TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought

Tommaso Legnaioli¹, Juan Cuevas¹
and Paloma Mas*

Department of Plant Molecular Genetics, Centre for Research in Agricultural Genomics (CRAG, CSIC-IRTA-UAB), Barcelona, Spain

Despite our increasing knowledge on the transcriptional networks connecting abscisic acid (ABA) signalling with the circadian clock, the molecular nodes in which both pathways converge to translate the environmental information into a physiological response are not known. Here, we provide evidence of a feedback mechanism linking the circadian clock with plant responses to drought. A key clock component (TOC1, timing of CAB expression 1) binds to the promoter of the ABA-related gene (*ABAR/CHLH/GUN5*) and controls its circadian expression. *TOC1* is in turn acutely induced by ABA and this induction advances the phase of *TOC1* binding and modulates *ABAR* circadian expression. Moreover, the gated induction of *TOC1* by ABA is abolished in *ABAR RNAi* plants suggesting that the reciprocal regulation between *ABAR* and *TOC1* expression is important for sensitized ABA activity. Genetic studies with *TOC1* and *ABAR* over-expressing and *RNAi* plants showed defective responses to drought, which support the notion that clock-dependent gating of ABA function is important for cellular homeostasis under dry environments.

The EMBO Journal (2009) 28, 3745–3757. doi:10.1038/emboj.2009.297; Published online 8 October 2009

Subject Categories: chromatin & transcription; plant biology

Keywords: abscisic acid (ABA); *Arabidopsis thaliana*; circadian clock; feedback loops; drought

Introduction

Plants, as sessile organisms, have evolved a number of ways to rapidly sense and adjust to the changing environmental conditions. One of the most studied mechanisms involved in these adjustments is the biological or circadian clock, which is able to maintain biological rhythms with a period of approximately 24 h (Más, 2005; McClung, 2008; Harmer, 2009). Circadian clocks were suggested to confer adaptive advantages to organisms (Ouyang *et al*, 1998; Green *et al*, 2002; Michael *et al*, 2003; Dodd *et al*, 2005) by means of not merely responding to the external signals but also

anticipating the predictable environmental variations that occur during the day–night transitions. The presence of an endogenous timing system thereby allows the synchronization of biological activities to occur at specific phase relationships with the environment, permitting the temporal separation of incompatible metabolic events (Más, 2005; Hotta *et al*, 2007; McClung, 2008; Harmer, 2009).

Intensive research efforts have been devoted in past years to decipher the molecular and biochemical mechanisms underlying circadian clock function. In plants, as in many other organisms, the circadian rhythmicity seems to rely on multiple negative feedback loops at the core the oscillator (Bell-Pedersen *et al*, 2005; Wijnen and Young, 2006). Molecular-genetic approaches in *Arabidopsis thaliana* have aided in the identification of clock components and mechanisms of regulation within the circadian oscillator (Más, 2008). The reciprocal regulation between a pair of Myb transcription factors, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) (Wang and Tobin, 1998) and LATE ELONGATED HYPOCOTYL (Schaffer *et al*, 1998) with a pseudo-response regulator TIMING OF CAB EXPRESSION 1 (TOC1 or PRR1) (Strayer *et al*, 2000; Makino *et al*, 2002) was initially proposed as a core feedback mechanism important for clock function (Alabadí *et al*, 2001). Computer modelling and experimental validation have subsequently confirmed the existence of additional, interconnected multiple loops that confer robustness and flexibility to the oscillatory activities (Rand *et al*, 2004; Locke *et al*, 2006; Zeilinger *et al*, 2006).

Despite the increasing knowledge of the molecular networks comprising the *Arabidopsis* circadian oscillator, little is known about the precise biochemical and molecular function of TOC1 within the clock. *TOC1* mutant and *RNAi* plants display a short-period phenotype for clock-controlled gene expression as well as day length-insensitive flowering time (Strayer *et al*, 2000). The constitutive high expression of *TOC1* leads to arrhythmia under constant light conditions in a number of clock outputs (Makino *et al*, 2002; Más *et al*, 2003a). TOC1 was also proposed to function between the environmental signals and the circadian and photo-morphogenic outputs (Más *et al*, 2003a). Further studies have evidenced that a very precise regulation of TOC1 gene and protein rhythmic expression is essential for proper clock function. Different mechanisms contribute to this regulation including changes in chromatin structure (Perales and Más, 2007), transcriptional regulation (Alabadí *et al*, 2001) and protein degradation by the proteasome pathway (Más *et al*, 2003b), which together, accurately control the 24 h rhythmic oscillation of *TOC1* expression and function.

The use of genome-wide approaches has provided some clues into the global transcriptional networks controlled by the clock. Circadian clock regulation of the *Arabidopsis* transcriptome is highly extensive although the precise fraction of the genome that is circadian regulated highly

*Corresponding author. Plant Molecular Genetics, Centre for Research in Agricultural Genomics (CRAG, CSIC-IRTA-UAB), C/ Jordi Girona 18-26, Barcelona 08034, Spain. Tel.: +34 93 400 6100/ext. 277; Fax: +34 93 204 5904; E-mail: paloma.mas@cid.csic.es

¹These authors contributed equally to this work

Received: 29 July 2009; accepted: 10 September 2009; published online: 8 October 2009

varies among the different studies (Harmer *et al*, 2000; Schaffer *et al*, 2001; Michael and McClung, 2003; Edwards *et al*, 2006; Covington and Harmer, 2007; Dodd *et al*, 2007; Covington *et al*, 2008; Mizuno and Yamashino, 2008; Michael *et al*, 2008a,b; Hazen *et al*, 2009). A recent study has integrated the information from multiple circadian datasets to get a better estimation of the expressed gene fraction that is circadianly regulated (Covington *et al*, 2008). The use of tiling arrays has also identified the circadian regulation of intergenic regions, introns and natural antisense transcripts, which has extended the pervasiveness of clock function far beyond protein coding genes (Hazen *et al*, 2009). The functional clustering of clock-regulated genes has also provided insights into metabolic and physiologic pathways that are under circadian control. Indeed, clock-regulated genes are over-represented among several plant hormone and stress-responsive pathways. This is consistent with the diurnal variations of phytohormone abundance (Robertson *et al*, 2009) and with the gated regulation of plant hormone sensitivity by the clock (Covington and Harmer, 2007; Dodd *et al*, 2007).

One of the hormones regulated by the circadian clock is abscisic acid (ABA). This phytohormone is essential in the regulation of many plant growth and development processes as well as in the control of plant responses to stressful environments (Leung and Giraudat, 1998; Finkelstein *et al*, 2002; Zhu, 2002). The significant number of ABA-related genes that are controlled by the clock has been reported in various studies (Covington and Harmer, 2007; Dodd *et al*, 2007; Mizuno and Yamashino, 2008). Furthermore, the regulation between ABA and clock signalling pathways is bidirectional as treatment with ABA lengthens the circadian period of gene promoter activity (Hanano *et al*, 2006). Evidence of a feedback loop between cADPR and the circadian oscillator also suggests a mechanism by which the circadian coordination of ABA-transducing components might be involved in clock-mediated regulation of plant responses to ABA (Dodd *et al*, 2007). The influence of the clock is not limited to gene expression but is also evidenced in the circadian regulation of physiological processes controlled by ABA. For instance, under water-deficit conditions, ABA induces the closure of the stomatal pore, and this closure was found to be gated by the clock. Indeed, ABA is less effective at closing the stomata in the morning than in the afternoon (Correia *et al*, 1995), which may ensure that stomata are closed in the heat of the afternoon if water supply is limited (Robertson *et al*, 2009).

The cellular changes in ABA concentration (Verslues and Zhu, 2007) trigger a downstream network of signalling cascades that ultimately regulate vegetative and reproductive processes and result in enhanced plant tolerance to the environmental stress (Hirayama and Shinozaki, 2007). Essential for ABA signalling is the perception of hormone changes by ABA receptors (Hirayama and Shinozaki, 2007). Among the possible hormone sensors, recent reports have proposed that regulators of ABA-related protein phosphatases 2C may function as ABA receptors within the ABA signalling pathway (Ma *et al*, 2009; Park *et al*, 2009). The H subunit of the magnesium-protoporphyrin IX chelatase (ABAR/CHLH/GUN5, At5g13630) was arguably proposed to function as an ABA receptor because of its ABA-binding activity and the

ABA-related phenotypes in germination, stomatal closure and responses to water-deficit conditions of ABAR mis-expressing plants (Shen *et al*, 2006). In broad bean (*Vicia faba*), ABAR is also involved in ABA-induced stomatal signalling and, equally to its homologous in Arabidopsis, was proposed to specifically bind ABA (Zhang *et al*, 2002). However, the possible function of ABAR as a receptor was questioned in a study reporting that in barley (*Hordeum vulgare* L.), the magnesium chelatase large subunit does not bind ABA (Muller and Hansson, 2009). This issue was addressed in a recent study by using a newly developed ABA-affinity chromatography technique, which convincingly showed the specificity of the ABA binding to ABAR (Wu *et al*, 2009). Furthermore, the authors also assigned ABA-related functions to particular ABAR protein domain (Wu *et al*, 2009). Altogether, the results in Arabidopsis consign an important role for ABAR within the ABA signalling pathway.

Here, we uncover ABA-related phenotypes for *TOC1* mis-expressing plants and confirm a role for ABAR in the ABA signalling pathway. *TOC1* is induced by ABA, and this induction is gated by the clock and determines the timing of *TOC1* binding to the *ABAR* promoter. Molecular-genetic studies show the existence of a negative feedback loop in which *TOC1* negatively regulates the expression of *ABAR*, whose activity is in turn necessary for *TOC1* activation by ABA. Our studies suggest that proper timing of this feedback loop is important for ABA-mediated changes in gene expression and plant responses to drought conditions.

Results

Genome-wide analysis of *TOC1* transcriptional networks

To gain insights into the mechanisms of *TOC1* function in the clock, we performed genome-wide transcriptomic analysis of wild-type (WT) and *TOC1* over-expressing plants (*TOC1* cDNA fused to YFP, herein denominated *TOC1-ox*) (Más *et al*, 2003a) synchronized under 12 h light:12 h dark (LD) cycles for 10 days. Statistical analysis showed a total of 245 significantly increased and 160 significantly decreased genes in *TOC1-ox* compared with WT (Supplementary Table S1). We also analysed transcriptomic data of WT and *TOC1* mutant plants (*toc1-2*) (Strayer *et al*, 2000) synchronized under LD cycles followed by 2 days under constant light (LL) conditions and found that about 75 genes were downregulated and 105 upregulated in *toc1-2* samples (Supplementary Table S2). Using a recently published circadian dataset (Hazen *et al*, 2009) we found that about 50% (in *TOC1-ox*, Fisher's exact test $P = 2.2 \times 10^{-16}$) and over 40% (in *toc1-2*, Fisher's exact test $P = 1.8 \times 10^{-7}$) of the mis-regulated genes showed circadian fluctuations in mRNA abundance (Figure 1A and Table I). Similar or even higher percentages were obtained when other circadian datasets (Covington *et al*, 2008) were examined (Table I). Among all of the mis-regulated genes, we were expecting to find a significant proportion of clock-related genes not directly regulated by *TOC1*. Noticeably, meta-analysis of mis-regulated genes using databases that contain a broad spectrum of transcriptomic profiles (<https://www.genevestigator.com/gv/index.jsp>) also showed a significant overlap with transcripts involved in ABA signalling pathways (Supplementary Figure S1). Functional clustering using specific ABA-related datasets (Matsui *et al*,

Figure 1 Genome-wide analysis of TOC1 transcriptional networks. (A) Percentages of cyclers and ABA-related genes at the whole-genome level, and in TOC1-ox and *toc1-2* transcriptomic datasets (***P*-value < 10⁻⁸; ****P*-value < 10⁻¹¹; *****P*-value < 10⁻¹⁵) (B) Venn diagrams showing the overlap between cyclers and ABA-related genes in TOC1-ox and *toc1-2* transcriptomic datasets. (C) Hierarchical clustering of *toc1-2* and TOC1-ox mis-regulated genes with transcripts involved in dehydration responses. Percentages of dehydration (D) at the whole-genome level, and in TOC1-ox and *toc1-2* transcriptomic datasets, and among these, the ABA-related genes (E) (**P*-value < 10⁻³; ***P*-value < 10⁻⁶; ****P*-value < 10⁻¹¹; *****P*-value < 10⁻¹⁵). Details of datasets used for analysis are described in Supplementary data.

Table I Meta-analysis of TOC1 mis-regulated genes with publicly available datasets of cycling, ABA- and drought-related genes

Hormone/ treatment/condition	TOC1-ox		<i>toc1-2</i>	
	Enriched genes (%)	<i>P</i> -value	Enriched genes (%)	<i>P</i> -value
Cyclers (Hazen <i>et al</i> , 2009)	48.14	2.2 × 10 ⁻¹⁶	42.22	1.7 × 10 ⁻⁷
Cyclers (Covington <i>et al</i> , 2008)	55.17	2.2 × 10 ⁻¹⁶	59.22	5.6 × 10 ⁻⁹
ABA (Matsui <i>et al</i> , 2008)	53.82	2.2 × 10 ⁻¹⁶	48.33	6.0 × 10 ⁻¹²
ABA (Matsui <i>et al</i> , 2008) Cyclers (Hazen <i>et al</i> , 2009)	54.58	1.36 × 10 ⁻⁷	54.02	6.2 × 10 ⁻⁴
Drought (Matsui <i>et al</i> , 2008)	38.51	2.2 × 10 ⁻¹⁶	37.22	3.4 × 10 ⁻¹⁰

2008) confirmed the highly significant number of ABA-responsive genes in TOC1-ox (53.82%, Fisher's exact test $P = 2.2 \times 10^{-16}$) and in *toc1-2* (48.33%, Fisher's exact test $P = 6.0 \times 10^{-12}$) (Figure 1A; Table I). Both, upregulated and downregulated set of genes were found to be associated with ABA transcriptional networks (Supplementary Figure S2A). The list of mis-regulated genes included relevant components of the ABA signalling pathway (Finkelstein *et al*, 2002) such as ABA1, Histone H1-3, RD29B, several late embryogenesis abundant proteins and ABAR/CHLH/GUN5 (Supplementary Tables S3 and S4). We also checked whether clock dysfunction in TOC1 mis-expressing plants was affecting other hormone transcriptional networks. However, functional clustering analysis showed that, with the exception of methyl jasmonate, no other hormone pathways were as significantly affected as the ABA (Supplementary Figure S2B and C). Furthermore, a significant proportion of these ABA-related genes show a circadian oscillation (54.58% in TOC1-ox, Fisher's exact test $P = 1.36 \times 10^{-7}$ and 54.02% in *toc1-2*, Fisher's exact test $P = 0.6 \times 10^{-4}$) (Figure 1B and Table I).

Our data analysis also showed that about 38% of the mis-regulated genes in TOC1-ox (Fisher's exact test $P = 2.2 \times 10^{-16}$) and in *toc1-2* (Fisher's exact test $P = 3.4 \times 10^{-10}$) were related to plant responses to dehydration (Figure 1D and Table I). These percentages are higher than the 18% of the

genes that are associated with drought responses in the genome (Matsui *et al*, 2008). This is noteworthy because the plant response to changes in water status is one of the many processes controlled by ABA. Consistently, the analysis of common elements in the promoters of the mis-expressed genes also showed a significant over-representation of motifs implicated in molecular responses to dehydration (Supplementary Table S5). Furthermore, two- and three-fold comparisons of circadian, ABA and drought-related genes showed that 81.41% (in TOC1-ox) and a 74.62% (in *toc1-2*) of the drought-related genes are regulated by ABA (Figure 1E). We also found an important overlapping among ABA and drought genes that were regulated by the circadian clock in both TOC1-ox (Supplementary Figure S2D) and *toc1-2* (Supplementary Figure S2E) datasets. Analysis by quantitative PCR (Q-PCR) confirmed the mis-expression of some selected genes in TOC1-ox and *toc1-2* mutant plants (Supplementary Figures S3 and S4). When other relevant dehydration-related genes were analysed by Q-PCR, we also found a significant mis-expression, suggesting that the drought-related transcriptional networks altered in TOC1 mis-expressing plants might be even more important than estimated by our microarray data. Altogether, these data led us to further examine the possible link between TOC1 and plant responses to drought mediated by ABA.

ABA-mediated responses to drought are impaired in *TOC1* mis-expressing plants

If the changes in gene expression correlate with functional alterations in physiological responses, then plants mis-expressing *TOC1* should display ABA-related phenotypes. Data analysis showed that many stress-responsive genes known to be involved in dehydration tolerance (Ingram and Bartels, 1996; Thomashow, 1999) were downregulated in *TOC1*-ox plants, suggesting that *TOC1*-ox plants might have reduced tolerance to drought. We therefore analysed responses to drought stress in WT, *TOC1*-ox, *toc1-2* and *TOC1 RNAi* plants (Más *et al*, 2003a) by examining percentages of recovery after dehydration using seedlings grown on agar plates (Fujita *et al*, 2005). Our results showed that most *TOC1*-ox plants were unable to recover from the drought stress, exhibiting a reduced survival rate (2%) after re-watering (Figure 2A and B). In contrast, approximately 50% of the *toc1-2* (not shown) and 52% of *TOC1 RNAi* plants completely recovered the drought (Figure 2A and B) whereas WT plants showed intermediate phenotypes and about 15% recovered after re-watering (Figure 2A and B). Thus, over-expression of *TOC1* significantly reduced the plant tolerance to drought

(P -value < 0.0001) whereas *toc1-2* and *TOC1 RNAi* plants responded better than WT to water-deficit conditions (P -value < 0.0001). The survival rates of *TOC1 RNAi* plants on dehydration are a long-term response that is unlikely because of altered clock phase in these plants.

The altered tolerance of *TOC1* mis-expressing plants to water stress might be due to deficient stomatal closure under dehydration conditions. Thus, we next examined stomatal guard cell movement that is controlled by both ABA and the circadian clock (Robertson *et al*, 2009). The experiments were performed at midday, a time when stomata are more responsive to ABA (Robertson *et al*, 2009). As expected, ABA treatment in WT leaves caused significant stomatal closure (Figure 2C and D). However, this effect was not as pronounced in *TOC1*-ox whereas stomata in *TOC1 RNAi* (not shown) and *toc1-2* mutant leaves were more effectively closed (Figure 2C and D). Compared with WT, the stomatal response to ABA was significantly altered in *TOC1*-ox (P -value < 0.001), *TOC1 RNAi* (P -value < 0.01) and *toc1-2* (P -value < 0.01). Consistent with these results, the stomatal conductance of *TOC1*-ox plants was not as responsive to the treatment with ABA as in WT plants (Figure 2E)

Figure 2 Altered responses to drought conditions of plants mis-expressing *TOC1*. (A) Plant survival to dehydration stress on agar plates. Data are means $\pm$ s.e.m. of duplicate experiments with at least 25 plants per genotype. (B) Representative photographs of *TOC1*-ox (left), WT (middle) and *TOC1 RNAi* (right) plants of the dehydration experiments. (C) Stomatal aperture in rosette epidermis of *TOC1*-ox, WT and *toc1-2* plants. Stomatal dimensions were measured after incubation for 2 h in a buffer containing 0, 1 or 5 μ M ABA. Data are means $\pm$ s.e.m. of duplicate experiments with at least 100 stomata per genotype and per treatment. (D) Representative images by light microscopy of the stomata guard cells. (E) Stomatal conductance of *TOC1*-ox, WT and *toc1-2* mutant plants. Gas exchange measurements were performed with at least 10 rosettes for genotype. (F) Water-loss rates of detached rosettes from WT, *TOC1*-ox and *toc1-2* plants. Data are means $\pm$ s.e.m. of triplicate measurements with at least five rosettes for genotype at each time point. Compared with WT, the phenotypic differences were statistically significant with P -values below 0.01 in all cases.

whereas *toc1-2* mutant plants showed an increased response (Figure 2E). Statistical analysis verified the significance of the differential ABA effects, with P -values < 0.001 . If the stomatal closure and conductance are impaired in *TOC1* over-expressing plants, then the leaf water-loss rates might also be affected. Thus, we next measured the water-loss rates of detached rosettes in *TOC1* mis-expressing plants. Our studies showed that 3 h after detachment, the leaves of *TOC1 RNAi* plants showed a 40% of water loss in contrast to the 54 and 75% of WT and *TOC1-ox* plants, respectively (Figure 2F). These differences were observed throughout the time course analysis (Figure 2F).

Collectively, our results indicate that proper *TOC1* expression is important for stomatal function and regulation of leaf transpiration rates, a notion that is reinforced by the opposite phenotypes of *TOC1* over-expressing and mutant plants. In addition to the previously reported and well-characterized clock phenotypes, our study shows an unpredicted role for *TOC1* in the control of ABA-mediated plant responses to drought.

TOC1 regulates the diurnal and circadian expression of *ABAR*

One of the mis-regulated genes in *TOC1* microarray datasets was *ABAR/CHLH/GUN5* (Shen *et al*, 2006; Wu *et al*, 2009). This was of interest because *ABAR* was initially reported as an ABA receptor and because preliminary studies in the laboratory had linked *TOC1* with *ABAR* (Riechmann JL and Mas P, unpublished results). Although a recent report in barley has questioned the function of *ABAR* in ABA signalling (Muller and Hansson, 2009), our phenotypic studies using different Arabidopsis *ABAR RNAi* lines with

decreased patterns of *ABAR* expression (Figure 3A) were consistent with previous reports (Shen *et al*, 2006; Wu *et al*, 2009). Indeed, compared with the effect of ABA on WT plants (Figure 3B and C), the stomata in different *ABAR RNAi* lines were almost insensitive to the treatment with the hormone (Figure 3B and C). Differences were statistically significant in the absence (P -value < 0.0001) and in the presence of ABA (P -value < 0.0001). Consistent with these and the previously described phenotypes (Shen *et al*, 2006; Wu *et al*, 2009), our studies also showed that the water-loss rates of *ABAR RNAi* plants were significantly higher than in WT (P -value < 0.005 at all time points) (Figure 3D). Furthermore, comparisons of *ABAR* mRNA expression with the severity of water-loss phenotypes in different *RNAi* lines showed a significant inverse correlation between *ABAR* mRNA abundance and water-loss rates (Figure 3E). Finally, our studies also showed that the impaired stomatal closure and water-loss rates also correlated with altered responses to dehydration conditions (Figure 3F) with a reduced survival percentage of the different *ABAR RNAi* plants. Altogether, our results are in concordance with the previous study in Arabidopsis and verify a role of *ABAR* within the ABA signalling pathway in Arabidopsis.

On the basis of the ABA-related phenotypes and the pattern of *ABAR* expression in *TOC1* microarrays, we next explored the possible link between the circadian clock and *ABAR* expression. To do so, we first performed time course analyses over a diurnal cycle of WT plants grown under short-day (ShD) and long-day (LgD) conditions. Our results showed that *ABAR* transcript abundance rhythmically oscillated with a peak of expression close to dawn (Supplementary Figure S5A). Similar oscillatory patterns were observed when

Figure 3 Altered responses to drought conditions of plants mis-expressing *ABAR*. (A) Analysis by Q-PCR of *ABAR* mRNA expression in WT and several *ABAR RNAi* lines. (B) Stomatal aperture in rosette epidermis after incubation for 2 h in a buffer containing 0 or 5 μ M ABA. Data are means $\pm$ s.e.m. of duplicate experiments with at least 100 stomata per genotype and per treatment. (C) Representative images by light microscopy of the stomata guard cells. (D) Water-loss rates of detached rosettes from WT and *ABAR RNAi* plants. Data are means $\pm$ s.e.m. of triplicate measurements with at least five rosettes for genotype at each time point. (E) Correlation between *ABAR* mRNA abundance and weight-loss changes of detached rosettes. Weight-loss changes were plotted relative to the WT value. (F) Plant survival to dehydration stress on agar plates. Data are means $\pm$ s.e.m. of duplicate experiments with at least 25 plants per genotype. Compared with WT, the phenotypic differences were statistically significant with P -values below 0.005 in all cases.

ABAR expression was examined under LL conditions (Supplementary Figure S5B) indicating that the circadian clock controls the expression of *ABAR*. As *TOC1* is an essential component of the Arabidopsis circadian system, we reasoned that the diurnal and circadian expression of *ABAR* might be controlled by *TOC1*. Indeed, our results showed that the waveform of *ABAR* mRNA was altered in *TOC1 RNAi* and *TOC1-ox* plants under LL. The phase of *ABAR* expression in *TOC1 RNAi* was advanced, leading to higher mRNA abundance at times when *ABAR* expression is at its minimum in WT plants (Figure 4A). The waveform of *ABAR* expression in *TOC1 RNAi* plants also suggests that *ABAR* might be complexly regulated in the absence of a functional

TOC1. In *TOC1-ox* plants, *ABAR* expression was clearly affected, with very reduced abundance throughout the circadian cycle (Figure 4B). Changes in *ABAR* expression were also observed in *TOC1-ox* and *TOC1 RNAi* plants growing under LD cycles (Supplementary Figure S5C and D). The alterations in *ABAR* expression were also observed when seedlings were treated with ABA (Supplementary Figure S5E and F).

Together, these results indicate that the circadian clock controls the timing of *ABAR* expression. Our studies also assign a key function for *TOC1* in modulating the phase and amplitude of *ABAR* diurnal and circadian waveform. The circadian period and/or diurnal phase changes in *toc1-2*

Figure 4 *TOC1* binds to the *ABAR* promoter and regulates *ABAR* circadian expression. (A, B) Northern blot analysis of *TOC1 RNAi* and *TOC1-ox* plants synchronized under LD cycles followed by 2 days under LL. The waveforms of *ABAR* expression after mRNA quantification are shown below the blots. CT, circadian time. (C) ChIP assays with *TOC1-ox* (right axis) and TMG plants (left axis) after *TOC1* immuno-precipitation with an antibody to YFP followed by Q-PCR amplification of the *ABAR* promoter. (D) Comparison of the antiphasic waveforms of *ABAR* expression by northern blot and *TOC1* binding to the *ABAR* promoter by ChIP assays. (E) Effects of ABA on the waveform of *TOC1* binding to the *ABAR* promoter. (F) Effects of ABA on the *ABAR* mRNA expression. ChIP abundance was plotted relative to the maximum value.

and *TOC1*-ox plants might affect the proper expression of *ABAR* and other ABA/drought-related genes, and this mis-regulation may alter the proper timing of plant responses to drought.

TOC1 is physically associated to the ABAR promoter

As DNA-binding events are crucial in ABA signalling pathways (Busk and Pagès, 1998), we next explored whether regulation of *ABAR* expression occurred through binding of *TOC1* to the *ABAR* locus. Chromatin immunoprecipitation (ChIP) assays were performed with *TOC1*-ox and *TOC1* minigen plants (TMG, expressing the *TOC1* gene under its own promoter) (Más *et al*, 2003a). The TMG plants more closely resemble the WT conditions (as compared with the arrhythmic *TOC1*-ox) although the TMG clock runs slower than in WT, with a delayed phase of rhythmic gene expression (Más *et al*, 2003a). Time course experiments throughout the 24 h cycle showed significant amplification, consistent with the *in vivo* binding of *TOC1* to the *ABAR* promoter (Figure 4C). Further analysis showed a rhythmic oscillation in the binding, with a peak at a time when *ABAR* expression is minimal (Figure 4C and D). Our results showed that *TOC1* binding is antiphasic to *ABAR* mRNA expression (Figure 4D) and this is consistent with the inverse correlation between abundance and phenotypes of *TOC1* and *ABAR*. The binding seems to be restricted to the *ABAR* promoter, and more specifically located around the transcription start site (Supplementary Figure S6). No evident amplification was observed with primers flanking a region in the fourth exon of the *ABAR* gene (Supplementary Figure S6). Binding ChIP assays with *TOC1*-ox plants showed significant amplification of the *ABAR* promoter at all times examined, indicating that *TOC1* protein remains bound throughout the cycle (Figure 4C). This constant binding (as opposed to the oscillatory waveform in TMG plants) correlates with a constant repression of *ABAR* (compare Figure 4B and C) and might account for the ABA-related phenotypes observed in *TOC1*-ox plants. On the basis of this notion, we expected that treatment with ABA should affect *TOC1* binding and/or *ABAR* expression. To check this possibility, we next performed a time course of ChIP assays in TMG samples treated with the hormone. We also compared by northern blot the pattern of *ABAR* expression in ABA-treated and untreated plants. Our results showed a transient change in the phase of *TOC1* binding to the *ABAR* promoter in ABA-treated TMG plants (Figure 4E). Concomitantly, we also observed a change in *ABAR* mRNA waveform, with a significant acute repression (P -value < 0.005) around midday, the time window when *TOC1* is preferentially bound to the *ABAR* promoter (Figure 4F). These results indicate a temporal coincidence between the ABA-mediated regulation of *ABAR* expression and *TOC1* binding to the *ABAR* promoter. The specificity of this association was confirmed by analysing *TOC1* binding to other relevant ABA-related loci. Our results showed that with the exception of *ABAR*, none of the selected loci was amplified (Supplementary Figure S7). Binding was not observed even after treating the seedlings with ABA (Supplementary Figure S8).

Altogether, our results show that *TOC1* binds to the *ABAR* promoter. This binding is regulated not only by the circadian clock but also by the ABA, which advances the phase of *TOC1* binding and acutely represses *ABAR* expression around

midday. On the basis of the phenotypes of *ABAR* and *TOC1* mis-expressing plants, this regulation is most likely important for ABA-mediated plant responses to drought.

TOC1 expression is acutely induced by ABA and this regulation is gated by the clock and requires a functional ABAR

Relevant to our studies was the observation that *TOC1* was included in the microarray dataset of genes regulated by ABA (Matsui *et al*, 2008). To examine this new regulatory mechanism, we monitored bioluminescence rhythms of plants expressing the luciferase gene (*LUC*) driven by the *TOC1* promoter (*TOC1::LUC*) in the presence of ABA. Our results showed that ABA treatments administered during the subjective day acutely induced *TOC1::LUC* expression (Figure 5A). The magnitude of induction was not constant but progressively increased throughout the day, reaching maximum values at 5–10 h after the subjective dawn (Figure 5A). Administration of ABA during the subjective night had no clear immediate consequences on *TOC1::LUC* amplitude or phase (Figure 5B). On subsequent days, the extended time with the hormone led to a decreased amplitude and slight delay in the phase of *TOC1::LUC* expression. The time-of-day-dependent regulation of *TOC1* expression by ABA indicates that the acute response is gated by the clock and correlates with the timing of *TOC1* binding and changes in *ABAR* expression by ABA.

The transient induction of *TOC1* is suggestive of a fine-tuned switch mechanism that regulates the ABA-mediated effects on *TOC1* expression. As feedback loops are common mechanisms for precise regulation of gene expression, we explored the possible function of *ABAR* in *TOC1* induction by ABA. We therefore examined the regulation of *TOC1::LUC* expression in *ABAR RNAi* plants, lacking a functional expression of the *ABAR* gene. Our results showed that in the absence of ABA, *TOC1::LUC* expression in *ABAR RNAi* plants was similar to the one observed in WT plants, although we found a slightly advanced phase of *TOC1::LUC* expression that was not reproducibly observed in all *ABAR RNAi* lines (Supplementary Figure S9). After treatment with ABA, the acute induction of *TOC1::LUC* in WT plants was completely abolished in *ABAR RNAi* plants (Figure 5C) with a slight delay in the oscillatory phase of *TOC1::LUC* expression and with bioluminescence signals lower than those of untreated *ABAR RNAi* samples (Figure 5C). Furthermore, upregulation of *TOC1::LUC* expression by ABA was not observed at any time during the circadian cycle. The role of *ABAR* in *TOC1* induction was also verified by northern blot analysis. Our results showed that in *ABAR RNAi* samples, *TOC1* mRNA steady state was not upregulated after ABA treatment as opposed to the gated induction observed in WT plants (Figure 5D and E; Supplementary Figure S10). Together, these results indicate that the ABA-mediated acute induction of *TOC1* requires the presence of a functional *ABAR*, which positively regulates the expression of *TOC1*. Noticeably, this regulation seems to have an important function only in the presence of ABA. In the absence of exogenous hormone, the waveform of *TOC1* expression is not clearly affected, with the possible exception of a slightly advanced phase. These results suggest that *ABAR* is important for regulation of *TOC1* expression within the ABA signalling pathway but not within the circadian pathway. Our results also indicate the

Figure 5 *TOC1* is acutely induced by ABA and this regulation is gated by the clock and requires a functional *ABAR*. (A, B) *TOC1::LUC* expression in WT plants treated with 25 μM ABA at the indicated circadian times (CT) during the subjective day and night. (C) *TOC1::LUC* expression in *ABAR RNAi* plants treated with 25 μM ABA at the indicated CT during the subjective day. Data are means ± s.e.m. of luminescence from 6–12 plants. (D) Northern blot analysis of *TOC1* mRNA expression in WT and *ABAR RNAi* plants. *TOC1* expression was compared in the absence or in the presence of ABA.

existence of a regulatory feedback loop involving the reciprocal regulation of *ABAR* and *TOC1*.

Biological relevance of *ABAR* and *TOC1* interaction

To further investigate whether the reciprocal regulation between *TOC1* and *ABAR* expression is important for ABA signalling, we conducted a genetic study in which the *TOC1*-ox and the *TOC1 RNAi* plants were transformed with the *ABAR RNAi* construct (Shen *et al*, 2006). Several lines for each genotype were selected and the decreased *ABAR* expression was verified by Q-PCR (Supplementary Figure S11). Initial studies were focused on water-loss rates, as analysis of different *ABAR RNAi* transgenic lines showed that the severity of the water-loss phenotypes correlated with the *ABAR* mRNA abundance (Figure 3). In our genetic studies, we predicted that if the *TOC1 RNAi* phenotypes were due to increased *ABAR* expression, then the significantly diminished transpiration rate of *TOC1 RNAi* plants should be overridden by the opposing phenotype of *ABAR RNAi* plants. Conversely, if the ABA-related phenotypes of *TOC1*-ox plants are mediated by the repressed *ABAR* expression, then the transpiration rates of *ABAR RNAi/TOC1*-ox plants should be similar to those of plants over-expressing the repressor *TOC1*. Consistent with these assumptions, our results showed that double *TOC1 RNAi/ABAR RNAi* plants displayed higher transpiration than WT plants, with similar water-loss rates to those of *ABAR RNAi* plants (Figure 6A). As predicted, the water-loss phenotype of *TOC1 RNAi/ABAR RNAi* clearly contrasted to the decreased transpiration of *TOC1 RNAi* (Figure 6A) indicating that the *TOC1 RNAi* phenotype is only apparent in the presence of a functional *ABAR*. Conversely, our results showed that the transpiration rate of *TOC1*-ox was not significantly affected by the reduced expression of *ABAR* in *TOC1*-ox/*ABAR RNAi* plants. These

plants displayed an increased transpiration rate, similar to the phenotype observed for single *TOC1*-ox (Figure 6B). The *ABAR RNAi* plants displayed intermediate phenotypes (Figure 6B) as *ABAR* expression was overall higher in these *RNAi* lines than in *TOC1*-ox/*ABAR RNAi* (Supplementary Figure S11). Similar genetic conclusions were obtained when we performed experiments of dehydration on plates. The high percentages of *TOC1 RNAi* plant survival after dehydration were completely overridden in the double *RNAi* plants, suggesting that *TOC1 RNAi* phenotypes are only evident in the presence of a functional *ABAR* (Figure 6C and D). Conversely, the reduced survival percentages of *TOC1*-ox plants were not clearly affected by the reduced *ABAR* expression in *TOC1*-ox/*ABAR RNAi* lines (Figure 6C and D).

Altogether, the genetic studies are in agreement with the molecular data and functionally connect the reciprocal regulation of *TOC1* and *ABAR* expression with plant tolerance to drought conditions (Figure 6E). We propose that increased ABA concentrations activate *TOC1* expression and this activation is gated by the clock and determines the timing of *TOC1* binding to the *ABAR* promoter and the changes in *ABAR* expression. Our results indicate that *ABAR* is required for *TOC1* induction by ABA. Proper timing of this feedback loop is important for ABA-mediated changes in gene expression and plant responses to drought conditions.

Discussion

Comparisons of circadian and ABA-responsive transcriptional profiles show that the genome-wide fraction of ABA-related genes controlled by the clock is around 40%, a significant percentage that is in agreement with previous studies (Covington *et al*, 2008) and with the notion that ABA signalling

Figure 6 ABAR and TOC1 interaction in plant responses to drought. (A, B) Water-loss percentages of detached rosettes from WT, *ABAR RNAi*, *TOC1 RNAi*, double *ABAR/TOC1 RNAi*, *TOC1-ox* and *TOC1-ox/ABAR RNAi* plants. Data are means $\pm$ s.e.m. of triplicate measurements with at least 10 rosettes for genotype at each time point. (C) Survival percentages of WT, *ABAR RNAi* (A-R), *TOC1 RNAi* (T-R), double *ABAR/TOC1 RNAi* T/A-R, *TOC1-ox* (Tox) and *TOC1-ox/ABAR RNAi* (Tox/A-R) plants subjected to dehydration on agar plates. (D) Representative photographs of plants in the dehydration experiments. (E) Schematic representation depicting the reciprocal regulation between TOC1 and ABAR and the implication of ABA and the circadian clock in this regulation.

is under circadian control. The connection of the circadian clock with ABA pathways was also proven in studies showing the overlap between the ABA-related signalling molecule cyclic adenosine diphosphate ribose (cADPR) and the circadian transcriptome (Dodd *et al*, 2007) and in studies in which a significant proportion of ABA-responsive genes was found to display diurnal oscillation (Mizuno and Yamashino, 2008). Together, these data imply that the ABA pathway, if considered as a clock output, should be altered when the circadian clock is not properly functioning. This is consistent with our studies showing a significant overlap among TOC1-regulated genes and ABA-related transcriptional networks.

Among the multiple roles of ABA along the plant life cycle, the hormone implications in plant responses to stressful environments are well established (Leung and Giraudat, 1998; Finkelstein *et al*, 2002; Zhu, 2002). Our studies show ABA-mediated drought phenotypes in *TOC1-ox*, *TOC1 RNAi* and *toc1-2* mutant plants. The stomata phenotypes in *toc1-2* and *TOC1 RNAi* plants were less severe than in *TOC1-ox*, suggesting a possible functional redundancy with other genes. This would be in consonance with a recent report showing that other members of the TOC1 family, the PRR9,

7 and 5 negatively regulate the biosynthetic pathways of ABA, chlorophyll, carotenoid and alpha-tocopherol (Fukushima *et al*, 2009). The physiological phenotypes of TOC1 mis-expressing plants establish a direct or indirect connection of TOC1 with plant responses to drought. In this sense, our results were in agreement with previous studies showing that proper rhythmic oscillation of stomatal opening is impaired in *toc1-1* (Somers *et al*, 1998; Dodd *et al*, 2005). In these studies, it was proposed that the clock might allow stomata to anticipate dusk, contributing to increased water-use efficiency. The differential plant responses to drought, stomatal aperture and water-loss rates of TOC1 over-expressing and mutant plants were thereby consistent with previous studies and validated our microarray data.

Our studies show that *ABAR* is mis-regulated in *TOC1-ox* and *TOC1 RNAi* plants. A recent study has questioned the function of *ABAR* as a receptor, reporting that in barley, mutant plants do not display ABA phenotypes (Muller and Hansson, 2009). However, this issue was convincingly addressed in a recent study (Wu *et al*, 2009). Furthermore, our own studies with *ABAR RNAi* plants were consistent with the phenotypes reported in *Arabidopsis*, which are also in

agreement with the presence of ABA-related motifs in the *ABAR* promoter and with the inclusion of *ABAR* as a gene regulated by ABA (Matsui *et al*, 2008). We found a significant hyposensitivity in *ABAR RNAi* plants to the ABA-mediated stomata closure, altered water-loss rates and decreased plant survival after dehydration. Furthermore, we observed an inverse correlation between *ABAR* mRNA abundance and water-loss rates, which highlights the important function of *ABAR* in the regulation of this plant response. *ABAR* function was also reinforced by our genetic studies in which the *TOC1 RNAi* phenotypes were completely reverted by the *ABAR RNAi* construct, which in addition to provide clues about *TOC1* and *ABAR* genetic interaction, also assign a function for *ABAR* in the regulation of plant responses to drought.

Our ChIP results suggest that regulation of *ABAR* expression by *TOC1* occurs through direct binding of *TOC1* to the *ABAR* promoter. A recent study has reported the physical association of *TOC1* with chromatin, most likely through interaction with a TCP clock-associated factor-denominated CHE (Pruneda-Paz *et al*, 2009). It would be interesting to examine whether *TOC1* forms protein complexes with CHE or other factors at the *ABAR* promoter. The mechanisms linking ABA with the circadian clock may also involve the ABA signalling factor ABI3, as it was shown earlier that ABI3 physically interacts with *TOC1* (Kurup *et al*, 2000). In our studies, we found *TOC1* binding in the absence of ABA, suggesting a role for *TOC1* in the control of *ABAR* circadian expression. The binding itself is controlled by the clock, judging by the rhythmic oscillation found in TMG plants. Interestingly, treatment with ABA advanced the binding of *TOC1* to the *ABAR* promoter and acutely repressed *ABAR* expression. Both advanced binding and *ABAR* repression occurred specifically during the subjective day, which is a highly relevant time in ABA-regulated processes. Indeed, the peak of ABA diurnal fluctuation, the maximal leaf transpiration rate and maximal effectiveness of ABA at closing stomata all occur at this specific time window (Robertson *et al*, 2009). Furthermore, *TOC1* expression is acutely induced by ABA, and this induction is gated by the clock at this particular time. A plausible explanation for all these results is that plants use the circadian clock to coordinate *TOC1* induction by ABA at a highly sensible phase. This induction rapidly shifts *TOC1* binding to the *ABAR* promoter, which acutely advances *ABAR* repression. The effects of ABA treatment on the circadian expression of other clock-associated genes were examined earlier (Hanano *et al*, 2006). The study showed that ABA lengthens periodicity of bioluminescence in seedlings expressing the promoter of CHLOROPHYLL A/B-BINDING PROTEIN (CAB2/LHCB1*1), COLD- AND CIRCADIALLY REGULATED 2 (CCR2/AtGRP7) and CCA1 (Hanano *et al*, 2006) fused to the luciferase. The acute effects of ABA, which we observe on *TOC1:LUC* expression, might not affect the other genes examined in Hanano's study. It is also possible that due to the different experimental designs, the acute changes were missed. In agreement with this notion, we also found that after the acute induction of *TOC1*, the bioluminescence signals decreased in amplitude with a slight delay in the phase of *TOC1:LUC* expression. It cannot be ruled out, however, a possible saturation of the response because of the extended time with the high concentration of the hormone.

Interlocked feedback loops are common mechanisms for precise regulation of gene expression within the circadian

oscillator (Rand *et al*, 2004). In the case of other metabolic or physiologic processes, feedback loops might also exert an important regulatory role. For instance, the ABA-insensitive 1 (ABI1) and 2 (ABI2) phosphatases were found to function in a negative feedback regulatory loop within the ABA signalling pathway (Merlot *et al*, 2001). It was suggested that this loop might be responsible for resetting the ABA signalling cascade, thereby allowing the cell to continuously monitor the presence or absence of ABA (Merlot *et al*, 2001). A similar mechanistic explanation may underlie the reciprocal regulation between *TOC1* and *ABAR*. It would be interesting to examine the mechanisms and identify other components implicated in this regulation. The existence of a feedback loop connecting the clock and ABA signalling pathways is also in line with previous studies showing that cADPR, which has an important function in molecular and physiological ABA responses (Juan-Pablo Sánchez, 2004), forms a feedback loop within the plant circadian clock (Dodd *et al*, 2007).

Our genetic studies are in agreement with the molecular data and functionally connect the reciprocal regulation of *TOC1* and *ABAR* expression with plant tolerance to drought conditions. Although we could not obtain double *ABAR-ox/TOC1-ox* or *ABAR-ox/TOC1 RNAi* plants, the results showing that the phenotypes of *TOC1 RNAi* plants were reverted in the double *ABAR/TOC1 RNAi* plants indicating that *TOC1 RNAi* phenotypes are only evident in the presence of a functional *ABAR*. Conversely, the drought-related phenotypes of *TOC1-ox* plants were not significantly affected by decreasing *ABAR* abundance, reinforcing the negative role of *TOC1* in the regulation of *ABAR* expression. Although our studies do not exclude the interaction of *TOC1* with other ABA signalling components, the feedback loop between *TOC1* and *ABAR* links a key oscillator component with drought-related responses. This mechanism might contribute to the clock-controlled gating of ABA activity at midday, a time when transpiration rate is higher and plants need a more precise control of hormone function if water supply is limited. Thus, the circadian gating of stomatal closure would ensure proper adjustments of guard cell closure in the heat of the afternoon, when this regulation is more needed. We propose that the reciprocal regulation between *TOC1* and *ABAR* might function as a fine-tuned switch that helps to modulate the plant sensitivity to ABA, which in turn favours the temporal regulation of plant responses to dry environments (Figure 6E). The direct involvement of an essential clock component in these responses also provides a plausible explanation on how the circadian clock confers an adaptive advantage to plants (Dodd *et al*, 2005). Detailed characterization of the molecular components involved in clock interaction with other hormones such as auxin (Covington and Harmer, 2007) or cytokinins (Hanano *et al*, 2006; Salome *et al*, 2006; Zheng *et al*, 2006) might be useful to get further insights into the gating mechanisms synchronizing hormone signalling with plant growth and metabolism.

Materials and methods

Plant material, growth conditions and bioluminescence analysis

The *TOC1 RNAi*, TMG-YFP (TMG), *TOC1-YFP-ox* (*TOC1-ox*) and the *toc1-2* mutant plants (Strayer *et al*, 2000; Más *et al*, 2003a) were provided by Prof. Steve A Kay (University of California San Diego, La Jolla, CA, USA). The *ABAR RNAi* construct (Shen *et al*, 2006)

was provided by Prof. Da-Peng Zhang (China Agricultural University, Beijing, China). The *TOC1 RNAi/ABAR RNAi* and *TOC1-ox/ABAR RNAi* plants were obtained after transforming the *ABAR RNAi* construct (Shen *et al*, 2006) by *Agrobacterium tumefaciens*-mediated DNA transfer (Clough and Bent, 1998). Seedlings were grown on Murashige and Skoog (MS) agar plates with 3% sucrose under controlled environmental conditions ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ of cool white fluorescent light at 22°C). Luminescence analyses were performed as described earlier (Portolés and Más, 2007). In experiments of hormone treatments, (+) ABA (A4906; Sigma-Aldrich) was added to seedlings at the indicated concentration at different times during the diurnal and circadian cycle. For microarray experiments, WT (C24) and *toc1-2* mutant seeds were stratified in the dark at 4°C for 4 days on MS plates with 3% sucrose. Plates were subsequently placed on a growing chamber under 12 h light:12 h dark cycles (LD) for 10 days followed by constant light conditions (LL). Samples were taken after 40 h in LL. WT (Col-0) and *TOC1-ox* samples were taken at Zeitgeber Time 16 (ZT-16) from plants synchronized under LD cycles for 10 days. Details of microarray hybridization and analysis are provided in Supplementary data. Microarray data were deposited at GEO (www.ncbi.nlm.nih.gov/geo/; GEO accessions: GSE18001 and GSE18007).

ChIP assays

ChIP assays were performed as described earlier (Perales and Más, 2007). Specific details of the procedure are described in Supplementary data. For hormone treatment, seedlings were sprayed 4 h before sampling with 7.5 ml of 0.001% Triton X-100 aqueous solution containing 100 μM ($\pm$) ABA and samples were analysed at the indicated time points. Q-PCR was performed with the SYBR Premix Ex Taq (Takara) in a 96-well Lightcycler 480 system (Roche) and quantified with the Lightcycler 480 software (Version 1.5.0.39, Roche). A list of primers used for locus amplification is shown in Supplementary data.

Analysis of gene expression by northern blot and Q-PCR

Northern blot analysis of *ABAR* and *TOC1* expression was performed with RNA from 12-day-old seedlings grown under the indicated conditions. For hormone treatment, seedlings were sprayed with 7.5 ml of 0.001% Triton X-100 aqueous solution containing 100 μM ($\pm$) ABA and samples analysed at the indicated time points. The northern blot of *TOC1* expression shown in Figure 5D was obtained after spraying seedlings with the hormone at CT5 (so that can be compared with the waveform of *TOC1::LUC* expression adding ABA at CT5 in Figure 5A). RNA was isolated using the Purelink Total RNA purification system (Invitrogen) and separated on 1.0% agarose/formaldehyde gels as described earlier (Perales and Más, 2007). *ABAR* probe was described earlier (Shen *et al*, 2006). *TOC1* probe was described earlier (Más *et al*, 2003a). Quantification analysis was performed on a Bio-Rad phosphor-imager using Quantity One software, version 6.1 (<http://www.bio-rad.com/>). For Q-PCR analysis, RNA was treated with RNase-free DNase (Ambion) and the single-strand cDNA was synthesized using Superscript III (Invitrogen) and a mixture of oligo-dT18 and random hexamers (Invitrogen) following the manufacturer recommendation. The cDNA was diluted 10-fold with nuclease-free water and Q-PCR was performed with the SYBR Premix Ex Taq (Takara) in a 96-well Lightcycler 480 system (Roche). The *IPP2* gene (isopentenyl pyrophosphate:dimethyl-allyl pyrophosphate isomerase) was used as a control (Hazen *et al*, 2005). Quantification was performed using Lightcycler 480 software (Version 1.5.0.39, Roche). Primers

were designed using the PrimerExpress 2.0 software (Applied Biosystems) with lengths of 18–25 nucleotides, PCR amplicon lengths of 80–180 bp, 40–60% G:C content and 60–65°C. See Supplementary data for the list of primers used for Q-PCR assays.

Measurements of guard cell dimensions, stomatal conductance and plant survival to drought conditions

Detached rosette leaves from 3-week-old plants grown under ShD conditions (8 h light:16 h dark) were incubated for 2 h in a buffer containing 50 mM KCl and 10 mM MES, pH 6.15 with cool white fluorescent light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$). ABA was subsequently added in the same buffer solution at different concentrations (0, 1 and 5 μM) and leaves were incubated for 2 more hours with the hormone. Abaxial epidermis were carefully taken with tweezers and mounted with the same buffer solution. Epidermis strips were observed with a Zeiss AxioPhot microscope with a $\times 63$ objective. Approximately 100–200 stomata per sample were photographed using an Olympus DP70 camera attached to the microscope. Stomatal aperture was scored as width:length pore ratio using CellD software (Olympus). For stomatal conductance measurements, plants were grown under ShD for 5 weeks and 60–65% relative humidity. A Licor 6400 photosynthesis system with a 6400-15 Arabidopsis leaf chamber attachment (Licor, NE) was used for gas exchange measurements. Temperature was maintained at 22°C, humidity to 62–67% and CO_2 to 400 $\mu\text{mol mol}^{-1}$. Stomatal conductance was monitored every 20 s until stabilization. For water-loss assays, rosette leaves of comparable size from 4-week-old plants grown under ShD were detached, placed abaxial side up on a Petri dish and weighted at different times after detachment. Assays were performed at 22°C and 60–65% relative humidity. For dehydration experiments and plant survival assays, 2-week-old plants grown on agar plates under ShD were transferred to filter paper on Petri dishes and left un-watered for 10–12 h under LL. Survival percentages were scored 2 days after re-watering the plants. Greenish, healthy-looking plants were counted as surviving. Experiments were repeated at least twice with about 20–40 plants per treatment and genotype. Statistical significance was evaluated using one- and two-way ANOVA.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Dr M Pagès, Dr T Stratmann, Dr M Yanovsky and members of P Más lab for helpful comments on the experiments and the manuscript. We are grateful to Dr DP Zhang (China Agricultural University, Beijing, China) for the *ABAR RNAi* construct and Dr T Altabella for help with the stomatal conductance measurements. This work was supported by grants to PM from the Spanish Ministry of Science and Education (MEC) (BIO2007-66068), from the EMBO YIP program and from EUROHORCS (European Heads Of Research Councils) and European Science Foundation (ESF) through the EURYI Award. TL is a recipient of a JAE-CSIC pre-doctoral fellowship; JCC is supported by a post-doctoral contract through the CRAG.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA (2001) Reciprocal regulation between *TOC1* and *LHY/CCA1* within the Arabidopsis circadian clock. *Science* **293**: 880–883
- Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat Rev Gen* **6**: 544–556
- Busk PK, Pagès M (1998) Regulation of abscisic acid-induced transcription. *Plant Mol Biol* **37**: 425–435
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**: 735–743
- Correia MJ, Pereira JS, Chaves MM, Rodrigues ML, Pacheco CA (1995) ABA xylem concentrations determine maximum daily leaf conductance of field-grown *Vitis vinifera* L. plants. *Plant Cell Environ* **18**: 511–521
- Covington M, Maloof J, Straume M, Kay SA, Harmer S (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biol* **9**: R130

- Covington MF, Harmer SL (2007) The circadian clock regulates auxin signaling and responses in *Arabidopsis*. *PLoS Biol* **5**: e222
- Dodd AN, Gardner MJ, Hotta CT, Hubbard KE, Dalchau N, Love J, Assie J-M, Robertson FC, Jakobsen MK, Goncalves J, Sanders D, Webb AAR (2007) The *Arabidopsis* circadian clock incorporates a cADPR-based feedback loop. *Science* **318**: 1789–1792
- Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ, Webb AA (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309**: 630–633
- Edwards KD, Anderson PE, Hall A, Salathia NS, Locke JC, Lynn JR, Straume M, Smith JQ, Millar AJ (2006) FLOWERING LOCUS C mediates natural variation in the high-temperature response of the *Arabidopsis* circadian clock. *Plant Cell* **18**: 639–650
- Finkelstein RR, Gampala SSL, Rock CD (2002) Abscissic acid signaling in seeds and seedlings. *Plant Cell* **14**: S15–S45
- Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* **17**: 3470–3488
- Fukushima A, Kusano M, Nakamichi N, Kobayashi M, Hayashi N, Sakakibara H, Mizuno T, Saito K (2009) Impact of clock-associated *Arabidopsis* pseudo-response regulators in metabolic coordination. *Proc Natl Acad Sci USA* **106**: 7251–7256
- Green RM, Tingay S, Wang ZY, Tobin EM (2002) Circadian rhythms confer a higher level of fitness to *Arabidopsis* plants. *Plant Physiol* **129**: 576–584
- Hanano S, Domagalska MA, Nagy F, Davis SJ (2006) Multiple phytohormones influence distinct parameters of the plant circadian clock. *Genes Cells* **11**: 1381–1392
- Harmer SL (2009) The circadian system in higher plants. *Annu Rev Plant Biol* **60**: 357–377
- Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* **290**: 2110–2113
- Hazen SP, Naef F, Quisel T, Gendron J, Chen H, Ecker J, Borevitz J, Kay S (2009) Exploring the transcriptional landscape of plant circadian rhythms using genome tiling arrays. *Genome Biol* **10**: R17
- Hazen SP, Schultz TF, Pruneda-Paz JL, Borevitz JO, Ecker JR, Kay SA (2005) LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. *Proc Natl Acad Sci USA* **102**: 10387–10392
- Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends Plant Sci* **12**: 343–351
- Hotta CT, Gardner MJ, Hubbard KE, Baek SJ, Dalchau N, Suhita D, Dodd AN, Webb AAR (2007) Modulation of environmental responses of plants by circadian clocks. *Plant Cell Environ* **30**: 333–349
- Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. *Annu Rev Plant Physiol Plant Mol Biol* **47**: 377–403
- Juan-Pablo Sánchez PDN-HC (2004) ABA activates ADPR cyclase and cADPR induces a subset of ABA-responsive genes in *Arabidopsis*. *Plant J* **38**: 381–395
- Kurup S, Jones HD, Holdsworth MJ (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing *Arabidopsis* seeds. *Plant J* **21**: 143–155
- Leung J, Giraudat J (1998) Abscissic acid signal transduction. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 199–222
- Locke JC, Kozma-Bognar L, Gould PD, Feher B, Kevei E, Nagy F, Turner MS, Hall A, Millar AJ (2006) Experimental validation of a predicted feedback loop in the multi-oscillator clock of *Arabidopsis thaliana*. *Mol Syst Biol* **2**: 59
- Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**: 1064–1068
- Makino S, Matsushika A, Kojima M, Yamashino T, Mizuno T (2002) The APRR1/TOC1 quintet implicated in circadian rhythms of *Arabidopsis thaliana*: Characterization with APRR1-overexpressing plants. *Plant Cell Physiol* **43**: 58–69
- Más P (2005) Circadian clock signaling in *Arabidopsis thaliana*: from gene expression to physiology and development. *Int J Dev Biol* **49**: 491–500
- Más P (2008) Circadian clock function in *Arabidopsis thaliana*: time beyond transcription. *Trends Cell Biol* **18**: 273–281
- Más P, Alabadi D, Yanovsky MJ, Oyama T, Kay SA (2003a) Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. *Plant Cell* **15**: 223–236
- Más P, Kim WJ, Somers DE, Kay SA (2003b) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis*. *Nature* **426**: 567–570
- Matsui A, Ishida J, Morosawa T, Mochizuki Y, Kaminuma E, Endo TA, Okamoto M, Nambara E, Nakajima M, Kawashima M, Satou M, Kim J-M, Kobayashi N, Toyoda T, Shinozaki K, Seki M (2008) *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol* **49**: 1135–1149
- McClung C (2008) Comes a time. *Curr Opin Plant Biol* **11**: 514–520
- Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J* **25**: 295–303
- Michael TP, Breton G, Hazen SP, Priest H, Mockler TC, Kay SA, Chory J (2008a) A morning-specific phytohormone gene expression program underlying rhythmic plant growth. *PLoS Biol* **6**: e225
- Michael TP, McClung CR (2003) Enhancer trapping reveals widespread circadian clock transcriptional control in *Arabidopsis*. *Plant Physiol* **132**: 629–639
- Michael TP, Mockler TC, Breton G, McEntee C, Byer A, Trout JD, Hazen SP, Shen R, Priest HD, Sullivan CM, Givan SA, Yanovsky M, Hong F, Kay SA, Chory J (2008b) Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules. *PLoS Genet* **4**: e14
- Michael TP, Salome PA, Yu HJ, Spencer TR, Sharp EL, McPeck MA, Alonso JM, Ecker JR, McClung CR (2003) Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* **302**: 1049–1053
- Mizuno T, Yamashino T (2008) Comparative transcriptome of diurnally oscillating genes and hormone-responsive genes in *Arabidopsis thaliana*: insight into circadian clock-controlled daily responses to common ambient stresses in plants. *Plant Cell Physiol* **49**: 481–487
- Muller AH, Hansson M (2009) The barley magnesium chelatase 150-kD subunit is not an abscisic acid receptor. *Plant Physiol* **150**: 157–166
- Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. *Proc Natl Acad Sci USA* **95**: 8660–8664
- Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow T-F, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu J-K, Schroeder JI, Volkman BF *et al* (2009) Abscissic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071
- Perales M, Más P (2007) A functional link between rhythmic changes in chromatin structure and the *Arabidopsis* biological clock. *Plant Cell* **19**: 2111–2123
- Portolés S, Más P (2007) Altered oscillator function affects clock resonance and is responsible for the reduced day-length sensitivity of CKB4 overexpressing plants. *Plant J* **51**: 966–977
- Pruneda-Paz JL, Breton G, Para A, Kay SA (2009) A functional genomics approach reveals CHE as a component of the *Arabidopsis* circadian clock. *Science* **323**: 1481–1485
- Rand DA, Shulgin BV, Salazar D, Millar AJ (2004) Design principles underlying circadian clocks. *J R Soc Interface* **1**: 119–130
- Robertson F, Skeffington A, Gardner M, Webb A (2009) Interactions between circadian and hormonal signalling in plants. *Plant Mol Biol* **69**: 419–427
- Salome PA, To JPC, Kieber JJ, McClung CR (2006) *Arabidopsis* response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. *Plant Cell* **18**: 55–69
- Schaffer R, Landgraf J, Accerbi M, Simon V, Larson M, Wisman E (2001) Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell* **13**: 113–123
- Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carré IA, Coupland G (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* **93**: 1219–1229
- Shen Y-Y, Wang X-F, Wu F-Q, Du S-Y, Cao Z, Shang Y, Wang X-L, Peng C-C, Yu X-C, Zhu S-Y, Fan R-C, Xu Y-H, Zhang D-P (2006)

- The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* **443**: 823–826
- Somers DE, Webb AAR, Pearson M, Kay SA (1998) The short-period mutant *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. *Development* **125**: 485–494
- Strayer CA, Oyama T, Schultz TF, Raman R, Somers DE, Más P, Panda S, Kreps JA, Kay SA (2000) Cloning of the Arabidopsis clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* **289**: 768–771
- Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* **50**: 571–599
- Verslues PE, Zhu J-K (2007) New developments in abscisic acid perception and metabolism. *Curr Opin Plant Biol* **10**: 447–452
- Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* **93**: 1207–1217
- Wijnen H, Young MW (2006) Interplay of circadian clocks and metabolic rhythms. *Annu Rev Genet* **40**: 409–448
- Wu F-Q, Xin Q, Cao Z, Liu Z-Q, Du S-Y, Mei C, Zhao C-X, Wang X-F, Shang Y, Jiang T, Zhang X-F, Yan L, Zhao R, Cui Z-N, Liu R, Sun H-L, Yang X-L, Su Z, Zhang D-P (2009) The Mg-chelatase H subunit binds abscisic acid and functions in abscisic acid signaling: new evidence in Arabidopsis. *Plant Physiol* **150**: 1940–1954
- Zeilinger MN, Farre EM, Taylor SR, Kay SA, Doyle FJ (2006) A novel computational model of the circadian clock in Arabidopsis that incorporates PRR7 and PRR9. *Mol Syst Biol* **2**: 1–13
- Zhang D-P, Wu Z-Y, Li X-Y, Zhao Z-X (2002) Purification and identification of a 42-kilodalton abscisic acid-specific-binding protein from epidermis of broad bean leaves. *Plant Physiol* **128**: 714–725
- Zheng B, Deng Y, Mu J, Ji Z, Xiang T, Niu Q-W, Chua N-H, Zuo J (2006) Cytokinin affects circadian-clock oscillation in a phytochrome B- and Arabidopsis response regulator 4-dependent manner. *Physiol Plant* **127**: 277–292
- Zhu J-K (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* **53**: 247–273