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1 Teaching Tools in Plant Biology Lecture Notes

2 Rhythms of Life: The Plant Circadian Clock

3

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11

12 **Abstract**

13 This teaching tool explores circadian rhythms in plants. This is an exciting and fast-moving area of
14 research, which requires us to think in terms of temporal biological dynamics, interconnected
15 networks of cellular components, and the relationship between plant molecular biology and
16 environmental adaptation. We present this topic as a series of concepts illustrated by examples,
17 including the architecture of circadian clocks, the connections between the oscillator and circadian-
18 regulated processes such as metabolism and control of flowering time, and consider how
19 understanding circadian rhythms could lead to crop improvement. We also explain some of the
20 techniques used to investigate circadian biology, as many of these may be unfamiliar. We do not
21 describe each component of the circadian oscillator; there are so many genes and mechanisms
22 involved that this resource would become unintelligible and not be useful to an undergraduate
23 audience. Instead, we consider it more important to think in terms of the overall organisation and
24 principles, rather than becoming lost in the details of individual components. For those interested in
25 finding out more, there are many excellent reviews on circadian rhythms, some of which we highlight
26 at the end of the article.

27

28 **Article contents**

29

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47 The importance of the circadian clock in plant biology

48 The architecture of circadian clocks

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54

55

56 Introduction

57

58 The rotation of the Earth on its axis causes cycles of day and night. This causes repetitive daily
59 changes in the environment that present living organisms with a variety of challenges. During each
60 24 h day, there are large fluctuations in light intensity and temperature, which also lead to daily
61 fluctuations in other aspects of the environment such as humidity and predator behaviour. The
62 requirement to co-ordinate biological processes with environmental light-dark and temperature
63 cycles and biological processes, has led to the evolution of circadian clocks (from the Latin *circa*
64 meaning about, and *dies* meaning day). Circadian rhythms are defined as biological oscillations
65 having a cycle of about 24 hours, which persist in the absence of external signals. The persistence of
66 the rhythms in conditions of continuous light (or dark) and temperature indicate that they are driven
67 by an endogenous biological process. For example, rodents have higher levels of wheel running
68 activity at night under normal light-dark cycles. However, if kept in continuous darkness there are
69 still rhythms of activity, but the period of these oscillations is regulated by the internal biological
70 oscillator so may not be exactly 24 hours. Human physiology is controlled extensively by an internal
71 circadian oscillator. The impact of the circadian oscillator is felt most keenly in jet-lag, where the
72 discrepancy between the time of day predicted by the internal oscillator and the external conditions
73 results in physiological stress. Circadian oscillators (circadian clocks) have evolved multiple times
74 independently, and are found in organisms from all kingdoms of life.

75

76 Plant circadian biology has a long history; for example, it the ancient Greeks are thought to have
77 been aware of rhythmic processes in plants. Detailed measurements of plant circadian rhythms were
78 made in 1729 by the French biologist Jean-Jacques d'Ortous de Marian in the heliotropic plant
79 *Mimosa pudica*. He observed changes in the folding of the leaflets of *Mimosa* under constant
80 environmental conditions. These leaf movements were referred to as 'sleep' movements, and occur
81 in a range of different plants. Charles and Francis Darwin also recorded daily changes in leaf position
82 in 'The Power of Movement in Plants' (1880). In 1751 Carl Linneaus proposed a model for a 'Floral
83 Clock', whereby the predictable opening and closing of flowers at particular times of day could be
84 used to indicate the time. Uncovering the molecular bases for these rhythms has been the focus of
85 a huge amount of research in recent years, and we now have a good understanding of how the
86 circadian clock regulates many aspects of plant physiology, molecular biology and development.

87

88 The architecture of circadian clocks

89

90 Most of the research on the functioning of plant circadian clocks has been performed in the model
91 plant *Arabidopsis thaliana*. Circadian clocks in plants, animals, insects, and fungi share similar
92 properties and features, although the specific genes involved are different. Most circadian oscillators
93 are based on transcriptional regulation, and the importance of post-transcriptional regulation is
94 becoming increasingly understood. The circadian clock in cyanobacteria is functionally different to
95 eukaryotic oscillators because it is based mainly upon post-translational regulation. Circadian clocks
96 may be an interesting example of convergent evolution, because they are thought to have evolved
97 independently on a number of occasions.

98

99 The circadian network needs to have three main properties to coordinate the activities of the plant
100 with the external environment:

101

102 1) It needs to generate a 24 h rhythm inside the cell that can be sustained in the absence of
103 external stimuli. This is accomplished by the genes and proteins of the 'circadian oscillator'
104 (sometimes called 'circadian clock').

105

106 2) The circadian clock needs to be kept synchronized with the environment. In other words, its
107 phase needs to match the phase of the environment. The synchronization process is known
108 as 'entrainment'.

109

110 3) Mechanisms must exist to link the circadian clock with aspects of the plant that have
111 circadian rhythms. These are known as 'output pathways' because they connect the output
112 of the clock- which is a measure of the time of day- with other aspects of plant cells. The
113 main way the circadian clock influences the cell is by regulating daily rhythms of transcription
114 of a large number of genes. In turn, this leads to circadian rhythms in biochemistry and
115 physiology (see later sections of the teaching tool).

116

117 There is an additional level of complexity in that the sensitivity of both the entrainment and output
118 pathways can change over the course of the day, which may be regulated by the circadian clock itself.
119 This is referred to as 'circadian gating' and is discussed in detail in later sections of the teaching tool.

120

121 A useful piece of terminology that is used in circadian biology, which we use in this article, is the
122 word 'subjective' in relation to events that occur during the day-night cycle. When a plant is placed
123 under constant conditions, it no longer experiences dawn and dusk. However, the time of day at
124 which dawn, dusk or night would have occurred, had the plant been in day-night conditions, is called
125 the 'subjective dawn' 'subjective night', etc.

126 **The circadian oscillator**

127 *NB: In this section we consider processes that give rise to circadian rhythms in plant cells. This is a*
128 *rapidly moving field and checking recently published literature or reviews is recommended, as models*
129 *of the circadian oscillator are updated frequently as new discoveries are made.*

130

131 In general, circadian oscillators are formed from transcription-translation feedback loops. The
132 conceptual example on the slides considers a very simple oscillator with two components, A and B.
133 A and B are genes that encode proteins that are part of this oscillator. Here, one of the genes
134 activates the other, and one gene represses the other, so they regulate each other in a cyclical
135 manner. Over a day, Gene A is expressed in the morning and the protein that it produces is an
136 activator of Gene B. Therefore, when a certain amount of Protein A has accumulated, Gene B is
137 turned on and expressed later in the day. However, Protein B is a repressor of Gene A, so as Protein
138 B accumulates, Gene A is switched off and Protein A levels decrease during the night. Since Protein
139 A is required to activate Gene B, as Protein A decreases, Gene B is turned off. This releases the
140 repression of Gene A by Protein B, and Gene A begins to increase again the following morning. The
141 biochemical kinetics of these processes introduce rate constants and delays into the oscillator,

142 causing the cycle to be completed in about 24 hours. Each plant cell is thought to have its own
143 circadian oscillator that can operate independently, but recent research has found that there is
144 communication between the circadian oscillators of different tissue types.

145

146 Early breakthroughs in understanding the plant circadian clock focused upon interactions between
147 the transcription factor genes *TIMING OF CAB EXPRESSION 1 (TOC1)*, *CIRCADIAN CLOCK ASSOCIATED*
148 *1 (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY)*. It was thought originally that TOC1 activates CCA1
149 and LHY expression, and CCA1 / LHY repress TOC1, establishing a negative feedback loop in a similar
150 manner to Gene A and Gene B in the hypothetical oscillator. Models of the oscillator have changed
151 significantly since then, but at their core all contain a negative feedback loop. A significant revision
152 to the early models is that we now know that TOC1 represses rather than activates CCA1 / LHY; care
153 should therefore be taken when reading around this subject as older models may well include
154 inaccuracies including the structure of the TOC1-CCA/LHY loop.

155

156 A large number of oscillator genes have been identified through a variety of different experimental
157 approaches. The oscillator genes are expressed at different times of day; for example CCA1 is
158 maximally expressed around dawn while *LUX ARRHYTHMO (LUX)* reaches a peak around 12 hours
159 after dawn. Current thinking about the structure of the plant circadian clock is that it is quite complex,
160 with several feedback loops of gene expression that interact with each other. The circadian oscillator
161 incorporates a main feedback loop linking CCA1 / LHY with TOC1, GI and LUX / ELF3 / ELF4. There is
162 also a morning loop involving CCA1, LHY, PRR7 and PRR9, and an evening loop involving the 'evening
163 complex' of ELF3, ELF4 and LUX.

164

165 An underlying feature of plant circadian oscillators is that they maintain a free running period of
166 about 24 h across a range of physiologically-relevant temperatures, meaning that the circadian
167 period is buffered against changes in temperature. This feature is called 'temperature compensation'
168 of the circadian oscillator. Given that the rate of enzyme activity is temperature-dependent, the
169 robustness of the circadian oscillator to changes in temperature is an intriguing aspect of circadian
170 regulation. Temperature compensation of the circadian oscillator appears involve temperature-
171 dependent regulation of CCA1 and LHY expression. Several mechanisms have been identified,
172 including roles for PRR7 and PRR9, a regulator of CCA1 transcription called FBH1, and temperature-
173 dependent alternative splicing of RNA.

174

175 In addition to transcriptional feedback loops, there are a number of post-transcriptional control
176 mechanisms that contribute to the correct functioning of the oscillator. These include chromatin
177 remodelling, regulation of protein degradation by the proteasome, phosphorylation of oscillator
178 proteins and the involvement of small signalling molecules such as cyclic ADP ribose (cADPR) and
179 cytosolic free Ca²⁺ (see Mas 2008 for a review of post-transcriptional processes).

180

181 The complexity of the oscillator means that circadian research is moving away from identification
182 and characterisation of individual oscillator components, and towards understanding overall
183 emergent properties of the gene network. Mathematical modelling studies (see later sections of
184 teaching tool) have suggested that the complexity and interconnectivity within the oscillator may
185 provide the oscillator with greater robustness or stability in the face of many fluctuating aspects of
186 the environment that change the regulation of components of the clock.

187 **Entrainment pathways**

188 Circadian clocks never have a period of precisely 24 h, so if the circadian clock was not resynchronized
189 with the environment every day it would not predict time accurately for the plant. Additionally,
190 entrainment allows small daily adjustments to the phase of the circadian clock to match the changes
191 in the time of dawn that result from the progression of the seasons (see Slide 14 for an example of
192 the seasonal changes in the time of dawn that occur in northern Europe). Several environmental
193 signals act as entrainment cues. Red and blue light, sensed by the phytochrome and cryptochrome
194 photoreceptors respectively, provide a strong resetting signal to the circadian clock and are
195 important for synchronization of the circadian clock with dawn. Phytochrome A acts to regulate the
196 clock mainly under low intensity red light, whereas Phytochrome B is important under high intensity
197 red light. Cryptochrome 1 is important for regulation of the clock under low and high intensity blue
198 light. It is thought that phyA and phyB act additively, whereas cry1 and cry2 act redundantly. The
199 circadian clock component ZEITLUPE (ZTL) also senses blue light and contributes to the response of
200 the circadian clock to light. Photosynthesis within chloroplasts produce sugars, which also entrains
201 the circadian oscillator (see later sections of teaching tool). Temperature fluctuations can also
202 entrain the circadian oscillator, however the molecular mechanisms of temperature entrainment are
203 poorly understood at present.

204 **The importance of the circadian clock in plant biology**

205 The circadian clock influences almost all aspects of plant biology, and confers a significant selective
206 advantage. *Arabidopsis thaliana* plants that have been mutated to abolish circadian rhythms (*CCA1-*
207 *ox*) have only 53% of the biomass of wild type plants. Wild type plants also accumulate significantly
208 less biomass when grown under either 20 or 28 hour days than under 24 hour days, indicating that
209 the period of the endogenous oscillator must match that of the external environment for optimal
210 growth. The correct functioning of the circadian clock therefore has very significant impacts on plant
211 performance.

212

213 The circadian clock influences plant biology at a range of different levels. Analysis of the
214 transcriptome indicates a significant proportion of the *Arabidopsis thaliana* genome is under
215 circadian control. The percentage of transcripts that are expressed rhythmically varies between
216 different studies, but meta-analyses of multiple microarray datasets indicate that around a third of
217 the transcriptome cycles in constant conditions. Similar patterns of circadian transcript abundance
218 have been found in other species including tomato, soybean, rice, sugarcane and poplar. A large
219 number of genes associated with metabolism are under circadian control, indicating that the clock
220 has a significant effect on plant biochemistry (see later sections of teaching tool). The clock also
221 controls physiological processes such as leaf gas exchange, with stomatal being more open in the
222 subjective day than subjective night, when plants are grown under continuous light.

223

224 Growth and development are also under the control of the circadian oscillator. Video imaging of
225 *Arabidopsis* seedlings under continuous light reveals that both the elongation of the hypocotyls of
226 seedlings, and changes in cotyledon position, are rhythmic. Whether the clock directly controls cell
227 division in higher plants is an open question, but circadian control of water and carbon availability
228 contributes to rhythmic patterns of growth. Gibberellin and auxin-dependent growth is also
229 regulated by the oscillator through a variety of mechanisms. Developmental transitions such as the

230 initiation of flowering are also under the control of the circadian clock; many circadian clock mutants
231 flower either earlier or later than wild type plants when grown under long days (see later section of
232 teaching tool for details of mechanisms). It is clear that the circadian clock controls many different
233 aspects of plant biology, and is essential for optimum plant performance.

234

235 The circadian clock provides organisms with a fitness advantage, explaining why circadian oscillators
236 have evolved independently multiple times across different kingdoms of life. In plants, this
237 advantage can be demonstrated through competition experiments in the model plant *Arabidopsis*
238 *thaliana*. When plants are grown under 20 hour long days (10 hours light, 10 hours dark), mutant
239 plants with a short circadian period (*toc1-2*) accumulate more biomass than mutants with a long
240 circadian period (*ztl-27*; see later sections of teaching tool for definition circadian period). However,
241 if grown under 28 hour long days (14 hours light, 14 hours dark) the situation is reversed, so mutant
242 plants with a long circadian period outcompete those with a short period. The same pattern was
243 seen when mortality rates were compared, indicating that having an internal circadian oscillator that
244 matches the dynamics of the external environmental conditions confers a significant fitness
245 advantage.

246 **Investigating circadian rhythms in the laboratory**

247 **Properties of circadian rhythms**

248 Circadian rhythms are self sustaining in the absence of signals from the environment. Therefore, a
249 common way to study circadian rhythms is to place the plant under constant conditions- constant
250 temperature and constant light or darkness- and monitor the circadian regulated aspect of
251 physiology or biochemistry that is of interest. The plant is typically grown for a period time under
252 cycles of day and night, then transferred to constant conditions. Under constant conditions, the
253 circadian clock is said to 'free run' and the experimental conditions are sometimes called 'free
254 running conditions'. For example, to study circadian rhythms of photosynthesis, the plant might be
255 cultivated in 24 h light-dark cycles for several weeks before being moved to continuous light for the
256 measurement of photosynthetic CO₂ uptake.

257

258 Circadian rhythms have a number of properties that can be quantified and are often used during
259 studies of plant circadian biology. In continuous conditions, the circadian period is the time taken by
260 the rhythm to complete one full oscillation. This is typically about 24 h in wild type plants. The phase
261 is the time after dawn when a specific point in the cycle occurs, such as the peak. For example, the
262 rhythm could have a dawn phase or a dusk phase, depending on the property that is being
263 investigated. The amplitude of the rhythm is the difference between the average (center) point in
264 the oscillation and the maximum or minimum of the oscillation. These properties can be quantified
265 with a variety of analytical tools, such as Fast-Fourier Transform.

266

267 The reason it is useful to measure these properties of circadian rhythms is because they can change
268 depending on the state of the circadian clock. For example, mutating genes that encode parts of the
269 circadian oscillator can cause circadian rhythms to adopt a period that is longer or shorter than 24 h.
270 Similarly, mutations to other parts of the circadian clock can change its phase or its amplitude.
271 Measuring these properties has allowed important progress to be made in the identification of

272 components of the circadian clock, understanding how the components interact, and understanding
273 which aspects of plant physiology are controlled by which component of the circadian clock. For
274 example, the central oscillator component TOC1 was first identified in a forward genetic screen for
275 plants with altered circadian periods of *CAB2:LUCIFERASE* activity (see below).

276 **Common measurement techniques for studying circadian rhythms**

277

278 To gain molecular insight into circadian rhythms it is common to collect a time-course of samples of
279 plant tissue from which mRNA is isolated to monitor circadian changes in mRNA transcripts encoding
280 proteins. Depending on the transcript studied, this can be used to investigate- for example- the
281 functioning of the circadian clock, the control of metabolism by the clock, or provide a read-out of
282 the operation of a signalling pathway associated with the clock. Experiments often use quantitative
283 RT-PCR to measure the amount of transcript, but also studies have monitored circadian changes in
284 either all or a substantial proportion of plant transcripts (the transcriptome) using microarray or
285 sequencing methods (Harmer et al. Science 2000 is a great example of this). In a similar manner,
286 tissue samples can be collected to monitor circadian rhythms in the quantity of certain proteins, the
287 activity of enzymes, or the concentration of metabolites.

288

289 Circadian experiments often involve repetitive measurements at regular intervals over a long period
290 of time. This is laborious, can involve antisocial hours- also increasing the chances of mistakes- and
291 makes large scale experiments difficult. In addition, taking regular plant tissue samples to measure
292 gene expression, enzyme activity or a metabolite requires considerable amounts of plant material to
293 be grown in order to obtain enough samples through the time-course for sufficient levels of
294 experimental replication. To address this, several non-destructive / non-invasive technologies have
295 been developed to allow relatively straightforward monitoring of plant circadian rhythms.

296

297 One non-invasive technique involves monitoring circadian rhythms of seedling growth using a
298 camera. The hypocotyl (stem) of seedlings grows faster at night, and an automated camera trained
299 carefully on the young seedling can capture the rhythms in the position of the leaves that result from
300 this pulsatile growth. Since the circadian rhythm of growth is controlled by the circadian clock, this
301 method again provides a way to study the operation of the circadian clock. More commonly the
302 positions of the leaves themselves can be recorded; in some species the leaves change position due
303 to the activity of the pulvinus at the base of the leaf, whereas in others (including *Arabidopsis*
304 *thaliana*) the 'leaf movement' response is in fact due to rhythmic patterns of growth. One advantage
305 of this approach is that it does not require the generation of transgenic plants (see below). To see
306 some growth rhythms of plants, check out the movies on the Plants in Motion website
307 (<http://plantsinmotion.bio.indiana.edu/plantmotion/starthere.html>).

308

309 While leaf movement imaging is useful as a circadian-dependent phenotype and is commonly used
310 to screen for or characterise circadian mutants, it gives little molecular insight into the oscillator. The
311 non-invasive bioluminescent reporter luciferase has revolutionized plant circadian biology and
312 underpinned the discovery of many parts of the circadian clock in the model plant *Arabidopsis*
313 *thaliana*. Luciferase is an enzyme derived from fireflies that catalyzes the biochemical reaction
314 causing fireflies to glow. If an optimized luciferase is introduced to plants by making a transgenic

315 strain, and the plant is supplied with the substrate of luciferase (called luciferin), the plants will emit
316 light when the luciferase gene is expressed. The light emitted from the plant, due to the luciferase,
317 can be detected with sensitive camera systems or a luminometer. If luciferase is expressed in plants
318 under the control of a circadian-regulated gene promoter from the plant such as CHLOROPHYLL A/B
319 BINDING PROTEIN 2 (*CAB2*), circadian rhythms occur in expression of luciferase (and hence there is
320 a rhythm in the amount of light emitted from the plant). Luciferase monitoring instruments for
321 circadian rhythms research are often automated, making experiments relatively straightforward. In
322 a typical experiment, the plants are illuminated, then every hour or so the lights turn off, luciferase
323 bioluminescence is measured, then the lights turn back on again, then the lights turn back on again
324 so the plants can photosynthesize.

325 **Advanced methods for studying circadian rhythms**

326 Circadian bioluminescence imaging has been adapted to address specific questions in plant circadian
327 biology. This type of imaging typically monitors the circadian rhythms in a whole seedling by
328 measuring luciferase bioluminescence from entire plants. However, by using a particularly sensitive
329 camera and appropriate lens, the variation in circadian rhythms across single leaves can be measured.
330 Circadian rhythms can even be measured in single tissue types by using a 'split luciferase' or
331 'bimolecular luminescence complementation' technique. In this, one half of the protein is expressed
332 with a tissue specific promoter (e.g. vascular tissue only) and the other half a circadian regulated
333 promoter- and the two halves of the enzyme only come together to emit light in the chosen tissue
334 at the right time! It is even possible to measure circadian rhythms in single cells of a leaf by firing
335 microscopic beads coated with luciferase gene at the leaf- they only penetrate some cells, which are
336 the ones that subsequently glow and from which the rhythm can be measured.

337

338 From the slides about the structure and function of the molecular circadian clock, you will see that it
339 is extremely complex. There are multiple feedback loops, many interconnected components with
340 both negative and positive regulation steps, and both transcriptional and post-transcriptional
341 regulation processes. Considering the number of components, it is difficult- if not impossible- to
342 envisage with a diagram how the plant circadian clock functions. As a result, circadian biologists have
343 turned to mathematical modelling to deepen their understanding of the plant circadian clock.
344 Mathematical modelling has helped to identify gaps in our knowledge of the circadian clock, test the
345 accuracy of our theories concerning the operation of the circadian clock, and provide adaptive
346 explanations for certain properties of the clock, such as its complexity. In molecular plant biology,
347 the field of circadian rhythms was one of the first to use mathematical modelling to understand a
348 complex gene network and it could be argued that circadian rhythms researchers had a pioneering
349 role in the more widespread adoption of 'systems biology' approaches in plant sciences.

350

351 **The circadian clock and plant metabolism**

352 **Primary metabolism**

353 Plant metabolism undergoes dramatic shifts under each day-night cycle, with photosynthesis
354 dominating during the day and starch degradation and nutrient assimilation occurring at night. This

355 is not simply a response to changes in light availability, but is also under the control of the circadian
356 clock.

357

358 The first circadian transcriptome studies revealed extensive control of metabolism by the circadian
359 oscillator. It regulates the transcription of large number of enzymes of primary metabolism, including
360 chlorophyll biosynthesis, photosynthetic electron transport, starch synthesis and degradation,
361 nitrogen and sulphur assimilation (see Farré and Weise, 2012 for review). In many cases the peak in
362 RNA abundance precedes that of the actual physiological process that the enzyme is involved in; for
363 example the expression of chlorophyll biosynthesis genes occurs at the end of the night, anticipating
364 the onset of dawn. In contrast, the peak of genes associated with starch catabolism is around
365 subjective dusk.

366

367 Care should be taken not to over-interpret circadian transcriptome analyses as there are several
368 examples where individual transcripts have a circadian rhythm but there is no corresponding
369 circadian rhythm in protein level or catalytic activity. This does not necessarily mean that circadian
370 rhythms of transcription are without physiological significance; it may be that circadian rhythms in
371 gene expression compensate for patterns in protein degradation, so the role of the oscillator is to
372 maintain a constant level of protein. Alternatively there may be additional levels of post-
373 transcriptional regulation that contribute to control of metabolism.

374

375 Metabolite concentrations in circadian mutants differ from those in wild type plants, indicating a link
376 between the circadian oscillator and metabolism suggested by the transcriptome data. A metabolite
377 analysis of a *prr9/7/5* triple mutant found that the concentration of citric acid cycle (TCA cycle/Kreb's
378 cycle) intermediates (e.g. malate, fumarate) was significantly higher in the mutant than in wild type
379 plants. This can be partially explained by the reduced levels of fumarase and 2-oxoglutarate
380 dehydrogenase expression in these mutants. The triple mutants also have high levels of shikimate,
381 which is a precursor molecule for many secondary metabolites. The metabolic phenotype of the *prr*
382 triple mutant was distinct from that of CCA1-ox despite both mutants being arrhythmic, indicating
383 different parts of the oscillator have different regulatory functions in metabolism.

384

385 One of the roles of the oscillator is to allow plants to predict light availability. At night plants are
386 unable to photosynthesise, so are in danger of starvation. During the day starch is synthesised as a
387 temporary store of carbon, which is then degraded through the night to support respiration and
388 growth. The rate of starch degradation is tightly controlled so that plants exhaust 95% of their starch
389 by the end of the night. The plant can adjust this rate to reflect the length of the night, so that starch
390 degradation occurs more slowly in long nights. Remarkably, the plant can also adjust the rate of
391 degradation immediately in response to an unexpected early onset of night. When plants grown
392 under 12h light: 12 h dark cycles experience darkness only 8 hours after dawn they reduce the rate
393 of nocturnal starch metabolism, thereby preventing starvation at the end of the night. *cca1/lhy*
394 mutants degrade starch 35% faster than wild type plants, meaning they run out of starch 3-4 hours
395 before the end of the night and enter a period of starvation. The inability to regulate starch
396 metabolism therefore also contributes to the reduced growth of circadian clock mutants.

397

398 As metabolism in plants is split between different organelles it is also important to consider how the
399 oscillator is spatially organised within the cell. There are circadian rhythms of gene expression within

400 the chloroplast, which seem to controlled by the nucleus. Gene expression of the nuclear-encoded
401 sigma factor SIG5 is controlled by the circadian oscillator, with maximal expression around dawn.
402 SIG5 is then imported into the chloroplast where it forms part of a chloroplast RNA polymerase to
403 control the expression of plastid genes including *psbD*, which encodes the D2 protein of Photosystem
404 II.

405

406 Early genetic models of the plant circadian system proposed that light and temperature entrained
407 the oscillator, which then controlled outputs such as metabolism and stomatal movements. However,
408 it is now clear that metabolic processes also regulate the circadian oscillator, so the connection
409 between the clock and metabolism is infact bi-directional. Young seedlings grown in the absence of
410 sucrose have very low amplitude circadian oscillations of *CCA1:luc* and *CAB2:luc* in continuous dark,
411 but these rhythms increase in amplitude in the presence of exogenous sucrose. The restoration of
412 rhythmicity is dependent on the central oscillator component GIGANTEA. Application of
413 metabolically active sugars (sucrose, glucose, fructose) shortens the period of circadian rhythms of
414 plants grown in continuous low light. Externally applied sugar entrains the central oscillator, but
415 rhythms entrained to sugar have different characteristics to those entrained by light, indicating that
416 sugar is an independent entrainment signal in the circadian network. Redox status and the co-
417 enzyme NAD⁺ also contribute to plant circadian rhythms. The relationship between the clock and
418 primary metabolism is therefore more complex than the early models suggested, with metabolism
419 both being controlled by and controlling the central oscillator.

420 **Secondary metabolism**

421 Secondary metabolism is also under the control of the oscillator. Secondary metabolites are
422 molecules that are not required directly for the survival of the organism. Secondary metabolites play
423 a variety of roles in plants, including pigments and herbivore defence compounds. Floral scent
424 molecules are volatile secondary metabolites, having a key role in attracting pollinators. For example,
425 *Petunia hybrida* cv. Mitchell releases volatile compounds at night to attract pollinators such as
426 hawkmoths, and this has been shown to be under the control of the circadian oscillator. There is a
427 circadian rhythm in the production of volatiles such as methyl benzoate in continuous darkness, but
428 no such rhythm occurs in continuous light. Volatiles are synthesised from phenylalanine via the
429 shikimate, phenyl-propanoid and benzenoid pathways. Many of the enzymes and transcriptional
430 regulators in this pathway are under circadian control at the level of transcription, with maximal
431 expression occurring during the subjective night. If the clock gene LHY is constitutively overexpressed
432 in *Petunia hybrida*, rhythms of volatile emission are disrupted.

433

434 The circadian clock also plays a role in the interactions between plants and herbivores. In one study
435 looking at the interaction between *Arabidopsis* and cabbage loopers it was found that if the plants
436 and insects were entrained to the same light-dark cycles, the plants were able to resist herbivory
437 under continuous darkness. However, if the plants and insects were entrained to opposite conditions,
438 the plants were vulnerable to attack by the caterpillars. The insects have a strong circadian rhythm
439 of feeding, with maximal feeding occurring during the subjective day. Plant herbivore defences are
440 induced by jasmonates, which were found to accumulate during the subjective day. The plant is
441 therefore actively protecting itself during the time of maximal insect feeding, representing an
442 additional benefit to having a circadian clock. Through influencing plant-animal interactions it is

443 therefore likely that the circadian clock contributes towards ecological dynamics, although this has
444 not yet been systematically been investigated.

445 **The circadian clock provides timing information to control photoperiodic flowering**

446 Annual changes in photoperiod provide an environmental cue that is used by plants to detect
447 changes in the seasons. Many plants use the changing photoperiod to control the season of flowering.
448 For example wheat (*Triticum aestivum*) flowers in late spring when the days become longer, whereas
449 rice (*Oryza sativa*) flowers in late summer when days become shorter. Photoperiod sensitive plants
450 can be divided into long day and short day plants. Long day plants flower when the dark period is
451 shorter than a particular duration, and will also flower if a long night is interrupted by a short
452 exposure to light. Short day plants flower when the dark period is longer than the critical length, and
453 are unaffected by interruptions to the night. Some plants are obligately photoperiodic (they only
454 flower under a specific photoperiod), while others are facultatively photoperiodic (flowering is
455 stimulated by photoperiod but the plant will eventually flower even under non-flowering
456 photoperiods). Here, we consider specifically the role of circadian regulation in the seasonal
457 regulation of flowering, though it is important to remember that other environmental cues (e.g.
458 temperature, abiotic stress) also influence the transition to flowering.

459

460 The “external coincidence model” was first proposed by Erwin Bünning in 1936 as way to explain
461 photoperiod-dependent flowering in long days. In the simplest version of the model there are two
462 components; a circadian dependent regulator which has its peak of expression between 10-14 hours
463 after dawn, and a downstream floral inducer. Flowering is only induced when expression of the
464 circadian dependent regulator and light coincide. In short days the expression of the regulator is low
465 during the day so the plant continues in vegetative growth. In long days the regulator is expressed
466 highly in the afternoon, therefore light and the regulator coincide and the floral inducer is activated.

467

468 The molecular basis of this process is now well characterised, with at least a dozen different proteins
469 playing a role in the mechanism. Flowering is a tightly controlled event which centres on the
470 regulatory protein FLOWERING LOCUS T (FT). FT is regulated at the level of transcription by a number
471 of different pathways including the photoperiodic pathway, and when its expression is stimulated
472 the protein moves from the leaves to the shoot apical meristem to induce flowering. One activator
473 of FT is CONSTANS (CO), a zinc-finger transcriptional activator which is indirectly regulated by the
474 circadian oscillator. These two proteins can be integrated into the external coincidence model, where
475 CO represents the circadian dependent regulator and FT the floral inducer.

476

477 *CONSTANS* expression is rhythmic, with the maximal level of *CO* mRNA being ~12-14 hours after
478 dawn. However, CO protein is unstable in the dark as it is targeted for degradation by the E3 ubiquitin
479 ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1). This provides a mechanism through which
480 photoperiod can be detected:

481 • In short days the peak in *CO* mRNA levels occurs at night, and the protein does not
482 accumulate, hence FT is not induced and the plant stays in the vegetative state.

483 • In long days the peak of *CO* mRNA occurs during the light, and CO protein is able to
484 accumulate. The stabilisation of CO protein in the late afternoon allows the expression
485 of *FT*, which can then travel to the meristem and induce flowering.

486

487 The same basic coincidence model is conserved between Arabidopsis and crop species, including rice
488 which is a short day plant. In rice the equivalent of *CO* is *Heading-date 1 (Hd1)*, and the equivalent
489 of *FT* is *Heading-date 3a (Hd3a)*. The major difference between the two models is that while in
490 Arabidopsis *CO* activates *FT* expression, in rice *Hd1* is a repressor of *Hd3a*. This means that when
491 coincidence between *Hd1* expression and light occurs in long days the expression of *Hd3a* is
492 suppressed, and the plant remains in the vegetative state.

493 **Circadian gating**

494

495 An important principle in circadian biology is circadian gating. Circadian gating is the process whereby
496 the circadian clock adjusts the sensitivity or response of another signalling pathway, so that the
497 outcome from the pathway depends on the time of day. In practice this means that when an identical
498 stimulus is applied at different times of day, there is a circadian rhythm of the strength of the
499 response to the stimulus. Essentially, the clock acts as a valve on another signalling pathway.

500

501 Circadian gating acts upon the entrainment of the oscillator itself, and also the responses of plant
502 cells to the environment. Firstly, the circadian clock gates its own response to entrainment signals.
503 Application of an entrainment cue (e.g. light) changes the phase of the circadian clock towards dawn.
504 Circadian gating of this response means that the clock is much more sensitive to light during the night
505 and around dawn than during the rest of the day. This is very important, because if the circadian
506 clock were equally sensitive to light throughout the entire day, it would be continuously reset to
507 dawn and so unable to maintain an accurate measure of the time of day. In addition, the light
508 intensity may change considerably over the course of the day due to cloud cover or shading by other
509 plants, so adjusting sensitivity to light levels through the day may confer benefits to plants in the
510 natural environment.

511

512 In addition to gating of the entrainment pathways, the circadian clock also gates the responses of
513 other environmental responses in plants. It is thought that this ensures that the nature of the
514 response to the environmental signal is appropriate for the time of day. Here are two examples of
515 circadian gating:

516

517 (1) The circadian clock underlies a daily rhythm in the sensitivity of plants to cold. The CBF family of
518 genes regulate processes that increase the freezing tolerance of Arabidopsis. When plants were
519 exposed to 4 °C at a range of times of day, the degree of upregulation of the CBF family genes (CBF2
520 on the slide) has a circadian rhythm. Overall, the CBF genes are more sensitive to upregulation by
521 cold at 4 h and 10 h after subjective dawn, rather than during the subjective night.

522

523 (2) The circadian clock regulates a rapid response of developing seedlings to shade. During seedling
524 establishment, rapid elongation of the hypocotyl positions the leaves optimally for photosynthesis.
525 If the seedling is shaded by other plants, the ratio of red to far red light reduces, because vegetation
526 absorbs red light and reflects/transmits far red. This is sensed by the seedling and causes rapid
527 elongation of the hypocotyl, to allow the seedling to over-top its neighbours and catch the light. This

528 response is gated by the circadian clock, such that the hypocotyl elongates much faster when
529 exposed to low red:far red light around subjective dusk than at other times of day .

530 **The potential for crop improvement using circadian-dependent traits**

531

532 Given that circadian regulation increases the fitness of plants at a number of levels and agronomic
533 traits are associated with certain circadian clock genes, there is significant interest in understanding
534 and optimising circadian networks in crop species. The overall architecture of the oscillator is broadly
535 conserved between the model system *Arabidopsis thaliana* and agronomically important crop
536 species. A number of circadian clock genes underlie QTLs (Quantitative Trait Loci) associated with
537 key agronomic traits, indicating that the circadian-related fitness advantages conferred to
538 *Arabidopsis* by circadian regulation may also be relevant to crops. Flowering time and biomass
539 accumulation are obvious target traits, but given the widespread influence of the oscillator there
540 may be other aspects of crop productivity that could be improved with increasing understanding of
541 circadian rhythms. One example might be to increase the latitudinal range over which some crops
542 can be grown, by manipulating the role of the circadian clock in photoperiodism. In the face of
543 climate change and associated increases in abiotic stress, the gating of environmental signalling
544 pathways may also become an area of interest to agronomists.

545

546 **Case study 1: A slower clock was selected for during the domestication of tomato**

547 Analysis of wild and cultivated tomato varieties identified that cultivated varieties have a longer
548 circadian period and delayed phase when compared to wild relatives. QTL analysis isolated two
549 genomic regions that independently controlled the period and phase phenotypes, and the phase QTL
550 was subsequently mapped to a homologue of an *Arabidopsis* light signalling protein (EID1). Genetic
551 analysis suggests delayed phase was selected for relatively early in tomato domestication, and long
552 period arose at a later stage. The late phase mutation results in late flowering and increased
553 chlorophyll content specifically in long days, both of which would have been advantageous as tomato
554 spread from Mesoamerica to more northerly latitudes.

555

556 **Case study 2: Photoperiodism in Barley is controlled by a circadian clock component**

557 Barley was domesticated around 8000 years ago in the fertile crescent (between the Mediterranean
558 Sea and Persian Gulf, including modern Iraq, Egypt, Lebanon, Jordan and Israel). Wild barley
559 (*Hordeum vulgare* ssp. *spontaneum* C. Koch.) is a long day plant, so flowering is accelerated in when
560 day lengths are longer than ~13 hours. Cultivated barley (*H. vulgare* ssp. *vulgare* L.) has two forms;
561 winter barley which is sown at the end of the autumn and is harvested in the spring, and spring barley
562 which is sown in early spring and is harvested in the summer.

563

564 Growing spring barley has the advantage that sowing occurs after winter, therefore the potential for
565 frost damage is reduced. On the other hand, winter barley has the advantage that harvesting occurs
566 before the height of summer when dehydration may affect yield. There are several differences
567 between the two varieties, one of which is a difference in sensitivity to photoperiod. Winter barleys
568 are photoperiod sensitive, i.e. flowering is accelerated in long days, which is the ancestral phenotype.
569 Spring barleys are photoperiod insensitive, i.e. flowering is not accelerated by long days therefore
570 flowering (and therefore harvest) occurs later in the year. Fine mapping of a cross between the

571 photoperiod sensitive variety 'Igrí' and the late flowering non-sensitive variety 'Triumph' identified
572 a genomic region containing a single pseudo-response regulator gene, which is most similar to the
573 *Arabidopsis* circadian clock gene *PRR7*. The recessive *ppd-H1* mutation causes a single amino acid
574 change in the protein which results in photoperiod insensitivity, although mutation of this gene does
575 not affect the free-running period of the circadian oscillator. Analysis of barley land races across
576 Europe shows that there is a shift from *Ppd-H1* (photoperiod sensitive) varieties in the south to *ppd-*
577 *H1* (photoperiod insensitive) varieties in the north, indicating that there has been selection pressure
578 at this locus during the spread of barley away from the fertile crescent.

579

580 As flowering time is such an important characteristic of cereal crop yield, the potential to fine tune
581 flowering time to environmental conditions and extend growing seasons is of great interest. *PRR7*
582 provides an example of difference between the oscillator model obtained from studies in *Arabidopsis*
583 *thaliana* and the clock in monocots. *AtPRR7* is not a major regulator of photoperiodism in natural
584 *Arabidopsis* populations, whereas natural mutations at *PRR7* in both rice and barley alter the
585 flowering time. Crop development based on the *Arabidopsis* model of the circadian clock will
586 therefore need to be done with care, to ensure any changes to the circadian clock in crops actually
587 target traits of importance for agriculture.

588

589 **Summary and Future Directions**

590

591 Plant circadian biology has seen extraordinary progress in recent years and is the focus of research
592 groups across the globe. It is now clear that the circadian clock influences almost all plant processes
593 in one way or another, which contributes to the significant fitness advantage conferred by the
594 circadian clock. Some of this control is direct, with circadian oscillator components directly
595 controlling output pathways, while other processes are controlled indirectly through circadian-
596 dependent gating of environmental signalling pathways. The complexity of the clock and the diversity
597 of processes that it controls means that we have to consider the circadian clock to be an extensive
598 control network within plant cells, where it is both difficult and arguably meaningless to consider any
599 individual clock component in isolation. The use of mathematical modelling approaches to studying
600 circadian rhythms is now widespread, and helps us analyse the emergent properties of the network
601 such as circadian period and gating.

602

603 There are many unresolved questions in circadian biology, some of which require technical
604 innovation to address. Some of the open questions in plant circadian biology that remain include:

- 605 • Is the circadian oscillator specialized at a molecular and functional level in each plant cell
606 type and organ, and do these oscillators communicate with each other?
- 607 • What are the molecular bases for circadian gating in plants?
- 608 • What is the contribution of circadian regulation to the performance of crop plants, and how
609 can we capitalize upon this to increase crop production?
- 610 • How does circadian regulation in plants contribute to the dynamics of ecosystems?
- 611 • How did the plant circadian oscillator evolve?

612

613 The number of papers published on plant circadian rhythms continues to increase every year as more
614 metabolic, physiological and developmental processes that the clock regulates are discovered. The
615 interface between circadian biology and downstream processes is still poorly understood in some
616 cases, particularly those where the gating of environmental signals plays a role. An increased
617 understanding of plant circadian rhythms may result in the optimization of agriculturally important
618 traits, so this aspect of plant biology is of significant future interest to both the scientific community
619 and commercial enterprise. As the circadian clock underpins so many processes in plants it
620 represents both a fascinating and powerful system for understanding coordination in biological
621 systems, and will continue to be actively researched for many years to come.

622

623 **Recommended Reading:**

624 This is a very large field of research, and is a fast-moving subject. We apologise to those whose work
625 we have been unable to include for reasons of space.

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