Hypocotyl Development in Arabidopsis and other Brassicaceae Displays Evidence of Photoperiodic Memory

James Ronald[1], Sarah C.L Lock[1], Will Claydon[1], Zihao Zhu[2], Kayla McCarthy[1], Elizabeth Pendlington[1], Ethan J. Redmond[1], Gina Y.W. Vong[1], Sanoj P. Stanislas[1], Seth J. Davis[1], Marcel Quint[2], Daphne Ezer[1]*.

[1]Department of Biology, University of York, Wentworth Way, Heslington, York YO10 5DD, UK.
[2]Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany.

*Corresponding authors:
Daphne Ezer (daphne.ezer@york.ac.uk)

Abstract

Sensing and responding to photoperiod changes is essential for plants to adapt to seasonal progression. Most of our understanding of how plants sense photoperiodic changes is through studies on flowering time. However, other aspects of plant development are regulated by the photoperiod, including hypocotyl elongation. Unlike flowering, hypocotyl elongation displays a greater plasticity to changes in the photoperiod with increases in daylength causing greater inhibition of growth until a threshold is met. Previous studies have only looked at hypocotyl development in the context of a stationary photoperiod. It is unknown if changes in the photoperiod during development influence hypocotyl elongation. Here, we developed a physiological assay to investigate this question. We have discovered that hypocotyl elongation is influenced by a memory of past photoperiod exposure in Arabidopsis and Brassicaceae cultivars used for microgreen agriculture. Photoperiodic memory persisted for multiple days, although it weakened over time, and the strength of the memory was dependent on the genetic background. We identified that phyB and ELF3, key regulators of hypocotyl development, were required for photoperiodic memory. Finally, we identified that the circadian clock is unlikely to function as a repository for photoperiodic memory as circadian rhythms quickly re-aligned with the new photoperiod. In summary, our work highlights for the first-time evidence of a photoperiodic memory that can control plant development.

Introduction

The daily rotation of the Earth generates cycles of light and dark. The magnitude change in these daily cycles across the year is influenced by the distance to the equator, with organisms in latitudes further from the equator experiencing greater extremes in photic and thermal change than those at the equator. Many organisms have adopted a suite of mechanisms to perceive and respond to these seasonal changes, particularly changes in the photoperiod. Adaptation to seasonal changes is critical for organisms to maximize their fitness and productivity.

In plants, photoperiodic responses have been best studied in the context of the transition from vegetative to floral growth. This has led to the establishment of the coincidence model to describe how the photoperiod controls the transition to flowering (Song et al., 2015). However, other aspects of plant development are also controlled by the photoperiod. This includes a recently reported metabolic photoperiod perception pathway that regulates growth under long-day (LD) photoperiods (Wang et al., 2024). Additionally, the development of the hypocotyl is regulated by the photoperiod (Krahmer and Fankhauser, 2023). Similar to photoperiodic regulation of flowering, a coincidence model has been proposed to explain how hypocotyl elongation is controlled by the photoperiod (Nozue et al., 2007; Niwa, Yamashino and Mizuno, 2009). Within

the hypocotyl coincidence model, PHYTOCHROME INTERACTING FACTOR4 (PIF4) and PIF5 function as positive regulators of hypocotyl elongation (Nozue et al., 2007). The timing of *PIF4/5* expression is regulated by the circadian clock in a photoperiod-dependent manner (Niwa, Yamashino and Mizuno, 2009). Maximal hypocotyl elongation occurs under short-day (SD) photoperiods as *PIF4/5* expression coincides with the dark, avoiding the repressive effect of light-activated photoreceptors (Leivar and Monte, 2014), and day and dusk-phased circadian proteins on PIF4/5 transcriptional activity (Nieto et al., 2015; Martín et al., 2018; Nohales et al., 2019).

Although flowering and hypocotyl development share a similar model to describe how the photoperiod is perceived and then acted upon, the effect of the photoperiod on the transition to flowering and hypocotyl development has fundamental differences. Photoperiodic-dependent induction of flowering is a binary process, whereby once the photoperiod meets the threshold flowering is initiated (Song et al., 2015). In contrast, hypocotyl development is more plastic to the photoperiod. Increasing the length of the photoperiod causes greater inhibition of hypocotyl elongation until a threshold is met where further increases in light duration has no additional inhibitory effect (Niwa, Yamashino and Mizuno, 2009; Anwer et al., 2020). The current photoperiodic model of hypocotyl elongation describes development exclusively in the context of a stable photoperiod (Nozue et al., 2007; Niwa, Yamashino and Mizuno, 2009). This model has not been tested under conditions where the photoperiod duration changes during development. Moreover, it is unknown if photoperiod transitions during development can influence hypocotyl development, a so-called "memory" of past-events. Such memory may have important implications in microgreen agriculture where hypocotyls function as the commercial product. Here, balancing the photoperiod in microgreen agriculture is important to maximize the nutritional value but also minimizing the energy requirements and associated costs from growth under LD photoperiods (Bhaswant et al., 2023; Filatov and Olonin, 2023; Hernández-Adasme, Palma-Dias and Escalona, 2023).

In this manuscript, we investigated whether plants retain memory of prior photoperiod exposure using hypocotyl development as a physiological system. We have designed a photoperiod shift assay to assess how prior photoperiod exposure influences hypocotyl elongation in *Arabidopsis thaliana* (Arabidopsis hereafter) seedlings and germinating seeds. We have found that Arabidopsis displays evidence of a photoperiodic memory. The strength of the memory was dependent on the direction of the photoperiod shift and the developmental age of the Arabidopsis plants when they were initially entrained to the first photoperiod. We have identified key roles for phytochromeB (phyB) and EARLY FLOWERING3 (ELF3) in regulating photoperiodic memory response. We also investigated whether circadian rhythms could encode information on prior

86 photoperiodic exposure. However, circadian rhythms quickly adapted to changes in photoperiod,
87 suggesting that other mechanisms are required for encoding historical photoperiod information.
88 Finally, we identified that several vertically farmed Brassicaceae (kale and radish) have stronger
89 photoperiodic memory than Arabidopsis. Ultimately, hypocotyl memory could have important
90 applications in developing energy-efficient growth regimes for microgreen commercial
91 applications.
92
93 **Methods**
94 Plant lines and husbandry.
95 The following *Arabidopsis thaliana* ecotypes were used in this work: Bayreuth-0 (Bay-0), Cape
96 Verde Island (Cvi), Sapporo (Sap), Shakdara (Sha), Tanzania-1 (Tnz-1) and Wassilewskija-2 (Ws-
97 2). All ecotypes harbored the *CCR2::LUC* reporter gene. Bay-0 *CCR2::LUC*, Cvi *CCR2::LUC*, Sha
98 *CCR2::LUC* and Ws-2 *CCR2::LUC* have been described previously (Doyle et al., 2002; Boikoglou et
99 al., 2011; Anwer et al., 2014). For the Sap and Tnz *CCR2::LUC* lines, the respective parent
100 backgrounds were transformed with the *CCR2::LUC* reporter by floral dip (Davis et al., 2009). A
101 luminesce transgenic plant was then identified and backcrossed to the respective parental line.
102 This backcrossed transgenic was then self-fertilized before a homozygous, single transgene
103 segregant was identified and bulked in the following generation. The following Arabidopsis
104 mutants in the Ws-2 background were also used: *elf3-4 CCR2::LUC*, *phyB-10 CCR2::LUC*, *elf3-*
105 *4/phyB-10 CCR2::LUC*. These mutants have been previously described in earlier work (Reed et al.,
106 1993; Hicks, Albertson and Wagner, 2001; Ronald et al., 2022). Prior to experiments, all lines
107 were propagated on soil in environmentally controlled growth chambers with a LD photoperiod
108 (16/8 light/dark) at a light intensity of 100 $\mu mol/m^{-2}/s^{-1}$ and a set temperature of 22°C.
109
110 Hypocotyl measurements.
111 For the collection of data presented in figure 1, figure 2 and supplementary figure 1-5, all seeds
112 were surface sterilized before being plated onto 1x Murashige and Skoog (MS) media with 1.5%
113 w/v phytoagar (Duchefa), 1% w/v sucrose (Sigma) and 0.5 g/L MES (Sigma). Seeds were then
114 stratified for 48-96 hours. After stratification, germination was induced by exposure to 75
115 $\mu mol/m^{-2}/s^{-1}$ of white light for 24 hours and a constant temperature of 19.5°C. After 24 hours,
116 germinating seeds were provided photoperiod information. They were either grown under SD
117 (6/18 light/dark) or LD photoperiods with a constant temperature of 19.5°C and a light intensity
118 of 75 $\mu mol/m^{-2}/s^{-1}$. After 48 hours, the photoperiod shifts were initiated. Seedlings were either
119 kept under the initial photoperiod as a control or transferred to the reciprocal photoperiod. All
120 photoperiod shifts occurred at ZT6, before lights off for the SD growth conditions. Measurements
121 were taken on day 5 (timepoint 1) or day 7 (timepoint 2) at ZT6. All experiments were repeated

122 twice with a similar result observed across the two experimental repeats. The presented data is
123 a combination of the two experimental repeats, with each individual experiment including at least
124 fifteen seedlings. Hypocotyl images were measured using ImageJ (Schneider et al. 2012). For
125 figures 1 and 2, hypocotyl measurements were scaled so that the median LD hypocotyl length was
126 0 and the median SD hypocotyl length was 1, using the formula: *Scaled length = (length – median*
127 *length LD)/(median length SD – median length LD).* Adjusted p-values were calculated using
128 TukeyHSD tests. We have included all statistical and raw hypocotyl measurements as a
129 supplementary dataset (Supplementary File).
130
131 Luciferase assay.
132 Arabidopsis Ws-2 *CCR2::LUC* WT seeds were surface sterilized and plated on 1x MS medium
133 containing 3% w/v sucrose (Sigma), 1.5% w/v phytoagar (Duchefa), 0.5 g/L MES (Sigma) and 15
134 µg/ml Hygromycin-B (Duchefa). After 5 days of stratification at 4°C, seeds were transferred to
135 the specified photoperiod for 6 days. For all photoperiods, a constant temperature of 20°C was
136 used and a light intensity of between 80 - 100 µmol/m^{-2}/s^{-1}. On day 6, seedlings were transferred
137 into a 96 well plate containing the same media as described above but additionally 15 µl of 1 mM
138 Luciferin. Plates were then placed back in the growth cabinet until they were then transferred to
139 the TOPCOUNT (TC) at dawn on day 7. Plates in the TC were grown under the stated photoperiod
140 with red plus blue light (2.4-3.6 µmol/m^{-2}/s^{-1} red light and 0.8-1.4 µmol/m^{-2}/s^{-1} blue light) and a
141 constant temperature of 20°C for a further 4 days. For samples undergoing a photoperiod shift,
142 this shift occurred on day 11, exactly 96 hours after being placed in the TC. The fda package in R
143 was used to smooth curves using B-splines and perform functional principal component analysis.
144
145 Continuous monitoring of hypocotyl elongation.
146 Sterilized Arabidopsis seeds were stratified in darkness at 4°C for 72 hours. To avoid contact of
147 seedlings with the media surface during hypocotyl elongation, two strips were cut and removed
148 from the cultivation medium. Solid *A. thaliana* solution (ATS) nutrient medium (Lincoln, Britton
149 and Estelle, 1990) with 1% w/v sucrose was used as the cultivation media for these experiments.
150 Seeds were sown in small indentations on the surface of the cut to prevent excessive movement
151 as described previously (Janitza et al., 2024). Plates were placed vertically at 21°C and constant
152 light (LL, 40 µmol m^{-2}s^{-1}) for one day to allow uniform germination of seeds, before being
153 transferred to the initial photoperiod: either LD or SD conditions. After two days, plates were
154 transferred to the alternative photoperiod (SD or LD, dawn-aligned), or were kept under the
155 initial photoperiod. The analysis of hypocotyl elongation then started 72 hours after sowing
156 (ZT0). Seedlings were illuminated by two infrared light emitting diodes and were imaged hourly
157 by a Panasonic G5 camera modified with a long pass filter (830 nm). To detect minor changes

158 between time points, image stacks were imported as "Image Sequence" in ImageJ. Each seedling
159 (n≳6) was analyzed separately, and the hypocotyl length was measured using the "Segmented
160 Line" tool, by slightly adjusting the control nodes between time points.
161
162 Hypocotyl Elongation in Microgreens
163 Microgreens (Supplementary Table 2) seeds were sown into trays containing a compost mix of
164 equal parts F2+S (Levington), coir and vermiculite supplemented with 0.7 g/L osmocote
165 fertilizer. Seeds were sown approximately 1 cm below the surface of the soil. Microgreens were
166 then grown under either a LD or SD photoperiod with a constant temperature of 19°C, 60%
167 humidity and 70 $\mu mol/m^{-2}/s^{-1}$ of light. Trays were inspected daily to determine when individual
168 hypocotyls had emerged from the soil. Once 50% of the respective microgreen cultivar had a
169 visible hypocotyl, seedlings were grown for three days under the initial photoperiod. This period
170 was determined as the initial photoperiod exposure. After this period, the microgreens were then
171 either switched to the reciprocal photoperiod or kept under the same photoperiod as a control
172 for a further 3 days. Trays were switched at ZT5 to ensure that most of the plants that underwent
173 photoperiod shifts had equal exposure to each photoperiod. Each experiment was carried out
174 three times with 40 individual plants imaged per variety per experiment. To image the
175 microgreen hypocotyls, leaves were firstly dissected before the hypocotyls were removed from
176 the soil and laid flat in an Epson scanner. High-resolution scans were then taken. Images were
177 imported into ImageJ for measurement. All imaging took place at ZT5.

178 **Results**

179 **Designing a photoperiodic memory experimental assay**

180 To understand whether hypocotyl development in Arabidopsis is influenced by prior exposure to
181 a given different photoperiod (Figure 1A) we designed the following assay. Stratified seeds were
182 firstly germinated under constant white light for 24 hours to synchronize germination before
183 seedlings were then provided two days of photoperiodic information, either SD or LD. On day 3,
184 seedlings were then either transferred to the reciprocal photoperiod or kept under the existing
185 photoperiod. Hypocotyl measurements were then taken on day 5 and day 7 to understand the
186 immediate and long-term impact of the shift, respectively. We transferred seedlings on day 3 as
187 hypocotyl development in de-etiolated Arabidopsis seedlings primarily occurs between three-
188 and four-days post-germination (Gendreau et al., 1997). Supporting this, we found minimal
189 difference in median hypocotyl length of Ws-2 seedlings grown under SD and LD on day 3 (Figure
190 1B). To compare the relative amount of photoperiodic memory between Arabidopsis ecotypes
191 and mutants with different hypocotyl lengths, we implemented a photoperiodic memory index
192 (PMI, Figure 1C). The PMI scales the hypocotyl length relative to the median hypocotyl length of

193 growth under LD and SD conditions. Thus, seedlings with a PMI closer to 1 have a hypocotyl
194 length similar to those grown under constant SD, while a score closer to 0 indicates development
195 similar to that of constant LD.
196
197 **Arabidopsis seedlings display photoperiodic memory**
198 We firstly analyzed the photoperiodic memory of wild-type Arabidopsis Ws-2 seedlings. As
199 discussed above, Ws-2 seedlings were exposed to the photoperiodic shift on day 3 (Figure 2A).
200 By day 5, hypocotyl development in the Ws-2 seedlings that underwent either photoperiod shift
201 had a PMI of ~0.45 (Figure 2B, Supplementary Figure 1), indicating that exposure to the initial
202 photoperiod does influence hypocotyl development. By day 7, hypocotyl development in the
203 seedlings that underwent the photoperiod shift had diverged from each other and were more
204 closely resembling those under constant conditions (Figure 2C). However, for neither
205 photoperiod shift did hypocotyl elongation mirror that of seedlings grown under a constant
206 photoperiod (Figure 2C). Together, these results suggest that hypocotyl development displays
207 evidence of a lasting photoperiodic memory.
208
209 Next, we investigated whether other Arabidopsis ecotypes also displayed photoperiodic memory
210 and the heterogeneity in response between these ecotypes. Ecotypes were picked to cover a range
211 of latitudes from the equator to northerly latitudes (Supplementary Table 1). For all ecotypes,
212 hypocotyl elongation again displayed evidence of photoperiodic memory and this memory
213 continued to persist on day 7 (Figure 2B-C, Supplementary Figure 1). However, for the Sha, Cvi
214 and Bay-0 ecotypes the memory of the initial photoperiod was weaker than Ws-2 for both
215 photoperiod shifts. We also observed that certain ecotypes displayed stronger memory of a
216 particular photoperiod shift. For example, Tnz had a stronger memory of the LD-SD than SD-LD
217 transitions on day 5, while Sap displayed the opposite behavior (Figure 2B). However, neither
218 effect persisted after an additional two days in the final photoperiod (Figure 2C). Importantly, for
219 all ecotypes tested except Ws-2, there was a difference in hypocotyl length between the different
220 photoperiod shifts on day 5 even though the seedlings were exposed to the same amount of total
221 light (Supplementary Figure 2-3). Therefore, it is the order of the photoperiod exposure that is
222 important in determining hypocotyl elongation.
223
224 **phyB and ELF3 regulate the rate that Ws-2 responds to changes in day length**
225 To understand the genetic pathways underpinning the response to the photoperiodic shifts, we
226 characterized different mutants in the Ws-2 ecotype background (wild type, WT). We firstly
227 focused on phyB and ELF3 as they are key regulators of hypocotyl elongation (Reed et al., 1993;
228 Zagotta et al., 1996). On day 5, the *elf3-4* mutant (*elf3* hereafter) had reduced photoperiodic

229 memory of both photoperiod shifts relative to WT (Figure 2D, Supplementary Figure 4-5). On day
230 7, hypocotyl elongation in the *elf3* mutant remained more sensitive to the SD-LD shift but had a
231 similar response to WT for the LD-SD shift (Figure 2E). For the *phyB-10* (*phyB* hereafter) mutant,
232 seedlings phenocopied the WT response to the LD-SD on day 5 but then displayed reduced growth
233 by day 7 (Figure 2D-E). Under the SD-LD shift, *phyB* mutants had a similar PMI to those kept under
234 constant LD (Figure 2D), indicating a weak memory of the initial SD photoperiod. This phenotype
235 became further enhanced by day 7, with *phyB* mutants moved from SD-LD now shorter than those
236 kept under continuous LD (Figure 2E). We also analyzed the *elf3/phyB* double mutant phenotype
237 to understand the genetic relationship between ELF3 and phyB. For the LD-SD shift, the *elf3/phyB*
238 mutant phenocopied the WT response at both timepoints (Figure 2D-E). Similarly, for the SD-LD
239 shift the *elf3/phyB* mutant had a similar response to WT at the first timepoint (Figure 2D).
240 However, at the second time point the *elf3/phyB* mutant had reduced photoperiodic memory than
241 WT (Figure 2E). Together, our results highlight a complex requirement of phyB and ELF3 in
242 regulating photoperiodic memory: ELF3 is necessary to regulate a plant's initial sensitivity to
243 changes in the photoperiod, while phyB prevents over-compensation for the SD-LD shift and
244 sensitizes plants to the shift to SD.
245
246 **The impact of photoperiodic memory is dependent on developmental age**
247 Hypocotyl elongation is dependent on the accumulation of PIFs at specific times of day. Maximal
248 hypocotyl elongation occurs under SD photoperiods as PIF accumulation coincides with the late
249 evening, where PIF activity displays minimal inhibition (Niwa, Yamashino and Mizuno, 2009;
250 Leivar and Monte, 2014). This led us to investigate how the dynamics of hypocotyl elongation
251 changes across time in seedlings that underwent the photoperiod shift. For this experiment, seeds
252 were stratified in water rather than on the surface of the agar-media for 3 days. This small change
253 in the protocol delayed the onset of hypocotyl emergence until day 3, the point at which the
254 photoperiod shift initiates. Thus, the perception of the initial photoperiod under these conditions
255 occurred exclusively in the germinating seed. This allowed us to subsequently investigate how
256 photoperiodic perception in seeds influences seedling development alongside the effects of the
257 photoperiod shifts on the timing of hypocotyl elongation.
258
259 Intriguingly, WT seedlings whose germinating seeds were exposed to LD, but then shifted to SD
260 had extremely rapid hypocotyl elongation, compared to all other conditions (Figure 3A). The
261 timing of growth in seeds that were shifted from LD-SD was consistent with those grown under
262 constant SD conditions (Figure 3B). However, the maximal growth rate in the seeds that were
263 shifted from LD-SD was much higher on the first night and remained elevated on the second night.
264 Thus, the shift from LD-SD caused a temporary acceleration of growth. For the SD-LD shift, WT

265 displayed a less pronounced memory of the initial SD exposure, with overall hypocotyl elongation
266 (Figure 3A) and the timing of growth (Figure 3B) closely mirroring seedlings that were only
267 grown under constant LD. In summary, WT seeds display photoperiodic memory of prior LD
268 exposure, and this results in sustained levels of increased hypocotyl elongation when shifted to
269 SD.
270
271 As ELF3 and phyB were required for photoperiodic memory in seedlings, we investigated
272 whether they contributed to photoperiodic memory in seeds. phyB is functional in seeds
273 (Piskurewicz et al., 2023) and the circadian clock is also active in imbibed seeds (Zhong et al.,
274 1998). The role of ELF3 in seeds remains to be determined. As in WT, the *elf3, phyB* and *elf3/phyB*
275 mutants' overall growth and the timing of growth showed weak memory of the SD-LD shift
276 (Figure 3A-B). For the LD-SD shift, the *elf3* mutant did not display enhanced growth on the first
277 night (Figure 3B). Instead, the enhanced growth was delayed until the second night. Furthermore,
278 the peak timing of this growth was shifted from late evening to dusk, where ELF3 functions within
279 the evening complex to repress *PIF4/5* expression (Nusinow et al., 2011). As with WT, *elf3*
280 mutants displayed two evenings of accelerated growth following the LD-SD shift before the rate
281 of hypocotyl elongation became more consistent with growth under constant SD (Figure 3B). In
282 the *phyB* mutant, there was no evidence of accelerated growth following from the shift from LD-
283 SD. Instead, growth displayed two smaller peaks at dusk and in the late evening. These two
284 smaller peaks of growth likely compensate for the absence of one predominant period of growth.
285 The *elf3/phyB* double mutant displayed similar phenotypes to the respective single mutants for
286 the LD-SD shift, with both a delay in the adjustment to the new photoperiod and an absence of a
287 major peak of growth (Figure 3B). Thus, phyB and ELF3 seemingly work independently to control
288 the acceleration of growth and adjustment to the new photoperiod, respectively, following the
289 LD-SD shift.
290
291 **Circadian clocks adapt to photoperiod shifts quickly in Arabidopsis seedlings**
292 Although our data suggests that seeds and seedlings have a memory of the prior photoperiod, it
293 is unclear how this photoperiodic information is stored. One possible mechanism is through the
294 circadian clock. The circadian clock has a critical role in regulating photoperiodic responses in
295 plants (Nozue et al., 2007; Niwa, Yamashino and Mizuno, 2009; Song et al., 2015; Wang et al.,
296 2024). Furthermore, the circadian clock is active in imbibed seeds (Zhong et al., 1998) and the
297 expression of *PIF4* and *PIF5* display photoperiod-dependent changes in the phase of their
298 expression (Niwa, Yamashino and Mizuno, 2009). Thus, we hypothesized that the circadian clock
299 may function as a repository for photoperiodic information.
300

301 To test this possibility, we analyzed the circadian waveform of the *CCR2::LUC* reporter in the WT
302 background. Here, seedlings were entrained for ten days under SD (8/16 light/dark) and then
303 shifted to LD for three days. As a control, seedlings were entrained and then kept under SD or LD
304 for the length of the experiment. For seedlings grown under either constant SD or LD conditions,
305 *CCR2::LUC* was phased to the early evening of each respective photoperiod as reported previously
306 (Strayer et al., 2000; Schultz et al., 2001). In seedlings that underwent the photoperiod shift, the
307 phase of *CCR2::LUC* was initially between the phase of *CCR2::LUC* in either constant SD or constant
308 LD seedlings (Figure 4A). However, by the second day post-shift, the phase of the *CCR2::LUC*
309 reporter became aligned with seedlings that were grown under constant LD. As hypocotyl
310 elongation displays memory of the photoperiod information for at least four days post-shift
311 (Figure 2-3), it is unlikely photoperiodic information stored by the circadian clock can sufficiently
312 explain the developmental responses we have observed.
313
314 Although it is unlikely that the circadian clock provides photoperiodic memory, we were
315 interested in understanding the heterogeneity of the response to the SD-LD shift. To investigate
316 this, we visualized the individual responses of each plant that underwent the shift using a
317 functional Principle Component Analysis (PCA). We included three timepoints for each individual
318 plant measured from the day before the shift, the day of the shift, and the day after the shift. Each
319 point represents the shape of the circadian rhythm across the respective day. This plot can be
320 interpreted in the same way as a traditional PCA plot, so days with similarly phased circadian
321 rhythms should group together. We found that there were individual plants that had rhythms that
322 resembled those under LD prior to the SD to LD shift and conversely there were individual plants
323 that had rhythms resembling SD plants two days after the shift (Figure 4B). However, this was a
324 very small proportion of the total plants evaluated. Therefore, the re-adjustment of the circadian
325 phase following the photoperiod shift is robust and uniform. In summary, hypocotyl
326 photoperiodic memory is unlikely to be directly controlled by the circadian clock.
327
328 **Other Brassicaceae display strong photoperiodic memory.**
329 Next, we investigated whether photoperiodic memory was present in other plants. Here, we
330 focused on microgreens that are grown in vertical farms as the hypocotyl is the primary
331 commercial product (Filatov and Olonin, 2023). Microgreens grown under LD photoperiods have
332 enhanced nutritional quality, but the longer photoperiods are associated with higher operational
333 energy costs. Alongside reducing energy consumption, shorter photoperiods will also induce
334 hypocotyl elongation, increasing the quantity of yield. We hypothesized that photoperiodic
335 memory may enable us to combine the benefit of growth under both photoperiods. To investigate
336 this possibility, we focused on kale and radish crops that are grown as microgreens within vertical

337 farms. As with Arabidopsis, kale and radish displayed evidence of photoperiodic memory (Figure
338 4C, Supplementary Figure 6). However, all three cultivars that were tested displayed stronger
339 photoperiodic memory than Arabidopsis. Seedlings shifted from SD-LD had a PMI closer to those
340 grown under constant SD, while those shifted from LD-SD had a PMI similar to those grown under
341 constant LD. Moreover, the plants that experienced a SD to LD shift were also less etiolated and
342 had greener coloration, which is a commercially advantageous trait (Supplementary Figure 6).
343 These results suggest that photoperiod shifts may have immediate uses in optimizing growth
344 conditions in vertical farms.
345
346 **Discussion**
347 Sensing and responding to photoperiodic changes are essential for plants to adapt to seasonal
348 progression. Hypocotyl elongation is strongly controlled by the photoperiod (Nozue et al., 2007;
349 Niwa, Yamashino and Mizuno, 2009), but it has remained unclear how changes in the photoperiod
350 impacted hypocotyl development. Furthermore, it is unknown if plants store information about
351 prior photoperiodic exposure. Here, we have found that hypocotyl development in different
352 Brassicaceae species displays clear evidence of a photoperiodic memory (Figures 2, 3). Our data
353 directly supports a memory response as there was minimal difference in Arabidopsis hypocotyl
354 length at day 3 between those grown under a SD or LD photoperiod (Figure 1). If there was no
355 memory of the prior photoperiod, then those shifted from SD-LD and vice-versa should closely
356 replicate growth from those under the final photoperiod. However, we did not observe this for
357 any experiment we have carried out. The memory of the initial photoperiod persisted for at least
358 four days in Arabidopsis (Figures 2, 3) and two days in microgreen crops (Figure 4). Different
359 Arabidopsis ecotypes displayed unique sensitivities to the photoperiod shift, but this did not
360 clearly correlate with latitude (Figure 2, Supplementary Table 1). One critical factor in the
361 memory response was the age of the plant when it experienced the photoperiod shift. For
362 instance, shifting germinating Ws-2 seeds from LD-SD resulted in greater hypocotyl elongation
363 relative to those grown under constant SD (Figure 3), a behavior that was not seen when
364 seedlings underwent a similar photoperiod transition (Figure 2). For the SD-LD shift, germinating
365 seeds had a poor memory of the SD-LD compared to seedlings that underwent the same transition
366 (Figures 2, 3). These results highlight how a plant's response to environmental stimuli needs to
367 be contextualized by the developmental stage.
368
369 In the natural environment, seasonal progression results in daylengths changing in the order of
370 minutes, rather than hours. Therefore, it is not clear why plants would need a mechanism for
371 responding to the kind of perturbations we have used in our assay. In natural environments,
372 plants may experience temporary dusk or dawn light disturbances caused by physical obstacles

373 or weather events, which may artificially shorten the day. Plants need a way to distinguish
374 between these kinds of short-term photoperiod fluctuations and the actual photoperiod
375 fluctuations resulting from seasonal progression. In this way, photoperiodic memory may
376 increase robustness to noisy environmental signals. It will be important to understand the effect
377 of smaller photoperiodic shifts and the effect of transient rather than permanent shortening and
378 lengthening of the photoperiod on hypocotyl elongation.
379
380 For hypocotyl elongation to be affected by the prior experience of photoperiod exposure, there
381 needs to be an internal mechanism by which information about prior photoperiod exposure is
382 encoded. The circadian clock has an essential role for photoperiodic responses, mediating
383 photoperiod regulation of flowering time (Song et al., 2015) and metabolic growth (Wang et al.,
384 2024). Similarly, the circadian clock has been established as a key regulator of photoperiodic
385 regulation of hypocotyl elongation (Niwa, Yamashino and Mizuno, 2009) and is active in imbibed
386 seeds (Zhong et al., 1998). This led us to hypothesize that the circadian clock may function as a
387 repository for photoperiodic information. However, we found that the circadian clock quickly re-
388 adjusted to the new photoperiod within two days of being transferred and these changes were
389 largely consistent across the population (Figure 4). As photoperiodic memory persisted for at
390 least four days in Arabidopsis seedlings (Figure 2) and seeds (Figure 3), it is unlikely that
391 photoperiodic information is encoded within transcriptional circadian rhythms. Therefore, other
392 mechanisms must exist to store past photoperiodic information to control hypocotyl elongations.
393
394 A variety of mechanisms have been described in the literature to explain short-term and long-
395 term memory of environmental stimuli (Murcia et al., 2022; Kambona et al., 2023; Auge et al.,
396 2023). This includes a recent example of how hypocotyl development is controlled by memory of
397 temperature exposure (Murcia et al., 2022). Here, changes in the subcellular localisation of ELF3
398 function as a device to store a memory of earlier temperature exposure. We have shown that ELF3
399 is required for photoperiodic memory and is particularly important in regulating the immediate
400 response to both photoperiod shifts (Figures 2, 3). ELF3 can form nuclear bodies at ambient
401 temperatures (Herrero et al., 2012; Anwer et al., 2014) and these are regulated by light,
402 temperature and time (Jung et al., 2020; Ronald, Wilkinson and Davis, 2021; Ronald et al., 2022;
403 Murcia et al., 2022). It will be interesting to investigate whether ELF3 localisation changes under
404 different photoperiods and the role for phyB within this. Additionally, it will be important to
405 further expand our understanding of the genetic pathway responsible for the long-term
406 adaptation to a new photoperiod. Here, phyB seems to have a critical role, with *phyB* mutants
407 displaying reduced growth following either the LD-SD or SD-LD shift (Figure 2). Phytochromes
408 are required for de-etiolation and the switch to photoautotrophic growth in seedlings (Li et al.,

2011) and regulate carbon resource allocation in adult plants (Yang et al., 2016). Thus, it will be important to understand the metabolic response of WT and *phyB* mutants to photoperiodic shifts.

Concluding statement

In summary, our work has uncovered a previously unknown mechanism of photoperiodic memory that controls hypocotyl development. Distinct pathways seem to operate to control the response to different directions of photoperiodic shifts, and the impact of the memory is dependent on the developmental age of the plant. The genetic pathway that regulates photoperiodic memory is unclear, but it seems to occur independently of transcriptional circadian rhythms. Photoperiodic memory may have immediate direct agricultural implications by allowing the optimization of nutritional quality, yield, and operational costs of vertical farms. Future work can explore whether photoperiodic memory persists in non-Brassicaceae species to understand the wider potential benefits of photoperiodic memory in other commercial applications.

Figures

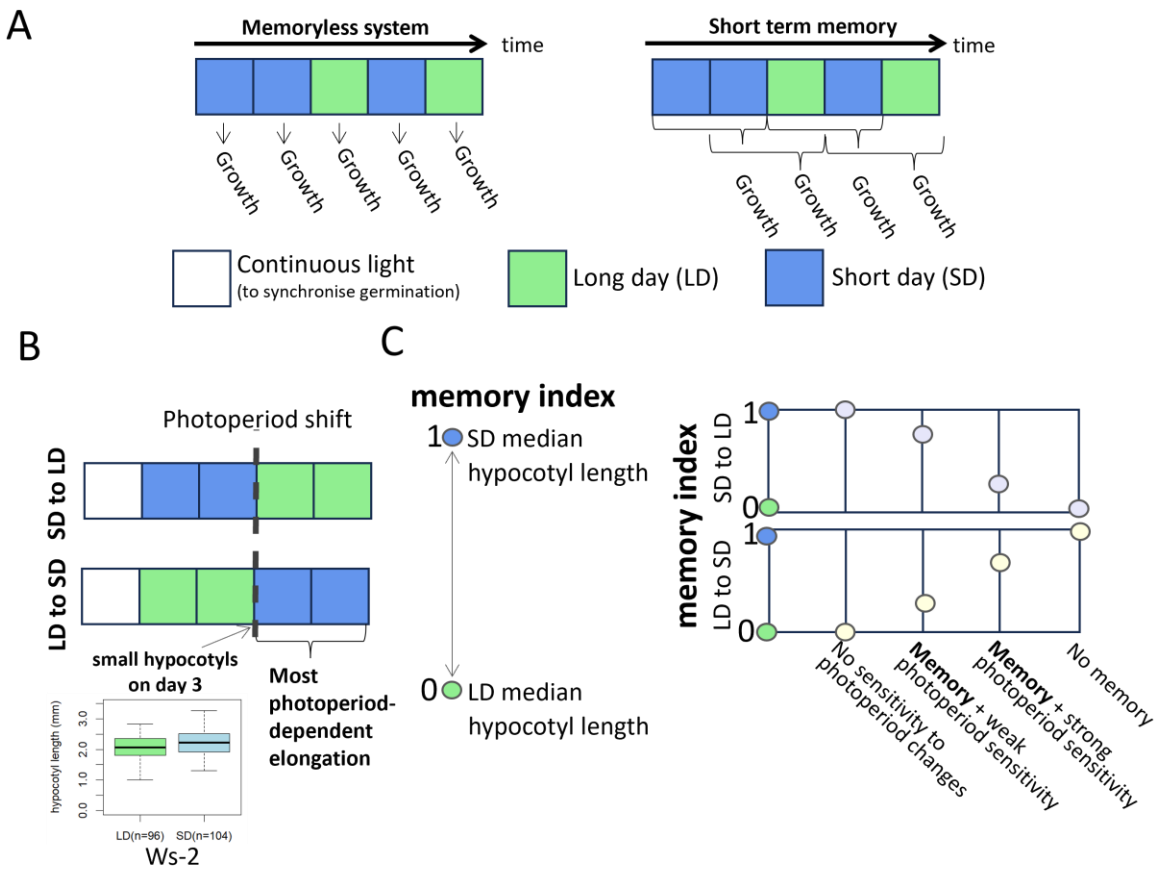
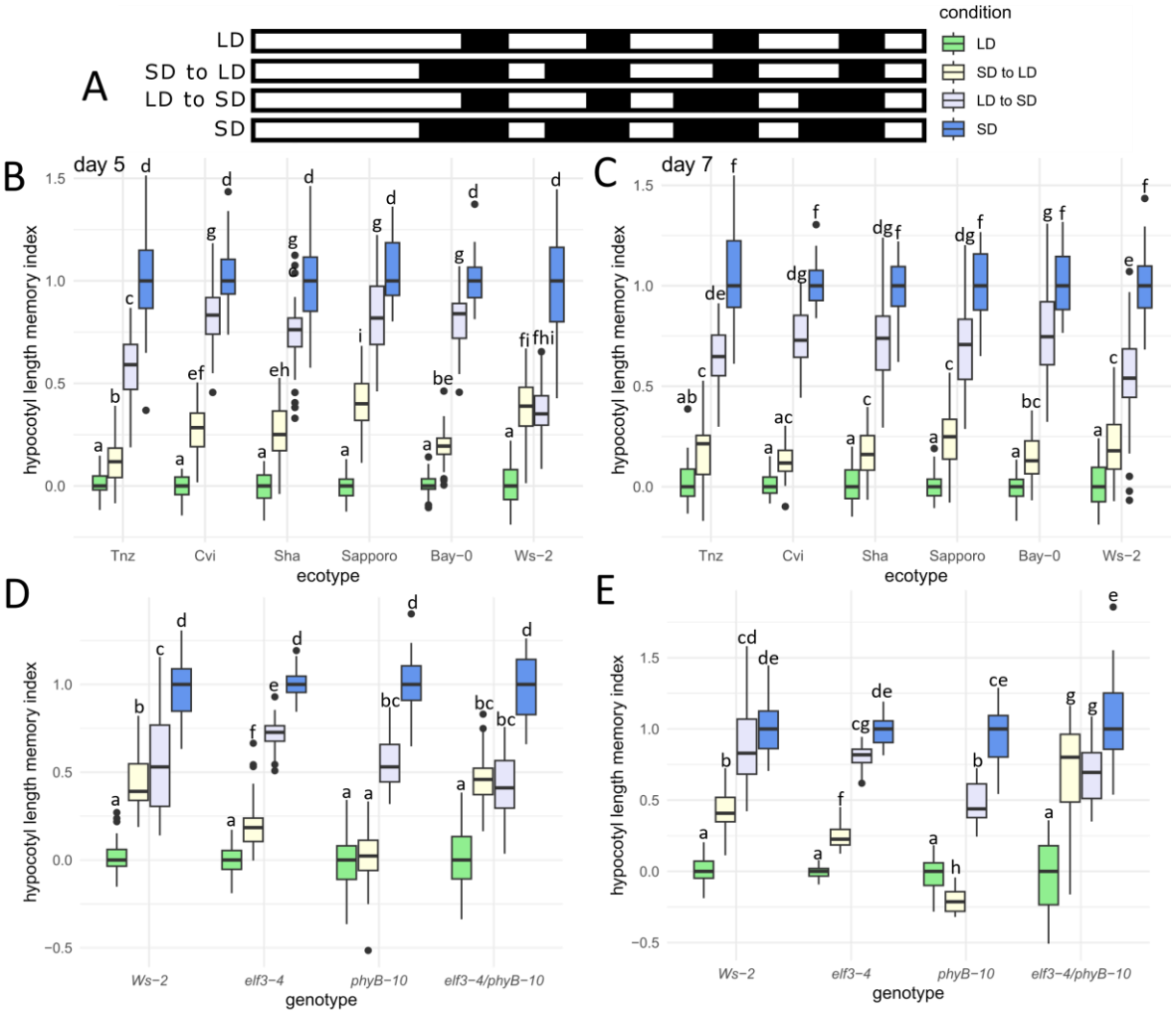


Figure 1 – Designing an assay to test for photoperiodic memory. (A) In the context of this manuscript, we define a 'memory-less system' as one whose growth only depends on the current

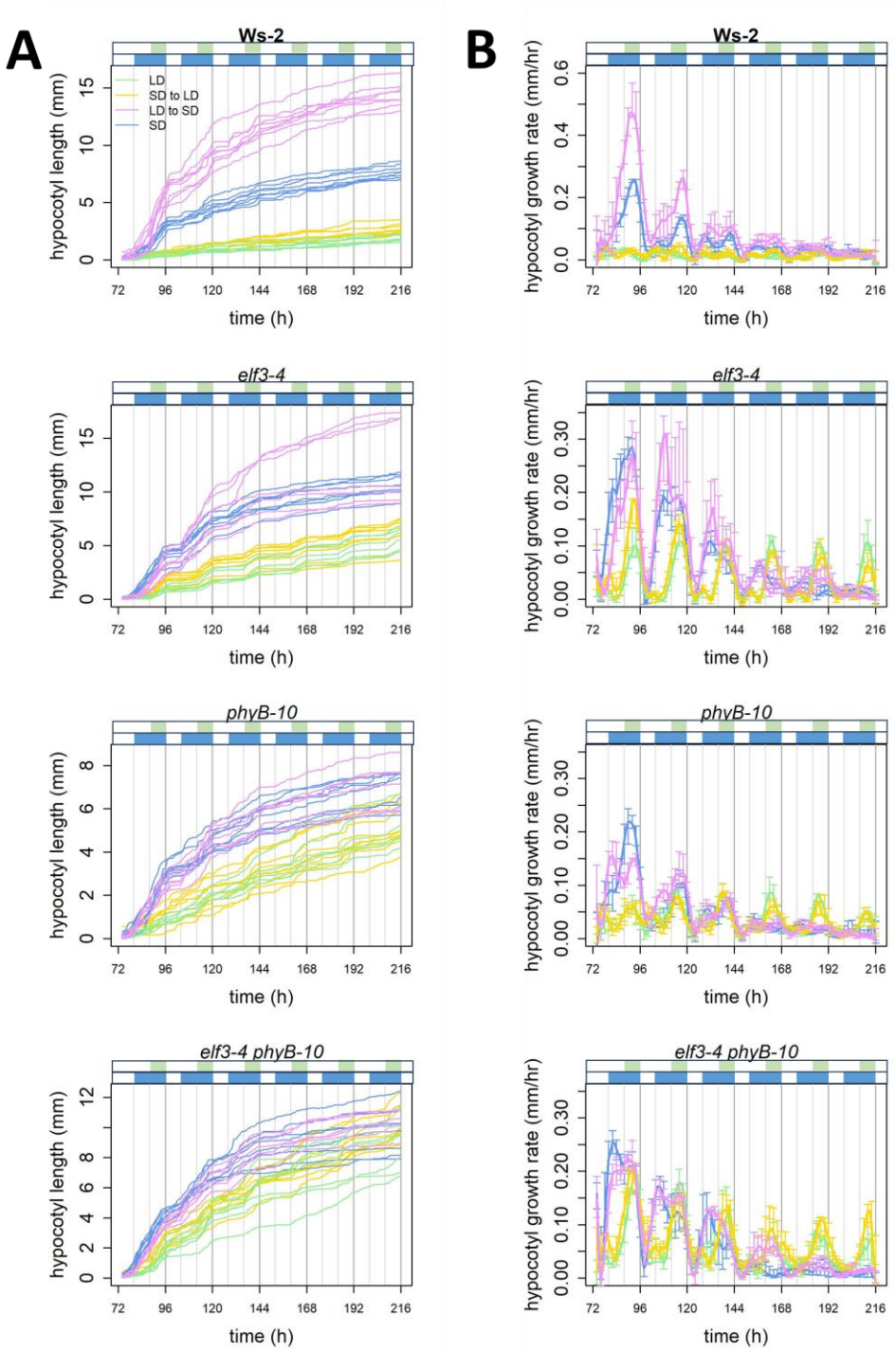
428 photoperiod. Conversely, 'memory' refers to growth that also depends on prior environmental
429 conditions. (B) Hypocotyl growth in Arabidopsis seedlings occurs predominantly during the
430 period after the photoperiod shift has occurred. (C) A hypocotyl memory index was used to
431 compare photoperiodic memory and the sensitivity to photoperiodic shifts between ecotypes and
432 mutants that have large magnitudes differences in hypocotyl elongation. Hypocotyl lengths were
433 scaled so that the median hypocotyl length at LD (16/8hr light/dark) is 0 and the median
434 hypocotyl length at SD (8hr/16hr light/dark) is 1.



435

436 **Figure 2 –** Hypocotyl development in Arabidopsis displays evidence of photoperiodic memory.
437 (A) Diagram explaining the four different photoperiodic growth conditions, the day phase is
438 white, and the night phase is black. Different Arabidopsis wild-type ecotypes were either
439 measured (B) two or (C) four days after the respective photoperiod shift or were kept under the
440 same photoperiod for the duration of the experiment. Photoperiodic memory response in *elf3-4*,
441 *phyB-10* and *elf3-4/phyB-10* mutants after either (D) two days or (E) four days following the
442 photoperiod shift. Letters signify a significant adjusted p-value of $p < 0.01$, determined by
443 TukeyHSD.

444



445

446 **Figure 3 –** Photoperiodic memory response is dependent on the developmental age of the plant.
447 (A) Hypocotyl length in Arabidopsis Ws-2 wild type, *elf3-4*, *phyB-10* and *elf3-4/phyB-10* seedlings
448 under different photoperiodic conditions across time. (B) Hypocotyl growth rate (mm/hr) in the
449 respective genotypes. Highlighted above the plots are the respective photoperiods, long-days in
450 green and short-days in blue. The color boxes highlight the dark phase under the respective
451 photoperiod. All measurements were taken after the photoperiod shift had occurred at ZT72.
452 Note that the seeds germinated later in this experiment than in the experiment depicted in Figure
453 2, with hypocotyl emergence occurring primarily on day 3.

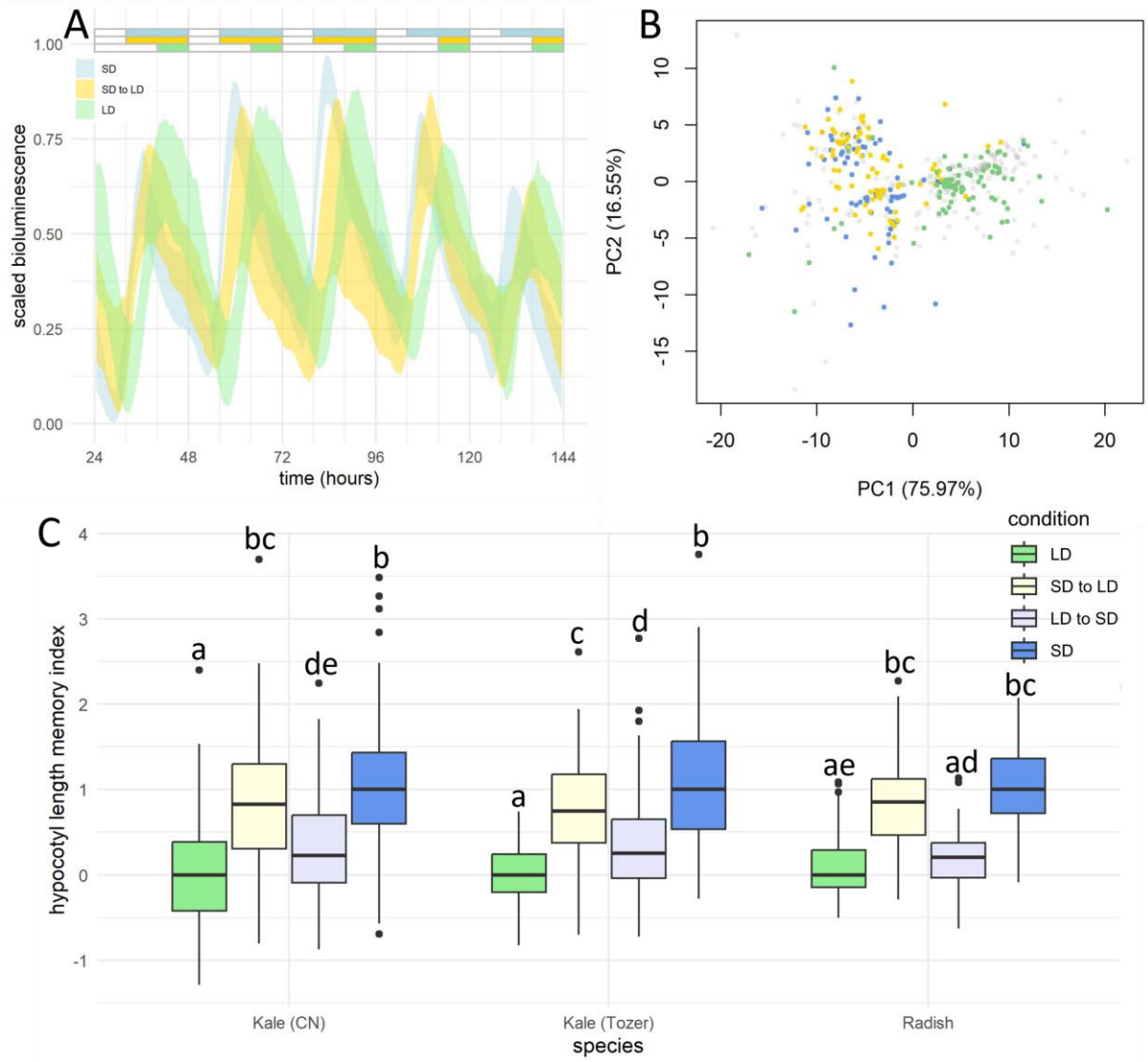


Figure 4 – The circadian clock is unlikely to store photoperiodic memory in Arabidopsis**.** (A) Waveform of *CCR2::LUC* bioluminescence in Ws-2 wild-type seedlings grown under continuous short-day (blue) or long-day (green) or in seedlings shifted from short-day to long-day (yellow). (B) A functional PCA (fPCA) plot, where each point represents a different bioluminescence curve for an individual plant, color-coded by their photoperiod condition that plant was exposed to, using a color-scheme consistent with (A). fPCA is interpreted in the same way as a standard PCA, as a projection of data (in this case, coefficients of a complex B-spline function) to a lower dimensional space. (C) Kale (CN seeds), Kale (Tozer seeds) and radish plants used in microgreen agriculture displayed evidence of photoperiodic memory. Letters signify a significant adjusted p-value of $p < 0.01$, determined by TukeyHSD.

Supplementary Figure 1 – Representative scans of seedlings from wild-type Arabidopsis ecotypes used in this work. Images are from day 5, two days after the photoperiod shift. Scale bar is 5 mm.

Supplementary Figure 2 – Hypocotyl length (mm) of wild-type ecotypes sorted by total light exposure (hours) on day 5. Dark blue is constant short-day, purple are seedlings shifted from long-day-short-day, yellow is seedlings shifted from long-day to short-day and green is constant long-day.

Supplementary Figure 3 - Hypocotyl length (mm) of wild-type ecotypes sorted by total light exposure (hours) on day 7. Dark blue is constant short-day, purple are seedlings shifted from long-day to short-day, yellow is seedlings shifted from long-day to short-day and green is constant long-day.

Supplementary Figure 4 – Representative scans of seedlings from wild-type Arabidopsis Ws-2, *elf3-4*, *phyB-10* and *elf3-4/phyB-10.* Images are from day 5, the first measurement point. Scale bar is 5 mm.

Supplementary Figure 5 - Hypocotyl length (mm) of wild-type Ws-2, *elf3-4*, *phyB-10* and *elf3-4/phyB-10* sorted by total light exposure (hours) on (A) day 5 and (B) day 7. Dark blue is constant short-day, purple are seedlings shifted from long-day-short-day, yellow is seedlings shifted from long-day to short-day and green is constant long-day.

Supplementary Figure 6 - Representative scans of microgreen seedlings. Microgreens are approximately 11 days old at the end of the experiment Scale bar is 15 mm.

Supplementary File – Raw data and statistical tests associated with the figures of this manuscript.

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Conflicts of Interest

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Supplementary Table 1 – Arabidopsis ecotypes used in this work.

Ecotype	Latitude	Longitude	Country
Wassilewskija-2 (Ws-2)	52.3	30.0	Belarus
Tanzania (Tnz)	-2.9	36.2	Tanzania
Bay-0	49.0	11.0	Germany
Shakdara (Sha)	38.4	68.5	Tajikistan
Cape Verde Island (Cvi)	15.1	-23.6	Cape Verde Island
Sapporo (Sap)	43.1	141.3	Japan

Supplementary Table 2 – Microgreen cultivars used in this work

Microgreen	Species	Variety
Kale (Tozer Seeds)	*Brassica oleracea*	Dwarf Green Curled
Kale (CN Seeds)	*Brassica oleracea*	Dwarf Blue
Radish	*Raphanus raphanistrum*	Sangrin

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