Environmental Stresses Modulate Abundance and Timing of Alternatively Spliced Circadian Transcripts in Arabidopsis

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ABSTRACT

Environmental stresses profoundly altered accumulation of nonsense mRNAs including intron-retaining (IR) transcripts in Arabidopsis. Temporal patterns of stress-induced IR mRNAs were dissected using both oscillating and non-oscillating transcripts. Broad-range thermal cycles triggered a sharp increase in the long IR CCA1 isoforms and altered their phasing to different times of day. Both abiotic and biotic stresses such as drought or Pseudomonas syringae infection induced a similar increase. Thermal stress induced a time delay in accumulation of CCA1 I4Rb transcripts, whereas functional mRNA showed steady oscillations. Our data favor a hypothesis that stress-induced instabilities of the central oscillator can be in part compensated through fluctuations in abundance and out-of-phase oscillations of CCA1 IR transcripts. Taken together, our results support a concept that mRNA abundance can be modulated through altering ratios between functional and nonsense/IR transcripts. SR45 protein specifically bound to the retained CCA1 intron in vitro, suggesting that this splicing factor could be involved in regulation of intron retention. Transcriptomes of nonsense-mediated mRNA decay (NMD)-impaired and heat-stressed plants shared a set of retained introns associated with stress- and defense-inducible transcripts. Constitutive activation of certain stress response networks in an NMD mutant could be linked to disequilibrium between functional and nonsense mRNAs.

Key words: Arabidopsis, alternative splicing, circadian clock, intron retention, nonsense-mediated mRNA decay (NMD), environmental stress


INTRODUCTION

Genome-wide mapping of cellular transcripts revealed that the extent of alternative splicing (AS) in eukaryotes, including plants, has been greatly underestimated. Between 42% and 61% of intron-containing genes in plants (Filichkin et al., 2010; Marquez et al., 2012; Reddy et al., 2013; Staiger and Brown, 2013) and up to 95% in humans (Pan et al., 2008; Sultan et al., 2008) are alternatively spliced. Intron-retaining (IR) events are prevalent in plants (Campbell et al., 2006; Wang and Brendel, 2006), whereas exon skipping is more widespread in animals (Sammeth et al., 2008). Retained intronic sequences alter localization, stability, and translation of transcripts (Jailion et al., 2008; Buckley et al., 2011; Wong et al., 2013). AS can be regulated by developmental stage, cell type, and environmental stress, and may yield transcripts harboring an in-frame premature stop codon.
termination codon (PTC) (Lewis et al., 2003; Lareau et al., 2007). AS that generates PTC-harboring isoforms frequently referred to as unproductive alternative splicing (UAS) (Lewis et al., 2003; Lareau et al., 2007). Depending on transcript features, mRNAs harboring in-frame PTCs may trigger a nonsense-mediated mRNA decay (NMD) pathway or may escape NMD detection. UAS could also lead to production of stable truncated proteins, which either may be detrimental to the cell or interfere with normal function of their full-length counterparts.

Circadian clocks in many organisms operate predominantly through interlocked transcriptional regulatory feedback loops (Pruneda-Paz and Kay, 2010). However, circadian oscillations can persist even in the absence of transcription, and require diverse post-transcriptional regulatory mechanisms including AS (Perez-Santangelo et al., 2013). Deficiencies in splicing machinery components can alter AS patterns and affect plant circadian rhythms. Mutation in SPliceosomal TIMEKEEPER LOCUS1 affects efficiency of intron splicing and induces a long circadian period phenotype in Arabidopsis (Jones et al., 2012). The components of the central circadian regulator represent a unique model system ideally suited for studies of cyclical UAS. First, several plant circadian clock regulatory genes undergo extensive AS to yield relatively abundant nonsense transcripts. Second, depending on transcript features, nonsense circadian mRNAs may be regulated by NMD or escape degradation (Filichkin and Mockler, 2012; James et al., 2012). Finally, environmental stresses can alter AS patterns of the central circadian oscillator components and perturb equilibrium of the PTC-containing and functional mRNAs (Staiger et al., 2003; Schoning et al., 2007; Filichkin et al., 2010; Sanchez et al., 2010; Filichkin and Mockler, 2012; Staiger and Brown, 2013). Splicing patterns of the master circadian regulators CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) pre-mRNAs have been shown to change in response to heat or cold stress (Filichkin et al., 2010; Filichkin and Mockler, 2012; James et al., 2012). Both CCA1 and LHY play important roles in the compensation mechanism of Arabidopsis central circadian regulator that sustains the pace of the circadian clock across a broad range of ambient temperatures (Gould et al., 2006; Salome et al., 2010). Recent data suggested that temperature-induced changes of CCA1 and LHY splicing patterns are also involved in the temperature compensation mechanism of the circadian clock (James et al., 2012).

Under normal physiological conditions, daily oscillations of the nonsense circadian transcripts are typically synchronized in time with the peak abundance of the productive mRNA (termed here symmetric cyclical UAS). In contrast, specific environmental stresses can alter symmetric oscillations of PTC isoforms to asymmetric out-of-phase mode. In this work, we investigate this phenomenon in detail. We present evidence that production of PTC-containing isoforms of mRNAs encoding circadian oscillator proteins is triggered by environmental factors such as thermal stress and pathogen infection.

The retention of a fourth CCA1 intron (referred to here as long or I4R event) in the mRNA is highly conserved across plant phyla, suggesting its functional significance (Filichkin et al., 2010). A homologous long (fifth) intron is also retained in Arabidopsis CCA1 homolog LHY. Increasing evidence points toward important regulatory roles the nonsense transcripts play in plant development, innate immunity, and stress response. However, the specific functions of PTC+ mRNAs in controlling the pace of the circadian oscillator remain poorly understood. This study investigates changes in the expression profiles of key alternatively spliced mRNAs of the circadian oscillator under optimal physiological conditions and under biotic or abiotic stress. AS of the circadian genes occurs in a rapid, cyclical, and reversible manner. In contrast to symmetric UAS, physiologically extreme thermocycles triggered a sharp increase in PTC-containing isoforms of CCA1 and REVEILLE 2 (RVE2) and altered their oscillation profiles to an asymmetric mode. Other environmental stresses such as drought or pathogen infection also sharply increased synthesis of IR CCA1 transcripts. Many circadian (Filichkin and Mockler, 2012; James et al., 2012) and non-circadian Arabidopsis transcripts retaining full introns (Kalyna et al., 2012; Marquez et al., 2012) escape NMD, whereas those with PTC-introducing exons or partial IR events can elicit NMD response (Kalyna et al., 2012; Göhring et al., 2014). Our data suggest that a steady oscillation of protein-coding circadian clock transcripts during environmental stresses can be maintained via altering levels of nonsense isoforms and/or via asymmetric UAS. Our results favor a hypothesis that the reversible channeling of pre-mRNA splicing toward unproductive isoforms allows rapid post-transcriptional adjustments in daily oscillations of functional mRNAs in response to environmental stresses.

RESULTS
Nonsense CCA1 Isoforms Accumulate at Substantial Levels, Escape NMD

AS of Arabidopsis CCA1 pre-mRNA generates two distinct PTC-containing isoforms resulting from the retention of the long (fourth) intron. In the major isoform, I4Ra, the entire long intron is retained, whereas in the shorter I4Rb variant a short intron is spliced via an alternative donor splicing site (Figure 1). To interrogate the daily oscillation profiles of the CCA1 transcripts, we designed several event-specific primer sets (Figure 1 and Supplemental Table 1) using a previously described approach (Filichkin et al., 2010; Filichkin and Mockler, 2012). Both RNA-seq data (Supplemental Figure 1) and the results of quantitative RT–PCR (qRT–PCR) (Figure 2A) suggested that IR CCA1 transcripts accumulate at substantial levels. Together, the I4Ra and I4Rb constituting approximately half of the copies of the functional mRNA (Figure 2). We monitored I4Rb transcript more closely because of its important feature: splicing of a short intron removes the only branch sequence in the long intron of I4Ra (Supplemental Figure 1A). This unique consensus branch site sequence was identified using neural network predictions of splice sites in Arabidopsis thaliana (Hebsgaard et al., 1996) and NetPlantGene Server, as described in Methods. Further analysis of the I4Rb sequence using NetPlantGene Server produced no predicted splicing signals/branch sites, suggesting that further splicing/recycling I4Rb into functional mRNA is improbable.

Arginine methylation of splicesosomal proteins is essential for correct splicing of many pre-mRNAs (Deng et al., 2010),
including several circadian transcripts (Sanchez et al., 2010; James et al., 2012). CCA1 intron retention appeared to be insensitive to the deficiency in ARGININE N-METHYLTRANSFERASE 5 as the ratios of the CCA1 transcripts were not significantly affected in the PRMT5-deficient mutant (Figure 2A). If PTC+ transcript elicits NMD response it would accumulate in NMD-impaired mutants. However, levels of CCA1 I4Ra and I4Rb transcripts were not significantly increased in an NMD-impaired upf1-5 mutant (Figure 2A), suggesting that both are not regulated by UPF1-dependent NMD. In fact, I4Rb transcripts were decreased or unchanged in both upf1-5 (Figure 2B) and upf3-1 mutants (data not shown). Similarly to CCA1, long IR transcript of LHY (a homologous event corresponding to CCA1 I4Ra) did not accumulate in upf1-5 mutant, and therefore was not detected by UPF1-mediated NMD (Supplemental Figure 1B and 1C). Unexpectedly, a cassette exon derived from a partially retained long LHY intron did not accumulate in upf1-5. This latter observation was consistent with the results of James et al. (2012). However, a small increase of the LHY cassette exon was detected the upf3-1 mutant (James et al., 2012), suggesting that this isoform can elicit weak NMD response. Thus, similarly to many other circadian and non-circadian IR transcripts (Filichkin et al., 2010; Filichkin and Mockler, 2012; James et al., 2012; Kalyna et al., 2012) the long IR CCA1 and LHY mRNAs escape UPF1-mediated NMD pathway even though they possess NMD-eliciting features.

Under Normal Physiological Conditions, Nonsense Circadian Transcripts Typically Oscillate in Phase with Functional mRNA

Under physiologically optimal temperature and light/dark cycles (LDHH condition; Supplemental Figure 2), oscillations of the CCA1 I4Rb and the fully spliced functional transcripts were phased to the same time of day (Figure 3). Substantial

Figure 1. Arabidopsis CCA1 Locus, Protein Domains, and Structures of Alternatively Spliced mRNA Isoforms.

Retention of the entire fourth intron yields splicing isoform I4Ra. Splicing of a short intron occurs via use of the long intron splicing acceptor site, and an alternative donor site results and yields isoform I4Rb. Primer sets specific for particular transcripts are indicated by arrows. Both the I4Ra and I4Rb transcripts contain multiple PTCs that preclude translation of the full-length CCA1 protein. A unique high-scoring branching sequence GTGTCATTAG TTTTTGTTT (branch point is underlined) was predicted using a NetPlantGene Server (http://www.cbs.dtu.dk/services/NetPGene/) configured for producing neural network predictions of splice sites in A. thaliana. The approximate positions of the functional protein domains are indicated by brackets. Exons and introns are indicated by the rectangles and lines, respectively. The gene model is not drawn to scale. For additional sequence analysis details, see Supplemental Figure 1A.

Figure 2A. Arabidopsis CCA1 locus, protein domains, and structures of alternatively spliced mRNA isoforms.
quantities and synchronized oscillations of PTC isoforms and constitutively spliced counterparts were also observed for several other alternatively spliced circadian genes (Supplemental Figure 3 [Filichkin and Mockler, 2012]). The symmetric cyclical UAS observed for Arabidopsis clock genes under normal environmental conditions was also conserved in phylogenetically distant plant species. A high-resolution quantitative RT–PCR time course of the rice CCA1/LHY homolog showed that the long IR mRNA also peaked in phase with its functional transcript (Supplemental Figure 3).

Cold Acclimation Thermocycles Induce Phase Delay in Peak Expression of the CCA1 I4Rb Isoform

In contrast to optimal conditions, in plants grown under cold acclimation thermocycles (condition LDHC-4°C, Supplemental Figure 2), the peak accumulation of the CCA1 I4Rb isoform was delayed by approximately 3 h relative to the fully spliced functional mRNA (Figure 3). A time delay of I4Rb peak occurred shortly after a transition from 4°C to 25°C, established within 24 h, and persisted during entrainment to cold acclimation cycles (Figure 3). A sharp increase in abundance of I4Rb peak overlapped a 3-h window of rapid decrease of the functional CCA1 mRNA to its lowest levels. Notably, at constant temperature this transition encompassed approximately 6 h. Phase delay of I4Rb was reversed to the symmetric mode after the plants were re-entrained under LDHH condition (data not shown). I4Rb transcript phase delay during cold acclimation thermocycles was reproduced in at least five independent time-course experiments. A cold-induced delay in I4Rb phasing persisted in both NMD-impaired mutants and in their crosses, with several knockout lines deficient in serine/arginine-rich (SR) splicing factors (Figures 3 and 4A; Supplemental Figures 4 and 5). Notably, the expression of CCA1 mRNA was always phased to the same time of day under all tested conditions, suggesting that its oscillations were temperature compensated.

Asymmetric Unproductive AS of CCA1 Persists During Broad Fluctuations of Temperature

Next, we investigated whether widening of thermal cycles to physiological extremes would further affect phasing of isoforms.
The rationale of this condition was to examine timing of CCA1 isoforms during cyclical transitions from low (mRNA up, PTC+ down) to elevated (mRNA down, PTC+ up) temperatures (Filichkin et al., 2010) and vice versa. Plants were entrained at 20°C (LDHH) and then transitioned to LDHC-xt condition (LDHC-xt, Supplemental Figure 2). A time delay in the phasing of the I4Rb isoform
relative to mRNA persisted under the broad temperature cycles (Figure 4). Similar to cold acclimation thermocycles, I4Rb production was reversed to a symmetric mode after re-entraining plants at 20°C (data not shown). The temperature-induced phase delay of I4Rb occurred in both prmt5 and upf1 mutants (Figure 4A), suggesting that neither gene is required for the switch to asymmetric mode. Conspicuously, under the LDHC-xt conditions, pre-mRNA and I4Ra transcripts

Figure 4. Physiologically Extreme Thermocycles (LDHC-xt) Induce Asymmetric UAS of the CCA1.
(A) An initial delay in phasing of the I4Rb isoform relative to the productive transcript (indicated by red arrow) occurred 12 h after the temperature transition from 37°C to 4°C. The delay was observed in WT Columbia 0 and in the NMD- and PRMT-deficient mutants.
(B) CCA1 pre-mRNA and PTC isoforms peak at different phases relative to the mature mRNA. Results were consistent in three biological experiments using two independent primer pairs designed to specifically amplify pre-mRNA.
Inverse Thermocycles Induce Extra I4Rb Peak at Abnormal Times of Day

To decouple possible interactions between light and temperature, daily hot/cold temperature cycles were inverted in respect to the subjective day and night. Elevated temperature (37°C) was maintained during the night whereas ambient temperature (20°C) was preserved during the day. This condition was designated as inverse thermocycles (LDHC-inv). Adaptation to heat stress during subjective nights resulted in the accumulation of substantial levels of the CCA1 I4Rb isoform (Figure 5). In contrast to LDHC-4°C and LDHC-xt conditions (Figures 3 and 4), phase delay of the I4Rb (3 h after dawn) was not observed under inverse thermocycles. Instead, it peaked in phase with the functional mRNA at its typical position (at dawn). LDHC-inv also induced an additional I4Rb peak (6 h before dawn). This peak comprised a substantial proportion of the typical I4Rb peak and was mirrored by a minor broad peak of the functional mRNA. Both peaks occurred at the time of day when production of CCA1 mRNA was typically suppressed under all tested diurnal conditions. Re-entrainment of plants under 20°C following LDHC-4°C, LDHC-xt, and LDHC-inv treatments reverted cyclical UAS of CCA1 to the typical symmetric mode (data not shown). Thus, both LDHC-inv and LDHC-xt conditions altered phasing of CCA1 I4R isoforms. In contrast, the main peak of fully spliced functional mRNA remained unbiased under broad temperature swings (Figure 6).

Stress-Induced Asymmetric UAS of Other Circadian Transcripts

To investigate whether asymmetric UAS is widespread among components of the circadian clock, we examined daily profiles of several other Arabidopsis circadian transcripts. The nonsense isoform of REVEILLE 2 (RVE2) mRNA is generated by retention of a short internal segment of the first intron, which introduces an in-frame PTC (Filichkin and Mockler, 2012). This type of AS event is conserved among mammalian SR splicing factors and is termed a “poison cassette exon” (PCE) (Lareau et al., 2007). The RVE2 PCE isoform elicited a strong NMD response, and under constant temperature oscillated in phase with fully spliced mRNA (Figure 7). Initial transition to 4°C drastically reduced relative levels of the PCE transcript and induced its phase advance (Figure 8). Further entraining under 4°C/25°C thermocycles (LDHC-4°C) resulted in an approximately 12-h phase shift of the PCE RVE2 transcript relative to the mRNA in both wild-type and upf1-5 plants. As expected, functional RVE2 mRNA showed sustained oscillations (i.e., peaked at midnight) regardless of temperature swings. Peaking of the PCE RVE2 isoform in counter phase to functional mRNA suggested that cold acclimation thermocycles induce the production of the PTC-NMD-sensitive transcript at the time of day when functional mRNA is typically fully suppressed.

In contrast to CCA1 and RVE2, no phase shifting was observed for the long IR isoform of LHY during cold acclimation (LDHC-4°C, Supplemental Figure 7). Temperature-induced phase shifting of PTC transcripts was also not observed for several other alternatively spliced transcripts including GLYCINE RICH PROTEIN (GRP) 7, GRP 8, LHY/CCA1-like 1, and other circadian genes described (Filichkin and Mockler, 2012 and data not shown). Temperature stress did, however, frequently result in an increase of the nonsense isoforms (Filichkin and Mockler, 2012 and see below). The latter indicated that environmental stress frequently alters an abundance of circadian nonsense isoforms, whereas the temperature-induced phase shifting of PTC transcripts occurs only in a subset of circadian genes.

Long IR CCA1 Transcripts Accumulate in the Nucleus

PTC-harbouring transcripts and other aberrant mRNAs can accumulate in the nucleus (Kim et al., 2009). We therefore quantified CCA1 isoforms both in isolated nuclei and in the cytosolic fraction using qRT–PCR and event-specific primer sets. The ratio of levels of the I4Rb isoform relative to those of the fully spliced transcript was significantly higher in the nucleus than in a total RNA (Supplemental Figure 8), suggesting the PTC CCA1 transcripts accumulate in the nucleus. Similarly, PTC-containing transcripts of other circadian (GRP7) and non-circadian (HSFA2) genes accumulated in the nucleus both under normal physiological conditions and under heat stress (Supplemental Figure 9). Unlike IR CCA1 transcripts, these PTC transcripts elicited a strong NMD response and, as expected, accumulated to higher levels in the upf1-5 mutant (Supplemental Figure 10).

Splicing Factor SR45Affects Accumulation of CCA1 Isoforms under Physiologically Extreme Temperature Cycles and Binds to the Long Retained Intron In Vitro

To identify factors that influence phasing of the I4Rb isoform, we screened a panel of Arabidopsis lines with mutations in known splicing and/or NMD components. A mutant plant deficient in the SR45 splicing factor (Ali et al., 2007) showed a statistically significant (p ≤ 0.05) decrease in accumulation of CCA1 pre-mRNA, fully spliced mRNA, and both I4R isoforms compared with the wild-type control at 20°C (LDHH) (Figure 9). We also quantified CCA1 isoforms in Arabidopsis transgenic lines in which alternatively spliced short and long versions of
Figure 5. qRT–PCR of CCA1 Transcripts during Inverse Thermocycles.

The plants were entrained under LDHC-inv conditions (light at 19°C for 12 h; dark at 37°C for 12 h) for 5 days followed by sampling at 3-h intervals starting at ZT 0 h (light to dark and normal to heat stress transition time points).

(A) Co-expression of constitutively spliced (CS) and I4Rb isoforms at constant temperature (LDHH).

(B) An additional I4Rb peak (shown by blue arrow) is in phase with a similarly positioned broad peak of the CS transcript. Delayed position of the I4Rb isoform peak relative to functional mRNA under broad thermocycles (LDHC-4°C and LDHC-xt conditions) is shown by red arrow.
the SR45 protein were expressed ectopically (Zhang and Mount, 2009).

Broad-range thermocycles (LDHC-xt) induced a significant increase in the \(I_4Ra\) and \(I_4Rb\) isoforms in the SR45 overexpressing plants when compared with either the \(sr45-1\) mutant or the wild-type control (Figure 9). In contrast, such an increase was not evident under constant temperature. The \(I_4Rb\) phasing was not affected in the SR45 overexpressing plants (data not shown). However, accumulation of the long IR \(CCA1\) transcripts was increased in the SR45 overexpressing plants. The latter raised the possibility that the SR45 protein may modulate levels of the \(CCA1\) IR isoforms, possibly via physical interactions with retained intron. To test this hypothesis, an RNA containing the sequence of \(CCA1\) intron 4 was transcribed in vitro and incubated with purified recombinant SR45 protein. Increasing concentrations of the SR45 protein proportionally increased the amount of the SR45–intron 4 complex (Figure 10). The binding of SR45 to the long intron was specific, since an excess amount of cold intron 4 RNA completely abolished binding to the labeled RNA, whereas a fragment containing constitutively spliced \(CCA1\) intron 7 did not bind to SR45. These experiments indicate that, like the partially retained \(SR30\) intron 10 (Day et al., 2012), the SR45 protein specifically binds to the retained long \(CCA1\) intron in vitro.

**Depletion of the IR \(CCA1\) Isoforms Decreases Levels of Functional mRNA**

To assess whether there is a relationship between the levels of the PTC and functional \(CCA1\) mRNAs, we constructed an RNA interference (RNAi) vector targeting transcripts with retained intron 4 sequence. We reasoned that if the intron-containing transcripts can be further spliced, then depletion of the \(I_4R\) isoforms would result in reduction of the functional mRNA pool. A chemically inducible short hairpin RNA interference (RNAi) vector was designed to target a region contained in both \(I_4Ra\) and \(I_4Rb\). Transgenic \(I_4R\)-RNAi and wild-type Arabidopsis plants were entrained under broad-range thermocycles (LDHC-xt) followed by a dexamethasone (Dex) treatment to induce shRNA expression. After 48 h, tissues were sampled over a 24-h time course. A transient induction by Dex substantially decreased levels of the targeted \(I_4R\) isoforms in the \(I_4R\)-RNAi transgenic plants but not in the wild-type control (Figure 11 and Supplemental Figure 11). Concomitantly, levels of fully spliced mRNA were also decreased in the Dex-induced transgenics but not in the wild-type control, suggesting that under extreme temperature swings, a depletion of the \(CCA1\) IR isoforms negatively affected accumulation of mRNA.
Similarly to Abiotic Stress, Pathogen Challenge and NMD Impairment Alter AS Patterns and Perturb Clock-Modulated Gene Expression

We used splicing event-specific quantitative RT–PCR to measure levels of the CCA1 and LHY isoforms over a time course of infection with pathogenic and non-pathogenic P. syringae pv. tomato DC3000 strains. The non-pathogenic strain is compromised in its ability to assemble a type III secretion system, a molecular machinery necessary for pathogenesis. Relative levels of the CCA1 I4Rb isoform increased sharply in response to bacterial infection. Both strains induced an increase in I4Rb accumulation relative to mock-inoculated control plants (Figure 12). The wild-type pathogenic strain also induced a significantly higher increase in I4Rb accumulation relative to plants infected with the non-pathogenic strain. Moreover, a delay in peak abundance of the I4Rb isoform was observed in plants infected with pathogenic P. syringae strain relative to the control plants. In contrast, the period and amplitude of the fully spliced CCA1 transcript were similar in both infected and untreated control plants (Figure 12). The bacteria-induced increase in I4Rb levels was similar in the upf1-5 mutant and wild-type plants (data not shown), confirming that I4Rb escapes UPF1-mediated degradation. P. syringae infection also caused a moderate increase of the PTC LHY cassette exon isoform (Supplemental Figure 12). Similar to CCA1, functional LHY transcripts with fully spliced long intron were maintained at nearly steady levels during the infection time course.

To identify differentially expressed and alternatively spliced transcripts elicited by bacterial infection, the NMD-impaired and wild-type host transcriptomes were profiled using RNA-seq. Wild-type and upf1-5 mutant plants were independently challenged with pathogenic (DC3000 COR-) or non-pathogenic P. syringae strains (DC3000 COR- ΔhrpS and DC3000 COR- avrPphB). Coronatine is a toxin produced by P. syringae; therefore, the necessity of coronatine in virulence was avoided by directly injecting bacteria into leaf tissues (Xin and He, 2013). The ΔhrpS non-pathogenic mutant is compromised in assembling the type III secretion system and thus elicits pattern recognition receptor (PRR)-mediated immunity, whereas the strain carrying avrPphB elicits a more robust layer of immunity called nucleotide-binding domain and leucine-rich repeat containing protein (NLR)-mediated immunity. Bacterial infection resulted in upregulation of the CCA1, LHY, and PRR9 mRNAs. Significant increases in expression were observed for several other genes associated with the circadian clock, including RVE2, RVE8, and TIC, upon infection with the pathogenic strains, and most transcripts that were up-regulated as a result of infection in wild-type plants were also up-regulated in the upf1-5 mutant (Supplemental Table 2).
The effect of pathogen challenge on levels of CCA1, LHY, and PRR9 transcripts was significantly greater in upf1-5 plants than in wild-type plants, suggesting that the NMD-impaired genotype contributes to upregulation of these transcripts. The genes co-expressed during pathogenesis were clustered using the BioLayout Express tool. RNA-seq read counts for differentially

Figure 8. Asymmetric UAS of RVE2 Transcripts during Adaptation to Cold Stress (LDHC-4°C).
A sharp decline in accumulation of the relative expression of the PTC isoform was observed during the initial 12 h of temperature transition from 20°C to 4°C. An additional peak in counter phase appeared during the entraining period under LDHC-4°C thermocycles. The dotted lines indicate a gap in sampling during an entrainment period. The bottom panel indicates temperature cycles. Normalized fold expression change was calculated using the ΔΔCt method. Light and dark periods are denoted by the open and black bars, respectively. Vertical bars indicate SEM.
expressed transcripts from seven combinations of genotypes and treatments were used as input. The analysis of 8996 differentially expressed genes produced four major and several additional small clusters. Profuse transcriptome-wide changes in expression levels were observed in wild-type plants infected with pathogenic \textit{P. syringae} strains (Supplemental Figure 13). A group of 1800 genes clustered with \textit{CCA1} based on co-expression profiles (Supplemental Figures 13 and 14; Supplemental Table 3). These genes were significantly enriched in defense- and immune-response-associated Gene Ontology (GO) terms (Supplemental Figure 14).

The \textit{CCA1} cluster included a core group of 17 genes that were co-expressed with \textit{CCA1} under all tested conditions regardless of bacteria and/or plant genotype. The majority of these genes were implicated in immune response (Supplemental Table 4). Strikingly, these 17 transcripts were also up-regulated in the untreated \textit{upf1-5} mutant, suggesting that NMD deregulation results in activation of some defense/immune pathways. Three of them, \textit{GRP7} (AT2G21660) and the RNA-binding proteins AT4G24770 and AT2G37220, were identified as potential targets of the type III effector HopU1 (Fu et al., 2007) and are thus implicated in plant immunity (Nicaise et al., 2013). The \textit{CCA1} co-expression cluster associated with untreated \textit{upf1-5} mutant genotype was enriched for GO terms associated with plant immune responses (Supplemental Table 4). Consistent with this observation, NMD-compromised \textit{upf1-5} and \textit{upf1-4} mutants conferred a partial resistance to \textit{P. syringae} infection (Supplemental Figure 15).

Transcriptomes of Heat-Stressed Plants and NMD-Impaired \textit{upf1-5} Mutant Share Overlapping Sets of IR Events

Because intron retention is a prevalent class of the AS events in plants, we reasoned that transcripts with fully or partially retained introns may accumulate differentially under stress and/or in NMD-impaired mutant plants. To test this assumption, we evaluated differential RNA-seq coverage of all TAIR10-annotated introns in 2-week-old \textit{Arabidopsis} seedlings subjected to heat stress. Transcriptome analyses of plants infected with bacteria suggested the untreated \textit{upf1-5} mutant was enriched not only with pathogen defense-associated mRNAs but also with transcripts encoding genes involved in the general abiotic stress responses. Therefore, we reasoned that global IR events in the \textit{upf1-5} mutant and in environmentally stressed wild-type plants may show similarities. Indeed, the transcriptomes of \textit{upf1-5} mutant and the heat-stressed wild-type seedlings shared an overlapping set of differentially expressed introns (Supplemental Figure 16 and Supplemental Table 5). Furthermore, the genes harboring the differentially expressed introns in both transcriptomes were associated with similar GO categories such as “RNA splicing” and “mRNA processing,” suggesting that the patterns of pre-mRNA splicing in the NMD-impaired mutant and in the heat-stressed plants share similarities. Strikingly, both transcriptomes were also enriched with “temperature stimulus,” “response to heat,” and “response to cold” GO categories. The latter suggested that at least some
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Figure 10. SR45 Protein Binds Specifically to the Retained CCA1 Intron 4.

Under identical binding assay conditions, including similar concentration range of the recombinant protein, SR45 binds to the retained long intron, but not to the constitutively spliced intron 7 sequence.

(A) Electrophoretic mobility shift assay (EMSA) analysis with radiolabeled CCA1 intron 4 RNA fragment (2 fmol) and increasing concentrations (400, 600, 800, and 1200 ng) of purified SR45 protein.

(B) The binding of SR45 to radioactive CCA1 intron 4 RNA is inhibited by excess cold RNA from the same intron. SR45 protein (1200 ng) was incubated with 2 fmol of labeled intron 4 RNA in the absence or presence of excess (100X) cold RNA.

(C) EMSA analysis with radiolabeled CCA1 intron 4 RNA (2 fmol) and 1200 ng of purified SR45 protein. Lane 1, probe without SR45 protein; lane 2, probe incubated with 1200 ng SR45 protein; lanes 3–5, probe was incubated with 1200 ng of SR45 protein in the presence of increasing concentrations (25X, 50X, and 100X) of cold CCA1 intron 4 RNA.

(D) EMSA analysis using radiolabeled CCA1 intron 7 fragment (5 fmol). A gradient of increasing concentrations (400, 600, 800, and 1200 ng) of purified SR45 protein is indicated by a triangle. RNA–protein complexes were analyzed on a 5% polyacrylamide native gel and visualized by phosphorimaging. Arrows and arrowheads indicate free probe and RNA–protein complex, respectively.

NMD-Regulated and NMD-Insensitive AS Events are Common among Circadian Transcripts

To analyze possible interdependency between the type of AS event and sensitivity to NMD, we constructed custom interactome maps of the Arabidopsis circadian clock components based on empirical protein–protein interactions identified from publicly available databases (Supplemental Figure 17 and Supplemental Table 6). Neither sequence homology nor similar frameworks of protein–protein interactions were predictive of NMD sensitivity. For example, the LHY transcript that contained a long intron escaped UPF1-mediated degradation; whereas the LHY isoform that retained an intron in long 3’ UTR was regulated by NMD (Supplemental Figure 17A (Filichkin and Mockler, 2012). RVE2 is a close homolog and physically interacting partner of CCA1 and LHY. However, in contrast to CCA1, the RVE2 PTC transcript elicited a strong NMD response (Figure 7). Our data from this and a previous study (Filichkin and Mockler, 2012) indicate that most full-length intron retention events among circadian mRNAs, as well as in other Arabidopsis transcripts (Kalyna et al., 2012; Göhring et al., 2014), are NMD insensitive. These results favor a hypothesis that similarly to CCA1, other IR mRNAs in plants may escape NMD through sequestration of their splicing intermediates in the nucleus.

DISCUSSION

Specific Environmental Stresses Can Induce Asymmetric UAS of Circadian Transcripts

Under normal physiological conditions, oscillations of PTC isoforms of most tested circadian genes mirrored daily profiles of the constitutively spliced transcripts. In contrast, specific environmental stresses can trigger a switch from symmetric to asymmetric oscillations of some PTC isoforms and generate out-of-phase oscillations of the alternatively spliced transcript relative to the constitutively spliced mRNA. Thus, under wide temperature swings, the PTC-containing CCA1 transcripts I4Rb and RVE2 oscillated with a phase delay of several hours relative to the fully spliced mRNAs. Such a delay suggests that the synthesis of IR and fully spliced functional CCA1 transcripts can be temporally decoupled. During extreme temperature swings, CCA1 pre-mRNA and I4Ra transcripts accumulated up to 6 h before the peaking of mature functional mRNA. This result suggests that extreme thermocycles introduce a temporal pause between transcription and maturation of CCA1. Recent studies suggest that availability of splicing components is normally not a limiting factor that determines the kinetics of pre-mRNA maturation. For example, mammalian LT-α and β-globin mRNAs are spliced with half-lives ranging from 0.4 to 7.5 min, depending on the intron (Audibert et al., 2002). Moreover, pre-mRNAs in yeast are spliced co-translationally (Moehle et al., 2014) prior to their 3’-end cleavage and polyadenylation (Alexander et al., 2010).

CCA1 Intron Retention Occurs in a Temperature-Independent Manner

One reasonable assumption would be that the long intron retention could be directly modulated by temperature as a consequence of inefficient recognition of the weak splicing signals. Such a mechanism was proposed for the Neurospora circadian regulator FREQUENCY. Thermal stress-regulated AS of the FRQ transcript was attributed to an inefficient recognition of alternative non-consensus splice sites at elevated...
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Three lines of evidence favor a hypothesis that out-of-phase peaking regulates stress-compensated oscillations of functional CCA1 mRNA. First, pre-mRNA accumulated several hours in advance relative to the functional mRNA during wide temperature swings (Figures 4 and 6). Assuming nearly instant kinetics of the pre-mRNA splicing (Audibert et al., 2002; Alexander et al., 2010), an observed phase delay of the mRNA peaking was not consistent with a model of co-transcriptional splicing. Therefore, it is reasonable to hypothesize that if a broad pre-mRNA peak reflects transcriptional oscillations, a narrow peak of mRNA is produced via a time-delayed maturation of pre-mRNA. This assumption is favored by an observation that the decline in pre-mRNA/I4Ra quantity intersects with a sharp increase in mRNA (Figure 6). Second, temperature stress shifted I4Rb peak from in-phase toward out-of-phase mode relative to stable mRNA oscillations observed under all tested conditions. A 3-h delay in I4Rb timing suggested that this most likely non-recyclable isoform could be involved in depleting an excess of the I4Ra/pre-mRNA at a time of day when mRNA is suppressed. Decrease in mRNA during a short-term induction of a hairpin RNA directed against the long CCA1 intron was consistent with the notion of a direct relationship between the levels of IR transcripts and mRNA. Considering the prevailing opinion that siRNAs are generally ineffective in the targeting of pre-mRNAs (Fire et al., 1998; Zeng and Cullen, 2002; Vickers et al., 2003) this result favors a hypothesis that the shunting of AS toward IR intermediates depletes the mRNA pool. Finally, inverse thermocycles abolished a phase delay of I4Rb and induced an additional peak synchronized with expression of functional mRNA. Sharply increased production of I4Rb at times of the day when mRNA is typically suppressed may indicate its role in depleting residual mRNA levels. The potential influence of I4Ra levels on oscillations of the CCA1 mRNA could be the opposite (see Supplemental Figure 18A for details). Decay of I4Ra overlaps with a steep increase in functional mRNA, suggesting that this intermediate may be further spliced into mRNA and/or I4Rb. Because a unique predicted branch point site is removed during splicing of the short intron (Figure 1), further conversion of I4Rb into fully spliced mRNA is improbable. Thus, conversion of the I4Ra into I4Rb may be part of a temperature compensation mechanism that precludes I4Ra overproduction during thermal swings. As a result, peak width and period of mRNA oscillations would be preserved during stress-induced transcriptional fluctuations of pre-mRNA. Importantly, the mRNA peak will be constrained to the same peak width independent of its amplitude, ensuring a correct temporal regulation of all CCA1-dependent networks. Exact mechanisms that coordinate temporal arrest and resumption of CCA1 splicing remain to be characterized. Theoretically it is possible that an additional NMD-dependent circuit may regulate CCA1 mRNA abundance via an AS event reported in the 5’ UTR (Kalyna et al., 2012; James et al., 2012). I4Rb-specific RNAi targeting and investigation of the transcriptional kinetics and splicing rates in real time can provide further insights into the functional roles of CCA1 isoforms.

SR4S Splicing Factor Binds to the Retained CCA1 Intron

Out-of-phase accumulation and binding of SR4S to the retained intron indicate that CCA1 pre-mRNA and I4Ra may be temporally
sequestered and their splicing delayed. This hypothesis is consistent with the recent finding that unspliced and partially spliced transcripts can be stored in nuclear speckles of the fern *Marsilea vestita* microspore in association with the exon junction complex protein Mago nashi (Boothby and Wolniak, 2011). Release and splicing of these mRNAs is triggered by spermatogenesis at a specific time in a spliceosome-dependent manner (Boothby et al., 2013). SR45 protein is a likely candidate for temporal sequestration of I4R

CCA1 transcripts with common 3′ UTR

SR45 protein also specifically binds to a retained 5′ portion of intron 10 of the SR30 pre-mRNA (Day et al., 2012). This result suggested that SR45 binding may include a broader scope of the IR targets. *Arabidopsis* SR45 splicing factor is a close homolog of mammalian RNSP1 protein (Zhang and Mount, 2009), a component of the exon junction complex that binds 20–24 nucleotides upstream of exon–exon junctions (Le Hir et al., 2000). RNSP1 communicates the exon junction complex location with components of the NMD machinery (Lykke-Andersen et al., 2001; Sakashita et al., 2004).

SR45 protein is localized exclusively to nuclear speckles (Ali et al., 2003, 2007) which serve as storage sites, delivering splicing factors to nearby transcriptional active sites (Misteli and Spector, 1998; Misteli, 2005). Re-distribution of the SR45 protein between the splicing speckles and nucleoplasm can be induced by transcriptional activity, protein phosphorylation, low and high temperature (Ali et al., 2003, 2007), and hypoxia (Koroleva et al., 2009). Because the CCA1 IR isoforms accumulate in the nucleus, establishing their co-localization to the nuclear speckles could provide important evidence of interaction with SR45 in vivo. SR45 physically interacts with other spliceosomal proteins such as U1-70K, U2AF35b (Golovkin and Reddy, 1999; Day et al., 2012), and SKIP (Wang et al., 2012), which regulate AS and maturation of pre-mRNAs including a subset of specific circadian clock transcripts. SR45 binding to the retained introns favors a hypothesis that SR45 can be responsible for sequestration of IR intermediates in the nucleus. Sequestration and release of IR intermediates can be synchronized with circadian rhythms and/or induced by particular
Similarly to Abiotic Stresses, a Bacterial Pathogen Triggers Accumulation of the IR CCA1 Isoform

Levels of PTC-containing CCA1 and LHY transcripts were upregulated in P. syringae-infected plants. Infection also resulted in a 3-h delay in the peak of CCA1 I4Rb oscillations relative to that observed in uninfected plants. This phenomenon was reminiscent of the phase delay of the CCA1 I4Rb isoform during cold adaptation and under broad-amplitude thermocycles, indicating that phase delay enables adaptation of the master clock regulator components to abiotic and biotic stresses. Several lines of evidence link the circadian clock to plant immunity. CCA1 is implicated in regulation of genes involved in defense pathways, including those controlling disease resistance (Bharadwaj et al., 2011; Wang et al., 2011). For example, the CCA1-null mutant has a compromised resistance to infection with downy mildew (Wang et al., 2011), and ectopic overexpression of CCA1 or LHY caused disruption of circadian rhythms and increased susceptibility to P. syringae (Zhang et al., 2013). Circadian regulation presumably synchronizes plant immune responses in anticipation of the infection timing (Wang et al., 2011).

Stress Response Transcripts Co-Expressed with CCA1 Are Associated with Pathogenesis and Upregulated in an NMD-Impaired Mutant

We identified 17 transcripts that were co-expressed with CCA1 across all treatments independently of the pathogenicity of the P. syringae strain and/or host genotype. As anticipated, this subset of co-expressed transcripts included mRNA encoding GRP7, a “slave” oscillator driven by CCA1/LHY and linking the central oscillator to clock output and defense responses (Staiger et al., 2003; Schöning et al., 2007, 2008; Streitner et al., 2010; Nicaise et al., 2013; Zhang et al., 2013). The core group of 17 transcripts was also up-regulated in the untreated upf1-5 mutant, suggesting that at least some immune response pathways in the NMD-impaired plants are pre-activated. This conclusion is consistent with the observations that several NMD-impaired mutants confer partial resistance to P. syringae (Supplemental Figure 15; Jeong et al., 2011; Rayson et al., 2012) and that AS of several resistance genes can modulate defense activation (Gassmann, 2008). For example, a reduced NMD in the smg7 mutant affected the turnover of nucleotide-binding, leucine-rich repeat mRNAs encoding immune receptors and resulted in plant autoimmunity (Riehs-Kearman et al., 2012; Gloggnitzer et al., 2014).

Analyses of RNA-seq data suggested that the transcriptome of an NMD-impaired mutant shares common features with the transcriptome of heat-stressed plants: both were enriched with similar GO categories related to pre-mRNA processing and stress response. It is possible that NMD deficiency results in de-regulation of balance between PTC and functional mRNAs and activates constitutive expression of networks regulating abiotic and biotic stress responses. This scenario is consistent with the partial resistance of NMD-impaired plants to P. syringae. Microarray profiling of the upf1-5 transcriptome also suggested that the transcripts targeted by the UPF1-dependent NMD pathway include an array of the defense-related genes (Rayson et al., 2012). We hypothesize that the shift of the master circadian regulator CCA1 pre-mRNA toward UAS compensates for pathogen-driven destabilization of the host cell defense networks controlled by the circadian clock.

A Broader Concept of UAS Role in Transcriptome Regulation

At least 17% of alternatively spliced multi-exon, protein-coding Arabidopsis genes produce isoforms that are targeted by NMD (Drechsel et al., 2013). The CCA1 model of UAS-compensated oscillations can be extended to a broader scope of NMD-regulated circadian pre-mRNAs (Supplemental Figure 18B). Recent evidence of UPF1 and UPF3 functioning in a translation-independent manner (Drechsel et al., 2013) raises a possibility that stable truncated polypeptides can be produced and are involved in regulatory circuits at the protein level (Supplemental Figure 18B). The expression levels of several non-circadian key regulatory genes, such as human SC35 (Dreumont et al., 2010) or Arabidopsis SR30 (Filichkin et al., 2010), also can be regulated via shunting of the splicing products to PTC+ isoforms. Increasing evidence suggests that intron retention plays a pivotal role in regulating transcript abundance in eukaryotes. Results of Braunschweig et al. (2014) suggested that “transcriptome tuning” reduces the levels of transcripts that are less or not required for the cell or tissue-type physiology in which they are detected. Such an adjustment of transcript abundance in humans may occur via channeling of IR mRNAs toward either degradation by NMD or nuclear sequestration (Braunschweig et al., 2014). Our model of reversible, on-demand switches toward UAS is likely to be broadly relevant for non-oscillating systems. Identification of factors controlling spliceosomal responses to the individual biotic and abiotic stresses will provide critical insights into the mechanisms of stress-regulated UAS.

METHODS

Arabidopsis Genotypes and Plant Growth Conditions

All A. thaliana lines used in this study had a genetic background of Columbia-0 ecotype. Homozygous upf1-4, upf1-5, and upf3-1 mutant lines were described previously (Aruga-Reyes et al., 2006) and were kindly provided by Brendan Davies (Centre for Plant Sciences, University of Leeds, UK). The lba1 (upf1-1) mutant (Yoine et al., 2006) was provided by Masato Yoine, Nagoya University, Japan. Arabidopsis plants were grown in a Conviron PGR15 growth chamber under LDHH conditions (12 h light, 12 h dark at 20°C and light intensity of 300 μmol m⁻² s⁻¹). Three-week-old plants were entrained for a week under the conditions described above prior to temperature stress treatments or inoculation with P. syringae. Growth, entraining conditions, and sampling schemes used in experiments for cold adaptation (LDHC-4°C) and physiologically extreme thermocycles (LDHXC-xt) are summarized in Supplemental Figure 2. Arabidopsis seedlings were grown on sterile MS agar plates (Murashige and Skoog basal medium supplied with vitamins and 1.5% sucrose, Phytotechnologies Laboratories) for 7 days under LDHH conditions. Seedling entrainment, stress treatments, and sampling were described previously (Filichkin et al., 2010; Filichkin and Mokler, 2012).

For drought treatments, the Populus trichocarpa cuttings were propagated in greenhouse conditions for 10 weeks. Prior to treatments,
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plants were entrained in a Conviron growth chamber for 1 week under LDHH conditions at 23°C. For drought treatments, watering was withheld until the moisture level in soil decreased to 0.1 m₃/m³ and maintained at 0.06–0.1 m₃/m³ for 2 weeks. Root samples from the control and drought-treated plants were collected 3 h after the dark/light transition. Three biological replicates represented by individual trees were maintained for each treatment and control. Total RNA from root tissues was extracted as described previously (Filichkin et al., 2007).

P. syringae Genotypes and Inoculation Conditions

Pathogenic strains included DC3000 COR- avrPphB (elicitor of NLR-mediated immunity) and DC3000 COR-. DC3000 COR- ΔhrpS (Yuan and He, 1996) was used as non-pathogenic (elicitor of PRR-mediated immunity) control. In the infection qRT–PCR time-course experiments, pathogenic and non-pathogenic strains were represented by DC3000 and DC3000 ΔhrcC (Deng et al., 1998) isolates, respectively. Both DC3000 ΔhrcC and ΔhrpS mutants (Wei et al., 2000) are impaired in the type III protein secretion system and were used as non-pathogenic controls. DC3000 COR- strain (deficient in phytotoxin coronatine) was used instead of DC3000 for the RNA-seq because the pathogenicity is generally not affected when using a leaf infiltration protocol described below. Prior to leaf infiltration, Arabidopsis plants were grown for 3 weeks in a Conviron PGR15 growth chamber (Conviron) at 20°C LDHH conditions. Bacteria were grown in King’s B media, and plants and Arabidopsis leaves were infiltrated using a needleless syringe as described (Thomas et al., 2009). Sterile 10 mM MgCl₂ solution was used as a mock control. The sampling strategy is summarized in Supplemental Figure 2. For the qRT–PCR time course, leaves were infiltrated 4 h prior to the light/dark transition (at Zeitgeber time [ZT] 8 h) and then sampled every 3 h for 24 h starting 4 h post-inoculation (ZT time 12 h). For RNA-seq experiments, leaves were infiltrated 8 h before the light/dark transition and collected 7 h post-inoculation. For RNA-seq analysis, three biological replicates derived from independently grown plants and bacteria were used for each treatment/genotype. Total RNA for qRT–PCR analysis was isolated from two biological replicates. The assay of Arabidopsis susceptibility to P. syringae was performed using infiltrations of bacterial cells essentially as described (Ishiga et al., 2011).

RNA Isolation and cDNAs and Quantitative RT–PCR

Seedlings or leaf tissues were pulverized in liquid nitrogen. For preparation of RNA-seq libraries, total RNA was extracted from infiltrated leaves as described previously (Filichkin et al., 2010) and enriched for mRNA by two cycles of purification using a Poly(A) Purist kit (Ambion). Integrity and concentration of the RNA were monitored using an ND-1000 spectrophotometer (Thermo Fisher Scientific) and BioAnalyzer 2100 (Agilent Technologies). Potential contamination with gDNA was eliminated by treatment with Turbo-DNase (Ambion). RNA-seq libraries were prepared essentially as described previously (Filichkin et al., 2010). For the qRT–PCR assays the first cDNA strand was synthesized using 200 ng of total mRNA and a Quantitect RT kit (Qiagen) according to the manufacturer’s protocol. Potential genomic DNA contamination was eliminated using subsequent treatments with Turbo-DNase (Ambion) and gDNA Wipeout (Qiagen). Prior to PCR amplification, the first-strand cDNA reaction was diluted threefold. PCR primers were designed in such a way that only the mature mRNA was amplified. qRT–PCR was performed using a Bio-Rad CFX96 Real-Time instrument and SYBR Premix Ex Taq master mix (Takara) as previously described (Filichkin and Mockler, 2012). The normalized expression was calculated using CFX Manager software (Bio-Rad) and the ΔΔC₅₀ method. The expression was normalized using AT3G25470 (hemolysin related), AT5G11980 (Golgi complex component related), and At5G60390 (EF1α) transcripts as internal housekeeping references. The purity and identity of the amplification products were monitored by melting curve analysis and sequencing, respectively. Genomic DNA contamination was monitored by the amplification of the genomic ACTIN2 (AT3G18780) sequence as described (Filichkin and Mockler, 2012). Primer sequences are listed in Supplemental Table 1. Amplification conditions used in all qRT–PCR assays were performed previously (Filichkin and Mockler, 2012). The calibration curves were produced using amplification dilutions of the verified amplification products in the range of 5.8 × 10⁻⁷ to 5.8 × 10⁻⁵ copies.

Construction of Dex-Inducible RNAi Vector and Induction Conditions

RNAi vector targeting PTC CCA1 mRNA was constructed as follows. First, a synthetic 370-bp double-stranded DNA fragment encompassing 312 bp of the CCA1 intron 4 sequence (Supplemental Table 1) was PCR amplified using primers 5’-gggagacaatggctaaacaaagggcagttaatttg-3’ (forward) and 5’-ggggacactttgtcagaaagctgtgcttc-3’ (reverse). Second, a 370-bp PCR fragment was recombined into pDONR201 (Invitrogen). Finally, the RNAi expression vector was generated through an LR recombination between the entry clone and popoff2 (Hg³⁺) plasmid (Wielopolska et al., 2005). Both RNAi and expression plasmids were transformed into Agrobacterium tumefaciens strain GV3101 (Filichkin et al., 2007). All final constructs were confirmed by Sanger sequencing of the plasmids isolated from transformed Agrobacterium. Arabidopsis plants of the Columbia 0 ecotype were transformed with Agrobacterium using the floral dip method (Clough and Bent, 1998).

Three-week-old plants were entrained under LDHC-xt condition for 3 days prior to Dex treatment. The working Dex solution was prepared by diluting a 20 mM Dex stock solution 1:1000 in dimethyl sulfoxide (DMSO) with sterile water. Plants were repeatedly sprayed using 20 μM Dex solution (Sigma) at ZT time 12 h. Negative control plants were treated with DMSO diluted in water instead of Dex/DMSO solution. Leaf tissues were harvested and frozen at −80°C. Activation of the bidirectional pOp6 promoter driving expression of the CCA1 inverted repeat RNA sequence and the β-glucuronidase gene (GUS) was monitored by staining for GUS activity 36 h after Dex induction as described (Wielopolska et al., 2005). Only plants showing extensive GUS staining were used for RNA extraction.

Preparation of RNA Probes and Competitor RNAs for Electrophoretic Mobility Shift Assay

The fragments encompassing the CCA1 introns 4 and 7 were obtained from genomic DNA with forward and reverse primers corresponding to the beginning and end of the respective introns. The SP6 promoter sequence was incorporated into the forward primers. Primers used for amplification of the CCA1 introns 4 and 7 are described in Supplemental Table 1. The underlined regions in forward primers correspond to the SP6 promoter. RNA probes were generated using an in vitro transcription system as described earlier (Day et al., 2012). Internally labeled RNA probes were prepared with 4.5 μl of 800 Ci/mmol [α-³²P]UTP (Perkin Elmer) using 1 μl of 20 μCi/ml SP6 RNA polymerase (Fermentas) in the presence of 500 μM ATP, 500 μM CTP, 50 μM GTP, 50 μM UTP, and 70GpppG using PCR-amplified DNA templates containing an SP6 promoter. Unlabeled competitor RNAs were similarly labeled except that the 70GpppG and radiolabeled nucleotide were excluded, and 500 μM UTP and 500 μM GTP were added.

Recombinant SR45 protein was purified as described earlier (Day et al., 2012). The internally radiolabeled RNA probes (2–5 fmol) of intron 4 or intron 7 were incubated with increasing amounts of purified recombinant SR45 protein in the presence of 20 units of RNase inhibitor, 0.15 mM spermine, and gel-shift buffer (15mM HEPES [pH 7.9], 8% glycerol, 100 mM KCl, and 2 mM MgCl₂) for 5 min at 30°C in a 14-μl reaction volume. Following incubation, 4 μg/μl of heparin sulfate (Sigma) was added to the reaction mixture. Samples were chilled for 5 min on ice and 3 μl of 6× loading dye (30% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol) was added. RNA–protein complexes were loaded on 5% native polyacrylamide gel and run at room temperature in 1× TBE buffer at 200 V for 2–6 h. Gels were dried,
exposed to a phosphor screen, and visualized using Storm 840 (Molecular Dynamics).

**Fractionation of Nuclei and Isolation of Nuclear RNA**

Nuclei from the leaves of 3-week-old Arabidopsis plants were purified using Percoll (Sigma-Aldrich) gradients essentially as described (Folta and Kaufman, 2006). The cytosolic fractions were retained after nuclei were pelleted by centrifugation at 1800 g for 10 min. The separation of nuclear and cytoplasmatic fractions was assessed by qRT–PCR using U2 snRNA and U3 snoRNA as nuclear markers as described (Kim et al., 2009). Total RNA from nuclear and cytoplasmatic fractions was extracted as previously described (Filichkin et al., 2007). RNA integrity was monitored using a 2100 Bioanalyzer (Agilent). The cDNA was prepared using 200 ng of total RNA and QuantiTect RT kit (Qiagen) according to the manufacturer’s protocol.

**Preparation of Illumina Libraries and Analyses of RNA-seq Data**

Poly(A)-enriched mRNA and ds cDNA were prepared from three independent biological replicates for each treatment as described above. The RNA-seq libraries were prepared and indexed using the TruSeq DNA Sample Preparation Kit (Illumina) according to the manufacturer’s protocol. The pooled libraries were sequenced using Illumina Genome Analyzer GAIx (heat stress treatments/NMD mutant) or HiSeq2000 (Pseudomonas treatments/NMD mutant) using 36 and 50 cycles, respectively. Sequencing was done at the Center for Genome Research and Bio-computing core facility at Oregon State University as described (http://www.cgrb.oregonstate.edu/). The RNA-seq data sets were mined for differentially expressed genes using the statistical NBP (negative binomial models for RNA-seq data) models implemented in the GENE-Counter tool. The introns were considered differentially expressed at the level of statistical significance of differential expression of the TAIR10 annotated introns was evaluated using the GENE-Counter tool. The introns were considered differentially expressed at the level of statistical significance if the P and Q values for normalized Illumina read coverage were less than or equal to 0.05. An additional filtering included a minimum twofold change in read coverage.

The RNA-seq data sets have been deposited at the National Center for Biotechnology Information databases under BioProject accession numbers PRJNA246022 (Pseudomonas/NMD-impaired mutant treatments) and PRJNA245833 (heat stress treatments/NMD mutants).

**Co-Expression Clustering and Gene Ontology Enrichment Analyses**

The co-expression clustering of gene expression in response to P. syringae infection was constructed using the RNA-seq datasets as input for the BioLayout Express tool (http://www.biolayout.org/). Only transcripts with RNA-seq counts two-fold greater or less than the control genotype or treatment were considered as differentially expressed. Two Arabidopsis genotypes (wild-type and upf1-5) and three P. syringae strains (DC3000 COR-, DC3000 COR- avrPphb, and DC3000 COR- ΔhrpS) were used in all possible combinations (i.e., host genotype/treatment). The analysis of GO terms enrichment was performed using the Parametric Analysis of Gene Set Enrichment method (Kim and Volsky, 2005) via the agrigo portal (http://bioinfo.cau.edu.cn) (Du et al., 2010). The fold change values were obtained from the RNA-seq read coverage (primary TAIR10 transcript models), log2-transformed, and used as an input to the GO analysis.

**Construction of Arabidopsis Circadian Clock Interactome**

A circadian gene interactome was constructed using gene expression data available at AraNet (http://www.functionalnet.org/) and at Arabidopsis Interactome Mapping Consortium (http://interactome.dfci.harvard.edu). AraNet constructs probabilistic functional gene interaction networks based on several integrated experimental and genetic interactions data sets (Lee et al., 2010). The map of empirically observed interactions between the key circadian clock components was constructed using the following data sets: yeast two-hybrid system (Arabidopsis Interactome Mapping Consortium, 2011; http://interactome.dfci.harvard.edu), AraNet (http://www.functionalnet.org/), and a custom literature curated database of protein–protein interactions in the Jaiswal laboratory (http://jaiswallab.cgrb.oregonstate.edu). The Arabidopsis Interactome Network is a set of experimentally validated binary protein–protein interactions derived from yeast two-hybrid data and from the other physical interactions reported to date (Arabidopsis Interactome Mapping Consortium, 2011). The AS events and their NMD sensitivity were inferred using empirical data reported here and in our previous work (Filichkin et al., 2010; Filichkin and Mockler, 2012).

**SUPPLEMENTAL INFORMATION**

Supplemental Information is available at Molecular Plant Online.

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