p=0.003$ (two-tailed t test) (Supplementary Fig. 5), whereas transcript *At3g22231* (*PCC1*) was not significantly altered between WT and *prn1* mutant backgrounds (not shown). The most downregulated transcripts, *At1G53480* (*MRD1*), and *At5G53740* (unknown function protein) (Supplementary Table 1) were also evaluated via qPCR but did not produce enough transcript and were classed as "undetermined" due to such low expression in *prn1* (data not shown). The lack of differences in Dc (no light at any time) of WT compared to *prn1* is not unsurprising given the lack of phenotypic differences observed in many respects reported in Orozco-Nunnelly et al., 2014.

Discussion

PRN1 Transcript and Protein Accumulate in 7-Day-Old Dark-Grown Seedlings, Perhaps as a Priming Mechanism to Prepare the Plant for Light-Directed Growth *PRN1* transcript accumulated to higher levels in 7-day-old Dc-grown WT seedlings, compared to 7-day-old Wc or 16:8-grown WT seedlings when examined at a morning time point (Fig. 1). Protein, however, for these same points, showed light-condition-specific banding patterns, with bands ranged from 34 to 52 kD, generally 3–4 bands or mainly 50–52 kD. Based on MG132 data, where the 50–52 kD band(s) hyper-accumulated in WT, we inferred that these size(s) are targeted to the proteasome, and the larger migration size may result from polyubiquitination. After translation, the ~32-kD protein may be modified in other ways that affect its running size and activity of the protein.

Under 12:12-entrained conditions, transcript was lowest as plants headed into the afternoon (4 p.m.), reaching a low point at night (8 p.m. and 12 a.m.) and was increased in accumulation before the transition to day. This pattern was not mirrored by protein accumulation, where overall bands were semi-quantitatively summed to reveal that except for longer periods in the dark (midnight and 4 a.m., i.e., in darkness 4 and 8 h, respectively), protein level corresponding to PRN1 remained high. Interestingly, similar-aged *prn1* mutants and overexpressor (35S::PRN1 in WT background) seedlings have white light-specific phenotypes (Orozco-Nunnelly et al. 2014), but there are no score-able "growth" phenotypes in Dc seedlings of the same age. For example, 6-day-old 16:8-grown *prn1* mutant seedlings are smaller in size and exhibit a shoot disorientation defect, compared to WT seedlings, but dark-grown *prn1* mutant seedlings of the same age do not show any phenotype, i.e., they are not different from comparable WT seedlings (Orozco-Nunnelly et al. 2014). In addition, 6-day-old 16:8-grown 35S::PRN1 (in WT) overexpressor seedlings also display a defect in shoot orientation and appear even smaller in overall size than the mutant, whereas dark-grown 35S::PRN1 seedlings appear similar to WT seedlings in these phenotypes (Orozco-Nunnelly et al. 2014). Hence, the light cues have a major effect on this protein and the subsequent physiological actions and functions.

One phenotype that Dc *prn1* mutant seedlings do have is that they overaccumulate quercetins (Orozco-Nunnelly et al. 2014). The 6-day-old *prn1* mutant dark-grown seedlings

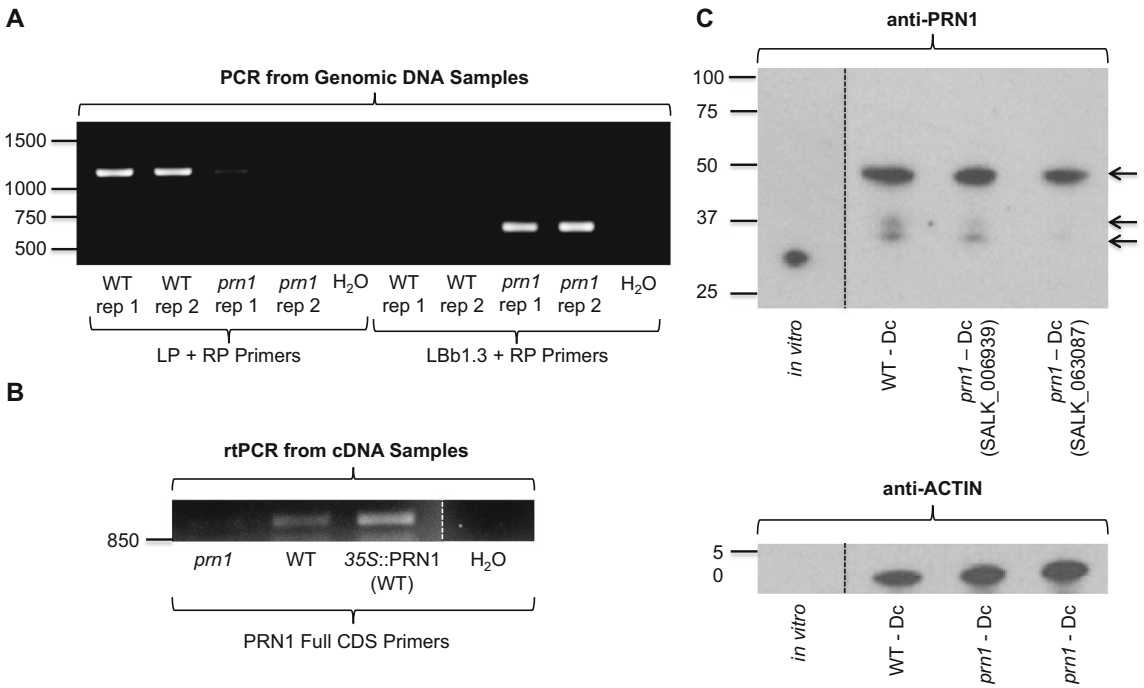

Fig. 4 *prn1* does not produce full-length transcript; antibody detects a different protein pattern. **a** PCR confirmation of T-DNA insertion in *prn1* mutant plants. To confirm the *prn1* T-DNA insertion, leaves of light-grown WT or *prn1* mutant seedlings were harvested into liquid nitrogen, genomic DNA was extracted and PCR reactions were performed with primer sets to amplify the WT version of PRN1 (LP + RP primers) or the T-DNA PRN1 insertion (LBb1.3 + RP primers) (n=2 independent samples, with leaves from five to ten different plants per sample). Both replicates are shown (rep 1 and rep 2), with DNA sizes listed on the left in bp. **b** PCR confirmation loss of full-length *PRN1* transcript in of *prn1* plants. To analyze *PRN1* transcript levels, RNA was extracted from light-grown *prn1* mutant, WT, or PRN1 overexpressing (*35S*::PRN1 [WT]) seedlings, and converted to cDNA. Reverse transcription PCR (RT-PCR) reactions were then performed using primers to determine presence of the full length *PRN1* CDS (n=2 independent samples, with leaves from five to ten different plants per sample). One replicate is shown, with DNA sizes listed on the left in bp. The *white dashed line* is used to separate parts of the gel that were pieced together for this composite image. **c** Changes in PRN1 protein banding patterns in *prn1* mutant versus WT seedlings. Aerial portions of 7-day-old dark-grown (Dc) *prn1* mutant (insertion lines SALK_006939, or SALK_063087) or WT seedlings were harvested into liquid nitrogen and prepared for immunoblotting as described in Fig. 1, probed with either an anti-PRN1 antibody (*top panel*) or an anti-ACTIN antibody (*bottom panel*). Blots were exposed for maximum time to ensure any bands could be visualized. In vitro-translated PRN1 (in vitro) was used here as a control. n=4 independent samples, with 500–750 seedlings per sample. One representative blot is shown with protein size indicated in kD. *Black arrows* emphasize PRN1 protein bands of interest, and *black dashed lines* are used to indicate lanes pieced together for this composite image

survive apoptosis-inducing levels of UV-C irradiation that kill WT seedlings (Orozco-Nunnelly et al. 2014) due to this specific defect, as one function of PRN1 protein is to cleave quercetin. These light-specific and Dc phenotypes indicate that PRN1 is playing an important role in the light-regulated seed-to-seedling developmental transition. The PRN1 transcript and protein may be specifically accumulating in specific long periods of darkness as a priming mechanism, to help prepare the seedling for the developmental switch once light is detected. A similar mechanism has been reported for some specific mitogen-activated protein kinases, in which mRNA and inactive proteins accumulate to help prepare the plant to more efficiently respond to different biotic and abiotic stresses (Beckers et al. 2009).

The PRN1 Transcript Displays Some Light Condition-Specific Regulation but Does Not Appear to Be Circadian Regulated In circadian free-running light (Supplementary Fig. 2) and free-running dark (Supplementary Fig. 3) conditions, the *PRN1* transcript appears to be expressed with small fluctuations indicated up or down but does not appear to follow distinct diurnal or circadian rhythms as exemplified by *CCA1*. Ultradian patterns of expression are not very common (Baskin 2007). *PRN1* transcript expression deviates little from 1.0, indicating that some transcript is always present in the seedling, regardless of day or night or circadian point. In all transcriptional analyses herein, *PRN1* expression is generally low but never as low as CCA in darkness (night) time points. The continuous presence (albeit low, relatively speaking) and variation in light responses indicate that *PRN1* transcript may respond to environmental changes, which often are sudden and without warning.

The steady-state level of transcript reflects a net result of the harvested tissue (hypocotyl and cotyledon), containing various cell types and the sum of compartments of the cell. Since PRN1 is localized to multiple cellular locations (Orozco-Nunnelly et al. 2014), different pools of PRN1 may exist, responding to local subcellular need or perceived

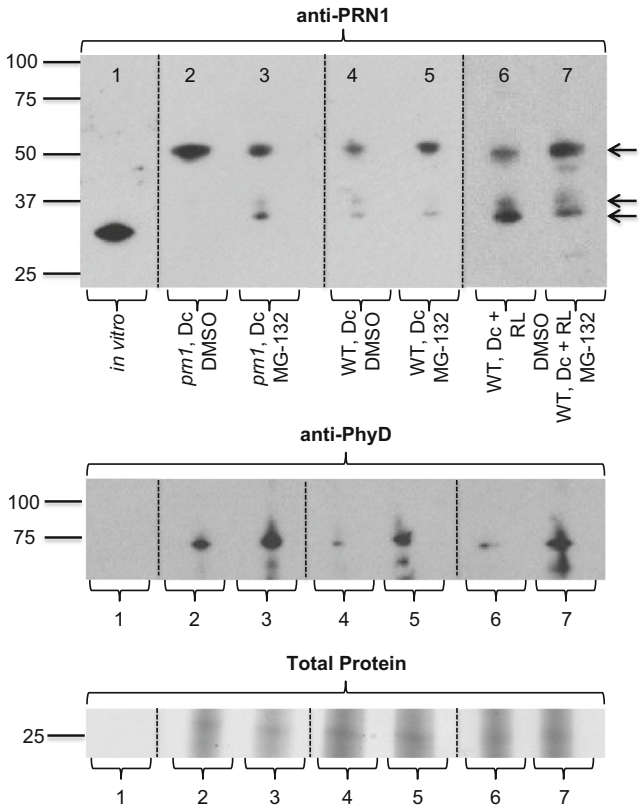

Fig. 5 Changes in PRN1 accumulation with MG132 treatment indicate proteasome targeting. After 6-day Dc seedlings were treated with MG132 or DMSO (control), they were returned to darkness or exposed to RL as described in the "Materials and Methods" section. Twenty-four hours after treatment period, aerial portions of 7-day-old DMSO- or MG132-treated (as described in the "Materials and Methods" section) dark-grown *prn1* mutant (*prn1*, Dc), dark-grown WT (WT, DC), or dark-grown with a red light treatment WT (WT, Dc + RL) seedlings were harvested into liquid nitrogen (10 a.m.). Protein was extracted, separated by gel electrophoresis, transferred to blotting membrane, and identical blots were probed with either an anti-PRN1 antibody (*top panel*) or an anti-PhyD antibody (used as an MG132 experimental control; middle panel), or stained for total protein (*bottom panel*). In vitro-translated PRN1 (in vitro) was used as a control. $n=2$ independent samples, with 500–750 seedlings per sample. One representative blot is shown with *lanes 1–7* (with antibody used to probe indicated) and protein size listed on the left in kD. *Black arrows* emphasize PRN1 protein bands of interest, and *black dashed lines* are used to separate parts of blots that were pieced together for this composite image

external signals. Indeed, one of PRN1's "activities" (co-transcription factor vs quercetinase) may occur in particular locations, or these activities may even be competing in certain areas of the cell or under certain environmental conditions, and certain posttranslational mechanisms may be required to achieve those activities. However, separation of actual activities, i.e., that of transcriptional regulator (Warpeha et al. 2007) versus quercetinase (Orozco-Nunnelly et al. 2014), and how these activities are reflected at the RNA level may be difficult to achieve. Adams et al. have suggested in a review paper that pirins may have a role in protecting transcription machinery from inhibitory effects of quercetin, often present within the nuclear and cell environment (Adams et al. 2007). When considered with data that show quercetin can affect transcription (Boege et al. 1996; Ruiz et al. 2007; Spencer et al. 2003; Ciolino et al. 1999; Xing et al. 2001), it is possible that the transcriptional co-factor and quercetinase actions of PRN1 may be linked. Further studies are in progress to better understand the relationships of these two activities. Studies on the possible posttranslational modifications are in progress in our laboratory.

PRN1 Protein Accumulation and the Length of Darkness Versus Light In free-running light (Supplementary Fig. 2B), bands detected by anti-PRN1 appear at all time points and range from ~30–52 kD. Conversely, in free-running dark (Fig. 3c), PRN1 detection occurs in early time points (0, 4, 8 h), with similar patterns of expression as free-running light, but by 12 h in darkness, only the 50–52-kD band is detectable, with some small exception at 20, 24, and 48 h. PRN1 may accumulate in young seedlings to help prepare the plant for the switch from skotomorphogenesis to photomorphogenesis (i.e., quercetin to scavenge free radicals created by chemical reactions in light, or screening of UV, or transcriptional regulation), but after the seedling is established, PRN1 may no longer be needed in long periods of darkness, or if light is not perceived over a particular length of time, transcription and/or translation may be disrupted. PRN1 has several predicted light-responsive cis-regulatory elements, multiple light-specific mutant and overexpression phenotypes (Orozco-Nunnelly et al. 2014), and acts as a B light-responsive co-transcription factor (Warpeha et al. 2007).

Posttranslational modifications and protein turnover rates may contribute to the observed fluctuations in PRN1 accumulation. This is a common feature of genes that have light regulation or interaction with circadian clock components (reviewed in Farré and Weise 2012; Harms et al. 2004; Gardner et al. 2006). PRN1 expression was indicated by different size and number of bands detected under varied light conditions—different lengths of light and dark and bands were equal or larger than in vitro-translated protein, possibly due to one or more posttranslational modifications, such as predicted N-glycosylation, N-myristoylation, and multiple phosphorylation (casein kinase II, tyrosine kinase, and protein kinase C) sites (http://prosite.expasy.org). The protein may be translated or expressed in select cells or in response to particular light signals or an environmental stimulus, as promoter analysis indicated that PRN1 may be capable of responding to a wide variety of signals (Orozco-Nunnelly et al. 2014). Likewise, since PRN1 appears to be expressed at many time points, but sometimes is not, it is likely undergoing synthesis and turnover, supported by the MG132 data herein, performed in Dc and Dc + RL. Based on the MG132 data, 50–52 KD may represent ubiquitinated-PRN1 forms, to target PRN1 to the proteasome for degradation via

the plant ubiquitin-proteasome pathway (Moon et al. 2004). Further research is currently being conducted to explore this possibility.

PRN1 Protein Presence Affects Its Own Expression, As Well As the Expression of Other Environmental, Structural and Metabolism-Related Genes Six-day-old dark-grown *prn1* mutants exhibit changes in regulation of multiple developmental and environmentally responsive genes, including the upregulation of PRN1's exon junctions that are not interrupted by T-DNA, in particular exon 6 (Supplementary Fig. 1; Supplementary Table 1). It is possible that absence of functioning PRN1 in the *prn1* mutant (most protein detected by anti-PRN1 in *prn1* appears to be ~50–52 kD, Fig. 5) elicits a compensatory upregulation of *PRN1*. It is not known if allosteric molecules or other proteins or even quercetin itself can bind to PRN1 or to an intermediate protein to regulate PRN1 function, and it is unclear how the absence of the PRN1 protein affects normal positive and negative feedback mechanisms.

While several of the differentially regulated genes in the RNA-seq *prn1* mutant to WT comparison (Supplementary Table 1) are of unknown function, those of known function indicate PRN1 involvement in a wide variety of roles. As hypothesized, many of the differentially regulated transcripts have reported actions in metabolism, such as carbohydrate metabolism (At3g01345; unnamed hydrolase of O-glycosyl compounds), oxime metabolism (At4g31500), oxidation/reduction reactions (At5g64120; At5g11330), lignin biosynthesis (At4g11190), and methionine metabolism (At1g53480), although the latter is so low expressed that in individual qPCR the transcript was not detectable. Pirins contain a highly conserved metal-binding motif, and several transcripts important in metal homeostasis and binding (At3g56240, At5g11330 and At2g38390) were downregulated in the *prn1* mutant background. Additionally, several genes with roles in auxin production (At4g31500 and At3g07390) were also downregulated in the *prn1* mutant background. This is consistent with the fact that PRN1 can cleave the antioxidant and UV-screening compound quercetin (Orozco-Nunnelly et al. 2014), which has reported roles as a regulator of auxin accumulation or transport (Brown et al. 2001; Murphy et al. 2000; Kuhn et al. 2011; Lewis et al. 2011). This may also help explain why *prn1* mutant and PRN1-overexpressing seedlings have defects in hypocotyl orientation, when grown in 6-day white light (16:8) (Orozco-Nunnelly et al. 2014). Interestingly, 6-day-old 16:8-grown seedlings that with lack or overexpress PRN1 are smaller in size, compared to WT seedlings (Orozco-Nunnelly et al. 2014). Transcripts involved in maintaining appropriate membrane (At4g15160, At2g07719, and At2g16005) and cell wall (At3g29030, At5g64120, At4g11190, and At1g31580) integrity and size were observed as downregulated in the *prn1* mutant background, but poorly detected. From the most stringent analysis, it seems that PRN1 does not impact many other genes as much as it impacts itself, but it also significantly impacts an enzyme that hydrolyzes O-glycosyl compounds, which could be related to the quercetin "skeleton" in terms of glycolsylation.

PRN1 Is a Regulator of Light-Directed Development From Seed, with Distinct Roles in Young Seedlings, Indicating That It May Be a Good Candidate to Use in Plant Breeding Studies The significance of multiple pirins in plants is still unknown. Unlike humans, who only possess one identified Pirin gene, Pirins are common in plants, with some species having six or more members in the gene family, as indicated by BLAST search (Supplementary Fig. 6). Including PRN1, there are at least four Pirin-related proteins in the Arabidopsis genome (AT1G50590, AT2G43120, AT3G59220, and AT3G59260) (http://www.arabidopsis.org), yet the eFP browser (Winter et al. 2007) indicates that other Arabidopsis pirins are not expressed at the same developmental stages or in response to the same environmental signals. Expression of the PRN1 protein appears to be important in the seed-to-seedling transition, as young *prn1* mutants and PRN1-overexpressing seedlings have distinct light-specific developmental phenotypes (Orozco-Nunnelly et al. 2014), and 6-day-old dark-grown *prn1* mutants exhibit changes in accumulation of multiple developmental and environmentally responsive genes. Hence, it does not appear that the other Arabidopsis Pirin family members can compensate for a lack of PRN1 in the seedling stage.

Due to PRN1's distinct environmental and developmental roles and effects on seedling size, it is a suitable candidate to use for plant breeding studies. By aligning PRN1 to its closest "BLAST" protein matches, phylogenetic trees indicate that several agriculturally important plants, such as rice, soybean, tomato, grape, chickpea, cucumber, and common bean plants, as well as clementine, papaya, peach, and cocoa trees, have high sequence homology to the PRN1 protein (within the 50 closest BLAST matches) (Supplementary Fig. 6A). Other economically important crops, such as corn, potato, sorghum, and strawberry, also possess proteins closely related to PRN1 by sequence homology (within the 100 closest BLAST matches) (Supplementary Fig. 6B). It may, therefore, be worthwhile to apply this knowledge of PRN1 function in early seedling establishment to the context of crop breeding and stress responsiveness.

From this study, PRN1 appears to be responsive to light and dark changes that may occur in the seed-to-seedling transition in *A. thaliana*. PRN1 is accumulating in dark-grown seedlings, likely to help the seedling make the drastic developmental shift from skotomorphogenesis to photomorphogenesis. Constitutive expression of the *PRN1* transcript indicates that PRN1 may be needed at short notice to regulate

multiple light-regulated functions in the cell, and when PRN1 is not needed (such as in long periods of darkness), protein translation may be inhibited or the protein itself be subject to targeted degradation. Moreover, from RNA-seq analysis, PRN1 appears to be impacting the regulation of itself of many other environmentally responsive and metabolism-related genes, highlighting PRN1's important role in early seedling development and responses to the environment.

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