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The evening complex is central to the difference between the circadian clocks of *Arabidopsis thaliana* shoots and roots

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The circadian clock regulates the timing of many aspects of plant physiology, and this requires entrainment of the clock to the prevailing day:night cycle. Different plant cells and tissues can oscillate with different free-running periods, so coordination of timing across the plant is crucial. Previous work showed that a major difference between the clock in mature shoots and roots involves light inputs. The objective of this work was to define, in *Arabidopsis thaliana*, the operation of the root clock in more detail, and in particular how it responds to light quality. Luciferase imaging was used to study the shoot and root clocks in several null mutants of clock components and in lines with aberrant expression of phytochromes. Mutations in each of the components of the evening complex (EARLY FLOWERING 3 and 4, and LUX ARRHYTHMO) were found to have specific effects on roots, by affecting either rhythmicity or period and its response to light quality. The data suggest that the evening complex is a key part of the light input mechanism that differs between shoots and roots and show that roots sense red light via phytochrome B.

Introduction

Circadian clocks have evolved in many organisms in response to the daily rotation of the earth and the resulting light:dark (LD) cycle. They drive rhythms at the molecular and cellular levels, regulate the timing of many aspects of physiology and behaviour and thus provide a fitness benefit (Green et al. 2002, Dodd and Salathia 2005). About one-third of the Arabidopsis genome is under circadian regulation (Michael and McClung 2003, Covington et al. 2008). The circadian clock can influence plant processes at multiple levels, from cell division to interaction with the environment (Fung-Uceda et al. 2018, Hubbard et al. 2018); at the physiological level it can control such processes as photosynthesis, leaf movement, hormone responses, stem extension and stomatal opening (McClung 2006, Harmer 2009, Pruneda-Paz and Kay 2010, Greenham and McClung 2015).

In *Arabidopsis thaliana* (Arabidopsis), the core circadian oscillator includes multiple interlocking feedback loops of gene expression, modulated by post-translational control at several levels (Harmer 2009, Nohales and Kay 2016, McClung 2019). The first loop to be discovered comprised the morning-expressed MYB transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and the evening-phased transcriptional repressor PSEUDO-RESPONSE REGULATOR 1 (PRR1, also known as TIMING OF CAB EXPRESSION 1, TOC1). Other key players include the day-phased transcriptional repressors PRR9 and PRR7, and the evening-phased components EARLY FLOWERING 3 (ELF3), ELF4 and LUX ARRHYTHMO (LUX) which interact to form a transcriptional repressor named the evening complex (EC). LUX is a GARP transcription factor while ELF3 acts as a hub on which the EC is assembled (Huang and Nusinow

Abbreviations – DD, constant dark; EC, evening complex; LD, light:dark; LL, constant light.

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2016). The EC both integrates multiple signal inputs to the clock and regulates multiple outputs from it. For example, ELF3 interacts with light-sensing components such as phytochrome B (PHYB), CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and PHYTOCHROME INTERACTING FACTOR 4 (PIF4). Chromatin immunoprecipitation sequencing has defined numerous EC-binding genes (Huang et al. 2016, Huang and Nusinow 2016, Ezer et al. 2017). Mutations in EC components are typically arrhythmic (Huang and Nusinow 2016, Nohales and Kay 2016).

Evidence for tissue specificity in the circadian clock predates any knowledge about the molecular basis of the clock (Hennessey and Field 1991). Recent work has provided ample evidence that different parts of a plant can oscillate with different free-running periods (Endo 2016). This could result from differences in the wiring of the clock network and/or in the sensitivity of the clock to environmental inputs. For example, roots have a longer free-running period than shoots owing to differences in light inputs and the presence of metabolic sugars (James et al. 2008, Bordage et al. 2016, Nimmo 2018, Greenwood et al. 2019). At higher resolution, cells in the root tip have a shorter period than cells in the rest of the tissue (Gould et al. 2018). Correspondingly, in seedlings under LD cycles there are differences in clock phase between different organs, with cotyledons and hypocotyls peaking before roots, apart from the root tips (Greenwood et al. 2019). Such observations raise the question of how timing is coordinated across the whole plant. Greenwood et al. (2019) have shown that organ-specific inputs and local cell–cell coupling can drive spatial waves of clock gene expression and account for global coordination of the clock across the plant. In addition, long distance signals and light piping may contribute to coordination (Takahashi et al. 2015, Nimmo 2018).

We have already shown in Arabidopsis that, at the level of whole organs, the main difference between the shoot and root clocks involves light inputs (Bordage et al. 2016). Given the fundamental importance of entrainment and of coordination of the clock across the plant it is clearly important to analyse the properties of the root clock in more detail. Here we have used the ability to image mature shoots and roots separately (Bordage et al. 2016) to study the effects of mutations in the evening complex and photoreceptors on the root clock. The data show that mutations in evening complex components can affect the shoot and root clocks in different ways and therefore implicate these components in the generation of root-specific properties of the clock.

Materials and methods

Plant material and growth

Seeds of *Arabidopsis thaliana* mutant *elf4-1* expressing CCA1:LUC (McWatters et al. 2007) and the double mutant *cca1-11 lhy-21* (Locke et al. 2005) expressing GI:LUC (both in the Ws background) were obtained from the Nottingham Arabidopsis Stock Centre (stock numbers N2107356 and N2107367, respectively). The *phyA-211* line expressing CCA1:LUC, *elf3-2* expressing CCA1:LUC (both in Col-0) and L*er* expressing the constitutively active phyB (YHB) have already been described (Jones et al. 2015, Huang et al. 2016, Battle and Jones 2020). *lux-4* expressing CCA1:LUC was generated by crossing *lux-4* CAB:LUC (Hazen et al. 2005) to a Col-0 background, screening for F2 seedlings with elongated hypocotyls and loss of the CAB:LUC reporter, then crossing to CCA1:LUC (Pruneda-Paz et al. 2009), and screening for long hypocotyl and bioluminescent *lux-4* CCA1:LUC seedlings. All EC mutants showed the expected long hypocotyl phenotype.

All seeds were surface-sterilised, stratified for 4 days at 4°C and sown on 1.2% agar in 0.5 strength Murashige and Skoog (MS) medium (Sigma-Aldrich) adjusted to pH 5.7 in 120 mm square vertical plates which were exposed to LD cycles (12 h white light provided by fluorescent tubes, 100 µmol m^{-2} s^{-1}, 12 h dark) at 20°C. Ten days after sowing, seedlings (two clusters of three plants per plate) were transferred to fresh plates in which the top 3 cm of agar had been replaced with 1.8% agar and 2% charcoal in 0.5 strength MS medium, readjusted to pH 5.7 after addition of charcoal. After a further 11 days, plants were sprayed with luciferin and the plates were sealed with new lids containing a black barrier which separate the shoot and root compartments and prevent cross-contamination of their signals (Bordage et al. 2016).

Luciferase imaging

Plants (3 weeks old) were sprayed with 60 mM D-Luciferin in 0.01% triton (300 µl per plate). Plates were kept at 20°C and illuminated by blue and/or red light provided by LEDs (Luxeon Star 447 nm and 627 nm, respectively) at 20 µmol m^{-2} s^{-1} unless stated otherwise. Bioluminescence was usually detected using a Photek 225/18 Intensified CCD camera with a 16 mm lens. The camera, LEDs and covering system were controlled using Photek IFS32 software. Images (15 min) were recorded every 1.5 h in photon counting mode, without any filters. Root and shoot regions were defined and luminescence data extracted using Photek IFS32 software. In some experiments, bioluminescence was detected with a

Photometrics Evolve 512 EMCCD camera; data was extracted using Image-Pro Plus software and a 3x3 median filter was applied to overcome background from cosmic rays (Greenwood et al. 2019). In both cases the luminescence for each time-point was normalised to the average luminescence over the corresponding time-course. Control experiments showed that the results from the two cameras after normalisation were essentially identical.

Data analysis

Normalised time-courses from imaging were analysed using Biological Rhythm Analysis Software System (BRASS; www.amillar.org), using the data from 24–96 h in constant conditions. Period and relative amplitude error (RAE) were analysed using the FFT-NLLS suite of programs. Differences in period were assessed by Student's *t*-test. The RAE is the ratio of the amplitude error to the most probable amplitude. RAE gives an indication of rhythm robustness: values close to 0 and 1 indicate robust and weak (if any) rhythms, respectively.

Results

The EC integrates multiple signals that communicate timing to the circadian clock (Huang and Nusinow 2016) and may therefore contribute to differences between the shoot and root clocks. To investigate this, rhythms in the shoots and roots of several EC null mutants were examined. *elf3* mutant seedlings have been reported to be arrhythmic in constant light (LL; Hicks et al. 1996, McWatters et al. 2000, Thines and Harmon 2010). The data in Fig. 1 compare luminescence traces from Col-0 and *elf3-2* plants over one light:dark cycle followed by constant light, for red (Fig. 1A,B) and blue (Fig. 1C,D) light. The data show that mature *elf3-2* roots retain rhythmicity in both constant red and constant blue light. In contrast mature *elf3-2* shoots are either very weakly rhythmic (with RAE > 0.5 and period estimates ranging from 19 to 35 h) or arrhythmic. Further analysis of *elf3-2* roots showed that this mutation shortens the period of the root clock and abolishes the red/blue difference in root period (Fig. 1E, Table S1). The low rhythmicity of *elf3-2* shoots was also observed in red + blue light, where plots of period against RAE show tight clustering of the data for Col-0 shoots and roots, and *elf3-2* roots, but widely scattered data for *elf3-2* shoots (Fig. S1). To assess the clock of the *elf3-2* mutant in a different way plants were shifted from 12 h:12 h light/dark cycles to 6 h:6 h cycles. If the LD cycle is close to half of the circadian period, plants with strong clocks will entrain to every second cycle (termed frequency demultiplication), whereas arrhythmic plants will show rhythms driven by the new photocycle (Nozue et al. 2007, Thines and Harmon 2010, Kolmos et al. 2011). Fig. S2 shows that under these conditions wild-type shoots and roots, and *elf3-2* roots, exhibit alternating strong and weak peaks of luminescence every 24 h, whereas *elf3-2* shoots immediately become driven by the new photocycle, confirming the lack of a functional clock in *elf3-2* shoots.

These unexpected results with *elf3-2* prompted an investigation of mutants in the other components of the evening complex. *elf4* and *lux* null mutant seedlings have also been reported to be arrhythmic in LL. The data in Fig. 2 panels A-D show luminescence traces of the shoots and roots of the Arabidopsis wild-type ecotype Ws and *elf4-1* plants; Fig. 2 E,F are plots of RAE against period for Ws and *elf4-1*. These results demonstrate that *elf4-1* shoots and roots retain rhythmicity in either red or blue light. *elf4-1* is a short period mutation and, like *elf3-2*, it abolishes the red/blue difference in root period (Table S2). Fig. S3 and Table S3 show that *elf4-1* also has a short period in red + blue light. The data in Fig. 3, comparing *lux-4* with Col-0 in red + blue light, show that *lux-4* shoots have a short period while *lux-4* roots are very weakly rhythmic. For different *lux-4* root traces, period estimates ranged from 20 to 35 h. Some 60% of traces were scored arrythmic or had RAE values greater than 0.5 (Fig. 3C).

Mutations in each of the components of the evening complex thus disrupt the rhythmicity of wild-type roots, by affecting either rhythmicity or period and its response to light quality. To test whether mutations in morning-phased components had similar effects, the short period double mutant *cca1-11 lhy-21* (Mizoguchi et al. 2002, Ding et al. 2007, Lu et al. 2009) was studied. In constant red + blue light, this mutant retained rhythmicity in both shoots and roots, though *cca1-11 lhy-21* shoots were less robust (i.e. had higher RAE values) than Ws shoots. *cca1-11 lhy-21* showed the expected shortening of period in both shoots and roots relative to the Ws wild type (Fig. 4, Table S4). Fig. S4 shows luminescence traces for the *cca1-11 lhy-21* mutant in either red or blue light. This indicates that, although root rhythms are less robust in either red or blue light alone than in red + blue light, the red/blue period difference in roots (Nimmo 2018) is maintained in this mutant. Thus, mutation of both CCA1 and LHY does not appear to have any effect specific to the root clock that is not seen with the shoot clock.

The discrepancy between our detection of rhythmicity in EC mutants and earlier reports of arrythmicity could result from the inclusion of charcoal in the upper part of the agar plates used in our imaging system (see Discussion). We therefore tested several lines using either charcoal-containing or plain agar plates, maintaining the other conditions described by Bordage et al. (2016).

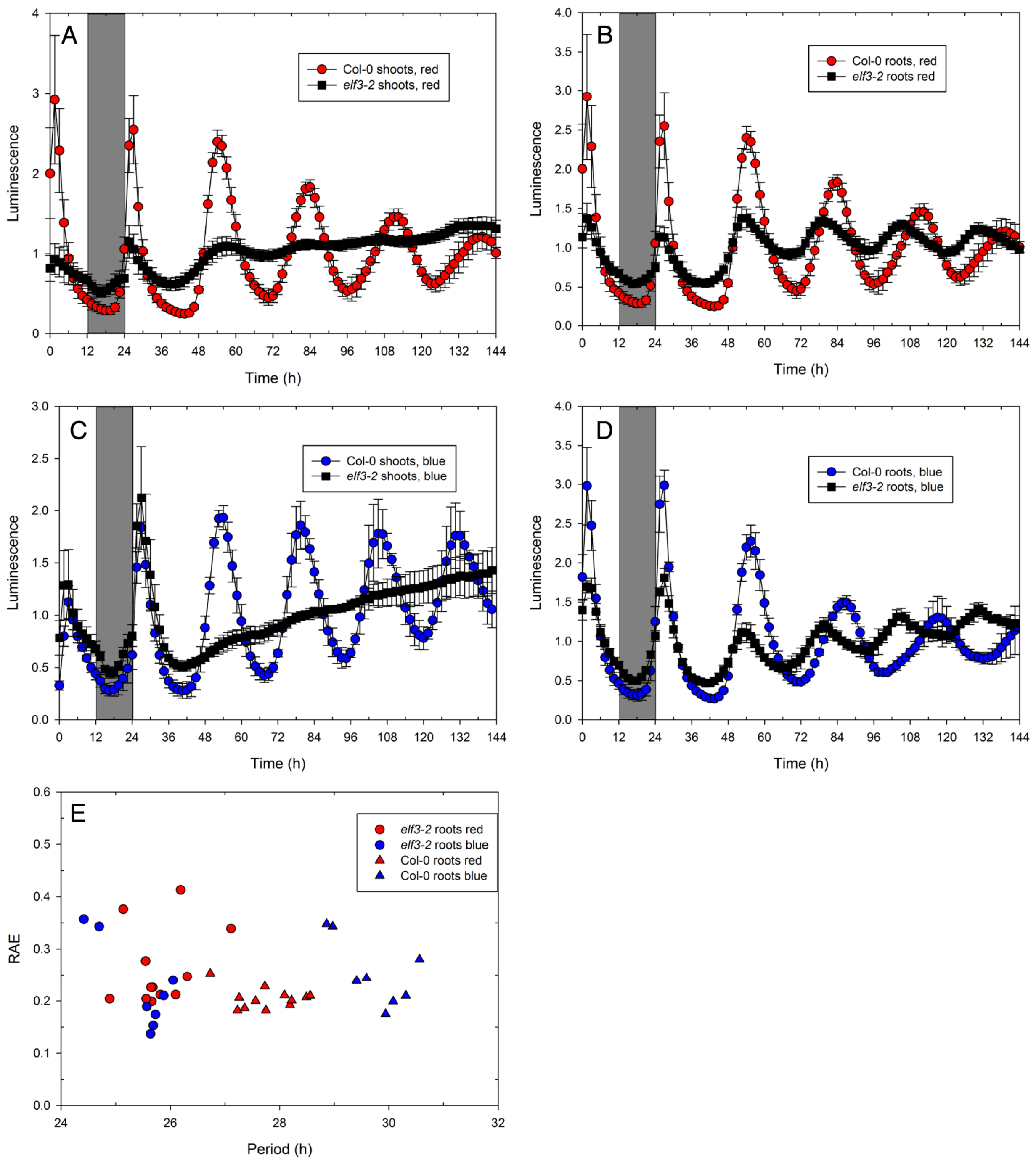

Fig. 1. *elf3-2* roots, but not shoots, retain rhythmicity. Col-0 and *elf3-2* plants expressing CCA1:LUC were imaged for 24 h in a light:dark cycle followed by 120 h in LL, in either red (A, B) or blue (C, D) blue light. Luminescence traces are means ± SD for n = 8–12 clusters of plants in three biological replicates. Panel (E) shows period and RAE values for individual traces. Mean period data are given in Table S1.

Fig. S5 shows representative luminescence time-course data from Ws and *elf4-1* shoots and roots. It is clear that the presence of charcoal has no effect on the behaviour of *elf4-1*. The luminescence of Ws roots on plain agar plates appears to run very slightly in advance of that on charcoal plates. This is probably due to slight spillover of the signal from Ws shoots which is prevented by the presence of charcoal. The signal from *elf4-1* shoots is

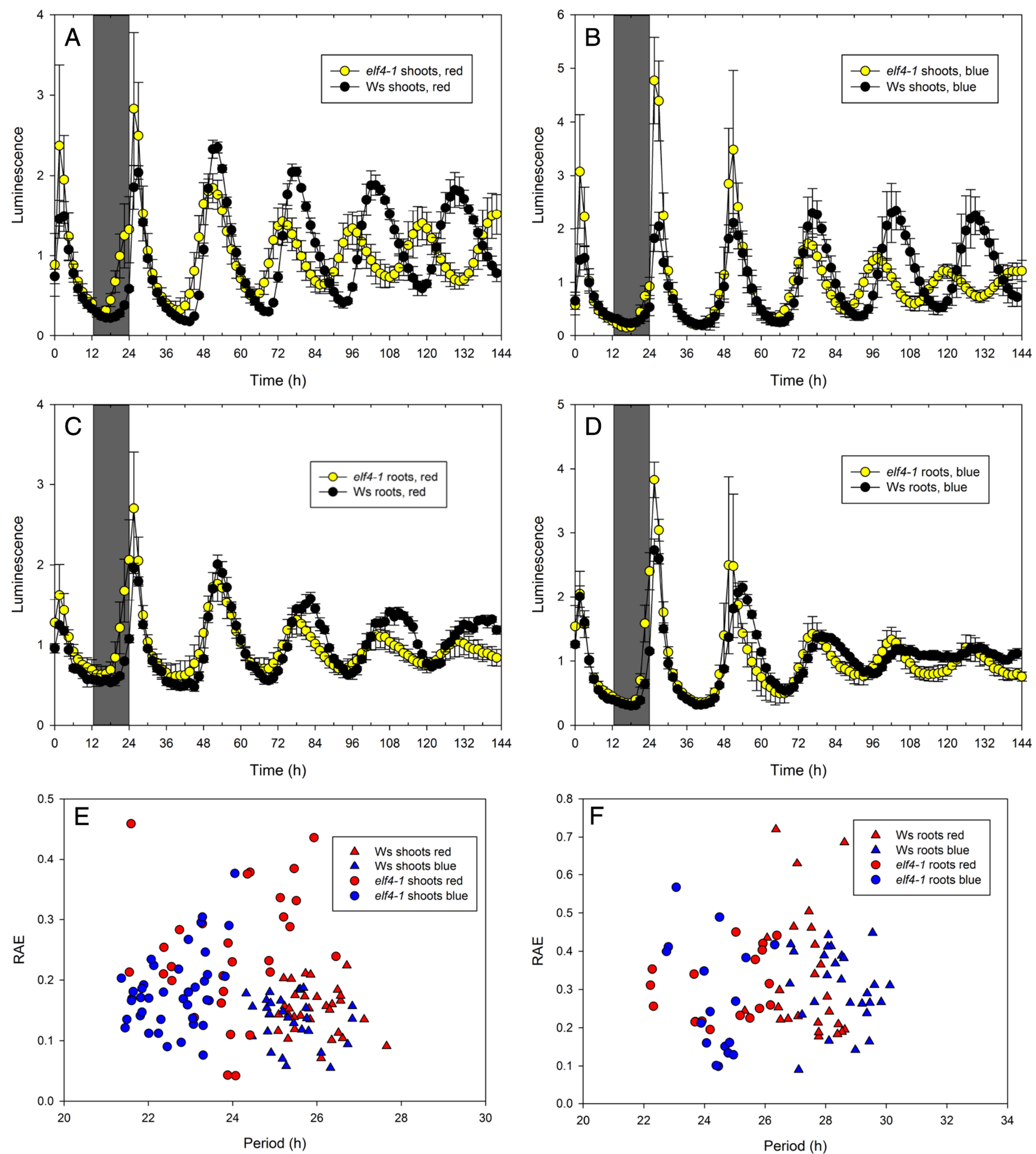

Fig. 2. *elf4-1* is a short period mutant that abolishes the period difference between shoots and roots. *elf4-1* and Ws plants expressing CCA1:LUC were imaged for 24 h in a light:dark cycle followed by 120 h in LL in either red (A, B) or blue (C, D) light. Luminescence traces are means ± SD for n = 16–26 clusters of plants in four to six biological replicates. Period and RAE values for individual traces are shown for shoots (E) and roots (F). Mean period data are given in Table S2.

appreciably lower than that from Ws shoots, so *elf4-1* root traces are not affected by spillover.

Previous work (Nimmo 2018) showed that the circadian clock in mature roots is sensitive to piped light, the red component of which is sensed by PHYA and/or PHYB. To distinguish between these phytochromes two approaches were used. First, plants that express YHB, a constitutively active form of PHYB (Jones et al. 2015),

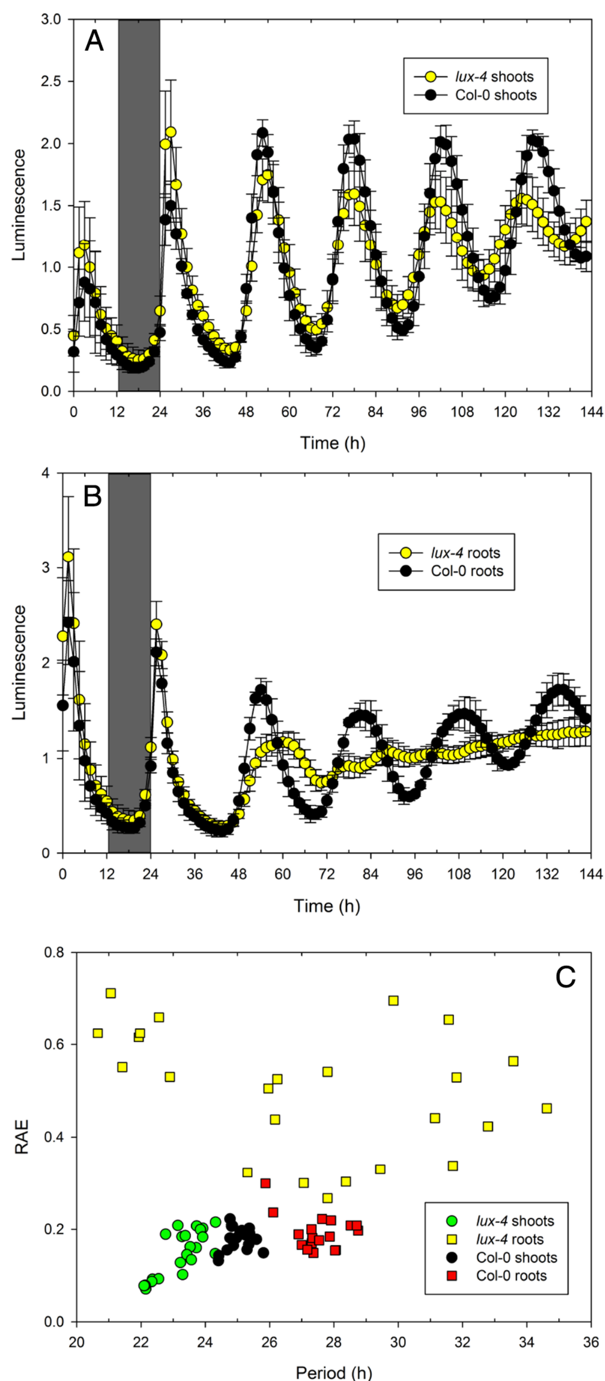

Fig. 3. Rhythmicity of *lux-4* shoots. Col-0 and *lux-4* plants expressing CCA1:LUC were imaged for 24 h in a light:dark cycle followed by 120 h in LL (red + blue light, total 25 µmol m^{-2} s^{-1}, equal intensities of each). Luminescence traces are means ± SD for n = 16–24 clusters of plants in four to six biological replicates for (A) shoots and (B) roots. Panel (C) shows period and RAE values for individual traces. The mean period for *lux-4* shoots (23.36 ± 0.08 h, n = 24) is shorter than that of Col-0 shoots (25.07 ± 0.38 h, n = 20), $P < 0.0001$, Student *t*-test.

were used. Plants were entrained to a 12 h:12 h LD cycle and then allowed to free run in constant dark (DD). Fig. 5A illustrates the effectiveness of YHB in mature shoots. In shoots of the wild-type L*er*, luminescence from CCA1:LUC loses rhythmicity very rapidly in DD, and the majority of traces are not scored rhythmic. In contrast rhythmicity in DD is maintained in the shoots of plants expressing YHB, though with lower amplitude than L*er* shoots in red LL. This control experiment, similar to the work of Jones et al. (2015) but with older plants, confirms the efficacy of YHB in mature tissue. Fig. 5B shows that, as reported for Ws by Bordage et al. (2016), mature L*er* roots maintain low amplitude rhythmicity in DD, but expression of YHB both shortens the period and increases the amplitude of root rhythmicity. The rhythm of YHB roots in DD has a period similar to that of L*er* plants in red LL, and is clearly more robust and of shorter period than Ler roots in DD (Fig. 5C). These data show that activation of PHYB is sufficient to allow roots to perceive red light. Second, the *phyA-211* mutant expressing CCA1:LUC was studied in constant red light. Like the Col-0 wild type, but unlike *phyAB* (Nimmo 2018), *phyA* exhibited robust rhythms and maintained the characteristic difference in period between shoots and roots (Fig. 5D).

Discussion

Based on early work on seedlings, mutants in each of the EC components are regarded as arrhythmic (Huang and Nusinow 2016, Nohales and Kay 2016, and references therein). However, some recently identified *elf3* mutants (*elf3-12*, *elf3-13* and *elf3-14*) maintain rhythmicity with a short period in LL (Kolmos et al. 2011, Kim and Somers 2019). In addition, *lux* mutant seedlings regain rhythmicity at lower temperatures (Jones et al. 2019) and the shoot apex tissue of *lux* mutants maintains rhythmicity (Takahashi et al. 2015). Clearly EC mutants are not necessarily arrhythmic. Nevertheless, it is surprising that the null EC mutants studied here, as mature plants on vertical plates, all retain robust rhythmicity, albeit at reduced amplitude, in either shoots (*lux-4*), roots (*elf3-2*) or both (*elf4-1*).

The work of Takahashi et al. (2015) on seedlings implies that *elf3-2* roots are arrhythmic. In contrast, the presence of a functioning clock in mature *elf3-2* roots was clearly shown here by both LL and T cycle experiments. ELF3 antagonises light input to the clock (Huang and Nusinow 2016), but the data reported here implies that this function is less important in roots than shoots, possibly because roots may perceive light through light piping (Nimmo 2018). The *lux-4* mutant showed the

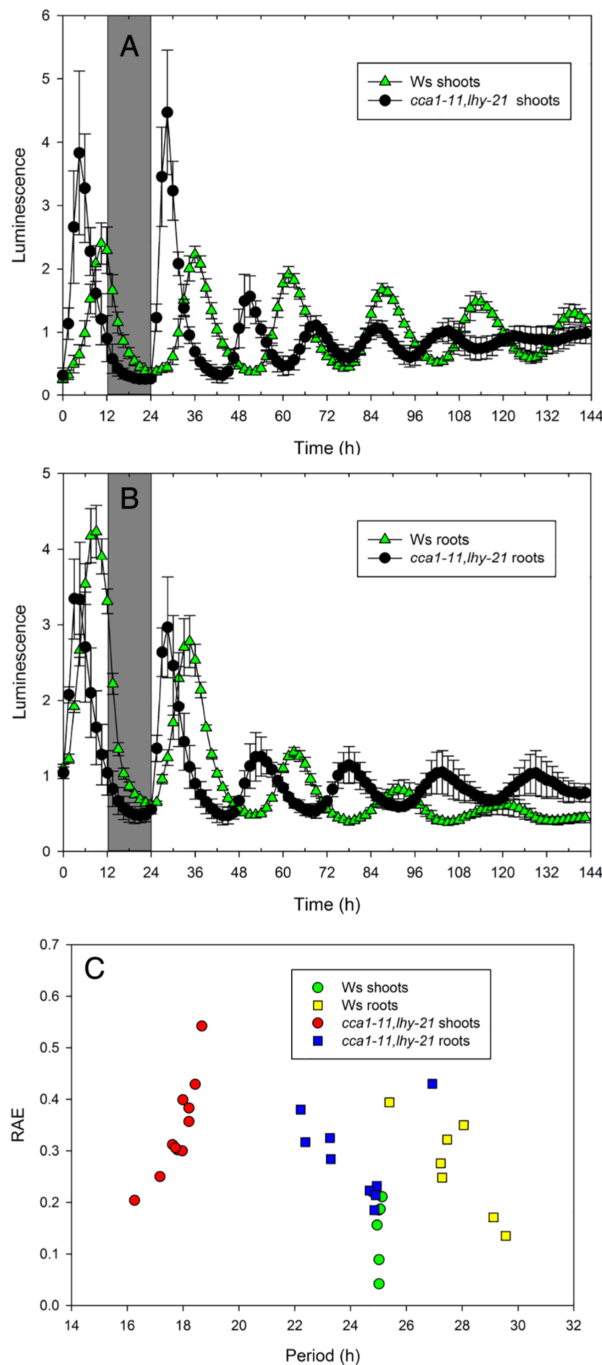

Fig. 4. Rhythmicity of *cca1-11,lhy-21* shoots and roots. Ws and *cca1-11 lhy-21* plants expressing GI:LUC were imaged for 24 h in a light:dark cycle followed by 120 h in LL (red + blue light, total 25 μmol m^{-2} s^{-1}, equal intensities of each). Luminescence traces are means ± SD for n = 6–9 clusters of plants in three biological replicates, for shoots (A) and roots (B). Panel (C) shows period and RAE values for individual traces. Mean period data are given in Table S4.

opposite behaviour to *elf3-2*, namely robust rhythmic behaviour in shoots but not roots. Arabidopsis contains a close homologue of LUX, BROTHER OF LUX ARRHYTHMO (BOA, also known as NOX); BOA and LUX are partially redundant (Huang and Nusinow 2016, Nohales and Kay 2016, McClung 2019). However, BOA is more highly expressed in roots than shoots (Dai et al. 2011), so the retention of rhythmicity in *lux-4* shoots but not roots is probably not linked to the expression of BOA. In recent work, Chen et al. (2020) reported that the roots of *elf4-1* seedlings are arrhythmic. Both this and the earlier work of Takahashi et al. (2015) involved detection of LUC activity using a luminometer. In contrast, we image mature plants on vertical plates with a photon counting camera, using several precautions to prevent cross contamination of shoot and root signals (Bordage et al. 2016). One of these precautions is the inclusion of charcoal in the upper section of the agar plates to prevent reflection of photons and cross contamination of signals. It could be argued that the presence of charcoal, which has the ability to bind to some small molecules, might affect the conclusions drawn from this method. Fig. S5 shows that this concern is not warranted. Hence our clear detection of rhythmicity in *elf4-1*, in both shoots and roots, suggests that our system may be more amenable to the detection of relatively low amplitude rhythms than previous work using seedlings. However, an alternative explanation, that the clock becomes more robust in older plants, cannot be ruled out at this stage. Low amplitude rhythmicity can be obscured if different cell types oscillate with different periods, so it would be interesting to test whether the recently developed single cell imaging system (Gould et al. 2018) allows detection of rhythmicity in EC mutants.

Bordage et al. (2016) implicated light inputs as a major cause of the difference in the properties of the shoot and root clocks. Strikingly, the data here show that mutations in each of the components of the EC affect the shoot and root clocks in different ways, whereas mutations in the dawn-phased components CCA1 and LHY do not. This suggests that the EC is a key part of the light input mechanism that differs between shoots and roots. Since the EC is known to interact with PHYB (Huang and Nusinow 2016) this is consistent with the finding that PHYB is responsible for red light input to the roots (Fig. 5). The data on EC mutants shown here also provide further evidence that the shoot and root clocks are wired differently. However, the effects of mutations in other clock components on the root clock also need to be examined. PRR7 is required for transmission of sugar signals to the clock

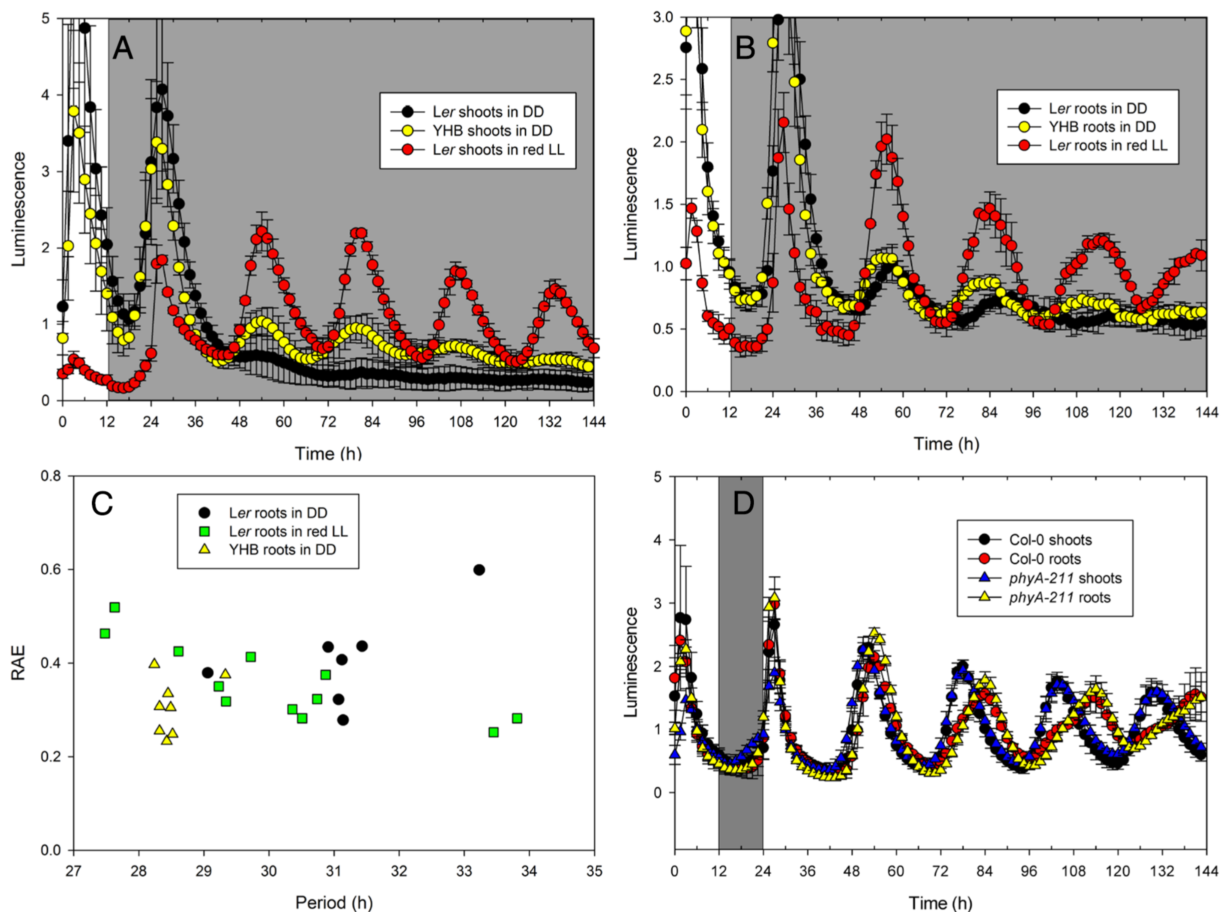

Fig. 5. Roots perceive red light via PHYB. *Ler* and YHB plants expressing CCA1:LUC were imaged for 24 h in a light:dark cycle followed by 120 h in DD. For comparison, data for *Ler* plants imaged in red LL at 25 µmol m^{-2} s^{-1} are also shown (red symbols). Luminescence traces are means ± SD for n = 8–12 clusters of plants in three biological replicates for (A) shoots and (B) roots. Panel (C) shows period and RAE values for individual root traces in DD. The mean period for YHB roots in DD (28.51 ± 0.34 h, n = 8) is shorter than that of *Ler* roots in DD (31.14 ± 1.21 h, n = 8), $P < 0.0001$ (Student *t*-test), and also shorter than that of *Ler* roots in red LL (30.59 ± 2.15 h, n = 12), $P < 0.05$. (D) Col-0 and *phyA-211* plants were imaged for 24 h in a light:dark cycle followed by 120 h in LL using red light. Data are means ± SD for n = 12 clusters of plants in three biological replicates.

(Haydon et al. 2013, Webb et al. 2019). Since sugar signalling may coordinate the shoot and root clocks (James et al. 2008) in addition to light piping (Nimmo 2018), the properties of the root clock in *prr7* mutants may prove particularly interesting.

Author contributions

H.G.N. conceived and designed the research; H.G.N and J.L. performed experiments; H.G.N. analysed data and wrote the paper; R.B generated *lux-4* CCA1:LUC lines under the direction of D.A.N. All authors read and approved the manuscript.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. *elf3-2* roots are rhythmic with a short period in red + blue light

Fig. S2. Behaviour of Col-0 and *elf3-2* plants in 6 h light:6 h dark

Fig. S3. *elf4-1* plants are rhythmic with a short period in red + blue light

Fig. S4. *cca1-11 lhy-21* is a short period mutant in red or blue light

Fig. S5. The presence of charcoal in imaging plates does not affect rhythmicity

Table S1. Circadian periods of Col-0 and *elf3-2* roots in red and blue light

Table S2. Circadian periods of Ws and *elf4-1* organs in red and blue light

Table S3. Circadian periods of Ws and *elf4-1* plants in red + blue light

Table S4. Circadian periods of Ws and *cca1-11 lhy-21* plants in red + blue light

Edited by E. Monte