Inhibition of RNA Degradation Integrates the Metabolic Signals Induced by Osmotic Stress into the Arabidopsis Circadian System

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Highlight

Post-transcriptional regulation of *PRR7* and *LWD1* transcripts by the exoribonuclease XRN4 enables the circadian system to respond to osmotic stress.
Abstract

The circadian clock system acts as an endogenous timing reference that coordinates many metabolic and physiological processes in plants. Previous studies have shown that the application of osmotic stress delays circadian rhythms via 3’-Phospho-Adenosine 5’-Phosphate (PAP), a retrograde signalling metabolite that is produced in response to redox stress within organelles. PAP accumulation leads to the inhibition of EXORIBONUCLEASEs (XRNs), which are responsible for RNA degradation. Interestingly, we are now able to demonstrate that post-transcriptional processing is crucial for the circadian response to osmotic stress. Our data show that osmotic stress increases the stability of specific circadian RNAs, suggesting that RNA metabolism plays a vital role in circadian clock coordination during drought. Inactivation of XRN4 is sufficient to extend circadian rhythms as part of this response, with PRR7 and LWD1 identified as transcripts that are post-transcriptionally regulated to delay circadian progression.

Keywords and Abbreviations

Arabidopsis
Circadian
Drought
Osmotic stress
RNA degradation
Post-transcriptional
Introduction

Drought is one of the primary contributors to the yield gap that exists between theoretical yields and those realised in the field (Gupta et al., 2020). However, plants’ responses to drought are complex and so our understanding of underlying signaling pathways remains limited. One of the initial steps in plants’ perception of drought stress is the induction of oxidative stress in the chloroplast (Chan et al., 2016a). These stresses lead to the inactivation of the redox-sensitive enzyme SAL1, resulting in the accumulation of 3’-PhosphoAdenosine 5’-Phosphate (PAP), a retrograde signalling molecule that indicates metabolic stress within the chloroplast (Chan et al., 2016a; Koprivova and Kopriva, 2016; Phua et al., 2018). PAP accumulation alters global patterns of transcription and RNA catabolism by inhibiting the activity of 5’-3’ exoribonucleases [XRNs; (Gy et al., 2007; Kurihara et al., 2012; Crisp et al., 2018)], while PAP also serves as a secondary messenger to promote abscisic acid (ABA) signalling (Pornsiriwong et al., 2017).

Higher order Arabidopsis mutants lacking all three Arabidopsis XRNs (XRN2, XRN3, XRN4; xrn234) have improved drought tolerance, similar to mutant lines that constitutively accumulate PAP (Hirsch et al., 2011). However, these higher order xrn234 mutants are unlikely candidates for crop improvement as such plants grow slowly and have a delayed flowering phenotype (Hirsch et al., 2011). By comparison, loss of XRN4 has modest effects on plant growth and physiology, although roles in seed germination and impaired responses to hormones including ethylene, ABA, and auxin have been reported (Basbouss-Serhal et al., 2017; Wawer et al., 2018; Windels and Bucher, 2018). Instead, XRN4 appears to have a greater role in mediating plants’ responses to abiotic factors including heat and salt stress by contributing to cytosolic RNA degradation (Merret et al., 2013; Merret et al., 2015; Nguyen et al., 2015; Kawa et al., 2020).

Mature mRNAs are protected from degradation by a 5’ 7-methylguanosine cap and the 3’ polyadenosine tail, with polyadenylation serving as the primary determinant of degradation rate (Decker and Parker, 1993; Sieburth and Vincent, 2018). Beyond these initial regulatory steps, cytosolic RNA degradation can occur in either a 3’→5’ or 5’→3’ direction. The exosome and SUPPRESSOR OF VARICOSE (SOV) contribute to 3’→5’ degradation, whereas XRN4 is the primary actor in 5’→3’ degradation; XRN2 and XRN3 are exclusively localized to the nucleus (Nagarajan et al., 2013; Sieburth and Vincent, 2018). These pathways are broadly conserved across metazoans and fungi, although animals and fungi utilize a functionally equivalent XRN4 orthologue (XRN1) for cytosolic 5’→3’ degradation (Kastenmayer and Green, 2000; Nagarajan et al., 2013). Degradation of cytoplasmic RNA via these pathways prevent the generation of siRNAs (Sieburth and Vincent, 2018; Zhang et al., 2015), although the function of these partially-degraded intermediates remain otherwise unstudied. Instead, recent reports demonstrate unanticipated relationships between these conserved RNA degradation pathways and other aspects of RNA metabolism and processing in yeast (Blasco-Moreno et al., 2019; Haimovich et al., 2013; Sun et al., 2013). In plants, at least a portion of XRN4-mediated degradation occurs co-translationally, leading to xrn4 seedlings having impaired translation of specific transcripts (Carpentier et al., 2020).
We have previously reported that the accumulation of PAP leads to a delay in the circadian system, and that comparable phenotypes are observed in *xrn234* seedlings (Litthauer et al., 2018). The circadian system is a molecular timekeeping mechanism that enables time-of-day to be integrated into plants’ responses to environmental signals (Millar, 2016). Timing information provided by the circadian system allows anticipation of regular environmental changes (such as dawn and dusk) whilst also modulating gene expression in response to stresses (Greenham and McClung, 2015; Grundy et al., 2015; Baek et al., 2020). Manipulation of the circadian system can improve drought tolerance (Legnaioli et al., 2009, Nakamichi et al., 2016) and alter water use efficiency (Simon et al., 2020). Due to its potential to improve agronomic traits, the circadian system has been proposed as a key target of breeding programmes (Bendix et al., 2015). We therefore sought to determine how osmotic stress contributes to the regulation of circadian timing.

The circadian system is multi-faceted but relies on interlocking transcriptional negative feedback loops that generate daily rhythms of approximately 24 hours (Millar, 2016). Morning-phased components, such as CIRCADIAN CLOCK ASSOCIATED1 (CCA1) work in combination with PSEUDO RESPONSE REGULATOR9 (PRR9), PRR7, and PRR5 to repress gene expression throughout the day (Alabadi et al., 2001; Nakamichi et al., 2010). At night, the Evening Complex [primarily comprising of EARLY FLOWERING3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX)] inhibits gene expression (Nusinow et al., 2011; Huang et al., 2016). These waves of repression are complemented by transcriptional activators including LIGHT-REGULATED WD1 (LWD1) and LWD2 that promote expression of morning-phased clock genes (Wang et al., 2011; Wu et al., 2016). Following transcription, proteins such as GIGANTEA contribute to the post-translational regulation of circadian timing (Kim et al., 2007; Cha et al., 2017)

Although primarily examined at the transcriptional level, the contribution of post-transcriptional regulation to the maintenance of circadian rhythms is becoming apparent. Alternative splicing, nuclear export, and Non-sense Mediated Decay (NMD) all contribute to circadian timing, and *CCA1* transcript has been reported to be less stable in the presence of light (Yakir et al. 2007; Jones et al., 2012; Wang et al., 2012; Macgregor et al., 2013; Kwon et al., 2014; Nolte and Staiger, 2015; Romanowski and Yanovsky, 2015; Mateos et al., 2018; Careno et al., 2022). Equally, post-transcriptional regulation is similarly recognized as contributing to plants’ responses to abiotic stress (Filichkin et al., 2015; James et al., 2018). In this study, we demonstrate that loss of XRN4 activity is sufficient to delay circadian timing, and that mannitol stress limits the degradation of *PRR7* and *LWD1*. Importantly, neither *prr7* nor *lwd1lwd2* seedlings are able to delay their circadian system in response to osmotic stress, demonstrating how signals from environmental stresses can be integrated into the circadian system.
Materials and Methods

Plant material, growth, and treatments

Plant genotypes used in this work are listed in Supplemental Table 1. Plants were germinated and grown on half-strength Murashige and Skoog (0.5 MS) media for 5-12 days as described before being transferred to either half-strength 0.5 MS media or 0.5 MS supplemented with 200mM mannitol as indicated. Plants were grown under 60 µmol m\(^{-2}\) s\(^{-1}\) white light in 12:12 light:dark cycles. Relative humidity and temperature were set to 60%-70% and 22°C, respectively.

Accession numbers

Genes examined in this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: APA1, At1g11910; APX3, At4g35000; ATP3, At2g33040; CCA1, At2g46830; CCR2, At2g21660; ELF4, At2g40080; ELF5A-2, At1g26630; GIGANTEA, At1g22770; IPP2, At3g02780; LHY, At1g01060; LWD1, At1g12910; LWD2, At3g26640; PDDPI, At2g21170; PRR7, At5g02810; PRR9, At2g46790; SALI, At5g63980; TOC1, At5g61380; XRN2, At5g42540; XRN3, At1g75660; XRN4, At1g54490.

Hypocotyl measurements

Seedlings were germinated on 0.5MS media and grown under 60 µmol m\(^{-2}\) s\(^{-1}\) white light in 8:16 light:dark cycles for three days prior to transfer to plates containing 200 mM mannitol or a mock-treated control. Hypocotyl length was measured at 7 days after germination using ImageJ (Abramoff et al. 2004).

Luciferase activity

Seedlings were grown in 12:12 light:dark cycles before being sprayed with 3mM d-luciferin in 0.01% Triton X-100 before being returned to entraining conditions for 24 hours. The age of seedlings used in each experiment is described in the respective figure legend. Luciferase imaging was completed under constant light conditions (20 µmol m\(^{-2}\) s\(^{-1}\) constant blue and 30 µmol m\(^{-2}\) s\(^{-1}\) constant red light) for 5 days. Images were taken every two hours with a QImaging Retiga LUMO Monochrome Camera controlled by a MicroManager 1.4 script. Circadian parameters were determined using the website biodare2.ed.ac.uk which used Fourier fast transform-nonlinear least squares to calculate circadian parameters (Moore et al., 2014).

Chlorophyll fluorescence imaging

Chlorophyll fluorescence parameters were recorded with a Fluorimager imaging system (Technologica) as previously described (Litthauer et al., 2015). Patterns of \(F_q/F_m^\prime\) were fitted to cosine waves using FFT-NLLS
(Plautz et al., 1997) to estimate circadian period length and additional circadian parameters. Sample size was chosen to achieve a power of 0.8 in a two-sample t test at \( \alpha = 0.05 \). Previously collected data were used to estimate \( \sigma = 0.6 \).

Assessment of PAP accumulation

Twelve-day-old seedlings grown on 0.5MS were transferred to 0.5MS medium supplemented with either 200mM mannitol or a mock control. Seedlings were returned to light-dark cycles for two days prior to harvesting on day 3 of osmotic stress at 4h intervals and stored at -80°C until processing. Plant tissue was ground using a TissueLyser (Qiagen-Retsch) and then incubated in 0.1M HCl for 15’. Particles were precipitated twice by centrifugation and the supernatant was added to CP buffer (620 mM citric acid, 760 mM Na₂HPO₄, pH 4). The samples were then derivatised with chloroacetyl-aldehyde at 80°C for 10’ prior to measurement using an HPLC system (Shimadzu) with a Phenomenex Luna 5µm C18(2) 100Å LC 150x4.6mm column. The column was equilibrated with 97%(v/v) Buffer A (5.7mM [CH₃(CH₂)]₄NHSO₄ and 30.5 mM KH₂PO₄, pH 5.8) and 3%(v/v) acetonitrile, after injection the concentration of acetonitrile rose to 33%(v/v) with a linear gradient across 43’20”, the column was then re-equilibrated with 97%(v/v) Buffer A and 3%(v/v) acetonitrile for 6’40”. Concentration of PAP was measured relative to commercially available standards and is presented relative to seedling dry weight.

Gene expression analyses

For gene expression, 10-15 seedlings were pooled. RNA isolation was performed using TriZol™ (Sigma) based on the manufacturers’ instructions, DNA contaminant was removed using DNase I - RNase-free (Thermo Scientific™) and cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) with Oligo dT or random hexamer as specified in the figure legend. The resultant cDNA was used as template for real-time PCR (primers listed in Supplemental Table 2) using StepOne™ Real-Time PCR System (Applied Biosystems™). Data were processed using the \( \Delta \Delta \text{C}_t \) method, and is presented relative to \( \text{APA1}, \text{APX3}, \text{and IPP2} \) which have previously been reported to be stable over circadian time (Nusinow et al. 2011).

Measurement of deadenylated RNA accumulation

Accumulation of deadenylated RNA was assessed as previously described (Nagarajan et al., 2019). Seedlings were preincubated in 3 mL incubation buffer (15 mM sucrose, 1mM PIPES pH 6.25, 1mM KCl, 1mM sodium citrate) with aeration (swirling 100 rpm) in petri dishes for 15 min. Transcription was inhibited by adding 3 mL of fresh buffer containing 1 mM cordycepin. Vacuum infiltration was performed for 15 mins. Tissue was collected 15 min after vacuum release, snap-frozen in liquid nitrogen, and stored at -80 °C prior to RNA extraction. Deadenylated RNA was extracted by retaining the unbound RNA from total RNA following a standard RNA magnetic bead-based oligo(dT) purification (Qiagen RNeasy Pure mRNA Bead Kit, (Nagarajan
et al., 2019). cDNA was synthesized using a random hexamer oligo. RNA steady-state accumulation is presented relative to \textit{GAMMA SUBUNIT OF MT ATP SYNTHASE (ATP3)}, \textit{EUKARYOTIC ELONGATION FACTOR 5A-2 (ELF5A-2)}, and \textit{PLASTID ISOFORM TRIOSE PHOSPHATE ISOMERASE (PDTPI)}.

**RNA stability assay**

RNA stability was assessed as previously described (Sorenson et al., 2018). Seedlings were preincubated in 3 mL incubation buffer (15 mM sucrose, 1mM PIPES pH 6.25, 1mM KCl, 1mM sodium citrate) with aeration (swirling 100 rpm) in petri dishes for 15 min. Transcription was inhibited by adding 3 mL of fresh buffer containing 1 mM cordycepin. Vacuum infiltration was performed for 15 mins. Tissue was collected 30, 60, 120, and 180 min after vacuum release, snap-frozen in liquid nitrogen, and stored at −80 °C prior to RNA extraction. cDNA was synthesized using a random hexamer oligo. RNA steady-state accumulation is presented relative to \textit{GAMMA SUBUNIT OF MT ATP SYNTHASE (ATP3)}, \textit{EUKARYOTIC ELONGATION FACTOR 5A-2 (ELF5A-2)}, \textit{PLASTID ISOFORM TRIOSE PHOSPHATE ISOMERASE (PDTPI)} and IPP2.

**Results**

**Circadian responses to osmotic stress occur at transcriptional and post-transcriptional levels**

We were interested how different aspects of the circadian system responded to osmotic stress. Therefore we utilised a catalogue of luciferase reporter lines and chlorophyll a fluorescence to monitor circadian rhythms following transfer to 200mM mannitol (Figure 1, Supplemental Figure 1). Osmotic stress significantly extends circadian free-running period (FRP) and reduces bioluminescence when assessed using luciferase reporters driven by the promoter of \textit{CCA1} or \textit{GI}, in line with our initial studies [Figure 1A, C, D, (Litthauer et al., 2018)]. However, it was noteworthy that \textit{pPRR9::LUC2} seedlings had a much reduced response, whereas \textit{pLWD1::LUC2} reporter lines did not demonstrate an extension of FRP following osmotic stress (Figures 1A, E, F). Although we were able to determine circadian parameters for \textit{pLWD1::LUC2} and \textit{pLWD2::LUC2} after the transfer to 200 mM mannitol these oscillations were greatly damped (and presented an increased Relative Amplitude Error; RAE), suggesting that expression of these genes is driven to functional arrhythmia in the presence of osmotic stress (Figures 1F-1G, Supplemental Figure 1G and 1I). On the other hand, \textit{pLWD2::LUC2} lines retained an extension of circadian period following the application of osmotic stress (Figures 1A and 1G). By contrast, rhythms of chlorophyll fluorescence were retained in the presence of 200 mM mannitol (Figures 1B and 1H). These data demonstrate that only a subset of the circadian system is disrupted following the application of osmotic stress.
The SAL1/PAP pathway delays circadian free-running period via cytosolic XRN4

3’ PhosphoAdenosine 5’-Phosphate (PAP) is a retrograde signal that accumulates in response to osmotic stress. Endogenous PAP levels increase as oxidative stress impairs the activity of SAL1, a redox-sensitive phosphatase that would otherwise degrade PAP in the chloroplast and mitochondria (Chan et al. 2016a, 2016b). sal1 seedlings have an extended circadian period (Litthauer et al., 2018), and so we were interested whether the accumulation of PAP during osmotic stress varied over the course of a day. PAP accumulation is modest in mock-treated wild-type plants, with little variation in PAP levels in Col-0 seedlings during the day (Figure 2A, p=0.268). However there was a significant increase in PAP accumulation in mannitol-treated wild-type plants compared to a mock-treated control (p<0.001). However, no significant difference in PAP accumulation was observed in plants carrying the fryl1-6 allele of SAL1 following application of osmotic stress. sal1 mutants constitutively accumulate PAP, with PAP accumulation substantially higher than that observed in both mock- and mannitol-treated wild-type seedlings (Figure 2A, p=0.24; (Litthauer et al., 2018)). These data suggest that PAP accumulation does not vary over the course of the day, either in the absence or presence of osmotic stress.

One of the biochemical consequences of PAP accumulation is the inhibition of the XRN family of exoribonucleases, with Arabidopsis expressing three XRN orthologues (Nagarajan et al. 2013). Previous studies have reported that xrn234 seedlings exhibit an extended circadian Free-Running Period (FRP), although they also exhibit a pleiotropic phenotype including impaired growth (Hirsch et al., 2011; Litthauer et al, 2018). We were thus interested in determining if specific XRN proteins were sufficient to link the SAL1/PAP signalling pathway into the circadian system. We examined the FRP of the single, double, and triple mutants of three arabidopsis XRN orthologues using chlorophyll fluorescence and luciferase assay. Neither xrn2-1, xrn3-3, nor xrn2-1 xrn3-3 seedlings have a significant FRP extension when assessed by chlorophyll fluorescence (Figure 2B, Supplemental Figure 2A). However, we observed that the ein5-1 allele of xrn4 displayed an impaired circadian system, with a circadian FRP 1 hour longer than wild type controls and was indistinguishable from xrn234 seedlings (Figure 2B, Supplemental Figure 2A). A similar extension of FRP was observed using a pCCA1::LUC2 reporter construct, with both ein5-1 and xrn4-3 alleles of xrn4 exhibiting an extended FRP compared to wild type (Figure 2C and Supplemental Figure 2C-H, τ=23.19±0.34 hours, 26.34±0.68 hours, and 26.70±0.60 hours in wild type, ein5-1 and xrn4-3 lines respectively). To further investigate whether the circadian system of xrn4 seedlings retained a response to osmotic stress, we assessed the response of ein5-1 and xrn4-3 seedlings to osmotic stress. Our results suggest that these seedlings continued to demonstrate a modest response to osmotic stress (p < 0.01), although this response was much less pronounced than in wild type seedlings (Figures 2C-F, Supplemental Figure 2E-H). These data suggest that XRN4 contributes to the extension of circadian FRP in response to osmotic stress, while also indicating that additional mechanisms contribute to the integration of osmotic stress into the circadian system.
Osmotic stress limits the degradation rate of \textit{LWD1}, \textit{LWD2} and \textit{PRR7} transcripts

Over 5500 genes are mis-regulated in \textit{xrn4} seedlings (Merret et al., 2015; Carpentier et al., 2020; Gregory et al., 2008) and approximately 2200 Arabidopsis transcripts have been proposed as XRN4 substrates following Parallel Analysis of RNA Ends or Genome-wide Mapping of UnCapped Transcripts (PARE and GMUCT respectively; Figure 3A, Nagarajan et al., 2019; Carpentier et al., 2020). Of these candidate substrates, only three (\textit{PRR7}, \textit{LWD1}, and \textit{LWD2}) are established components of the circadian system although \textit{COLD CIRCADIAN RHYTHM2/GLYCINE RICH PROTEIN7} (\textit{CCR2/GRP7}), part of a slave sub-oscillator, is also a candidate \textit{XRN4} substrate (Figure 3A; Heintzen et al., 1997; Farré EM et al., 2005; Wu et al., 2008). In order to validate these RNAseq data, we assessed the stability of \textit{LWD1}, \textit{LWD2}, and \textit{PRR7} transcripts in the presence or absence of osmotic stress. Experiments were completed in the presence of cordycepin to inhibit transcription (Supplemental Figure 3A). Degradation of \textit{LWD1} was reduced relative to controls following the application of osmotic stress in both wild type and \textit{ein5-1} seedlings (Figures 3B and 3C). However, the rate of \textit{LWD1} degradation was comparable in both wild-type and \textit{ein5-1} seedlings, suggesting that factors beyond \textit{XRN4} contribute to the degradation of this transcript (p > 0.66, Figures 3A-3C). Similarly, the stability of \textit{LWD2} was enhanced by osmotic stress in both genotypes examined, with little difference in degradation rate between wild type and \textit{ein5-1} seedlings (Figures 3D and 3E). In contrast to \textit{LWD1} and \textit{LWD2}, the rate of \textit{PRR7} degradation was comparable to the stable control transcripts in mock conditions and suggesting that \textit{PRR7} RNA is relatively stable (Figures 3F and 3G). Indeed, \textit{PRR7} RNA appeared more stable than controls following the application of osmotic stress in wild type plants (Figure 3F, p < 0.01). Our data show that osmotic stress limits the degradation rate of \textit{LWD1}, \textit{LWD2} and \textit{PRR7} but highlight that additional factors work with \textit{XRN4} to regulate degradation of these transcripts.

Both transcription and RNA degradation contribute to RNA abundance within cells, with evidence that disruption of cytosolic RNA degradation can influence transcription via ‘RNA buffering’ [Figure 4A; (Sorenson et al., 2018; Sieburth and Vincent, 2018)]. \textit{XRN4} plays a key role in these processes, with roles in both co-translational decay and cytosolic 5'-3' RNA decay (Figure 4A; Kastenmayer and Green, 2000; Nagarajan et al., 2019; Carpentier et al., 2020). Since \textit{XRN4} degrades deadenylated RNAs we next assessed the accumulation of partially degraded RNA targets (i.e. RNA without an adenylated tail, Figure 4A) compared to control RNAs that have not been defined as \textit{XRN4} targets (Figure 4, Supplemental Figure 3, Sorenson et al. 2018). Accumulation of deadenylated \textit{CCA1} (which is not a proposed \textit{XRN4} target) was consistent across our experiment and did not vary between wild type and \textit{ein5-1} (Supplemental Figure 3B). Deadenylated \textit{CCR2} levels were significantly increased in \textit{ein5-1} seedlings compared to wild-type controls (p < 0.01), with the number of deadenylated transcripts decreasing over time in \textit{ein5-1} seedlings (presumably as a consequence of exosome-mediated degradation, p < 0.0002, Figures 4A and 4B). Deadenylated \textit{LWD1} transcripts were similarly elevated in \textit{ein5-1} seedlings (despite being less pronounced than for \textit{CCR2}, p < 0.01), although in this case there was no significant difference in degradation rate between wild type and \textit{ein5-1} (p = 0.0504, Figure 4C). By contrast, patterns of deadenylated \textit{LWD2} accumulation were complex, with deadenylated \textit{LWD2} being more stable than control RNAs following the application of cordycepin (Figure 4D). However, we did not observe any significant differences in deadenylated \textit{LWD2} accumulation between wild type or \textit{ein5-1} samples at any time point. Levels
of deadenylated PRR7 were consistently elevated in ein5-1 seedlings compared to wild type, although there was no difference in degradation rate between the two lines (p<0.01, Figure 4E). Deadenylated PRR7 RNAs also increased over time relative to control deadenylated RNAs (p<0.001, Figure 4E). These data suggest that XRN4 contributes to degradation of deadenylated CCR2, PRR7, and LWD1. However, the relative contribution of other degradation pathways (such as the exosome) appears to vary, with deadenylated LWD2 and deadenylated PRR7 in particular being more stable than control RNAs.

We next examined how the application of osmotic stress altered the accumulation of deadenylated RNAs (Figure 4F-4I). Interestingly, the accumulation of deadenylated CCR2 was greatly reduced in ein5-1 mutants in the presence of 200mM mannitol compared to the mock control, in agreement with the enhanced accumulation of the full-length transcript during drought stress (Figure 4F, p < 0.001, Carpenter et al., 1994). By contrast, ein5-1 seedlings continued to accumulate more deadenylated LWD1 than wild type controls (Figure 4G, p < 0.01). Application of osmotic stress increased the accumulation of deadenylated LWD2 in both wild type and ein5-1 seedlings, with deadenylated LWD2 continuing to accumulate throughout cordycepin treatment (Figure 4H). Although deadenylated PRR7 continued to accumulate during the experiment, there was no difference in either deadenylated PRR7 accumulation (p = 0.06) or degradation rate (p = 0.897) between wild type and ein5-1 samples during osmotic stress (Figure 4I). Overall, our data demonstrate that osmotic stress affects the accumulation of deadenylated transcripts, but that additional factors beyond XRN4 contribute to this phenotype.

Since osmotic stress delays circadian progression and alters transcript stability we were interested whether the accumulation of polyadenylated and total RNA fractions were altered in stressed seedlings over circadian time. Osmotic stress was applied using 200 mM mannitol, and mRNA was extracted to generate cDNA using oligo-dT (to assess polyadenylated mRNA) or a random hexamer (to capture RNA decay intermediates in addition to polyadenylated mRNA; Figure 5, Supplemental Figure 4). We first examined polyadenylated transcript levels and were interested to note that CCA1 polyadenylated mRNA accumulation remained robust following application of 200 mM mannitol (Figure 5A). This contrasts with the general reduction of luciferase bioluminescence observed following osmotic stress (Supplemental Figure 1). Additionally we found that steady state levels of LWD1 or LWD2 polyadenylated mRNA were extremely low in either the presence or absence of mannitol despite pLWD1::LUC and pLWD2::LUC displaying circadian rhythms [Figures 1A, 1F-G, 5B-C, (Wang et al., 2011)]. Furthermore, polyadenylated PRR7 transcript peak levels were comparable between mock and osmotically stressed seedlings (Figure 5D).

We next compared the relative accumulation of polyadenylated RNA and total RNA over circadian time (Figure 5). Interestingly, we observed that patterns of polyadenylated RNA did not always align with total RNA within cells. These differences were particularly prominent for LWD1 and LWD2 transcripts, with total RNA of LWD1 and LWD2 displaying circadian rhythmicity as previously demonstrated by luciferase reporter lines, whereas polyadenylated LWD1 and LWD2 species did not accumulate (Figures 1F, 1G, 5B-C). The discrepancy between
polyadenylated and total LWD1 and LWD2 RNA highlights a strong post-transcriptional regulation of these transcripts. Additionally, we observed a dramatic increase in the total mRNA of LWD1 and LWD2 in both wild type seedlings and ein5-1 seedlings subjected to osmotic stress (Figures 5B and 5C). Conversely, these increases in total RNA following osmotic stress were not apparent in PRR7 or CCA1 species (Figures 5A and 5D), suggesting that LWD1 and LWD2 total RNA species are particularly sensitive to osmotic stress. These differences between promoter activity, polyadenylated mRNA accumulation, and total RNA (Figures 1 and 5) demonstrate how different aspects of transcript metabolism vary over circadian time.

We were next interested in whether the loss of XRN4 altered the accumulation of total RNA over circadian timescales. Despite the loss of XRN4, the rhythmic pattern of LWD1 and LWD2 total RNA was retained in ein5-1 plants, with a slight increase in peak levels of both LWD1 and LWD2 total RNA in ein5-1 seedlings in mock conditions (Figures 5B and 5C). Similar to wild type seedlings, the application of mannitol stress elevated the accumulation of both LWD1 and LWD2 total RNA. However, it was noteworthy that LWD1 total RNA levels were lower in ein5-1 seedlings compared to wild type following the application of osmotic stress. In contrast to LWD1 and LWD2 total RNA, only modest differences in CCA1 and PRR7 total RNA were observed in ein5-1 seedlings compared to wild type controls (Figures 5A, 5D). These observations of total RNA accumulation suggest that circadian accumulation of LWD1 and LWD2 total RNAs are greatly influenced by XRN4 activity but also indicate a complex effect beyond simply degrading the target transcripts, potentially involving RNA buffering mechanisms (Siebert and Vincent, 2018).

PRR7 and LWD1 enable the circadian response to osmotic stress

Given the role of osmotic stress in the degradation of LWD1, LWD2, and PRR7 (Figures 3 and 4) we were interested if disruption of these genes was sufficient to alter plants responses to osmotic stress. We first assessed whether prr7, lwd1, lwd2, lwd1 lwd2, and xrn4 seedlings retained a hypocotyl extension phenotype in response to osmotic stress (Supplemental Figure 5). All genotypes exhibited significantly shorter hypocotyls following the application of 200 mM mannitol), suggesting that osmotic stress is still experienced by each of these genotypes (p<0.001; Šidák’s multiple comparisons test, Supplemental Figure 5). We next assessed if PRR7, LWD1, or LWD2 were necessary for the extension of circadian period observed in response to mannitol treatment (Figure 6, Supplemental Figure 6). lwd1 pCCA1::LUC2 seedlings have a short period phenotype in the absence of osmotic stress (Airoldi et al., 2019) but retain the extension of circadian period following the application of mannitol (Figures 6A-6C). lwd2 pCCA1::LUC2 seedlings demonstrated a longer FRP than wild type during both control and osmotic stress treatments, although the lengthening in FRP in response to osmotic stress remained (Figures 6A and 6D). lwd1 lwd2 pCCA1::LUC2 seedlings displayed a pronounced shortening of FRP (Wang et al., 2011), but did not display an increase in FRP in response to osmotic stress (p > 0.05, Figures 6A and 6E). We then investigated the contribution of PRR7 towards the circadian response to osmotic stress. Interestingly, although robust circadian rhythms were maintained in prr7-3 pGI::LUC2 seedlings transferred to 200 mM mannitol, we did not observe an extension in FRP (Figure 6F-H). These data demonstrate that PRR7 is
necessary to maintain the proper response of circadian system to osmotic stress, and suggest that LWD1 and LWD2 redundantly contribute towards the extension of FRP as part of this response.

Discussion

Post-transcriptional regulation distinguishes the accumulation of polyadenylated mRNA from circadian patterns of promoter activity and RNA decay intermediates

Our initial experiments using luciferase bioluminescence reporters suggested that individual luciferase reporters within the circadian system were differentially regulated in response to osmotic stress, with LWD1 and PRR9 promoter-driven lines presenting a diminished circadian response to the application of osmotic stress (Figure 1). The divergence between different luciferase reporter behavior have previously been reported and may arise in part from tissue-specific expression patterns (Endo et al., 2014, Nimmo et al., 2020, Hall et al., 2002, Haydon et al., 2013). Our data suggest that CCA1-, GI-, and LWD2-driven luciferase activity is primarily derived from tissues that respond to osmotic stress, whereas PRR9- and LWD1-driven rhythms are predominant in less-responsive tissue. It will be of great interest to determine whether these differences are reflective of the circadian system adapting to osmotic stress.

Although modern circadian molecular biology is founded upon luciferase reporter constructs that reveal circadian rhythms of reporter activity (Millar et al., 1992), post-transcriptional regulation of some transcripts has been apparent for several years. For example, uniform levels of LIGHT HARVESTING CHLOROPHYLL BINDING PROTEIN (LHCB1*3) transcript are maintained despite luciferase activity driven from this promoter being rhythmic (Millar and Kay, 1991; Millar et al., 1992). Conversely, NITRATE REDUCTASE2 is transcribed constantly, and yet has rhythmic mRNA accumulation (Pilgrim et al., 1993). In our study, it is apparent polyadenylated LWD1 RNA is arrhythmic in constant light, despite a pLWD1::LUC reporter line and LWD1 total RNA displaying circadian regulation (Figures 1 and 5). A similar phenotype is observed for LWD2 transcripts (Figures 1 and 5). The length of the polyadenylated tail correlates negatively with gene expression in Arabidopsis (Parker et al., 2020), while removal of the polyadenylation signal is an important initial step in RNA degradation (Figure 4A, Siebruth and Vincent, 2018). The lack of significant accumulation of polyadenylated LWD1 and LWD2 in constant light may therefore indicate that LWD1 and LWD2 are rapidly transcribed and degraded, although additional experimentation will be required to test this hypothesis. We were also interested to note that peak accumulation of PRR7 total RNA preceded the phase of peak polyadenylated PRR7 RNA by several hours (Figure 5D). Differences in post-transcriptional processing presumably contribute to disparities we observe between luciferase reporter activity and steady-state transcript accumulation in response to osmotic stress (Figures 1, 5 and 6, Supplemental Figures 1 and 5).
RNA degradation via XRN4 contributes to the maintenance of circadian rhythms

3’-PhosphoAdenosine 5’-Phosphate (PAP) accumulates during osmotic stress, leading to the inhibition of exoribonuclease activity [Figure 2A, (Dichtl et al., 1997; Estavillo et al., 2011; Litthauer et al., 2018)]. The accumulation of PAP precipitates an extension of circadian period through the inactivation of XRN exoribonucleases, although it was not apparent from previous studies whether this phenotype was due to increased RNA Polymerase II 3’ read-through resulting from the nuclear-localised XRN2 and XRN3 or instead arose from catabolism in the cytosol via XRN4 (Crisp et al., 2018; Litthauer et al., 2018; Carpentier et al., 2020). Our studies, along with other recent work in this field demonstrate that the loss of XRN4 is sufficient to induce extension of circadian FRP (Figure 2B-F; Careno et al., 2022). This highlights the contribution of cytosolic RNA degradation to the maintenance of circadian rhythms. Loss of XRN4 was sufficient to induce accumulation of deadenylated LWD1 and PRR7 transcripts although circadian rhythms of PRR7, LWD1, and LWD2 total RNA were still observed in ein5-1 seedlings [Figures 3, 4 and 5, (Nagarajan et al., 2019)]. While we cannot exclude a role for XRN4 in the degradation of additional circadian transcripts, further timepoints harvested throughout the diel cycle will be necessary to obtain a comprehensive dataset describing all clock genes regulated by XRN4, as XRN4 accumulation is constant in both long-day and short-day conditions (Litthauer et al., 2018). Indeed, it will also be of interest to understand the contribution of XRN4 towards the translation of circadian proteins during osmotic stress, given the contribution of XRN4 to co-translational decay (Merret et al., 2015; Yu et al., 2016; Carpentier et al., 2020).

The application of osmotic stress induces changes in both steady-state RNA accumulation and the accumulation of partially degraded RNAs (Figures 3, 4, and 5). Although the differences in total RNA accumulation may be mediated in part via the PAP-mediated inhibition of XRN4 activity, it is apparent that XRN4 is not the sole contributor to RNA stability (Figures 3, 4, and 5). For example, the unanticipated reduced accumulation of LWD1 and LWD2 total RNA at peak times in ein5-1 seedlings during osmotic stress indicate that additional factors (such as transcriptional regulation or compensatory RNA degradation mechanisms) are also perturbed by the loss of XRN4 activity (Figure 5; Liu and Chen, 2016). In yeast, the XRN4 functional orthologue XRN1 couples transcription with RNA decay, shuttling into the nucleus as part of a feedback mechanism to regulate mRNA accumulation and translation (Blasco-Moreno et al., 2019; Haimovich et al., 2013; Sun et al., 2013). While this latter mechanism has not been explicitly reported in plants, global analysis of RNA decay in sov seedlings reveals communication between cytoplasmic decay and the transcriptional machinery (Sorenson et al., 2018; Sieburth and Vincent, 2018). Although the activity of XRN4 only accounts for a portion of the phenotypes observed, these data support the hypothesis that the circadian system adapts to osmotic stress through post-transcriptional regulation. Further research is necessary to understand which of these changes are directly precipitated by osmotic stress rather than being an indirect consequence of circadian perturbation.
**PRR7 and LWD1 contribute to circadian responses to osmotic stress**

Although little has been reported regarding the contribution of LWD1 and LWD2 to abiotic stress, PRR7 is increasingly recognized as a crucial circadian component that contributes metabolic information to the molecular timekeeper (Liu et al., 2013, Webb et al., 2019). Since prr7-3 and lwd1 lwd2 seedlings demonstrate impaired circadian responses to osmotic stress [Figure 6, (Nagarajan et al., 2019)] it is possible that altered accumulation of total LWD1, LWD2 and PRR7 transcripts following osmotic stress (Figure 5) contributes to the observed extension of circadian FRP. However, the biological function of these RNAs remains to be determined given that changes in polyadenylated RNA remains modest in constant light (Figure 5).

Previous work has reported enrichment of PRR7 targets that are responsive to abiotic stress (Liu et al., 2013). Indeed, the preponderance of reports linking PRR7 to abiotic stress responses suggest that PRR7 is a central component of plants’ responses to abiotic stresses such as heat, shade and drought (Liu et al., 2013; Kolmos et al., 2014; Blair et al., 2019; Zhang et al., 2020). A relatively high percentage (28%) of PRR7 targets are also ABA-regulated, with more than a third of PRR7 target genes possessing ABA-responsive elements (Liu et al., 2013). It is therefore possible that regulation of PRR7 by XRN4 provides an additional pathway for PAP to modulate ABA-induced signaling (Pornsiriwong et al., 2017). This idea would also align with the proposed role of PRR7 as a dynamic integrator of photosynthetic performance into the circadian system (Webb et al., 2019) and underscore the importance of PRR7 as an integrator of environmental signals.

**Supplementary Data**

**Supplemental Figure 1.** Raw and Normalised bioluminescence waveforms of data presented in Figure 1.

**Supplemental Figure 2.** Assessment of circadian rhythms in xrn seedlings.

**Supplemental Figure 3.** Assessment of deadenylated RNAs following osmotic stress.

**Supplemental Figure 4.** Relative abundance of polyadenylated RNA following application of osmotic stress.

**Supplemental Figure 5.** Hypocotyl lengths of seedlings in the presence or absence of osmotic stress.

**Supplemental Figure 6.** Normalised bioluminescence waveforms of data presented in Figure 5.

**Supplemental Table 1.** Plant genotypes used in this work.

**Supplemental Table 2.** Oligos used for qRT-PCR.
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Author Contributions

PP: Formal analysis, Investigation, Methodology, Visualisation, Writing – Review and editing; SL: Formal analysis, Investigation, Methodology, Visualisation, Writing – Review and editing; FV: Formal analysis, Investigation, Methodology, Visualisation; MWB: Formal analysis, Investigation, Methodology, Visualisation, Writing – Review and editing; MWW: Formal analysis, Investigation, Visualisation; XL: Formal analysis, Investigation, Methodology, Visualisation, Writing – Review and editing; CD: Investigation, Methodology; MAJ: Conceptualisation, Formal analysis, Funding Acquisition, Investigation, Methodology, Project administration, Supervision, Visualisation, Writing – Original draft preparation and Review and editing.

Conflict of Interest

The authors are unaware of any conflict of interest.

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Data availability

The genetic lines generated during this study and data in support of its findings are available from the corresponding author on request.
Literature Cited


Figure 1. Luciferase reporter constructs highlight varied responses to osmotic stress. (A) Circadian free-running period of pCCA1::LUC2, pGI::LUC2, pPRR9::LUC2, pLWD1::LUC2, pLWD2::LUC2 reporter constructs in the presence or absence of osmotic stress. Plants were grown on 0.5 MS media in 12:12 light:dark cycles before transfer to 0.5MS in the presence or absence of 200mM mannitol 24 hours prior to imaging in constant red and blue light (30 μmol m⁻² s⁻¹ and 20 μmol m⁻² s⁻¹ respectively). A Mann-Whitney Multiple t-test was used to assess differences in circadian period between treatments. (B) Circadian free-running period of chlorophyll fluorescence in the presence or absence of osmotic stress. Seedlings were treated as described in (A) prior to transfer to constant light for imaging. (C-H) Assessment of rhythmic robustness (Relative Amplitude Error, RAE) against circadian free-running period for data presented in (A) and (B). An RAE of 0 is indicative of a perfect fit whereas an RAE of 1 represents the mathematical limits of rhythm detection (Plautz et al., 1997).
**Figure 2.** XRN4 contributes towards the extension of circadian free-running period in response to osmotic stress. (A) Accumulation of 3’-PhosphoAdenosine 5’-Phosphate (PAP) in Col-0 and fry1-6 seedlings. Plants were grown on 0.5MS media for 12 d prior to transfer to 200mM mannitol. Seedlings were maintained in entraining conditions prior to harvest on the third day after application of osmotic stress (48-72 hours after transfer). Data are the mean of three biological replicates and analysed using paired t-test; s. e. m. is shown. (B) Circadian free-running period of Col-0 (wildtype), xrn2-1, xrn3-3, xrn2-1xrn3-3, xrn2-1xrn3-3xrn4-3, and ein5-1 seedlings were assessed using chlorophyll fluorescence. Seedlings were grown as described in (A) prior to imaging in the absence of osmotic stress. Data were analysed using one-way ANOVA and Tukey’s multiple comparisons test, s. e. m. is shown. (C) Circadian free-running period of xrn4 alleles expressing a pCCA1::LUC2 reporter construct. Seedlings were grown on 0.5 MS media in 12:12 light:dark cycles for 5 days before transfer to 0.5MS in the presence (blue) or absence (grey) of 200mM mannitol. Data were analysed using two-way ANOVA (D-F) Assessment of rhythmic robustness (Relative Amplitude Error, RAE) against circadian free-running period for data presented in (C), with a threshold set to 0.6.
Figure 3. Stability of circadian transcripts is increased by osmotic stress. (A) Comparison of putative XRN4 degradation targets and characterized components of the circadian system. Putative XRN4 targets were previously identified by Parallel Analysis of RNA Ends or GMUCT (PARE; Nagarajan et al. 2019, Carpentier et al. 2020), while defined circadian components were drawn from a previous review (Hsu and Harmer 2014). (B-C) Assessment of LWD1 total transcript stability in Col-0 (B) and ein5-1 (C) seedlings in the presence or absence of osmotic stress. (D-E) Assessment of LWD2 total transcript stability in Col-0 (D) and ein5-1 (E) seedlings in the presence or absence of osmotic stress. (F-G) Assessment of PRR7 total transcript stability in Col-0 (F) and ein5-1 (G) seedlings in the presence or absence of osmotic stress. Seedlings were grown on 0.5MS for 6 days prior to transfer to a mock control media or media containing 200 mM mannitol. Sampling and application of 0.5 mM cordycepin was completed at ZT4 on day 7; 28 hours after transfer to experimental conditions. Data are reported relative to the average accumulation of ATP3, ELF5A-2, PDTPI, and IPP2 transcripts. A simple linear regression applied for each combination of genotype and treatment was applied from t=0.5; p-values are shown when the slope is significantly different from 0. Data are the mean of at least three independent experiments, n>10. Error bars indicate s. e. m.
Figure 4. mRNA degradation is modulated by osmotic stress and XRN4 activity. (A) Outline of RNA degradation pathways in Arabidopsis. Following deadenylation, RNAs are degraded in a 3’-5’ direction by the exosome or SOV (note that Col-0 is an sov mutant; Zhang et al. 2010). XRN4 degrades RNA in a 5’-3’ direction following endonucleic cleavage or 5’ decapping. Compensatory adjustments between these parallel pathways occur following mutation of ribonucleases, and changes in transcription rates in these cases (‘RNA buffering’) have also been reported (Sorenson et al. 2018). Created with BioRender.com. (B-E) Assessment of deadenylated transcript accumulation in wild type and ein5-1. CCR2 (B), LWD1 (C), LWD2 (D), and PRR7 (E) RNAs were monitored. Application of 0.5 mM cordycepin and subsequent sampling was completed at ZT4 on day 7. (F-I) Assessment of deadenylated transcript accumulation in wild type and ein5-1 following the application of osmotic stress. CCR2 (F), LWD1 (G), LWD2 (H), and PRR7 (I) RNAs were monitored. Seedlings were grown on 0.5MS for 6 days prior to transfer to 200 mM mannitol, cordycepin was added at ZT4; 28 hours after application of osmotic stress. Data are reported relative to the average accumulation of ATP3, ELF5A-2, and PDTPI transcripts. Data are the mean of at least three independent experiments, n>10. A simple linear regression for each combination of genotype and treatment was applied from t=15; colour-coded p-values are shown when the slope is significantly different from 0. Separately, post-hoc Mann-Whitney tests were applied to compare initial deadenylated RNA levels at t=15. Data are the mean of at least three independent experiments, n>10. Error bars indicate s. e. m.
Figure 5. Relative polyadenylated and total RNA abundance of selected circadian clock genes following application of osmotic stress. Wild-type and ein5-1 seedlings were grown on 0.5 MS media in 12:12 light:dark cycles for 5 days before transfer to 0.5MS in the presence (red) or absence (black) of 200mM mannitol. Seedlings were returned to entraining conditions for 24 hours prior to transfer to continuous white light (60 μmol m⁻² s⁻¹). Fold-change in CCA1 (A), LWD1 (B), LWD2 (C), and PRR7 (D) is presented relative to three circadian reference genes listed in Supplemental Table 1. cDNA was synthesized using either an Oligo dT primer or random hexamer to obtain PolyA+ and total transcript, respectively. Data were normalised using 2⁻ⁿ⁻dCt method. Data are representative of at least three independent experiments (n > 10). Error bars indicate s. e. m.
Figure 6. Loss of *LWD1*, *LWD2*, or *PRR7* perturbs circadian responses to osmotic stress. (A) Circadian free-running period of pCCA1::LUC2 in wildtype, ein5-1, lwd1, lwd2, and lwd1lwd2 backgrounds. p-values for the difference in circadian free-running period following osmotic stress is shown (Mann-Whitney Multiple t-test). (B-E) Assessment of rhythmic robustness (Relative Amplitude Error, RAE) against circadian free-running period for data presented in (A), with a threshold set to 0.6. (F) Circadian free-running period of pGI::LUC2 reporter lines in wildtype and prr7-3 backgrounds. p-values for the difference in circadian free-running period following osmotic stress is shown (Mann-Whitney Multiple t-test). (G,H) Assessment of rhythmic robustness (Relative Amplitude Error, RAE) against circadian free-running period for data presented in (F), with a threshold set to 0.6. Plants were grown on 0.5 MS media for 5 days and transferred to 0.5MS with or without 200 mM mannitol 24 hours before imaging under constant constant red and blue light (30 µmol m⁻² s⁻¹ and 20 µmol m⁻² s⁻¹ respectively).