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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

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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 (Jaillon 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

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termination codon (PTC) (Lewis et al., 2003; Lareau et al., 2007). AS that generates PTC-harboring nonsense isoforms is 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-Santángelo 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; Schöning 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; Salomé 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 constituted 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 spliceosomal proteins is essential for correct splicing of many pre-mRNAs (Deng et al., 2010),