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Circadian clock adjustment to plant iron status depends on chloroplast and phytochrome function

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Plant chloroplasts are not only the main cellular location for storage of elemental iron (Fe), but also the main site for Fe, which is incorporated into chlorophyll, haem and the photosynthetic machinery. How plants measure internal Fe levels is unknown. We describe here a new Fe-dependent response, a change in the period of the circadian clock. In *Arabidopsis*, the period lengthens when Fe becomes limiting, and gradually shortens as external Fe levels increase. Etiolated seedlings or light-grown plants treated with plastid translation inhibitors do not respond to changes in Fe supply, pointing to developed chloroplasts as central hubs for circadian Fe sensing. Phytochrome-deficient mutants maintain a short period even under Fe deficiency, stressing the role of early light signalling in coupling the clock to Fe responses. Further mutant and pharmacological analyses suggest that known players in plastid-to-nucleus signalling do not directly participate in Fe sensing. We propose that the sensor governing circadian Fe responses defines a new retrograde pathway that involves a plastid-encoded protein that depends on phytochromes and the functional state of chloroplasts.

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Introduction

Chloroplasts sustain most life on earth. They are the site of photosynthesis, where solar energy is converted to oxygen and chemical energy stores in the form of sugars. The advent of the green lineage dates back over one billion years ago when a single-cell eukaryote engulfed a cyanobacterium with which it formed an endosymbiotic relationship (Dyall *et al*, 2004). Since the establishment of this symbiosis, the ancestral cyanobacterial endosymbiont has undergone a severe genome reduction, with gene loss or transfer to the host nuclear genome. Most of the proteins found in modern chloroplasts are encoded in the nucleus, produced in the cytoplasm and then translocated through plastid membranes, while the remaining proteins are synthesized inside the chloroplasts. All chloroplast components are coordinately transcribed across chloroplasts and nucleus via the plastid-to-nucleus retrograde signalling pathway to ensure stoichiometric balance (Woodson and Chory, 2008). Multiple pathways monitor the functional state of chloroplasts and control the expression of photosynthesis-associated nuclear genes (PhANGs). These pathways measure signals such as reactive oxygen species and 3'-phosphoadenosine 5'-phosphate, generated by exposure to bright light (Lee *et al*, 2007; Estavillo *et al*, 2011; Kindgren *et al*, 2012); the accumulation of a signalling molecule repressing PhANGs, possibly the tetrapyrrole Mg-protoporphyrin IX, in damaged chloroplasts (Strand *et al*, 2003), as well as a positive signal in the form of haem (Woodson *et al*, 2011). Finally, plastid transcription and protein translation initiate the plastid gene expression (PGE) pathway (Woodson and Chory, 2008). All pathways are thought to converge in chloroplasts onto the master regulator GENOMES UNCOUPLED 1 (GUN1), a pentatricopeptide protein of unknown function (Koussevitzky *et al*, 2007). Activation of GUN1 leads indirectly to the proteolytic cleavage of PHD TYPE TRANSCRIPTION FACTOR WITH TRANSMEMBRANE DOMAINS (PTM), which is associated with the chloroplast envelope (Sun *et al*, 2011). Once released, the N-terminus of PTM translocates to the nucleus, where it binds to the promoter of the *ABSCISIC ACID INSENSITIVE 4* (*ABI4*) gene, a downstream target of the retrograde pathway (Koussevitzky *et al*, 2007; Sun *et al*, 2011).

Chloroplasts play a central role in plant metabolism. In addition to photosynthesis, chloroplasts are the site of starch synthesis, assimilation of nitrate and sulphate, biosynthesis of fatty acids, purines and pyrimidines, chlorophyll, haem and other tetrapyrroles, and iron-sulphur clusters as well as initiation of abscisic acid, gibberellin and oxylipin biosynthesis (Neuhaus and Emes, 2000). About half of the iron (Fe) content in *Arabidopsis* seedlings is found in the shoot (Colangelo and Guerinot, 2004; Long *et al*, 2010), and about 70–90% of total cellular Fe in leaves localizes to chloroplasts (Terry and Abadia, 1986; Shikanai *et al*, 2003), which therefore constitute a major Fe sink during plant development. The photosynthetic machinery makes abundant use of Fe. Cytochrome b_6f and PSI are particularly Fe rich, requiring 6 and 12 Fe atoms, respectively (Wollman *et al*, 1999; Vassiliev *et al*, 2001; Kurisu *et al*, 2003). Leaves of Fe-deficient plants are yellow due to low chlorophyll levels, a likely consequence of impaired protochlorophyllide biosynthesis catalysed by the Fe-requiring enzyme CRD1/CHL27 (Moseley *et al*, 2002; Tottey *et al*, 2003). Other hallmarks of Fe deficiency include slower growth rates, associated with reduced photosynthetic rates and loss of grana stacks, as well as decreased plastid transcription and translation (Price and Carell, 1964; Stocking 1975; Spiller and Terry, 1980; Spiller *et al*, 1987). Mutants impaired in Fe uptake share the symptoms of Fe-deficient plants, and die as seedlings unless watered with a Fe-chelate solution (Vert *et al*, 2002; Colangelo and Guerinot, 2004).

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Arabidopsis, as other vascular plants except the grasses, utilizes Fe uptake strategy I. It relies on rhizosphere acidification to increase the solubility of ferric iron and reduction to Fe^{2+} by the root surface ferric chelate reductase FRO2 (FERRIC REDUCTASE OXIDASE 2), before transport across the plasma membrane of root hairs by the high-affinity Fe transporter IRON-REGULATED TRANSPORTER 1 (IRT1). Plants must face the low solubility of ferric iron, severely exacerbated in calcareous soils that make up about a third of the world's arable land (Morrissey and Guerinot, 2009). Limiting bio-availability of Fe to plants therefore has two major consequences for human health: it decreases photosynthetic output and thus crop yield for human consumption, but also lowers dietary Fe intake. Indeed, at least one third of the world population suffers from Fe deficiency (WHO/UNICEF/UNU, 2001). Following uptake in roots, Fe is distributed throughout the plant by several transporters (Rogers and Guerinot, 2002; Lanquar *et al*, 2005; Kim *et al*, 2006). The control of Fe distribution throughout the plant must be coordinated with Fe uptake by the roots, and involves a long-distance signal produced in leaves that induces Fe deficiency responses in roots (Enomoto *et al*, 2007). However, the exact consequence of Fe deficiency for leaves beyond a loss of chlorophyll is not well understood.

The expression of many nucleus-encoded plastid genes is under the control of the circadian clock. The clock, which affects 30% of the *Arabidopsis* transcriptome, supports the anticipation of daily transitions such as changes in light or temperature, as well as potential pathogen attacks (Harmer, 2009; Pruneda-Paz and Kay, 2010; McClung, 2011). The importance of a properly functioning circadian clock for fitness has emerged based on results from cyanobacteria (Ouyang *et al*, 1998), *Arabidopsis* (Dodd *et al*, 2005) and mammals (Wyse *et al*, 2011; Sahar and Sassone-Corsi, 2012).

A connection between the circadian clock and cell metabolism came into focus in animals after the mammalian clock protein REV-ERBα, an orphan nuclear receptor, was found to bind haem. Haem serves as a prosthetic group in haemoglobin and many enzymes acting in respiratory metabolism; in addition, haem levels are thought to follow a circadian rhythm, as the rate-limiting enzyme, ALAS1, is under clock control (Kaasik and Lee, 2004). Genes under the control of REV-ERBα are repressed upon haem binding to the nuclear receptor; these include gluconeogenic factors that play a role in glucose production. Together, this places haem as an output of the circadian clock, but also as input for metabolism and as a central clock component via its interaction with REV-ERBα (Raghuram *et al*, 2007; Yin *et al*, 2007). Most recently, an RNAi screen in *Drosophila* revealed that genes involved in Fe metabolism were important for a functional circadian clock, while a role for haem in the fly clock was not immediately clear (Mandilaras and Missirlis, 2012).

Metabolism also influences the circadian clock of plants (Dodd *et al*, 2005; Covington *et al*, 2008; Ni *et al*, 2009; Farré and Weise, 2012), but the nature of the signalling molecules that connect metabolism and oscillator is not known. Photosynthetic output constitutes a major readout of plant metabolism, and will be influenced by chloroplast functional state. We provide evidence here that Fe could act as such a molecule, based on the response of the *Arabidopsis* circadian clock to a modulation in Fe supply. Fe deficiency caused a strong period lengthening in free-running conditions, affecting both central clock components and output genes with roles in photosynthesis and metabolism. Under entraining cycles, low Fe levels were accompanied by a delay in the phase of circadian transcription. Several lines of evidence suggest that the circadian Fe sensor may be located in chloroplasts, and that its action is blocked by plastid translation inhibitors but not by photobleaching agents. Finally, our results indicate that the circadian clock in etiolated seedlings is initially uncoupled from Fe sensors, and that the protein HEMERA as well as the red-light photoreceptors phyA and phyB play a major role in the initiation and coordination of chloroplast development to adjust the pace of the circadian clock to available Fe supply.

Results

Free-running period under Fe deficiency

Fe-deficient seedlings are chlorotic and remain small (Figure 1A; Spiller *et al*, 1982; Vert *et al*, 2002). Although Fe deficiency can be induced by adding the Fe^{2+} chelator ferroZine to the growth medium, we elected to use Hoagland's medium with washed agar, as we discovered that ferroZine could accumulate in plant tissues (see Supplementary Figures 1 and 2 for details). FerroZine is structurally related to 2,2'-dipyridyl, commonly used as an inhibitor of chlorophyll and haem biosynthesis to disrupt plastid-to-nucleus signalling (Woodson *et al*, 2011). Under our conditions, Fe deficiency was accompanied by a lengthening of free-running period (FRP) of the circadian clock. This was observed with several luciferase reporter lines in which luciferase transcription was placed under the control of the promoters of the clock genes *CCA1* (CIRCADIAN CLOCK ASSOCIATED 1), *LHY* (LATE ELONGATED HYPOCOTYL), *PRR7* (PSEUDO RESPONSE REGULATOR 7) and *TOC1* (TIMING OF CAB2 1), or the clock-controlled genes *CAT2* (CATALASE 2), *CAB2* (CHLOROPHYLL A/B BINDING 2), *CAT3* and *FKF1* (FLAVIN-BINDING, KELCH REPEAT, F-BOX 1) (Figures 1B and C; Supplementary Figure 3). The period lengthened by up to 3 h between Fe-replete and Fe-deficient conditions, and followed an approximately linear relationship with respect to the log of Fe concentrations with all reporters tested (Figures 1D and E; Supplementary Figure 3). Thus, FRP reflected the level of external Fe supply levels experienced by seedlings in a dose-dependent manner.

Period and phase of seedlings grown without Zn, Cu or Mn were largely normal (Supplementary Figure 4D), indicating that not all micronutrients affect circadian period. An excess of Cu or Mn did not affect FRP, even though seedlings exhibited moderate symptoms of toxicity (Supplementary Figures 4A–C). The main high-affinity root Fe uptake transporter IRT1 can also transport Zn (Rogers *et al*, 2000), and exposure to excess Zn can cause Fe deficiency symptoms (Fukao *et al*, 2011; Haydon *et al*, 2012). Consistent with these observations, excess Zn caused a modest period lengthening that was rescued by supplying additional Fe in the medium (Figures 1F and G).

Fe^{3+} solubility decreases dramatically under alkaline conditions such as those encountered by plants growing on calcareous soils. Raising the pH of the growth medium from 5.8 to 7.4 led to a lengthening of FRP at high Fe concentrations.

Indeed, although the circadian clock still responded to various Fe supply, a concentration of 50 μM FeHBED (N,N'-di-(2-hydroxybenzoyl)-ethylenediamine-N,N'-diacetic acid) at pH 7.4 was less effective at shortening FRP than 20 μM FeHBED was at pH 5.8 (Supplementary Figure 5). Finally, modulation of FRP by the Fe supply was a rapid process, as Fe-deficient seedlings shortened their FRP within 24 h of transfer to Fe-replete conditions (Supplementary Figures 6A and B). Similarly, Fe-sufficient seedlings lengthened their FRP within 24 h of transfer to a medium containing ferroZine to impose quick Fe deficiency (Supplementary Figures 6A and C). From these results, we conclude that bio-available Fe concentrations in the growth medium dynamically modulate circadian period, and refer to this Fe modulation of FRP as a circadian Fe response.

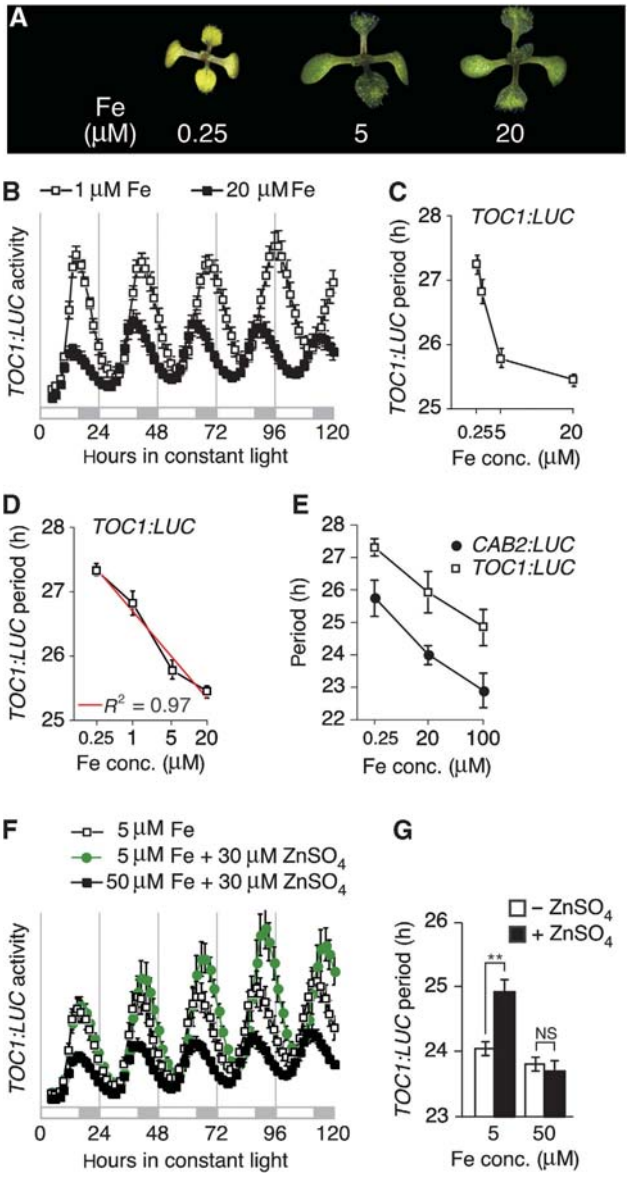

Figure 1 The period of the circadian clock reflects available Fe concentrations. (**A**) Fe deficiency induces chlorosis in seedlings. Photographs of Col-2 seedlings grown for 10 days on Hoagland medium containing 0.25, 5 or 20 μM FeHBED. (**B**, **C**) Inverse relationship between free-running period length and Fe supply. *TOC1:LUC* activity (**B**) and period length (**C**) of seedlings grown on Hoagland medium with various Fe concentrations. (**D**, **E**) Free-running period as a linear function of the log of Fe concentrations. (**D**) *TOC1:LUC* period length plotted against log(Fe concentration) follows a linear trend, with $R^2 = 0.97$. (**E**) *TOC1:LUC* and *CAB2:LUC* period maintains a linear relationship to log(Fe concentration) from 0.25 to 100 μM FeHBED. (**F**, **G**) Period lengthening under Zn excess is rescued by increased Fe supply. *TOC1:LUC* activity (**F**) and period length (**G**) for seedlings grown on Hoagland medium containing 5 μM FeHBED and 1 μM (as control) or 30 μM (for excess) $ZnSO_4$, or 50 μM FeHBED and 30 μM $ZnSO_4$. **Significantly different (two-tailed Student's *t*-test, $P < 0.001$). NS, not significant.

Circadian phase under Fe deficiency

During entraining cycles, the period of the oscillator will be very close to 24 h. An altered circadian period in free-running conditions can manifest itself during entrainment as a lagging phase (for long FRPs) or a leading phase (for short FRPs, Somers *et al*, 1998; Salomé and McClung, 2005). We determined circadian phase during entrainment as a function of Fe supply for three reporters that peak at different times of the day, *CCA1:LUC*, *PRR7:LUC* and *TOC1:LUC*. The phase of all three lagged in a similar manner between low and high Fe conditions (Figures 2A and B). Taking advantage of the lagging phase seen during entrainment, we also determined that the *elf3-1* mutant, which is arrhythmic in constant light (Hicks *et al*, 1996),

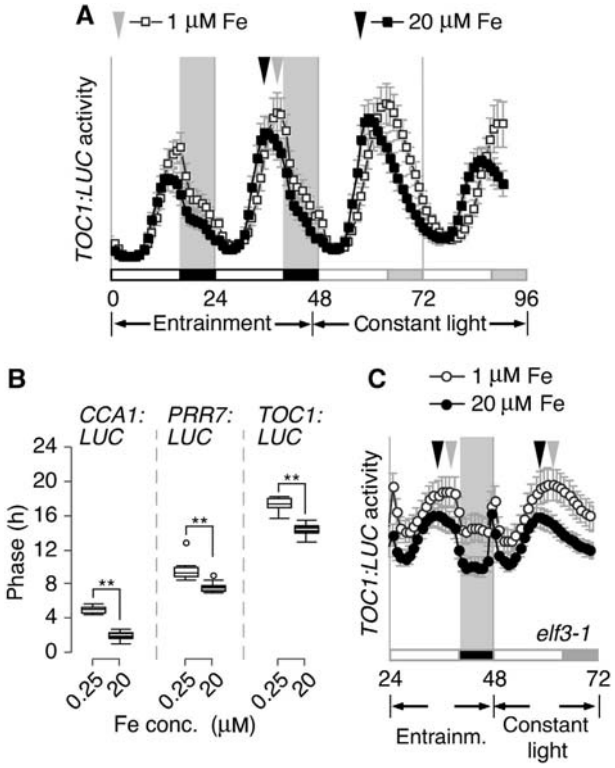

Figure 2 Lagging circadian phase in Fe-deficient seedlings during entrainment. (**A**) Low Fe supply results in a shift in circadian phase to a later time under entraining regime. *TOC1:LUC* activity in Col-2 seedlings subjected to light-dark cycles (0–48 h), and later released into constant light (48–96 h). Seedlings were grown on Hoagland medium containing low (1 μM) or high (20 μM) FeHBED. (**B**) The reporters *CCA1:LUC*, *PRR7:LUC* and *TOC1:LUC* share the same lag in phase under low Fe conditions. **Significantly different between low and high Fe conditions (two-tailed Student's *t*-test, $P \leqslant 0.002$). (**C**) The *elf3-1* mutant can respond to Fe levels before becoming arrhythmic. *TOC1:LUC* activity for Col-2 and *elf3-1* seedlings during light-dark cycles (24–48 h), and released into constant light (48–72 h).

responded normally to Fe levels, as circadian phases during entrainment and during the first cycle after entrainment were shifted later under low Fe conditions (Figure 2C). These results indicated that Fe deficiency likely shifts the phase of the oscillator and clock-controlled processes to a later time during the day.

Effects of the haem oxygenase HO1/HY6 on the circadian Fe response

In addition to *elf3-1*, we tested other circadian mutants (Supplementary Table 1; Supplementary Figure 7) for their ability to respond to various Fe supply. FRP lengthened when mutants were grown on low Fe concentrations, which ruled out a possible indirect effect of Fe deficiency via inactivation of a known clock protein with a Fe cofactor. The *tic-1* mutant similarly could adjust its period to Fe levels, indicating that TIC does not play a role in the modulation of FRP by Fe levels, despite its known involvement in the control of *FERRITIN 1* expression (Duc *et al*, 2009). It is possible that a circadian protein that requires Fe for function has yet to be identified.

Three mutants did not behave as wild-type seedlings did. A mutant lacking the Fe transporter IRT1 (*pam42*, Varotto *et al*, 2002) had a long period at intermediate, but not under low Fe concentrations, conditions under which both *irt1* and Col-2 seedlings are Fe deficient (Supplementary Figure 8). The circadian defect displayed by the *irt1* mutant was largely rescued by supplementation with high Fe concentrations (Supplementary Figures 8B and C). A mutant with a defect in the transcription factor FIT1, responsible for the induction of *IRT1* and *FRO2* during Fe deficiency, displayed similar circadian defects (Supplementary Figures 8D and E). MS (Murashige and Skoog) growth medium (Murashige and Skoog, 1962), used by many plant circadian labs for seedling growth (including during mutant screens), supplies 100 μM FeNaEDTA. In agreement with the conditional nature of period lengthening in *irt1* and *fit1-2* mutants, growth on MS medium completely abolished the long period of *irt1* and *fit1-2* seedlings (Supplementary Figures 8F–H). Circadian Fe responses in *irt1* and *fit1* mutants were thus a direct consequence of their internal physiological Fe status.

A third mutant that affected circadian Fe responses was *hy6*, defective in the main chloroplast-localized haem oxygenase, HO1, involved in phytochrome (Phy) chromophore biosynthesis from haem (Chory *et al*, 1989; Davis *et al*, 1999). FRP in *hy6* was similar to that seen in Fe-sufficient seedlings and remained constant over a wide range of Fe concentrations (Figures 3A and B). Circadian phase during entrainment was not shifted later during Fe deficiency conditions (Figure 3C), in contrast to the parental line (Figures 2A and B). The strong chlorosis of *hy6* mutants did not stem from a general Fe deficiency or homeostasis defect: unlike *irt1*, watering *hy6* plants with additional Fe was unable to rescue the small stature and low chlorophyll content of the mutant (Figures 3D and E). In addition, the expression of Fe-homeostasis markers (*IRT1* and *FRO2* for Fe deficiency, *FER1* for Fe sufficiency; Gaymard *et al*, 1996; Vert *et al*, 2002) was comparable to those seen in wild type under all Fe concentrations tested (Figure 3F), indicating that other Fe deficiency responses were normal in *hy6*.

Mutations in *HO1/HY6* impact several metabolic and developmental pathways during early seedling development: (1) *hy6* is defective in haem catabolism and thus accumulates haem (Davis *et al*, 1999; Muramoto *et al*, 1999, Woodson *et al*, 2011); (2) *hy6* deregulates chloroplast-to-nucleus retrograde signalling (Susek *et al*, 1993); and (3) *hy6* lacks active phytochromes due to its defect in chromophore biosynthesis (Chory *et al*, 1989; Davis *et al*, 1999; Muramoto *et al*, 1999). We therefore explored the potential contribution of each pathway to circadian Fe responses.

Role of haem in the plant circadian clock

In animals, haem acts as a ligand for the clock protein REV-ERBα, thereby modulating the transcription of downstream genes (Raghuram *et al*, 2007; Yin *et al*, 2007). Because haem biosynthetic enzymes are under the control of the clock in mammals (Kaasik and Lee, 2004) and plants (Supplementary Figure 9; Covington *et al*, 2008), we measured non-covalently bound haem in Col-0 wild-type plants over a circadian cycle. Haem levels showed a weak oscillation with a peak 16 h after light onset (Supplementary Figure 9D), but remained relatively constant over the range of Fe concentrations used, and did not correlate with FRP (Supplementary Figure 9E).

If haem accumulation in *hy6* resulted in a short period, then haem feeding would be expected to shorten FRP in Fe-deficient seedlings. High haem concentrations were effective in shortening FRP in Fe-deficient Col-2 seedlings (Figures 4A and C), as well as in rescuing the associated chlorosis (Figure 4B). Haem feeding was, however, unable to rescue either the long *TOC1:LUC* FRP or the chlorosis of *irt1* seedlings (Figures 4B and C). In addition to these two leaf phenotypes, we tested root surface ferric chelate reductase activity, a well-established biochemical marker for Fe deficiency. Root ferric chelate reductase activity gradually decreased in seedlings supplemented with increasing Fe (Figure 4D). Enzyme activity in seedlings treated with equivalent concentrations of haem remained higher than with FeHBED as Fe source. Haem may thus act as a poor Fe source for Fe-starved seedlings.

Haem is produced by the conversion of protoporphyrin IX and Fe to haem by ferrochelatases, encoded by two genes in *Arabidopsis* (Woodson *et al*, 2011). Loss of FC2 function had a drastic effect on seedling and plant development (Supplementary Figures 10A and B), but a very modest effect on circadian Fe responses (Supplementary Figures 10C and D). *fc2* seedlings clearly retained the ability to adjust FRP to various Fe supply, suggesting that lower haem production (or carbon monoxide (CO) release from haem catabolism) might not be involved in circadian Fe responses.

Growth in the presence of biliverdin IXα can rescue the long hypocotyl and low chlorophyll levels of *hy1* (Parks and Quail, 1991). *hy6* and *gun2-1* seedlings grown on low Fe and supplied with 250 μM biliverdin IXα recovered the ability to respond to Fe levels (Supplementary Figures 10E–H), demonstrating that the failure of *hy6* seedlings to respond to Fe supply is reversible, while not affecting the production of CO or haem catabolism. Last, we tested a T-DNA insertion allele for *HY2*, encoding the enzyme catalysing the conversion of biliverdin IXα to phytochromobilin (Figure 4E; Kohchi *et al*, 2001). The $hy2^{Wisc}$ allele displayed the characteristic long hypocotyl phenotype (Figure 4F), as well as a short FRP that did not adjust to various Fe supply (Figures 4F and G). Taken together, these results demonstrate that modulating haem levels (via catabolism or synthesis) in seedlings may not participate in circadian period control. Rather, the defect in

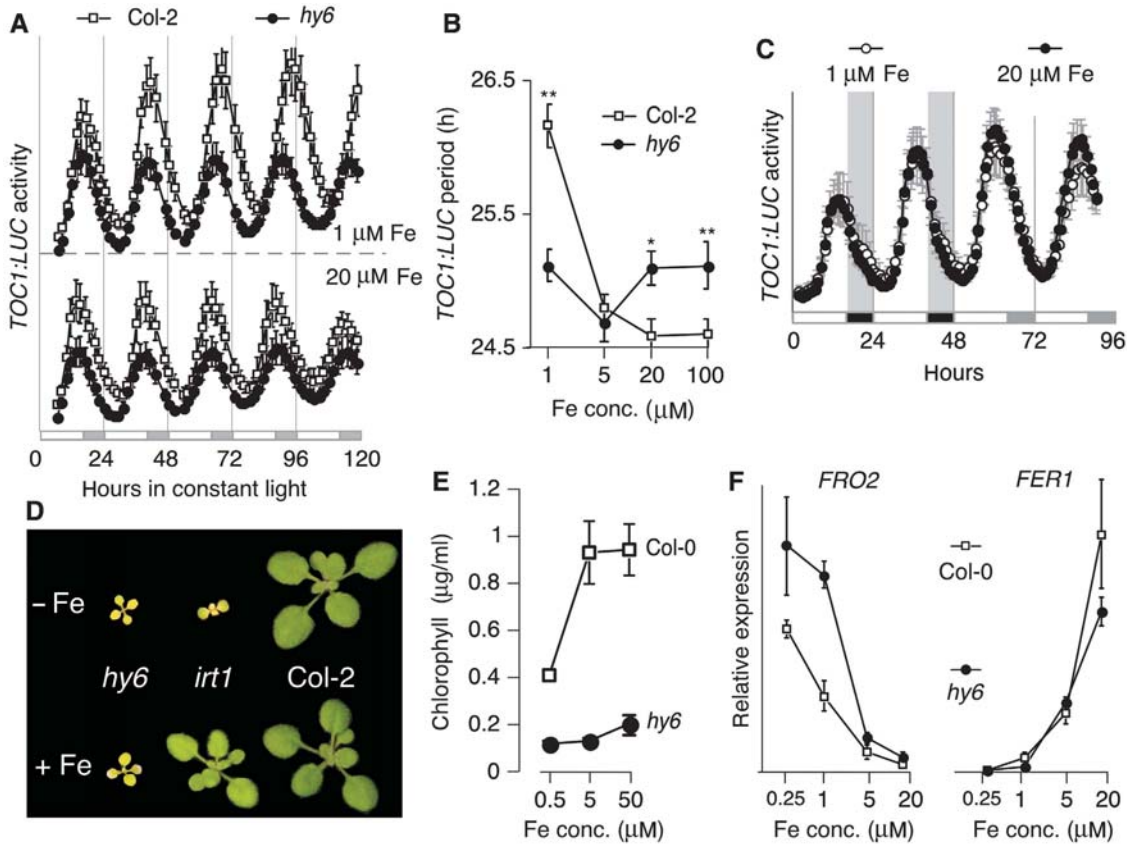

Figure 3 Free-running period in *hy6*, a mutant in the chloroplast haem oxygenase HO1, is independent of Fe supply. (**A**, **B**) Free-running period remains constant in *hy6*, regardless of the Fe concentrations in the growth medium. (**A**) *TOC1:LUC* activity in Col-2 and *hy6* seedlings grown on Hoagland medium containing 1 or 20 μM FeHBED. (**B**) *TOC1:LUC* period length in Col-2 and *hy6* seedlings grown on Hoagland medium and various Fe concentrations. *,**Significantly different (two-tailed Student's *t*-test, $*P = 0.09$; $**P \leqslant 0.002$). (**C**) Circadian phase in *hy6* is not affected by Fe concentrations in the growth medium. *TOC1:LUC* activity in *hy6* grown on Hoagland medium containing 1 or 20 μM FeHBED, during light-dark cycles (0–48 h) and in constant light (48–96 h). (**D**) Chlorosis in *hy6* is not due to a defect in Fe uptake. Representative images of 2-week-old seedlings grown on untreated soil (− Fe) or Fe-supplemented soil (+ Fe) soaked with 0.5% (w/v) sequestrene. (**E**) Increasing Fe supply does not rescue the low chlorophyll content in *hy6*. Chlorophyll content of Col-0 and *hy6* seedlings grown on Hoagland medium with various Fe concentrations. (**F**) Equivalent responses of Fe status marker *FRO2* and *FER1* transcript levels in *hy6* and Col-0 seedlings. *FRO2* and *FER1* transcript levels were determined by qPCR and normalized to *UBIQUITIN 10* levels (see Materials and methods for details).

Phy chromophore biosynthesis seen in *hy6* may be responsible for insensitivity to various Fe supply.

Role of GUN genes and chloroplast protein translation in circadian Fe responses

We first tested whether other mutants impaired in plastid-to-nucleus signalling shared the phenotype observed in *hy6*. In the absence of the herbicide norflurazon (4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3- (2H)-pyridazinone) (NF), the mutants *gun4-1* and *gun5-1* responded normally to various Fe supply, as did the strong *GUN1* allele *gun1-9* (Supplementary Figures 12 and 13). Surprisingly, *gun1-9* became insensitive to Fe supply only when treated with NF; the reason for this insensitivity is not clear, but might reflect changes in PGE in *gun1-9* (see Supplementary data for further discussion of this aspect of circadian Fe responses in *gun1* mutants). We conclude that a block in tetrapyrrole biosynthesis did not prevent circadian Fe responses, and that GUN1 was unlikely to play a general role in seedlings grown on various Fe supply.

We then tested whether plastid protein translation participated in circadian Fe responses with inhibitors of organellar protein translation. Kanamycin inhibits translation by binding to the plastid 16S rRNA, while lincomycin, erythromycin and chloramphenicol bind to the 23S rRNA (Harris *et al*, 1989; Rosellini *et al*, 2004). Treating wild-type seedlings grown on MS medium with 50 μg/ml kanamycin lengthened FRP by about 2 h, and caused a moderate drop in amplitude of the *CCA1:LUC* or *TOC1:LUC* reporters (Supplementary Figures 16A, B and D). In contrast, the herbicide glufosinate ammonium (an inhibitor of glutamine synthetase) had a modest effect on period, although luciferase activity was very severely compromised (Supplementary Figure 16C), indicating that period lengthening was not an indirect consequence of antibiotic or herbicide sensitivity. The effect of kanamycin on FRP was Fe dependent: seedlings grown on Hoagland medium with various Fe supply maintained a long FRP when kanamycin was added irrespective of Fe concentrations (Supplementary Figures 16F and G).

Other plastid translation inhibitors resulted in a similar loss of FRP adjustment to various Fe supply without affecting

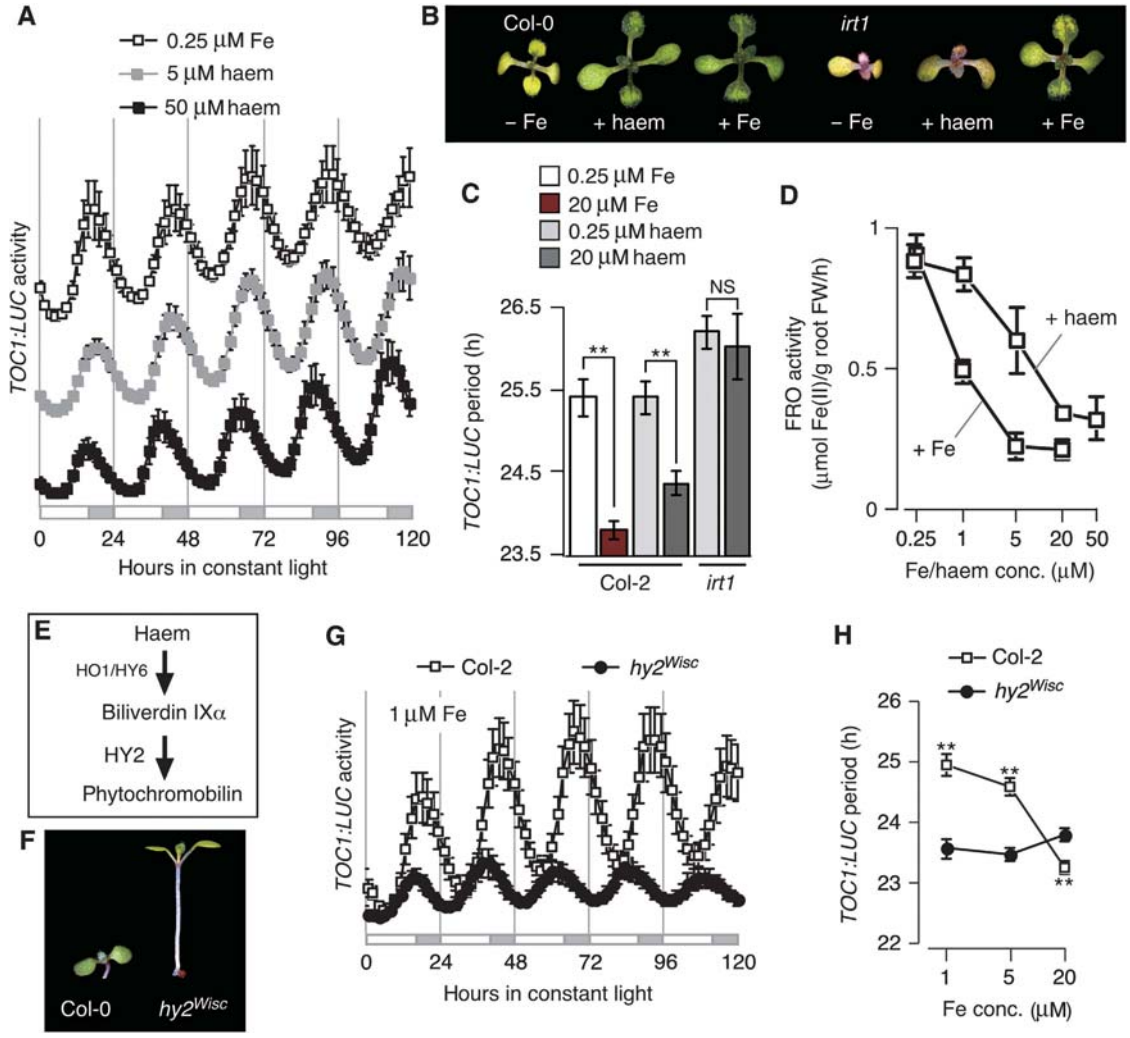

Figure 4 Assessment of haem as a potential circadian signalling molecule. (**A**) Supplementation with high concentrations of haem shortens circadian period of Fe-deficient seedlings. *TOC1:LUC* activity in Col-2 seedlings grown on Hoagland medium containing 0.25 µM FeHBED only, or supplemented with 5 or 50 µM haem. (**B**) Haem feeding rescues chlorosis caused by Fe deficiency in Col-0, but not in *irt1*. Representative 10-day-old seedlings grown on Hoagland medium containing 0.25 µM FeHBED (−Fe), 50 µM hemin (+haem) or 50 µM FeHBED (+Fe). (**C**) Haem feeding does not shorten *TOC1:LUC* period in *irt1*. *TOC1:LUC* period length in Col-2 and *irt1* seedlings grown on Hoagland medium containing 0.25 or 20 µM FeHBED or hemin. **Significantly different (two-tailed Student's *t*-test, $P \leqslant 0.01$). NS, not significant ($P > 0.5$). (**D**) Root surface Fe^{3+} chelate reductase activity responds less strongly to haem than to Fe supplementation. Activity was measured of whole roots of 10-day-old Col-0 seedlings grown on Hoagland medium containing 0.25–20 µM FeHBED (+Fe), or containing 0.25 µM FeHBED and 0.25–50 µM hemin (+haem). (**E**) The biosynthetic pathway leading from haem to phytochrome chromophore. (**F**) The new *hy2* allele $hy2^{Wisc}$ displays a typical long hypocotyl phenotype. Seven-day-old Col-2 and $hy2^{Wisc}$ seedlings grown on Hoagland medium containing 5 µM FeHBED. (**G**) $hy2^{Wisc}$ displays a short period under low Fe conditions. *TOC1:LUC* activity in Col-2 and $hy2^{Wisc}$ seedlings grown on Hoagland medium containing 1 µM FeHBED. (**H**) $hy2^{Wisc}$ shares the circadian Fe insensitivity of *hy6*. *TOC1:LUC* period lengths in Col-2 and $hy2^{Wisc}$ seedlings grown on Hoagland medium with various Fe supply. **Significantly different (two-tailed Student's *t*-test, $P < 0.01$).

amplitude. Erythromycin-treated seedlings displayed a weaker shortening of period length in response to increasing Fe supply (Figure 5B), while lincomycin treatment completely abolished adjustment of FRP (Supplementary Figure 17). Chloramphenicol acts upon both plastid and mitochondrial protein translation (Smith-Johannsen and Gibbs, 1972), and was very effective at blocking circadian Fe responses (Figures 5D and E). Finally, we noted that some, but not all, inhibitors (including lincomycin and kanamycin) caused a slight period shortening in seedlings grown under Fe deficiency (Supplementary Figures 16F, G and 17C). Inhibiting plastid translation under strong Fe deficiency conditions may allow the redistribution of cellular Fe to non-photosynthetic proteins (caused by reduced quota in these chloroplasts) to other Fe-requiring proteins, thereby decreasing the physiological Fe deficiency.

Conditionality of all Fe responses on light

Transcription and translation are very low in etioplasts, and will only be activated upon transfer into light. We tested whether Fe responses were dependent on light exposure. Low Fe supply did not prevent hypocotyl elongation in wild-type or *irt1* etiolated seedlings (Figures 6A and B). Etiolated seedlings accumulated much lower levels of *FRO2* mRNA than light-grown seedlings, even under Fe deficiency and in the Fe transporter mutant *irt1* (Figures 6C and D). Etiolated

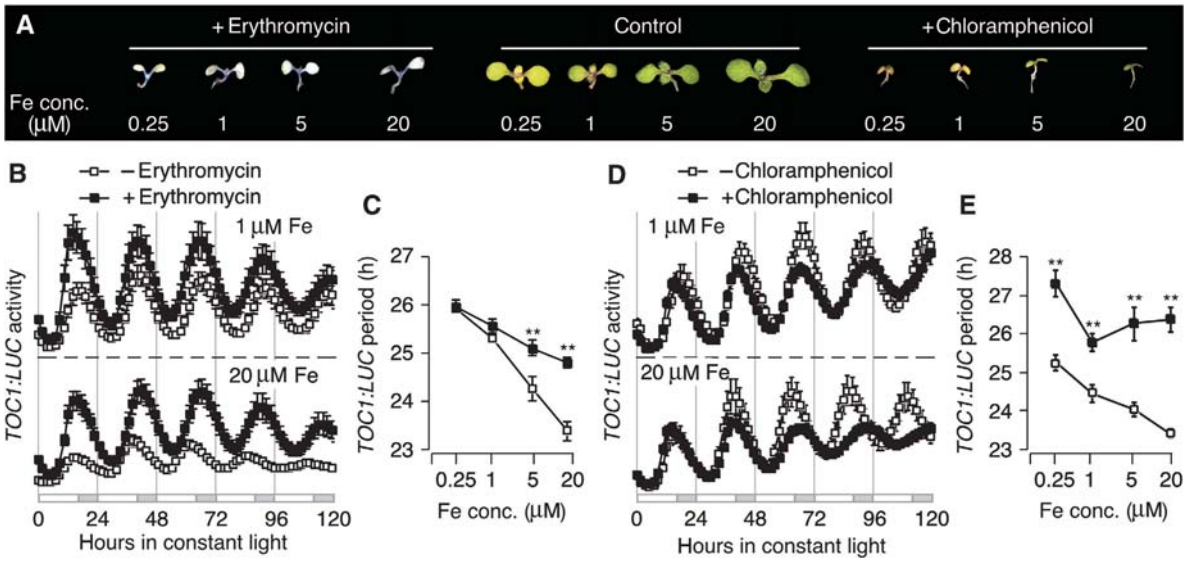

Figure 5 A block in chloroplast protein translation impairs circadian Fe responses. (**A**) Col-0 seedlings grown on Hoagland medium containing various Fe supply, alone or in combination with 50 μM erythromycin or 10 μg/ml chloramphenicol. (**B**, **C**) Circadian period length in seedlings treated with the plastid-specific protein translation inhibitor erythromycin responds less to Fe sufficiency. *TOC1:LUC* activity (**B**) and period lengths (**C**) in Col-2 seedlings grown on Hoagland medium containing various Fe supply and 50 μM erythromycin. (**D**, **E**) The plastid and mitochondria protein translation inhibitor chloramphenicol abolishes circadian Fe responses and results in a constitutive long period. *TOC1:LUC* activity (**D**) and period lengths (**E**) in Col-2 seedlings grown on Hoagland medium containing various Fe supply and 10 μg/ml chloramphenicol. **Significantly different between control and treated seedlings (two-tailed Student's *t*-test, $P \leqslant 0.02$).

seedlings also failed to accumulate *FER1* mRNA, even under high Fe supply (Figure 6D). Together, these results indicated that etiolated seedlings did not show common responses to Fe deficiency.

We next measured FRP of etiolated seedlings grown on various Fe supply. All seedlings were entrained by thermocycles (12 h 16°C/12 h 23°C) to ensure synchronization between individuals (Salomé *et al*, 2008); luciferase activity was then measured in constant darkness and a constant temperature of 23°C. FRP in etiolated seedlings was not affected by Fe levels. Proper entrainment was apparent, as all individuals were synchronized and displayed their expected phase after the last cold-to-warm transition (Figures 6E and F), suggesting that circadian function was intact in etiolated seedlings, but that circadian Fe responses were not active, or not coupled to the circadian clock. We found further support of the latter hypothesis when examining circadian Fe responses in etiolated *hy6* seedlings: FRP in *hy6* did not vary with Fe supply, and was comparable to Col-2 etiolated seedlings (Figures 6E and F). The short FRP seen in *hy6* under low Fe conditions was therefore conditional and light dependent.

Coupling of the circadian clock to the circadian Fe sensor

From our results, circadian Fe responses do not appear to rely on haem or the retrograde pathway. *hy6* mutants also lack active phytochromes, and both *hy6* and etiolated wild-type seedlings fail to adjust their FRP to Fe supply. We measured FRP in light-grown *phyB-9* single and *phyA-211 phyB-9* (*phyAphyB*) double mutant seedlings. Only the *phyAphyB* mutant behaved in a manner similar to *hy6*, with a short FRP irrespective of Fe supply (Figure 7A; Supplementary Figure 18). In addition, *phyAphyB* seedlings displayed a leading phase in *TOC1:LUC* activity, with a peak 5–6 h earlier than wild-type seedlings (Figure 7C). This phenotype was independent of Fe supply and unique to *phyAphyB*, as phase values in wild-type and *hy6* seedlings were identical (Figure 3), possibly due to feedback from haem accumulation in *hy6*. A leading phase was previously observed with *phyB* single mutants when grown on MS medium (Hall *et al*, 2002; Salomé *et al*, 2002).

Phy translocation to the nucleus is dependent upon the function of the nuclear- and chloroplast-localized protein HEMERA. *hmr* mutants are small and lack chlorophyll, and do not degrade the negative regulators of photomorphogenesis PIF1 and PIF3 (PHYTOCHROME INTERACTING FACTOR) upon light exposure (Chen *et al*, 2010). FRP in *hmr*-2 shortened to a much lesser extent with various Fe supply than in Col-2 (Figures 7D and E). Circadian Fe responses in *hmr*-2 seedlings were therefore partially compromised, and more similar to those seen in etiolated seedlings, consistent with the role of HMR in the switch to photomorphogenesis (Chen *et al*, 2010; Galvao *et al*, 2012). Period shortening in light-grown *hmr-2* seedlings in response to various Fe supply also reveals that an additional player might be involved in the coupling of the circadian clock to Fe responses. This hypothesis would be consistent with the short hypocotyl phenotype displayed by most *hmr* alleles, evidence that photomorphogenesis is initiated (Chen *et al*, 2010; Galvao *et al*, 2012).

The clock genes *CCA1* and *LHY* are direct targets of members of PIF transcription factors (Leivar *et al*, 2009; Oh *et al*, 2012), offering a simple and elegant model for circadian Fe responses. However, loss-of-function mutations in either *CCA1* or *LHY* did not affect circadian Fe responses (Supplementary Table 1; Supplementary Figure 7). Several *cca1lhy* double mutants were tested, but they could only

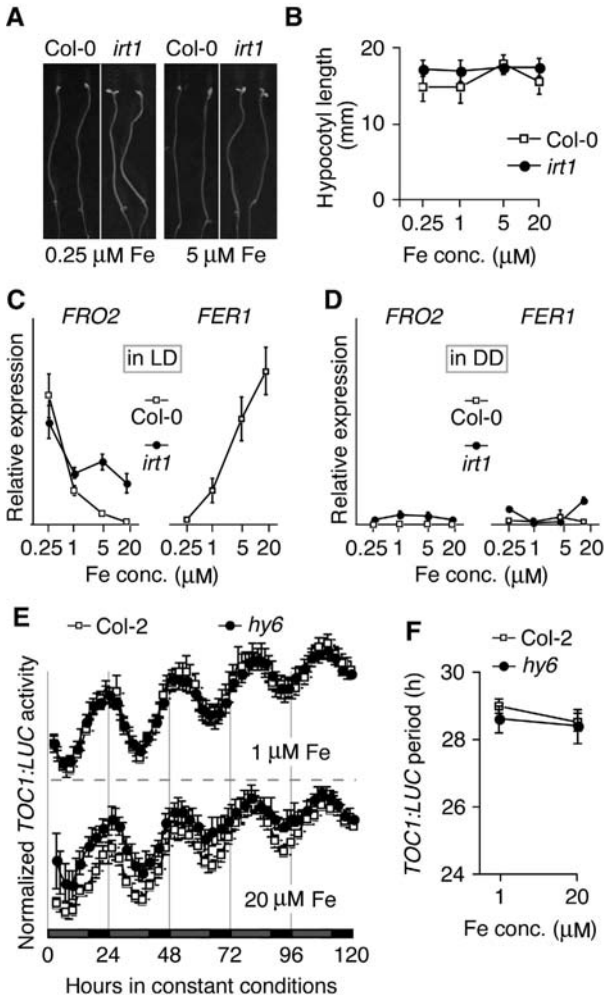

Figure 6 Non-circadian and circadian Fe responses are light dependent. (**A**, **B**) Hypocotyl elongation is not affected by Fe deficiency. (**A**) Representative Col-0 and *irt1* etiolated seedlings grown in the dark for 7 days on Hoagland medium containing 0.25 or 5 µM FeHBED. (**B**) Hypocotyl lengths of Col-2 and *irt1* etiolated seedlings as a function of Fe supply. (**C**, **D**) Expression of *FRO2* and *FER1* is induced by Fe deficiency (*FRO2*) or sufficiency (*FER1*) only in light-grown seedlings (**C**), but not in etiolated seedlings (**D**). (**E**, **F**) The circadian clock of Col-2 and *hy6* etiolated seedlings does not adjust its period to match the Fe supply. *TOC1:LUC* activity (**E**) and period lengths (**F**) in Col-2 and *hy6* etiolated seedlings grown on Hoagland medium containing various Fe supply. Period lengths for Col-2 and *hy6* are not significantly different ($P > 0.4$).

sustain a single cycle in constant conditions before becoming arrhythmic when grown on Hoagland medium (Supplementary Figure 19). Nevertheless, the initial phase of all reporters exhibited a leading circadian phase when seedlings were grown on high Fe supply (Supplementary Figure 19). In addition, seedlings carrying only a single functional copy or either *CCA1* (in a *lhy-20* background) or *LHY* (in a *cca1-1* background) were rhythmic and responded to various Fe supply (Supplementary Figure 20), suggesting that a single copy of either *CCA1* or *LHY* was sufficient to mediate proper circadian Fe responses and rhythmic behaviour on Hoagland medium. These results demonstrated that CCA1 and LHY were indispensable for rhythmicity on minimal growth medium. However, they might not be required to mediate circadian Fe responses, as mutants with reduced CCA1 and LHY activity retained the ability to adjust FRP to Fe supply.

Circadian Fe responses in an A. thaliana relative

Arabidopsis seeds are small and only accumulate Fe along the vasculature in roots and cotyledons. *Cardamine hirsuta*, another member of the Brassicaceae family, has much bigger seeds with a larger Fe pool in the vasculature and cotyledons (Supplementary Figure 21A). Nevertheless, *C. hirsuta* experienced typical Fe deficiency symptoms, with chlorosis and smaller size under low Fe levels (Supplementary Figure 21B). FRP, measured with the *A. thaliana LHY* reporter in stably transformed *C. hirsuta* T_2 transgenic lines, lengthened under low Fe conditions, and showed the same linear relationship to the log of Fe concentrations as in *Arabidopsis* (Figure 1; Supplementary Figures 21E and F). Circadian Fe responses are therefore likely to be conserved in species other than *Arabidopsis*, and will accompany the classical signs of Fe deficiency.

Discussion

Photosynthetic output is a measure of chloroplast functional state and development, and a major contributor to plant fitness (Ruckle *et al*, 2007, 2012). We uncovered a new signalling cascade between chloroplasts and the circadian clock that responds to changes in Fe levels in the growth medium. Seedlings grown on low Fe displayed clear signs of chlorosis caused by weak chlorophyll synthesis, but also showed a long period for several circadian reporters (Figure 1; Supplementary Figure 3). FRP followed a linear relationship with the log of Fe concentration, and was up to 3 h longer under limiting Fe compared to Fe-replete conditions (Figure 1). That circadian period responded in such a quantitative fashion to available Fe supply strengthens the notion that plants actively measure cellular Fe, in support of earlier observations of the graded responses to Fe deficiency experienced by *Chlamydomonas* (Moseley *et al*, 2002). This response is rapid, as Fe-deficient seedlings adjusted their circadian clock within 24 h of transfer to Fe-replete conditions (Supplementary Figure 6). Poor seedling health brought upon by Fe deficiency may in part contribute to period lengthening; however, we believe that the quantitative and reversible adjustment of FRP to Fe supply is indicative of a specific response to available Fe levels.

Our genetic analysis of circadian Fe responses demonstrates that this novel phenotype did not result from the inactivation of a known Fe-dependent clock protein, or from a modulation of plastid-to-nucleus signalling. Indeed, although the retrograde pathway would be an attractive candidate, inactivation of this pathway in *gun* mutants seldom interfered with the ability of seedlings to adjust FRP to Fe supply. One exception was *hy6/gun2*, lacking activity in the main chloroplast-localized haem oxygenase HO1 (Chory *et al*, 1989; Davis *et al*, 1999; Muramoto *et al*, 1999). *hy6* seedlings maintain the short FRP characteristic of Fe-sufficient seedlings even under low Fe supply (Figure 3). The circadian phenotypes seen in *hy6* suggested three potential hypotheses for the modulation of FRP by Fe supply, the first of which would advance haem as a signalling molecule linking the circadian clock and metabolism, as in animals

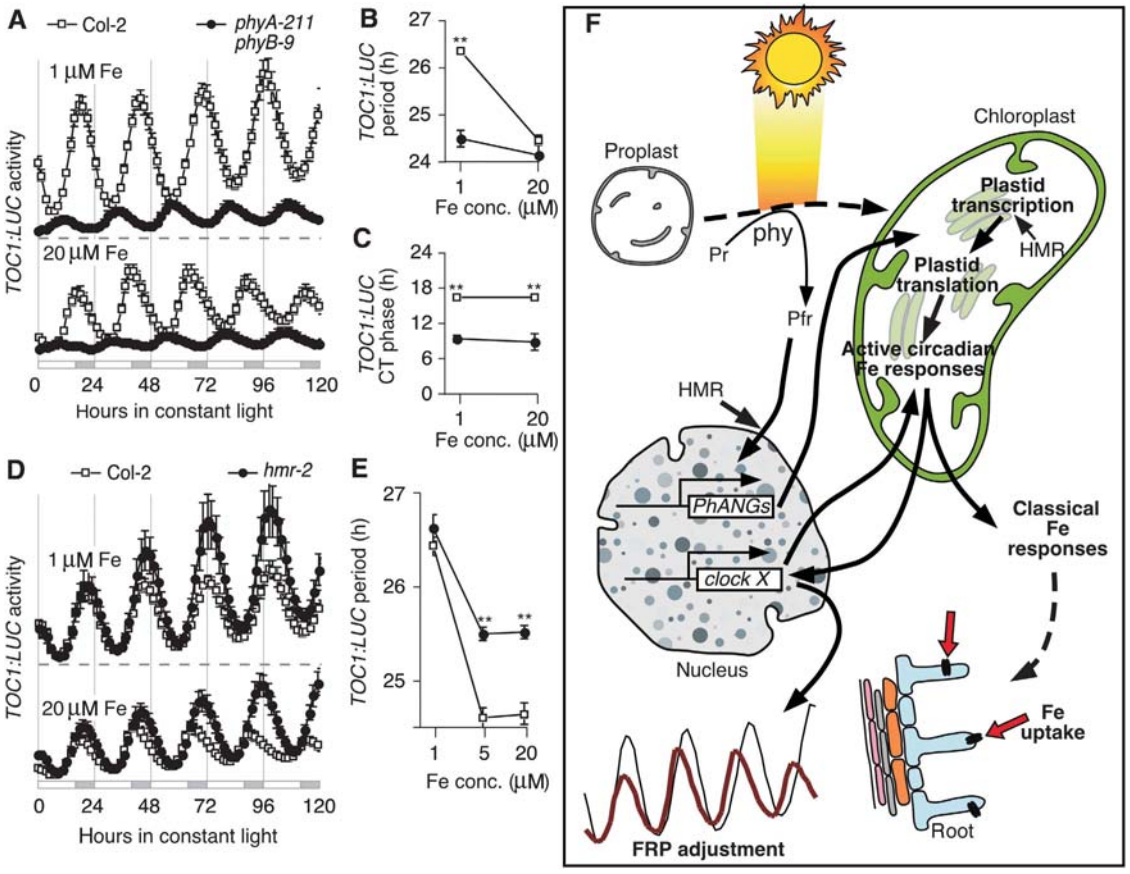

Figure 7 Coupling of the circadian clock to the circadian Fe sensor depends on phytochromes and HEMERA. (**A**–**C**) The photoreceptor mutant *phyA-211 phyB-9*, like *hy6*, maintains a short FRP even under low Fe conditions. *TOC1:LUC* activity (**A**), period lengths (**B**) and CT phase values (**C**) of Col-2 and *phyA-211 phyB-9* seedlings grown on Hoagland medium containing 1 or 20 µM FeHBED. (**D, E**) Blocking early phy signalling with a mutant in HEMERA compromises circadian Fe responses. *TOC1:LUC* activity (**D**) and period lengths (**E**) of Col-2 and *hmr-2* seedlings grown on Hoagland medium with various Fe supply. **Significantly different between Col-2 and mutant seedlings (two-tailed Student's *t*-test, $P \leqslant 0.02$). (**F**) A model for circadian Fe responses in *A. thaliana*. See text for details. The red arrows represent Fe uptake.

(Kaasik and Lee, 2004; Raghuram *et al*, 2007; Yin *et al*, 2007). Haem feeding did rescue the long period seen in Fe-deficient Col-2 seedlings, but not in an *irt1* mutant (Figure 4). Haem was consistently less effective in shortening FRP or lowering root ferric reductase activity than the FeHBED chelate, raising the possibility that exogenous haem might be used as a Fe source by Fe-deficient seedlings (Figure 4). The loss of most haem oxygenase activity in *hy6* seedlings will be accompanied by an accumulation of haem (Woodson *et al*, 2011), as well as an expected reduced release of CO. Proper FRP adjustment in a strong *fc2* ferrochelatase mutant argues against a potential signalling role for CO in circadian Fe responses, as this mutant would also be expected to display reduced CO production. CO releasers like CORM-2 (Motterlini *et al*, 2002) used in the animal field will prove very valuable to tease apart the individual contributions of haem, CO and other products of haem catabolism in circadian Fe responses.

We found that loss of Phy function, either through inactivation of *HY2*, *HY6* or simultaneous mutations in *PHYA* and *PHYB*, led to a constant FRP that did not adjust to Fe supply (Figures 3, 4 and 7). Based on the circadian Fe responses displayed by light-grown *hy6*, *phyAphyB* and *hmr* seedlings, as well as etiolated wild-type seedlings, which also lack photoactivated phytochromes, we propose a model in which the circadian clock of etiolated seedlings is first uncoupled from Fe sensing (Figure 7H). Upon light exposure, phytochromes relocate to the nucleus with the help of HMR and other factors to initiate photomorphogenesis and chloroplast development. As chloroplasts divide and multiply, they create a sink for Fe that is signalled to the root for increased Fe uptake, as well as to the nuclear circadian clock to adjust circadian period. In the absence of HMR, the clock fails to become coupled to circadian Fe responses and effectively remains largely blind to Fe supply. A second role of HMR in chloroplasts is also possible, as the protein shows dual nuclear and plastid localization (Chen *et al*, 2010). Other factors may also participate in the initial coupling of the clock to Fe responses, as *hmr* mutants are only partially insensitive to various Fe supply (Figures 7D and E). Similarly, most *hmr* alleles exhibit the short hypocotyl of light-grown seedlings, although they accumulate PIF1 and PIF3 proteins, a consequence of Phy mislocalization (Chen *et al*, 2010; Galvao *et al*, 2012).

An absence of FRP adjustment might arise from three distinct effects: (1) the clock is uncoupled from Fe responses (as in etiolated seedlings); (2) Fe responses are connected to the clock but are unable to generate or transduce the necessary signals, presumably originating from chloroplasts;

(3) the clock is coupled to Fe responses, but the functional state of chloroplasts results in a weaker Fe sink that is not interpreted as reaching Fe deficiency. Based on our results, we believe that Fe responses in etiolated wild-type seedlings and *hmr* mutants are partly uncoupled from the clock, while they are either not coupled to the clock at all or coupled but unable to transduce the information in *hy6* and *phyAphyB* seedlings if the signal signifies Fe deficiency. Alternatively, a Fe sufficiency signal may be constitutively produced in *hy2*, *hy6* and *phyAphyB* seedlings, resulting in a constant and short FRP irrespective of Fe supply. Classical Fe deficiency responses, such as induction of root ferric chelate reductase activity or *IRT1*, are largely unaffected in *hy6*, suggesting that *hy6* seedlings are fully able to perceive their internal physiological Fe supply and that their defect may lie in the transduction of Fe status. It is also possible that circadian Fe responses are mediated by a dedicated Fe sensor that is distinct from the one controlling classical Fe responses, in which case the defect in *hy6* and *phyAphyB* seedlings may also include the production of the signal itself. In either case, the Fe signal depends on Phy function to relay the information on Fe status to the nucleus.

What might be the nuclear targets of the Fe signal? PIF transcription factors are negative regulators of chloroplast development; their abundance of PIF transcription factors is controlled by phyA and phyB in light-grown seedlings, and their loss in etiolated seedlings supports partial grana formation (Leivar *et al*, 2009; Stephenson *et al*, 2009). The clock genes *CCA1* and *LHY* are direct targets of PIF4 (Leivar *et al*, 2009; Oh *et al*, 2012), but they do not participate in the response to circadian Fe levels: although *cca1lhy* double mutants did not sustain oscillations on our minimal medium, the initial phase of several luciferase reporters adjusted properly to Fe supply, as seen in wild-type seedlings (Figures 2, 7F and G). In addition, seedlings carrying a single functional copy of either CCA1 or LHY responded normally to various Fe supply (Supplementary Figure 20). The modulation of FRP as a function of Fe supply therefore does not rely on the modulation of *CCA1* or *LHY* gene expression levels, and the identity of the Phy-dependent nuclear targets of Fe status remains open. It should be noted that all circadian reporters used in this study responded to Fe supply by shortening FRP, although not all contained the known circadian motifs CCA1-binding site or Evening Element (Harmer *et al*, 2000; Michael and McClung, 2002), in agreement with active circadian Fe responses in seedlings with reduced CCA1 and LHY activity (Supplementary Figures 19 and 20).

Our results provide hints about the identity and behaviour of the circadian Fe sensor. The circadian Fe sensor may in theory sense Fe deficiency or Fe sufficiency exclusively, or perceive and integrate both signals into a unified Fe signal. Circadian Fe responses were seen in light-grown seedlings, but not in etiolated seedlings or seedlings lacking Phy function (Figures 3, 6 and 7). We therefore speculate that the Fe sensor might be a protein whose expression is induced by phytochromes. Most Fe storage and Fe use take place in chloroplasts, so it follows that chloroplasts might also house the Fe sensor (Price and Carell, 1964; Morrissey and Guerinot, 2009). However, because NF-treated seedlings retain their ability to sense and respond to Fe supply (Supplementary Figures 13 and 14), the Fe sensor is unlikely to be encoded by a PhANG, and more generally might not be under the control of the described plastid-to-nucleus pathways. Indeed, mutations in *gun4* and *gun5* did not disrupt FRP adjustment to Fe supply (Supplementary Figure 12), although both affect chlorophyll biosynthesis and lead to the accumulation of the chlorophyll precursor magnesium-protoporphyrin IX, a negative regulator of PhANGs expression (Strand *et al*, 2003; Woodson and Chory, 2008).

The loss of circadian Fe responses in seedlings treated with plastid translation inhibitors strongly suggests that the Fe sensor might be either a nucleus-encoded protein that is later translocated to the chloroplast and subject to Fe-dependent post-translational modifications, or a plastid-encoded protein. We found that only plastid-localized proteins encoded in the chloroplast genome were adversely affected by the protein translation inhibitors erythromycin and kanamycin (Supplementary Figure 15). Of all the mutants tested, only *hy6* and *phyAphyB* mutants failed to adjust their FRP in response to Fe levels, although they were able to sense Fe supply and respond accordingly for other Fe responses such as induction of the Fe uptake machinery under limiting Fe supply (Figure 3). This suggests that the adjustment of FRP to low Fe supply might be achieved by the modulation of a period lengthener that is absent in these mutants. Alternatively (but not excluding), the Fe sensor might be a period shortener that is always present in *hy6* and *phyAphyB*, whose activity might rise with Fe supply in wild-type seedlings and blocked by plastid translation inhibitors. Taken together, our results describe the genetic, molecular and biochemical signatures of the Fe sensor, a necessary first step towards its identification.

A biological function for circadian period adjustment as a function of Fe supply is not clear, but there are at least two possible scenarios for the potential adaptive significance of our results. Many photosynthetic proteins contain Fe, and under limiting Fe supply, excess light not channelled through photosynthetic electron transport chains might lead to an accumulation of reactive oxygen species. A lagging phase in PhANGs transcription might thus act as a coping mechanism targeted at the effects of Fe deficiency on photosynthetic capacity to minimize oxidative damage by delaying the accumulation of the photosynthetic apparatus. Production of H_2O_2 in plants follows a diurnal and circadian rhythm that is closely matched by a rhythm in catalase (CAT) activity, and is under the control of CCA1 (Lai *et al*, 2012). Alternatively, the leading phase under Fe-sufficient conditions might shift the peak in H_2O_2 production earlier during the day and better match the window of maximal CAT activity, thus acting as a protective measure against Fe ions, which are strongly redox active. In a perhaps more selfish scenario, more efficient Fe uptake or homeostasis in one individual might provide an advantage over its neighbours in the form of a slightly shorter FRP that will more closely resonate with the 24-h daily cycle. This slight growth advantage might over time contribute to a higher biomass in individuals with more efficient Fe uptake or larger root systems, resulting in larger seed sets and the propagation of alleles more effective at scavenging micronutrients from the environment. Ultimately, it will be important to extend our analyses to soil, in which Fe availability can be altered by changing the pH (Vert *et al*, 2002; Colangelo and Guerinot, 2004; Jeong *et al*, 2008), and where plant density can be modulated to inspect the consequences on plant biomass and FRP adjustment across individuals.

Materials and methods

Plant material

Luciferase reporter lines were generated in the Col-2 accession: *CCA1:LUC*, *LHY:LUC*, *TOC1:LUC*, *PRR7:LUC* (Salomé and McClung, 2005); *CAT2:LUC* (Salomé *et al*, 2008); *CAT3:LUC*, *FKF:LUC* (Michael and McClung, 2002). The *CAB2:LUC* reporter line was first generated in the C24 accession, and introgressed into Col-2 for six generations (Millar *et al*, 1995). Mutants tested here have been published; a complete list is provided in Supplementary Table 1. The *gun4-1*, *gun5-1* mutants and *Cardamine hirsuta* plants were transformed by the floral dip method (Clough and Bent, 1998) with a binary construct carrying the *A. thaliana LHY:LUC* reporter (Salomé and McClung, 2005). Primary transformants were selected on soil for resistance to the herbicide glufosinate ammonium, and allowed to self. Several T_2 lines were characterized and all behaved in a similar fashion. Circadian behaviour in the *gun1-7*, *gun1-9* and *gun1-9 gun5-1* mutants was analysed with the *CAB2:LUC* reporter (conferring resistance to kanamycin) that these mutants carry (Koussevitzky *et al*, 2007). *irt1*, *fit1-2*, *hy2*^{Wisc} (WiscDsLoxHs147_05H) and *hmr-2/HMR* plants were crossed to *TOC1:LUC*, and a segregating F_2 population was used for all assays. The *phyA-211 phyB-9* double mutant was crossed to *TOC1:LUC*, F_2 seedlings with long hypocotyls and closed cotyledons were selected and allowed to self; all assays were then performed with two F_3 lines.

Growth conditions

Seeds were surface sterilized by the vapour phase method (Clough and Bent, 1998) before being plated on Hoagland medium deficient for one of the four micronutrients Fe, Zn, Cu or Mn, and supplemented with 1% (w/v) sucrose and 0.5% (w/v) Fe-free agar (EDTA (ethylene diamine tetraacetic acid)-washed, see Supplementary data for details). The formulation of our minimal medium is provided in Supplementary Table 2. Micronutrient solutions for each micronutrient deficiency were prepared from the same stocks, omitting Fe and Zn, Cu or Mn. When appropriate, Fe or Fe and Zn/Cu/Mn were added prior to pouring plates. For all assays, micronutrient concentrations were Fe = 0.25, 1, 5, 20 and 100 μM FeHBED; Cu = 0, 0.1, 0.5 and 1.5 μM $CuSO_4$ (and 5 μM FeHBED); Mn = 0, 5 and 100 μM $MnSO_4$ (and 5 μM FeHBED); Zn = 0, 5 and 30 μM $ZnSO_4$ (and 5 μM FeHBED). Seeds were stratified for 2 days at 4°C in the dark before being released in long days (LD; 16 h light: 8 h light) at 23°C. Lights were provided by fluorescent bulbs, for a fluence of ~100 μM/m^2/s. For treatment with plastid translation inhibitors and herbicides, all chemicals (listed in Supplementary Table 3) were added from the time of germination.

Luciferase assays

Following 7 days in LD at 23°C, seedlings were transferred to 96-well plates containing 200 μl of the identical growth medium (Hoagland medium with varying concentrations of the micronutrient under investigation) and 30 μl of D-luciferin (potassium salt, PJK GmbH, Germany). Plates were kept for one additional LD cycle before release in constant light at 23°C. For luciferase measurements in etiolated seedlings, seeds were placed directly into 96-well plates with growth medium and D-luciferin, 1–2 seeds per well, and stratified for 2 days at 4°C in the dark. Plates were then given 12 h of white light (~100 μM/m^2/s) to induce germination, before being transferred back to constant darkness. Circadian entrainment was provided by thermocycles (16 h at 23°C, followed by 8 h at 16°C).

Luciferase activity was measured as described (Salomé and McClung, 2005) on a Perkin Elmer Topcount plate reader for 5 days. White light (~25 μM/m^2/s) was provided by four fluorescent light bulbs (gro-lux, Sylvania). Circadian parameters (period, phase, amplitude and relative amplitude error (RAE)) were determined by Fast-Fourier Transform, Non-Linear Least Square analysis as previously described (Plautz *et al*, 1997; Salomé and McClung, 2005). All seedlings had an RAE lower than 1. Unless otherwise specified (Supplementary Figure 12 for *gun4-1* and *gun5-1*, Supplementary Figure 13 for *gun1* mutants and Figure 6 for etiolated seedlings), all circadian traces are shown as mean absolute luciferase activity; amplitude and luciferase activity levels can therefore be directly compared between conditions. When indicated, luciferase activity of individual seedlings was normalized to the mean luciferase activity of that seedling over the entire time course. All experiments were conducted at least three times with similar results, with 12–24 seedlings per genotype and growth condition. All luciferase activity traces, period lengths and circadian phase values are shown as means (± standard error of the mean, with $n = 12$–24).

Gene expression analysis

Seedlings were grown in LD for 10 days, or in constant darkness for 7 days, on Hoagland medium with various FeHBED concentrations and supplemented with 1% (w/v) sucrose and 0.5% (w/v) EDTA-washed agar, at which point whole seedlings were collected and quickly frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (InVitrogen); first-strand cDNA synthesis was carried out on 0.5 or 1 μg total RNA using the RevertAid first strand cDNA synthesis kit (Fermentas) with an oligoT primer for nuclear gene expression analysis, or a random hexamer for nuclear and PGE analysis. Expression levels were determined by real-time quantitative PCR with SYBR Green (Molecular Probes) on an Opticon continuous fluorescence detection system (MJ Research) as described (Salomé *et al*, 2010). Relative expression levels were normalized to *UBQ10*. All experiments were repeated twice, and the means from two biological replicates of a representative experiment are shown (± standard error of the mean, $n = 4$). Primer sequences for qPCR are given in Supplementary Table 4.

Chlorophyll and haem measurements

Chlorophyll content was determined by measuring the absorption of extracts in 80% (v/v) acetone at 645 and 663 nm (Arnon, 1949). Soluble, free haem was extracted by the acid acetone method (Stillman and Gassman, 1978; Czarnecki *et al*, 2012). Haem levels were measured based on the reconstitution of holo-peroxidase from apo-peroxidase (Supplementary Table 3), as described (Takahashi *et al*, 2008), with a CCD camera (ORCA II, BTG2 model; Hamamatsu, Hamamatsu City, Japan). The experiment was conducted twice, and the results are given as means ± standard error of the means ($n = 3$–5) from one representative experiment.

Ferric-chelate reductase assays

Measurements of FRO activity were performed as described (Yi and Guerinot, 1996). Roots from three replicate pools of eight 10-day-old seedlings were washed twice in deionized H_2O before incubation in a ferroZine (Supplementary Table 3) solution (0.3 mM ferroZine and 0.1 mM FeNaEDTA in H_2O) for 30 min at room temperature and in the dark. The absorbance of the $Fe^{(II)}$-ferroZine complex was measured at 562 nm, and normalized to root fresh weight. All measurements were conducted at least twice, and the results are given as means ± standard error ($n = 3$) from one representative experiment.

Supplementary data

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

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Author contributions: PAS and UK designed the experiments PAS and MO performed experiments. PAS analysed the data. PAS and UK wrote the paper, with contributions from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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