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REVIEW ARTICLE

Circadian clock-dependent gating in ABA signalling networks

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Abstract Plant growth and development are intimately attuned to fluctuations in environmental variables such as light, temperature and water availability. A broad range of signalling and dynamic response mechanisms allows them to adjust their physiology so that growth and reproductive capacity are optimised for the prevailing conditions. Many of the response mechanisms are mediated by the plant hormones. The hormone abscisic acid (ABA) plays a dominant role in fundamental processes such as seed dormancy and germination, regulation of stomatal movements and enhancing drought tolerance in response to the osmotic stresses that result from water deficit, salinity and freezing. Whereas plants maintain a constant vigilance, there is emerging evidence that the capacity to respond is gated by the circadian clock so that it varies with diurnal fluctuations in light, temperature and water status. Clock regulation enables plants to anticipate regular diurnal fluctuations and thereby presumably to maximise metabolic efficiency. Circadian clock-dependent gating appears to regulate the ABA signalling network at numerous points, including metabolism, transport, perception and activity of the hormone. In this review, we summarise the basic principles and recent progress in elucidating the molecular mechanisms of circadian gating of the ABA response network and how it can affect fundamental processes in plant growth and development.

Keywords ABAR/CHLH · ABRE · Abscisic acid · CBF/DREB1 · Circadian clock gating · PIF · PRR · PYR/PYL/RCAR · TOC1

Introduction

Being sessile organisms, it is imperative for the survival of plants that they constantly monitor and adapt to their surroundings. The large variety of environmental challenges requires an equally broad range of interconnected perception and dynamic response mechanisms. Plant responses to environmental variables are predominantly mediated by hormonal networks. The fundamental processes of seed dormancy and germination, stomatal movements, the mechanisms of osmotic stress tolerance, cold adaptation and flowering are all regulated primarily by plant hormones. Although most processes are orchestrated by a suite of hormones, each individual hormone appears to play a dominant role in a specific set of processes. Abscisic acid (ABA), for example, is critically important in plant responses to osmotic stresses resulting from water deficit, salinity and freezing, and in responses to wounding and pathogen attack (Huang et al. 2008; Ton et al. 2009; Cutler

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et al. 2010; Cao et al. 2011; Hauser et al. 2011). It also plays a dominant role in the regulation of growth and development in processes such as seed germination (Penfield and King 2009) and sexual reproduction (Domagalska et al. 2010). Under conditions of water deficit or salinity stress, plant ABA levels increase dramatically, restricting water loss by stimulating stomatal closure and protecting cellular machinery against dehydration damage by promoting the accumulation of osmo-compatible solutes (Robertson et al. 2009). It has been estimated that in *Arabidopsis*, ABA regulates about two thirds of water stress-responsive genes, a much higher proportion than for other phytohormones (Huang et al. 2008; Fujita et al. 2011). Given the central importance of an ability to cope with fluctuating water availability, it is perhaps unsurprising that orthologues of genes encoding key ABA biosynthesis, signal transduction and response elements have been highly conserved through the evolution of land plants (Hauser et al. 2011).

Although plants must remain constantly vigilant, evidence suggests that hormonal signalling networks, including ABA signalling, are modulated, or 'gated', by endogenous circadian rhythms (Hotta et al. 2007; Robertson et al. 2009; de Montaigu et al. 2010). The circadian clock is entrained largely by daily light/dark oscillations, which act as a 'zeitgeber' or time giver (ZT) that establishes the clock's diurnal synchrony. Clock-dependent gating is thought to anticipate dawn and dusk signals and other diurnal changes in the environment, thereby maximizing metabolic efficiency and hence increasing the reproductive fitness of the plant (Webb 2003; Dodd et al. 2005b; Hotta et al. 2007; de Montaigu et al. 2010). It is possible that the circadian clock modulates the action of a broad range of phytohormones in an equally broad range of fundamental physiological processes throughout growth and development (Mizuno and Yamashino 2008; Robertson et al. 2009). Evidence has also emerged that the output of the circadian clock is in turn modulated by hormonal signalling networks, feeding back data from various metabolic and environmental variables and creating a complex network of regulatory interactions (Robertson et al. 2009; Edwards et al. 2010; Harmer 2010; Pruneda-Paz and Kay 2010; Umezawa 2011).

In this short review, we focus on ABA signalling in the context of circadian gating. Recent discoveries of ABA receptors have provided a firm basis for an emerging understanding of the core ABA signalling network (this work has been described in several recent reviews: Cutler et al. 2010; Hubbard et al. 2010; Raghavendra et al. 2010; Umezawa et al. 2010; Weiner et al. 2010). In parallel to the work on ABA, important advances have been made in elucidating the molecular machinery of the circadian clock (Harmer 2009; de Montaigu et al. 2010; Pruneda-Paz and Kay 2010; McClung and Gutierrez 2010; Li et al. 2011; Thines and Harmon 2011; Wenden et al. 2011). Here we briefly review the basic principles and recent progress in elucidating the molecular mechanisms of ABA signalling and the circadian clock machinery, and explore how circadian gating of ABA signalling operates in fundamental growth and developmental processes.

The circadian clock: molecular machinery and role in physiological processes

The circadian clock acts as a biochemical oscillator that attunes whole-plant physiology to the cycles of rhythmic, predictable changes in the environment (Dodd et al. 2005b; Hotta et al. 2007; de Montaigu et al. 2010; Thines and Harmon 2011). Many of the fundamental physiological processes in plants are linked to the regulatory activities of the endogenous circadian clock. These include diurnal variations in gene expression (Mas 2005; Covington and Harmer 2007; Michael et al. 2008a, b; Mikkelsen and Thomashow 2009; Khan et al. 2010; Dong et al. 2011), calcium ion fluxes (Dodd et al. 2007; Xu et al. 2007), metabolic activity (Hotta et al. 2007; Fukushima et al. 2009; Proels and Roitsch 2009), hormone signalling (Mizuno and Yamashino 2008; Robertson et al. 2009), osmotic and cold stress responses (Lee et al. 2006; Huang et al. 2008; Penfield 2008; Mizuno and Yamashino 2008; Legnaioli et al. 2009; Nakamichi et al. 2009; Dong et al. 2011), stomatal and leaf movements (Tallman 2004; Mishra et al. 2006; Hotta et al. 2007; Robertson et al. 2009) and the transition to flowering (Domagalska et al. 2010). Transcriptome profiling experiments in *Arabidopsis* indicate that up to a third of the genes in the genome are subject to circadian clock regulation (Covington and Harmer 2007; Michael et al. 2008a, b). In the maize inbred line, B73, around 10% of approximately 13,000 transcripts examined displayed expression patterns consistent with circadian regulation (Khan et al. 2010). Although ABA is typically associated with responses to random environmental challenges, some challenges recur with rhythmic diurnal periodicity (Hotta et al. 2007; Robertson et al. 2009). The evidence for gating suggests that plants anticipate these rhythmic environmental challenges , potentially enabling a more focussed use of resources (Dodd et al. 2005b; Hotta et al. 2007; Legnaioli et al. 2009; Robertson et al. 2009; de Montaigu et al. 2010; Wilkins et al. 2010).

At the core of the clock, transcriptional loops comprising both positive and negative regulators combine to form the robust interlocking self-regulatory circadian clock mechanism (see recent reviews in Harmer 2009, 2010; McClung 2006, 2008, 2009; McClung and Gutierrez 2010; Pruneda-Paz and Kay 2010). Most functional studies into the molecular machinery of the plant circadian clock have been carried out in *Arabidopsis*. Although individual components vary among different species, evidence to date indicates that the overall architecture of the mechanism is well conserved. The central transcriptional feedback loop ('central loop') generates a self-sustaining rhythm formed by the Myb-like transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which interact with the nuclear-localised PSEUDO-RESPONSE REGULATOR TIMING OF CAB EXPRESSION1/PSEUDO-RESPONSE REGULATOR1 (TOC1/PRR1, Fig. 1). The CCA1 and LHY proteins bind to the promoter of *TOC1* and repress its expression; TOC1 in turn indirectly promotes expression of *CCA1* and *LHY*, partly by inhibiting the transcription factor CCA1 HIKING EXPEDITION (CHE, Pruneda-Paz et al. 2009). A 'morning loop' transcriptional feedback loop is formed by the TOC1 homologues PRR5, PRR7 and PRR9, which inhibit expression of *CCA1* and *LHY* (Nakamichi et al. 2010). CCA1 and LHY proteins in turn promote expression of *PRR7* and *PRR9* (Nakamichi et al. 2010). The light-regulated WD1 and WD2 (LWD1 and LWD2) proteins, that have previously been shown to be involved in the photoperiodic regulation of flowering, have now been shown to be components of the clock, as they form a positive regulatory feedback loop with *PRR9* (Wu et al. 2008; Wang et al. 2011). In a third 'evening loop', the evening-expressed, GIGANTEA (GI), acts as a positive regulator of *TOC1* and the BROTHER OF LUX ARRHYTHMO (BOA) activates *CCA1* by binding to its promoter. Reciprocally, CCA1 binds to an evening element (EE) in the *BOA* promoter, negatively regulating its expression (Dai et al. 2011). Direct and indirect interactions between each of these elements reinforce the robustness and stability of the clock and its ability to maintain regular diurnal rhythms in the face of random, short-term environmental perturbations.

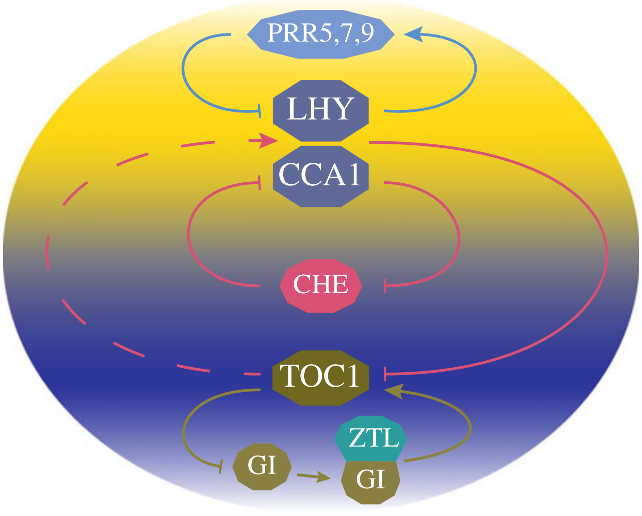

Fig. 1 Simplified model of the circadian central oscillator mechanism. The simplified model consists of three main interdependent positive-negative feedback loops. The 'central' loop (*middle*), the 'morning' loop (*top*) and 'evening' loop (*bottom*). *Dashed arrow* signifies unverified clock interaction

Several elements of post-transcriptional and post-translational control of the circadian clock have also been identified. For example, oligomerization of TOC1 and PRR5 has been shown to promote the nuclear localization of TOC1, presumably through enhanced nuclear import (Wang et al. 2010). TOC1 and PRR5 protein stability has also been shown to be actively regulated. ZEITLUPE (ZTL), a blue-light photoreceptor F-box protein, contains a light-regulated protein–protein interaction domain LIGHT, OXYGEN OR VOLTAGE (LOV) that is able to bind TOC1 and PRR5 and target them for proteasome-dependent degradation (Kim et al. 2007; Pokhilko et al. 2010). During the day, however, blue light induces a modification of the LOV domain that promotes binding to the GI protein, protecting TOC1, PRR5 and ZTL from degradation. Similar interactions occur between GI and the ZTL homologs, FLAVIN BINDING, KELTCH REPEAT, F-BOX1 (FKF1) and LOV KELCH PROTEIN2 (LKP2, Baudry et al. 2010). In addition to these post-translational modifications, evidence has begun to emerge that post-transcriptional modifications, including pre-mRNA splicing, polyadenylation, RNA decay and contributions from microRNAs also play important roles in regulating the circadian system (Mas 2008; Staiger and Koster 2011).

Given its fundamental importance, it is perhaps unsurprising that genes encoding the basic components of the circadian clock have remained highly conserved throughout plant evolution (Harmer 2009). As observed with hormone metabolism and signalling, many of the key components of the clock predate major events in plant speciation. For example, the closely related clock genes *PRR3*, *PRR5*, *PRR7* and *PRR9* diverged into three clades as a result of chromosomal duplication events before the speciation of monocots and eudicots (Takata et al. 2010). Orthologs of the *PRR* genes have also been identified in the moss *Physcomitrella* (Hauser et al. 2011; Satbhai et al. 2011) and close homologs of the *Arabidopsis TOC1*, *LHY* and *CCA1* have been identified in the green algae *Chlamydomonas* and *Ostreococcus* (Satbhai et al. 2011; Troein et al. 2011). Key clock components such as *LHY1* and *LHY2*, and *CCA1* are highly conserved among angiosperms, although the number of the genes per genome may vary, presumably as a result of independent chromosomal duplication events (Takata et al. 2009). Conservation of these clock components indicates the importance of the clock mechanism and suggests that targets of the clock are also conserved.

Entrainment of the circadian clock by ambient light and temperature cues

Just as it is critical for circadian clock oscillations to be robust and self sustaining, it is important that the clock is able to synchronise with key rhythmic environmental indicators. The clock is primarily entrained by and synchronised with regular diurnal rhythms of ambient light and temperature. These environmental cues have been shown to act on components in the clock's central loop (McWatters and Devlin 2011), although it appears that in roots the transcriptional activities of CCA1/LHY are inactive and the clock is instead synchronised by a photosynthesis-related signal from the shoot (James et al. 2008; McClung and Gutierrez 2010). Sucrose signalling mediated by the oscillator gene *GI*, for example, is known to provide entraining inputs (Blasing et al. 2005; Dalchau et al. 2011). In *Arabidopsis*, members of three families of photoreceptors have been shown to be involved in light entrainment of the clock: the Red/Far Red (R/FR) sensing phytochromes PHYA-E; the blue-light sensing cryptochromes CRY1 and CRY2; and a family of three F-box proteins including ZTL (Hotta et al. 2007; Li et al. 2011; Wenden et al. 2011). The pathway of transduction of the light signal from these photoreceptors to the central oscillator remains unclear, although it appears to be regulated via at least several mechanisms (Li et al. 2011; Wenden et al. 2011). Recently, three positive regulators of the PHYA signalling pathway, FAR-RED ELONGATED HYPOCOTYLS 3 (FHY3), FAR-RED-IMPAIRED RESPONSE 1 (FAR1) and ELONGATED HYPOCOTYL 5 (HY5), were shown to interact with clock components, indicating a role in feeding light environment information into the clock (Li et al. 2011). At dawn, the CCA1 and LHY proteins suppress expression of the central oscillator component, *EARLY FLOWERING 4* (*ELF4*), and throughout the following day FHY3, FAR1 and HY5 directly activate *ELF4* expression (Li et al. 2011).

Temperature cycles with an amplitude of only ~4°C are also able to entrain the clock (Salome and McClung 2005). At the same time, the robustness of the clock means that it is resistant to irregular changes in temperature from 12°C to 27°C (Hotta et al. 2007). Additional elements of the pathway of rhythmic temperature entrainment of the clock have been isolated recently. The EARLY FLOWERING 3 (ELF3), PRR7 and PRR9 proteins, for example, have been shown to be involved in temperature entrainment of the clock (Thines and Harmon 2010; McWatters and Devlin 2011). Through the evolution of a circadian clock and mechanisms for its entrainment, plants have developed a system for optimally matching physiology with predictable diurnal and seasonal variables.

Molecular mechanisms of circadian gating of ABA signal transduction

The key features of the clock are its self-stabilising and reinforcing nature, its ability to synchronise with key environmental indicators, and its pervasive capacity to influence plant physiology. Temporal information from the circadian clock is conveyed to downstream targets through at least four mechanisms (Thines and Harmon 2011). First, TOC1 expression has been shown to be associated with rhythmic modifications of chromatin structure through acetylation of histone H3 (Perales and Mas 2007). Second, core clock proteins bind to regulatory regions of output genes, including those involved in hormone signalling networks. TOC1, for example, binds to the promoter of the putative ABA receptor, *ABA-RELATED/ H SUBUNIT OF THE MAGNESIUM-PROTOPORPHYRIN IX CHELATASE/ GENOMES UNCOUPLED 5* (*ABAR*/*CHLH*/*GUN5*), regulating its diurnal expression (Shen et al. 2006; Legnaioli et al. 2009; Castells et al. 2010). Interestingly, *TOC1* expression is induced by ABA in the presence of a functional ABAR/CHLH/GUN5 protein (Legnaioli et al. 2009). Third, clock proteins interact directly with proteins of hormonal signalling pathways. TOC1, for example, interacts with transcription factors of the bHLH PHYTOCHROME INTERACTING FACTOR (PIF) family and with the CBF/DREB1 transcription factors, which are involved in drought and low temperature responses (Kidokoro et al. 2009; Koini et al. 2009). The pervasive TOC1 also interacts with ABA INSENSITIVE3 (ABI3), which has roles in seed germination, ABA signalling and drought tolerance (Kurup et al. 2000; Khandelwal et al. 2010). Finally, the circadian clock elicits rhythmic expression of transcription factors (Covington et al. 2008) and other regulatory components of the cellular machinery that modulate genes outside the clock. The clock-regulated expression of *PIF4* and *PIF5*, for example, in turn regulates gene networks that modulate hypocotyl elongation and photomorphogenesis (Fujimori et al. 2004; Nozue et al. 2007; Leivar et al. 2008).

Interactions between hormones and the circadian clock are clearly not unidirectional. Evidence has also emerged for the reciprocal impact of hormones on the amplitude, period and phase of the circadian clock. Perhaps the most direct demonstration has been provided by promoter: *luciferase* (*LUC*) assays using *Arabidopsis* plants expressing circadian-clock marker genes under free-running continuous light or continuous darkness conditions (Hanano et al. 2006). In these assays, exogenous ABA lengthened the period of *CCA1* and of a marker gene for clock output *CHLOROPHYL A*/*B-BINDING PROTEIN2* (*CAB2*) by ~2 h in continuous light compared with controls. Conversely, BR application shortened the period of the clock-marker gene *COLD- AND CIRCADIAN-REGULATED2* (*CCR2*) by ~1 h. Application of the synthetic auxin, NAA, reduced the amplitude of circadian cycling of *CCR2* and disrupted the precision of the clock. The cytokinins 6-benzylaminopurine (BA) and trans-zeatin delayed the circadian phase of *CCA1* and *CAB2* by 1–3 h and shortened the period (Hanano et al. 2006). No evidence has yet been found indicating that ethylene is able to affect the clock or its rhythmic outputs (Robertson et al. 2009). Given the central roles played by these two pervasive systems, it is unsurprising that the evidence is pointing to an interdependence between the hormonal and circadian clock regulatory systems.

Components of ABA metabolism and their circadian gating

Phytohormone-induced responses are regulated by the amount of biologically active hormone present in a cell or tissue and by the activity of the signal perception, transduction and response systems. As outlined in Fig. 2, the amount of biologically active ABA within cells and tissues is determined by a combination of the rates of ABA biosynthesis, activation/deactivation, catabolism and transport (Hauser et al. 2011; Ji et al. 2011; Jiang and Hartung 2008). Cellular levels are also influenced by the uptake of ABA from the apoplast by ABC transporters, such as ABCG40 and ABCG25 (Kang et al. 2010; Kuromori et al. 2010). As one of the canonical 'stress hormones', ABA is rapidly synthesised *de novo* in response to environmental stimuli such as drought (Iuchi et al. 2001), cold (Ji et al. 2011) and pathogen attack (Adie et al. 2007). However, although it is synthesised rapidly in roots in a drying soil and transported in the xylem stream to the shoot as a water-stress signal (Zhang and Davies 1989; Hartung et al. 2002), it is thought that large pools of ABA do not move around the plant and that the ABA produced *de novo* in affected organs is the most important for triggering physiological responses (Finkelstein and Rock 2002).

ABA is synthesised from β-carotene, primarily in the plastid, although the final steps that convert xanthoxin to ABA occur in the cytosol (Seo and Koshiba 2002). During salinity and drought stress, the rate-limiting step for ABA synthesis is the cleavage of 9-cis-epoxycarotenoid into xanthoxin by the enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) (Qin and Zeevaart 1999; Iuchi et al. 2001). Transgenic plants over-expressing NCED have high levels of ABA (Qin and Zeevaart 2002). Internally regulated, rhythmic diurnal fluctuations in ABA levels have been reported in leaves (Fukushima et al. 2009; Nováková et al. 2005), with the major peak occurring at the start of the dark period. NCED is similarly diurnally regulated, with transcripts most abundant at the end of the day (Thompson et al. 2000), correlating with the peak in ABA levels. Direct evidence for circadian regulation of ABA biosynthesis has been shown by metabolomic analysis of an *Arabidopsis* arrhythmic triple mutant *d975* which has mutations in three genes encoding the central clock components, *PRR9*/*7*/*5* (Fukushima et al. 2009). This study revealed that the *d975* mutations suppress the carotenoid and ABA biosynthetic pathways and, whereas ABA levels in wild-type plants were low at dawn (ZT0) and increased ~2-fold later in the day (ZT12), those in the *d975* mutant remained constantly high.

ABA is irreversibly inactivated through the degradation of the hormone to phaseic acid. The key enzymes in the process are the cytochrome P450 ABA 8′-hydrolases (Kushiro et al. 2004). In *Arabidopsis*, mutations in genes encoding these enzymes, such as members of the *CYP707A*

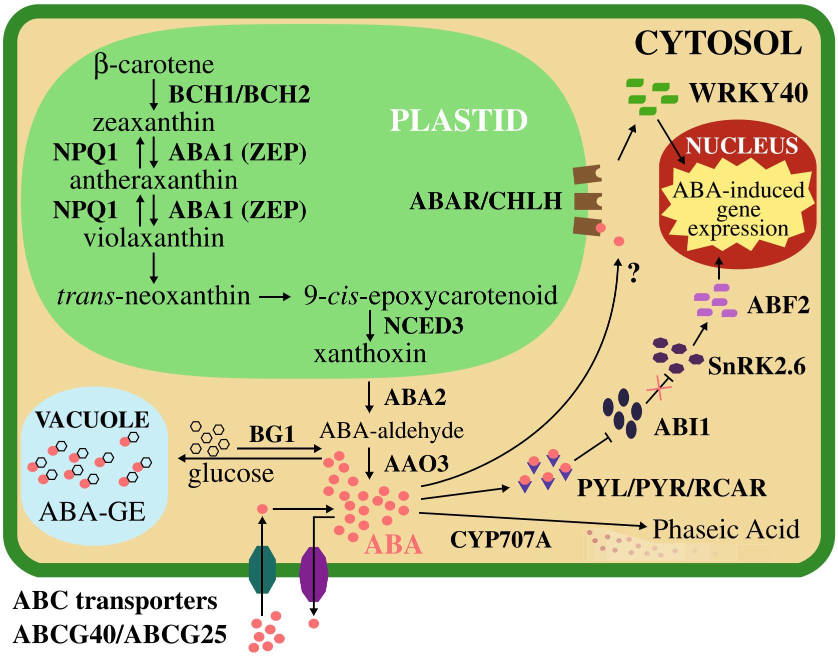

Fig. 2 Model of known components of the *Arabidopsis* ABA metabolism, transport and signal transduction network

family, cause multiple phenotypes that include an over-accumulation of ABA and increased seed dormancy (Kushiro et al. 2004). There is a clear evidence of diurnal and circadian regulation of ABA biosynthetic enzymes (Thompson et al. 2000) and some evidence for circadian regulation of ABA catabolic enzymes (Fig. 3). CYP707A4 is the only member of the CYP707A family that has been shown to be diurnally regulated (Pan et al. 2009). It is possible that diurnal regulation of ABA levels is more associated with conjugation/deconjugation than irreversible degradation. Large reserves of ABA are stored in vacuoles in the inactive glucose conjugate form, ABA-glucose ester (ABA-GE) (Lee et al. 2006). ABA-GE is cleaved into biologically active ABA by the ER-localised, β-glucosidase *At*BG1 (Lee et al. 2006). Diurnal fluctuations in biologically active ABA levels have been shown to correlate with the levels of the polymeric active form of *At*BG1 (Lee et al. 2006).

Circadian gating of ABA core signalling pathways and downstream targets

The elucidation of the core regulatory components of the ABA signalling pathway followed rapidly on from the discovery of candidate ABA receptors. PYRABACTIN RESISTANCE 1 (PYR1) and REGULATORY COMPONENT OF ABA RECEPTOR 1 (RCAR1) were the first members of the PYR/PYL/RCAR protein family shown to have the ability to directly bind ABA and to inactivate the previously identified negative regulators of ABA signalling, ABI1, AB12 and HAB1 (Ma et al. 2009; Park et al. 2009). Single and multiple gene knockout mutants in *PYR*/*PYL*/*RCARs* are ABA-insensitive in processes such as seed germination, root growth (Park et al. 2009) and stomatal conductance (Nishimura et al. 2010). The PYR/PYL/RCAR family consists of 14 members in *Arabidopsis* (Nishimura et al. 2010). Nine members of the family have so far been shown to interact with ABI1 (Nishimura et al. 2010). ABI1, ABI2, and HAB1 are protein phosphatase 2Cs (PP2Cs). Crystal structures of several PYR/PYL/RCAR family members have shown that ABA-binding triggers a conformational change in the PYR/PYL/RCAR ABA-receptor complex, facilitating the docking and inhibition of the PP2Cs (Melcher et al. 2009; Miyazono et al. 2009; Santiago et al. 2009). In the absence of ABA, PP2Cs dephosphorylate and consequently inactivate the SNF1-related kinases (SnRK) (Yamaguchi-Shinozaki and Shinozaki 2006; Fujii et al. 2009). Active, phosphorylated SnRK enzymes have the capacity to phosphorylate and activate the ABA RESPONSIVE ELEMENT (ABRE)-BINDING bZIP TRANSCRIPTION FACTOR2 (ABF2) which drives the expression of genes that contain an ABRE in their promoter (Yamaguchi-Shinozaki and Shinozaki 2006).

Despite the elucidation of the PYR/PYL/RCAR – PP2C pathway, a complete understanding of ABA signal transduction is yet to be achieved. Evidence suggests that there is an independent pathway that involves the transmembrane ABA-binding ABAR/CHLH/GUN5 protein that spans the chloroplast envelope (Shen et al. 2006). It is still the subject of debate whether ABAR/CHLH/GUN5 itself is an ABA-binding receptor (McCourt and Creelman 2008; Tsuzuki et al. 2011), however, it has been shown to interact directly with a set of WRKY transcription factors (i.e. WRKY40), which regulate important ABA-responsive genes, including ABA INSENSITIVE5 (ABI5) (Shen et al. 2006; Shang et al. 2010). ABI5 is a basic leucine zipper transcription factor that physically interacts with ABRE sequences, driving ABA-induced gene expression (Finkelstein and Lynch 2000; Carles et al. 2002). Because this functional ABA signalling pathway operates with another set of key players, it is possible that it plays a role in mediating a different set of ABA responses to the PYR/PYL/RCAR pathway. The ABAR/CHLH/GUN5 pathway has been shown to interact with components of the circadian clock (Legnaioli et al. 2009). This pathway is also related to the expression of a gene encoding an enzyme involved in fatty acid β-oxidation, 3-KETOACYL-CoA THIOLASE 2 (KAT2) (Jiang et al. 2011). KAT2 is a positive regulator of ABA signalling responses and is thought to be located downstream of ABA-induced TOC1-mediated circadian expression of *ABAR*/*CHLH*/*GUN5* (Legnaioli et al. 2009). The data presented here and in Fig. 3 clearly suggest that many components of the ABA biosynthesis, perception, and response network are gated by the circadian clock. Downstream ABA responsive genes are also clearly circadian regulated (Supplemental Table 1), either directly or via the circadian gating of ABA response networks.

SnRK2 kinases have been shown to regulate the expression of transcription factors such as AREB1, AREB2 and ABF3 during drought (Yoshida et al. 2010). These, in turn, drive expression of various downstream water stress-related genes, including those encoding the LATE-EMBRYOGENESIS-ABUNDANT (LEA) proteins (Yoshida et al. 2010). LEAs are a group of hydrophilic proteins that accumulate during seed maturation and osmotic stress (Battaglia et al. 2008). Most *LEA* genes contain an ABRE in their promoters (Hundertmark and Hincha 2008). Whereas the majority of the ABA responsive genes described in the literature contain an ABRE in their promoters and are, therefore, likely to be directly regulated by ABA, not all stress-inducible genes are regulated directly by the hormone. Many stress genes contain the DEHYDRATION-RESPONSIVE ELEMENT (DRE)/C-

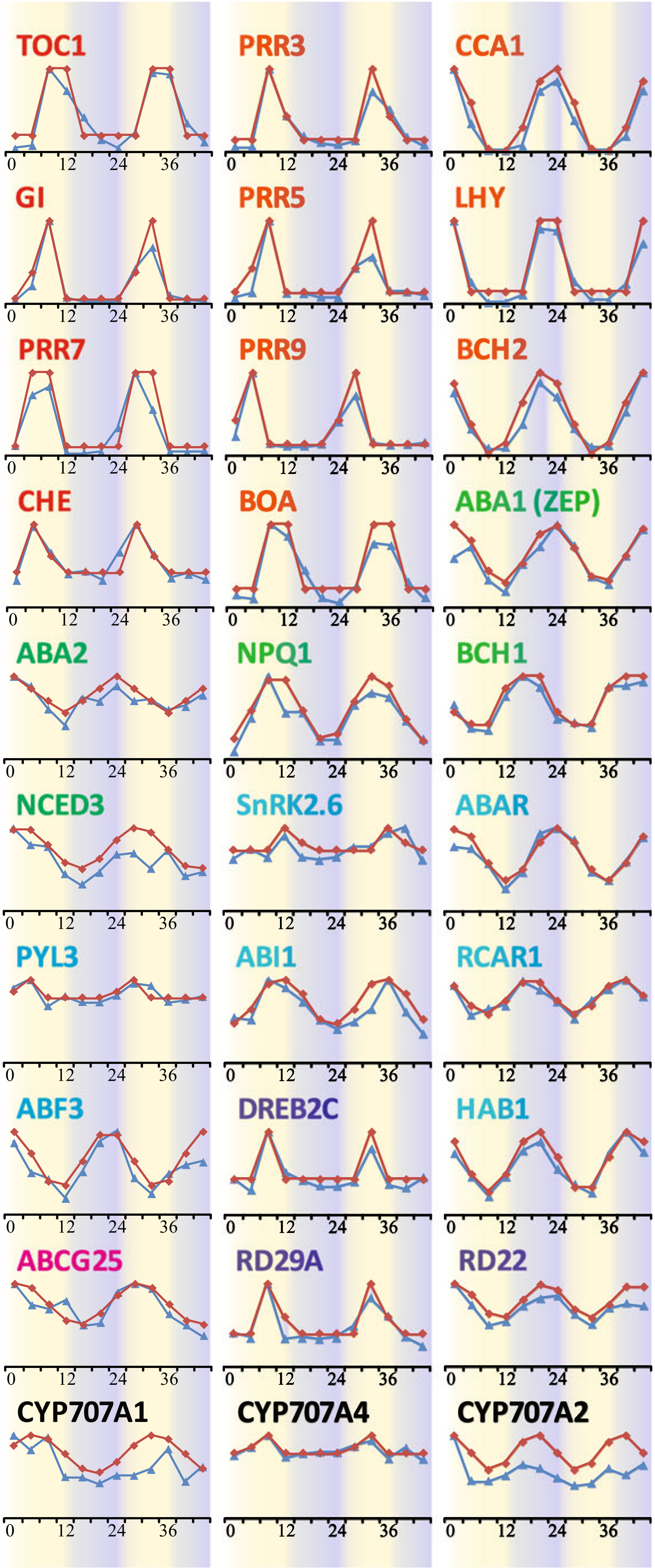

Fig. 3 Circadian clock regulated expression of key clock, and ABA metabolism, perception and response genes. Published genes were analysed for circadian regulation using the Diurnal Search Tool (http://diurnal.cgrb.oregonstate.edu/) to identify circadian regulation [LL23(LDHH)] (Mockler et al. 2007). In this experiment, plants were entrained under 12 h light/ 12 h dark cycles and then place in constant light [LL23(LDHH)] (Mockler et al. 2007). Time 0= lights on. The shaded areas mark the approximate division of subjective light and dark. Gene names in orange=circadian clock components, green=ABA biosynthesis, blue=ABA signal transduction, purple=ABA response genes, red=ABA transporter, and black=ABA degradation pathway components

REPEAT ELEMENT (DRE/CRT) in their promoter (Yamaguchi-Shinozaki and Shinozaki 2005). The DRE/CRT element is bound by the transcription factors CBF/DREB1 and DREB2 (Nakashima et al. 2009) and although the pathway is not directly responsive to ABA, CBF1 transcript levels can be influenced by ABA (Knight et al. 2004) and DREB2C can physically interact with the ABRE-binding, ABF2 protein (Lee et al. 2010). Importantly, expression of *DREB1C* is regulated by the circadian clock since it is repressed by the transcription factor PIF7, and PIF7's activity is regulated by a TOC1 interaction (Kidokoro et al. 2009). Some genes, such as the strongly drought-, cold- and salt-inducible *RESPONSIVE-TO-DEHYDRATION 29A* (*RD29A*) gene have both the DRE/CRT and ABRE in their promoters (Yamaguchi-Shinozaki and Shinozaki 1994). The *LEA*, early-response-to-dehydration *ERD10* genes are also regulated by both ABA and DREB1 (Kasuga et al. 2004; Kiyosue et al. 1994), again pointing to circadian control (Kidokoro et al. 2009). By contrast, the *RD22* gene is highly induced by ABA, but contains no ABRE in its promoter (Abe et al. 2003). Instead, its expression is driven by the MYC2 and MYB2 transcription factors, indicating the presence of yet another pathway for ABA responses.

Role of circadian-gated ABA signalling in plant growth and development

Seed dormancy and germination are good examples of developmental processes that are regulated by integrating environmental inputs with hormonal signalling and response outputs. The key hormones that regulate seed dormancy and germination are ABA, generally recognised as promoting dormancy, and its antagonist gibberellic acid (GA) (Koornneef et al. 2002). The capacity to germinate can be thought of as being regulated by a balance between the influence of ABA and GA (Finkelstein et al. 2008; Penfield and King 2009). ABA levels are actively maintained at high levels in dormant seeds and reduced in non-dormant and germinating seeds. In dormant seeds, imbibition, the passive uptake of water by seeds, leads to increased expression of the circadian regulated ABA biosynthetic *NCED* and *ABA1* genes and *de novo* ABA biosynthesis (Penfield and Hall 2009). ABA in turn blocks germination through the action of the ABA INSENSITIVE3 (ABI3) transcription factor (Piskurewicz et al. 2009). Conversely, imbibition in non-dormant *Arabidopsis* seeds leads to the expression of the ABA catabolic CYP707A2 enzyme (Millar et al. 2006). As with ABA, the GA signalling pathway has been shown to be related to the circadian clock (Arana et al. 2011) and to regulate seed dormancy and germination. The molecular mechanism has been studied in depth (Achard et al. 2008; Penfield et al. 2006a,b; Seo et al. 2006). Penfield and King (2009) have proposed a model for seed dormancy and germination where the clock PRR proteins (i.e. TOC1), PHYTOCHROME INTERACTING FACTOR (PIF) bHLH proteins, ABI3, and the GA response inhibitor, DELLA, proteins regulate dormancy and germination. Although the details are beyond the scope of this review, briefly, DELLA proteins are degraded in the presence of GA (Tyler et al. 2004), however, PIF proteins are able to bind to DELLAs, which inhibits their degradation (Feng et al. 2008). Penfield and King (2009) propose that in a dormant seed, ABI3 would be bound to PRR proteins and PIF proteins would be bound to the DELLAs, inhibiting the degradation of both ABI3 and DELLA proteins. Germination inducing environmental signals would promote the formation of a PIF-PRR complex. This would lead to the liberation and degradation of ABI3 and DELLA proteins. The system they suggest would be self reinforcing if the liberated PIF protein or the PIF-PRR complex induced GA biosynthesis. More GA would then further decrease the DELLAs, leading to a reinforcement of the system and germination. If the PRR, PIF, ABI3 and DELLA proteins are all integrated in the regulation of seed dormancy and germination, the system provides a good model for unravelling the complexities of the interdependent relationship between hormones and the circadian clock.

Another complex system where hormones and the circadian clock are known to play integrative roles is the control of stomatal aperture. As it is the principle point of control for plant water relations and for CO_2 uptake, in many situations stomatal aperture control is also the principal determinant of plant productivity. Environmental factors such as soil water status, humidity, CO_2 concentration and light all factor in the regulation of stomatal aperture (Israelsson et al. 2006; Hirayama and Shinozaki 2007; MacRobbie and Kurup 2007; Underwood et al. 2007; Neil et al. 2008). Much is known about the system. Stomatal aperture is regulated through a complex, dynamically integrated system of biochemical processes that include increases in cytosolic pH and free [Ca^{2+}] (Marten et al. 2007; Kim et al. 2010; Geiger et al. 2011), decreases in cytosolic K^+, Cl^- (Garcia-Mata et al. 2003) and malate^{2-} (Meyer et al. 2011), the production of reactive oxygen species (Jannat et al. 2011) and NO (Saito et al. 2009), and the rearrangement of the cytoskeleton (Higaki et al. 2010). The circadian clock (Dodd et al. 2005a) and ABA (Correia et al. 1995) play central roles by orchestrating these biochemical effectors. Stomatal aperture is capable of anticipating dawn and dusk signals (Hotta et al. 2007) and is more sensitive to ABA in the afternoon than in the morning (Correia et al. 1995). Cytosolic free [Ca^{2+}], one of the principal actors in stomatal aperture control has also been shown to be regulated by the circadian clock (Dodd et al. 2005a). Evidence has also emerged for roles for ethylene, cytokinin, auxin, brassinosteroids, jasmonic and salicylic acids and interactions between these players in their regulation of stomatal aperture (Acharya and Assmann 2009). It remains to be seen whether these hormones act in concert with the circadian clock in stomatal aperture control or whether they act only by overriding clock regulation during environmental stress responses.

Future perspectives

Increasing the level of complexity of developmental regulation and environmental responsiveness through an integration of multiple hormone response pathways and the circadian clock presumably increases the robustness and plasticity of response. Given the substantial evidence for circadian clock regulated gating of ABA and the other plant hormones (i.e. Covington and Harmer 2007; Michael et al. 2008a; Arana et al. 2011), it also clearly increases the fitness of plants. Evidence will undoubtedly continue to accumulate for the gating of hormonally regulated physiological processes. Cold tolerance (Harmer et al. 2000; Knight et al. 2004; Kidokoro et al. 2009; Robertson et al. 2009), dark growth (Nozue et al. 2007), and the transition to flowering (Turck et al. 2008) have all been shown already to be regulated by both hormones and the circadian clock. Using germination, stomatal conductance, or one of these models to dissect the network of responses will help us to understand at a deeper level how plants deal *in situ* with their ever changing environment.

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