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Cryptochrome mediated magnetic sensitivity in *Arabidopsis* occurs independently of light-induced electron transfer to the flavin

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Cryptochromes are highly conserved blue light-absorbing flavoproteins which function as photoreceptors during plant development and in the entrainment of the circadian clock in animals. They have been linked to perception of electromagnetic fields in many organisms including plants, flies, and humans. The mechanism of magnetic field perception by cryptochromes is suggested to occur by the so-called radical pair mechanism, whereby the electron spins of radical pairs formed in the course of cryptochrome activation can be manipulated by external magnetic fields. However, the identity of the magnetosensitive step and of the magnetically sensitive radical pairs remains a matter of debate. Here we investigate the effect of a static magnetic field of 500 μT (10× earth's magnetic field) which was applied in the course of a series of iterated 5 min blue light/10 min dark pulses. Under the identical pulsed light conditions, cryptochrome responses were enhanced by a magnetic field even when exposure was provided exclusively in the 10 min dark intervals. However, when the magnetic stimulus was given exclusively during the 5 min light interval, no magnetic sensitivity could be detected. This result eliminates the possibility that magnetic field sensitivity could occur during forward electron transfer to the flavin in the course of the cryptochrome photocycle. By contrast, radical pair formation during cryptochrome flavin reoxidation would occur independently of light, and continue for minutes after the cessation of illumination. Our results therefore provide evidence that a magnetically sensitive reaction is entwined with dark-state processes following the cryptochrome photoreduction step.

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Introduction

Cryptochromes are blue light-absorbing photoreceptors that participate in the control of numerous aspects of plant growth and development.[1,2] In *Arabidopsis*, there are two homologous genes, cryptochrome 1 (cry1) and cryptochrome 2 (cry2), which have been implicated in physiological roles including blue-light dependent hypocotyl growth inhibition, leaf and petiole expansion, chloroplast development, photosynthetic pigment biosynthesis, entrainment of circadian rhythms, floral induction, photoperiodism, response to biotic and abiotic stress, and hormone signaling pathways such as auxin and cytokinins.[1,3–6] In addition to cry1 and cry2, an additional cryptochrome from the so-called Cry-DASH family has been identified as cryptochrome 3 (cry3) in *Arabidopsis*. Cry3 is localized to both choloroplasts and mitochondria but appears to have no clearly defined signaling role in plants.[1,7]

Photochemical activation of cryptochrome

Structurally, cryptochromes are globular flavoproteins with significant homology to photolyases, an evolutionarily conserved class of flavoenzymes that catalyze light-dependent repair of UV-damaged DNA.[1,2] Cryptochromes are homologous to photolyases within their N-terminal domains which non-covalently bind FAD within a hydrophobic pocket. Unlike photolyases, cryptochromes have in addition a C-terminal domain (CCT) of up to several hundred amino acids necessary for biological activity, which is however poorly conserved between different family members.

Cryptochromes have retained from photolyases their capacity to undergo light-dependent redox reactions of the

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flavin cofactor.[1,2] In the case of both cry1 and cry2, the flavin occurs in the oxidized (FADox) redox state in the dark, and upon blue-light illumination undergoes photoreduction involving both electron and proton transfer to form neutral radical ($FADH^{\circ}$) and reduced ($FADH^{-}$) redox state intermediates. This photoreduction reaction occurs as a consequence of electron transfer to the excited state flavin through the intermediary of a chain of 3 highly conserved tryptophan residues leading from the surface of the Cry protein to the flavin. Thus, the immediate effect of illumination is flavin photoreduction, which occurs on a time scale of milliseconds, to form relatively stable reduced ($FADH^{\circ}$ and $FADH^{-}$) intermediates with lifetime on the scale of minutes.[8] The resting state (FADox) is restored by a slow process of flavin reoxidation which occurs independently of light, requires molecular oxygen, and produces reactive oxygen species (ROS).[9]

Flavin photoreduction has been linked to cryptochrome signaling in numerous studies of both *Arabidopsis* and *Drosophila* cryptochrome.[10–13] The *in vivo* biological response characteristics (wavelength and intensity dependence) of *Arabidopsis* cryptochrome-mediated phenotypes match those of the flavin redox state of the cryptochrome receptor *in vivo.*[10,12,14,15] Furthermore, the initiation of conformational change *in vitro*, particularly of the C-terminal domain necessary for signaling, has been shown to require flavin photoreduction for both *Arabidopsis* and *Drosophila* cryptochromes.[12,16–18] Mutations in the trp triad that result in diminished flavin photoreduction *in vitro* furthermore profoundly reduce biological cry function *in vivo* using a variety of different biological readouts.[18,19]

It should be mentioned in this context that there are contradictory views in the literature regarding the role of flavin reduction for the signaling mechanism of *Arabidopsis* cryptochromes. This results from recent studies which have reported that mutations in the trp triad pathway do not affect cryptochrome biological light sensitivity *in vivo*[20,21] (see also discussion in ref. 13). In one such study, the authors report that cry2 trp triad mutants show biological activity *in vivo*, stating that this contradicts the view that flavin photoreduction is a requirement for cry2 activation. However, the authors also show that these same mutations confer constitutive biological activity even in the complete absence of light.[20] This can only result from likely structural perturbations which render them constitutive, non-light responsive cry2 mutant phenotypes that are useless for analysis of light sensing mechanisms. To the contrary, in this same study, one sole cry2 phenotype was described which does retain light sensitivity in the trp triad mutants (namely light-dependent cry2 protein degradation). This phenotype indeed showed a marked decline in biological activity (reduced or absent Cry2 degradation) in trp triad cry2 mutants as compared to wild type, consistent with flavin photoreduction as an activation mechanism for Cry2.[20] Similarly, a second study reporting that Cry1 trp triad mutants had little effect on *in vivo* biological activity was also interpreted as inconsistent with flavin photoreduction as a mechanism for Cry1 activation.[21] However, in the supplement of this same study, a fluence response curve at multiple physiologically relevant blue light intensities was presented for one of the Cry1 mutant phenotypes, namely blue light dependent hypocotyl growth inhibition (Fig. S11[21]). This dose response curve compared growth inhibition of wild type Cry1 overexpressor seedlings (the relevant control) with mutant trp triad overexpressing seedlings at a range of blue light intensities. The data showed that although trp triad mutants were responsive to blue light, they required 20 to 50 fold higher light intensities than wild type to elicit the same response. This indicated an apparent 20–50-fold decrease in light sensitivity in Cry1 Trp triad mutants for seedling growth inhibition phenotype, as compared to the corresponding wild type (Cry1 overexpressing) control seedlings.[13,21] The phenotypic data comparisons presented in the main text, and on which the conclusions of the study were based, was shown at only a single blue light intensity well above the saturation point of the cry1 response (Fig. S11[21]), and was therefore not suitable for comparisons of relative activity. To the contrary the data at the appropriate (non-saturating) blue light intensity showed significantly reduced biological activity in Cry1 trp triad mutants,[21] consistent with prior work that supports flavin photoreduction as a means for cry activation.[10–19]

In summary, to our knowledge, the experimental data published in the literature to date supports a mechanism of flavin reduction to achieve the activated 'lit' redox state of the *Arabidopsis* cryptochrome receptor. The light-induced redox state transition at the flavin should then initiate all subsequent signaling functions of cry (see below).

Biological signaling and *in vivo* functional assays for cryptochrome

Cry1 and cry2 are nuclear localized proteins that interact with master regulators of de-etiolation including the Spa family of transcriptional regulators (suppressors of phyA) as well as transcription factors implicated in expression of light-regulated, stress responsive, and flowering genes.[6,22,23] It is likely that much of cryptochrome signaling in plants is through interaction with partner proteins mediating transcriptional responses, as shown by the profound impact of cryptochrome in global analyses of nuclear gene expression.[22] There is also evidence that enzymatic production of ROS (reactive oxygen species) by cryptochromes in the course of light absorption may have a signaling role,[24,25] possibly by modification of redox sensitive transcription factors and/or signaling pathways. Mechanistically, multiple studies have shown that both cry1 and cry2 proteins undergo conformational change in response to blue light, suggesting that the mechanism of activation of this class of proteins involves changes in the protein surface permitting conformationally activated cryptochrome to interact with partner proteins.[6,16–18] Studies from *Drosophila* cryptochrome have further pinpointed how altered charge in the flavin binding pocket resulting from flavin reduction alters hydrogen bonding and electrostatic interactions within the protein, leading to surface structural change and cytoplasmic exposure of the C-terminal domain.[12] Similar mechanisms appear to hold for plant crys.[18]

One widely used assay for directly detecting the degree of activation of the cryptochrome receptor is assessing the *in vivo* phosphorylation state of the C-terminal domain upon illumination.[6] C-terminal phosphorylation of both cry1 and cry2 follows from exposure of this domain to cytosolic kinases, presumably after undergoing light induced conformational change, and can be detected on western blots as an upward mobility shift in the migration of cryptochrome within minutes of the onset of illumination. Phosphorylation occurs primarily on serines and threonines which are particularly enriched within the C-terminal domain of both cry1 and cry2.[26,27] Phosphorylation is thought to be related to both biological function and protein stability. Another relatively rapid and quantitative biological assay for cryptochrome responsivity in plants is blue-light dependent inhibition of hypocotyl elongation (stem growth) in developing seedlings. This response results in shortened hypocotyls and expanded cotyledons in seedlings grown in the light as compared to the elongated growth in full darkness. It is primarily due to the action of cryptochromes in blue light as evidenced by the elongated hypocotyls of cryptochrome mutants in blue light. Plant growth inhibition follows from interaction of the light-activated cryptochrome with multiple cellular factors that regulate plant de-etiolation and growth responses.[1,6,22,23] The fact that growth inhibition is relatively rapid (few days growth) and shows a proportional relationship between growth inhibition and blue light intensity, has provided a sensitive assay for small, quantitative changes in cryptochrome biological activity.

Cryptochrome sensitivity to the electromagnetic fields

A particularly interesting feature of cryptochrome function has been their implication in the perception of electromagnetic fields over a wide range of organisms.[1] This idea originated in studies of migratory birds, in which orientation in the migratory direction occurs by sensing the inclination of the earth's magnetic field by a process that requires blue/green light.[28] Cryptochrome, which is present in the bird's retina in structures that orient it in a manner consistent for magnetoreception, has therefore been proposed as a possible magnetosensor.[28,29] Subsequently, cryptochrome-mediated responses have been investigated in a variety of organisms and found to be altered by the presence of weak magnetic fields. For example, cryptochrome-dependent plant growth, flowering time, gene expression, and cry protein C-terminal phosphorylation have all been shown to be altered in the presence of weak magnetic fields.[30–33] Similarly, in *Drosophila* and other insects, cryptochrome-dependent responses were shown to be modified in the presence of a magnetic field.[34–36] This has provided support for the suggestion that cryptochromes may function as biological magnetoreceptors, and that magnetic fields can in some way alter how the cryptochrome receptors respond to light.

Currently, a theoretical basis concerning how magnetic fields can impact on a biological response has been derived from quantum physical calculations of the effect of magnetic fields on the spins of unpaired electrons, which may be generated as intermediate states during biochemical reactions. This has led to the so-called radical pair hypothesis, which provides a mechanism whereby weak magnetic fields may alter the rate of biochemical reactions in living organisms.[37] Briefly, this mechanism requires the formation of paired radical intermediate states in the course of a biochemical reaction. The magnetic field may then act upon the electron spin (singlet/triplet interconversion rate) of these radicals, in this way influencing the rate of product formation.

In biological terms, this means that a response to a biological signal (for instance light) by a receptor that forms radical pair intermediates could be enhanced (accelerated) or diminished (slowed) by exposure to a magnetic field. As a result light of a given intensity (photons per m^2) would appear either brighter or darker to the organism in the presence of a magnetic field.

Possible magnetically sensitive radical pairs formed by cryptochromes

In the case of the cryptochrome photocycle, there are at least two known steps at which radical pair intermediate states have been suggested to be formed.[37,38] One of these steps occurs during forward light-driven electron transfer (flavin photoreduction), involving the formation of trp°/FADH° radical pairs. This step occurs exclusively under illumination, as the radical pairs are short-lived when compared with the lifetime of the reduced FADH° or $FADH^-$ states and disappear within at most a few milliseconds after the end of the light period. Another reaction step where radical pairs have been suggested occur is during flavin reoxidation (FADH°–FADox), which restores the resting state of the flavin. This reaction occurs independently of light and could involve the formation of flavin (FADH°) and reactive oxygen radical intermediates.[9] Since altering the rate of either forward (photoreduction) or reverse (reoxidation) reaction would alter the concentration of flavin in the activated (FADH°) state under a given illumination condition, either mechanism would lead to an effect of the magnetic field on cryptochrome-dependent biological activity. The current challenge is therefore to determine which, if any, reaction intermediate occurring in the course of the cryptochrome photocycle could mediate the magnetic sensitivity.

Light pulse experiments suggest the magnetically sensitive radical pair is formed in the dark

To date, the light-driven forward photoreduction reaction of cryptochrome has received the most attention as the possibly magnetically sensitive step.[37,38] This is because both theoretical parameters and studies with isolated proteins suggest that radical intermediates formed during trp triad mediated electron transfer may be sensitive to magnetic stimuli. However, recent biological studies under flickering (pulsed) blue light conditions have called this idea into question, by showing that magnetic sensitivity can occur during the dark intervals between light pulses. For example, birds were exposed to flickering light where the magnetic field was

applied only during the dark interval between the light pulses. Under these conditions, the birds were perfectly oriented and responsive to the magnetic field direction.[39] Similar studies were performed in plants, where cryptochrome responses were assayed under intermittent light/dark illumination conditions. When the magnetic field was applied only during the dark intervals between light pulses, the plants were perfectly responsive to the field, even though the onset of the magnetic field was delayed by ten seconds after the end of the light pulse.[33] Since the trp°/FADH° radical pair formed by cryptochrome is very short-lived and does not persist for more than a few milliseconds after the end of illumination, the observed magnetic sensitivity could not be explained by the mechanism of forward electron transfer. It was concluded that magnetic sensitivity under such pulsed light conditions could only ensue by the action of the magnetic field on radical pairs formed independently of light in the course of flavin reoxdiation, likely involving reduced flavin states and reactive oxygen species.[33,39]

However, neither of these studies eliminate that possibility that forward electron transfer could still be implicated to some degree in cryptochrome dependent magnetosensing, especially under the natural conditions of continuous illumination, and may complement mechanisms based on radical pairs formed during extended dark intervals.

In this study, we seek to resolve the remaining question of whether forward light-driven electron transfer may contribute in any way to magnetic sensitivity of cryptochrome responses in *Arabidopsis*. To do so, we have here investigated the effect of exposing plants to a magnetic field only during a relatively short light interval of 5 minutes, followed by 10-minute dark intervals in the absence of the field. Under these conditions, plants show a robust magnetic field response when the magnetic field is applied only during the dark intervals,[33] indicating that the cryptochrome redox cycle is fully responsive to magnetic fields under these conditions. However, since the illumination period is too short to allow full flavin re-reoxidation of activated cryptochrome,[14] a magnetic response under these conditions would more likely affect forward electron transfer, whereas absence of magnetic response, would exclude this mechanism *in vivo*.

Results and discussion

In order to detect the effects of an applied magnetic field at specific stages of the cryptochrome photocycle, we have used an assay developed in a previous study in which *Arabidopsis* seedlings were exposed to repetitive light/dark cycles consisting of 5 min blue light at 60 μmoles m^{-2} s^{-1} intensity followed by 10 min of darkness. Under these conditions, cryptochrome-bound flavin is photoreduced to the relatively stable activated FADH° intermediate only during the 5 min illumination phase, which means that radical pairs formed during the forward electron transfer reaction are not present during the subsequent 10 min dark interval. Once activated, the FADH° redox state of cryptochrome then persists for several minutes during the ensuing dark period before it is reoxidized to the resting (FADox) redox state. This reoxidation reaction also generates radical pair intermediates. Since biological activity of cryptochrome is directly related to the concentration of the FADH° redox state, any change either in the efficiency of flavin photoreduction during the illumination period, or in the stability of the FADH° during the dark interval (rate of reoxidation) would alter the ensuing biological activity of cryptochrome response (see discussion in ref. 33). These are the reaction steps at which magnetic sensitivity is analyzed.

Biological assays for plant cryptochrome

We have used two independent biological assays for cryptochrome activation. One of these involves the light-dependent C-terminal phosphorylation of cry1, which can be detected on western blots as an upward mobility shift of the Cry protein band on polyacrylamide gels after 6 cycles (90 minutes total) of the pulsed light/dark illumination cycle (Fig. 1a). Phosphorylation occurs as a result of conformational change triggered by flavin photoreduction, in turn triggering exposure of the C-terminal domain to cytoplasmic kinases.[33] The extent of phosphorylation is therefore a direct measure of cry1 biological activation. In addition, we have used a seedling growth assay using cry1 overexpressing seedling lines as a sensitive measure for cryptochrome activation. Under the illumination conditions used in this study (Fig. 1b), which consists of light pulses interleaved with dark intervals over a period of five days, there is reduced growth inhibition in *Arabidopsis* seedlings that are mutated in the CRY1 gene and thereby lack cry1 protein function (the *CRY1* mutant).[10] Wild type seedlings, containing wild type levels of Cry1 photoreceptor, show enhanced blue light dependent growth inhibition under blue light illumination, indicating that this response is due to the action of Cry1 (Fig. 1c). However, due to the low total number of photons to which the plants are exposed over the illumination period, the extent of plant growth inhibition under these pulsed blue light conditions is considerably less than can be observed under conditions of continuous light at the same intensity.[11] For this reason, we utilized seedlings that overexpressed Cry1 to obtain a stronger growth inhibition phenotype in response to blue light. This phenotype in the Cry1 overexpressor results as a direct consequence of the activation of cry1 and shows dependence on blue light intensity of the magnitude used in this study (Fig. 1c). Phosphorylation and plant growth inhibition were therefore examined for responsivity to a weak applied magnetic field of 500 μT, which is approximately 10× the intensity of the local earth's geomagnetic field.[33]

Multiple independent prior studies have shown that static magnetic fields applied exclusively during the dark interval under pulsed illumination conditions result in altered cryptochrome biological activation.[31,33,39] This has been taken as evidence that magnetically sensitive radical pairs, if generated by cryptochromes, are formed during the period of flavin reoxidation of the cryptochrome photocycle. However, these

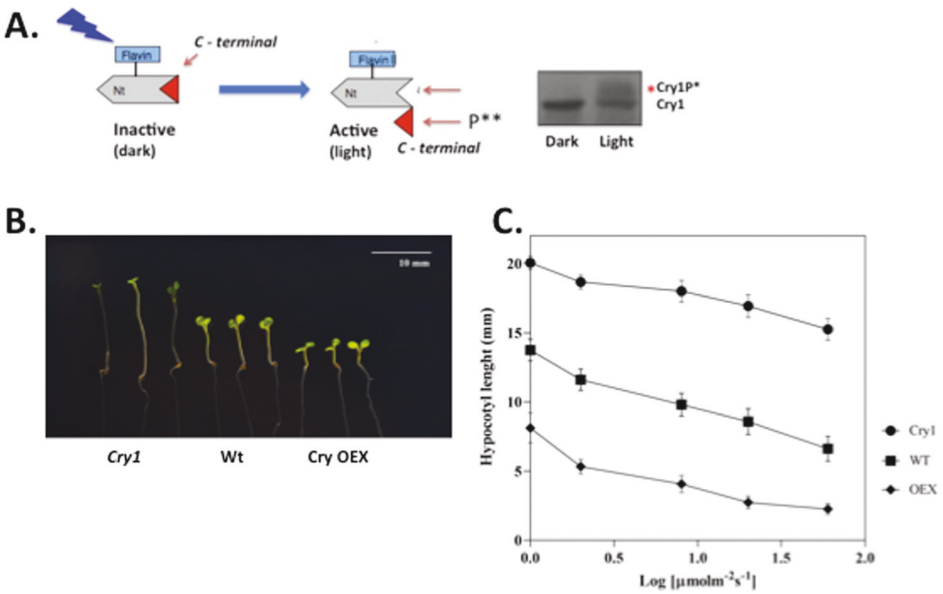

Fig. 1 Biological Assay for Magnetic field effects on *Arabidopsis* Cry1. A. Illumination of Cry1 leads to conformational change that exposes the C-terminal domain (red triangle) to cellular kinases. The phosphorylated Cry (Cry1P*) can be detected by higher-shifted band on western blots (right panel). B. Image of *Arabidopsis* seedlings grown under a pulsed illumination cycle of 5 min at 60 μmoles m^{-2} s^{-1}, followed by 10 minutes of darkness for 5 consecutive days. *Cry1* – cry1 deficient mutant; Wt – wild type; Cry1 OEX is seedlings overexpressing the Cry1 receptor. C. Blue light fluence dependence of seedling growth inhibition in pulsed blue light. Error bars represent SE.

prior studies did not assess the effect of applying the magnetic field only during the light interval (illumination period), which induces alternate, short-lived radical pairs in the course of flavin photoreduction, or that several alternate pathways are involved in cry-dependent magnetosensitivity. To categorically eliminate (or not) the forward electron transfer step as involved in cryptochrome-dependent magnetosensing, we therefore addressed the question of whether plants showed magnetic sensitivity when exposed to the magnetic field simultaneously with the 5 minutes light pulse, but not during the dark interval.

Phosphorylation assay for cryptochrome magnetosensitivity under 5 min illumination condition

Firstly, we performed the cryptochrome phosphorylation assay to quantitate the concentration of activated cryptochrome at a given illumination condition. Four-day old dark-grown *Arabidopsis* seedlings on Petri plates were exposed to 6 cycles of blue light pulses consisting of 5 minutes blue light at 60 μmoles m^{-2} s^{-1}, followed by 10 minutes of darkness, for a total time of 90 minutes per experiment as previously described.[33] In the test condition, seedlings were exposed to a 500 μT static magnetic field exclusively during the 5-minute illumination periods. The field was then turned off during the ensuing 10-minute dark intervals and western blot analysis was performed to evaluate the phosphorylation state of the cryptochrome protein (Fig. 3, MF light only). As a control condition, seedlings were compared to those that had not been exposed to a magnetic field under the identical illumination conditions (Fig. 3, MF control condition). Under these exposure conditions, cryptochrome showed no responsiveness to the magnetic field, as the extent of C-terminal phosphorylation was unchanged as compared to the control condition (Fig. 3). This was in marked contrast to the seedlings that were exposed to the magnetic field exclusively during the dark interval, which resulted in 20% increase in cryptochrome response at p-value < 0.001 (Fig. 3, MF dark only)[33] All experiments were of a minimum of five biological repeats in comparison to control and also to mock treated experimental conditions – see ref. 33 for details of experimental protocol and statistical analysis. Thus, the cryptochrome phosphorylation response to a static magnetic field occurs exclusively during the dark interval between blue light pulses.

Plant growth assay for cryptochrome-dependent magnetosensitivity under illumination conditions

We next investigated blue-light dependent plant seedling growth under these same illumination conditions. Plant growth experiments were performed double blind and as previously described,[33] with seedlings grown for five days on Petri plates under illumination cycles consisting of 5 minutes blue light at 60 μmoles m^{-2} s^{-1}, followed by 10 minutes of complete darkness, for a total of 5 complete days per experiment. In the test condition, seedlings were exposed to a 500 μT static magnetic field exclusively during the 5 minutes illumination periods. The field was then turned off during the ensuing 10 minutes dark intervals. After 5 days, the hypocotyl lengths of 15 seedlings from the test condition were harvested and measured to establish growth inhibition (Fig. 4, MF light only), which is dependent on cryptochrome biological activity

(see Fig. 1b and c). As a control condition, seedlings were compared to those that had not been exposed to a magnetic field under the identical illumination conditions (Fig. 4, MF control condition). Under these exposure conditions, there was no effect of the static magnetic field on plant growth (Fig. 4, MF light only) as compared to the control condition (Fig. 4, control). In marked contrast, there was 20% enhanced growth inhibition in seedlings exposed to a magnetic field exclusively during the dark interval between light pulses (Fig. 4, MF dark only).[33] These results are consistent with those obtained for the cryptochrome phosphorylation experiments (Fig. 3), and indicate that magnetic sensitivity occurs independently of illumination.

Advances with respect to prior work

In prior work[33,39] it was demonstrated that magnetic sensitivity of *Arabidopsis* and avian cryptochrome does not require illumination of the receptor simultaneously with application of the magnetic field, and instead occurs during a light-independent phase of the cryptochrome photocycle corresponding with the flavin reoxidation reaction (from FADH° to FADox) (Fig. 4). Since flavin reoxidation in *Arabidopsis* occurs over a time scale of several minutes after the end of the light period,[11,14] this result has been interpreted as that radical pairs necessary for magnetic field sensitivity are formed during the process of flavin reoxidation. It has furthermore been speculated that reactive oxygen species (ROS) together with flavin FADH° may form the basis for the magnetosensing radical pair. Since response to the magnetic field also occurs under continuous illumination, it is excluded that competing reactions may be occuring in the light that would somehow mask the magnetic sensitivity under our pulsed light conditions.[33]

However, what these prior studies did not fully exclude was the possibility that light-driven forward electron transfer to the flavin may also contribute in some quantitative manner to magnetic field perception. This is why we performed the present study which provides the magnetic field simultaneously with brief illumination periods, but switches it off during the ensuing longer dark intervals. Since no magnetic field effects could be detected in the plants, this data effectively eliminates any role of Trp° or Tyr°/FADH° radical pairs in magnetic sensitivity of plant cryptochromes. We note that the flavin reoxidation reaction (FADH° to FADox) also occurs during the period of illumination, as soon as reduced flavin (FADH°) starts to appear. However the illumination time is relatively short in comparison to the estimated lifetimes of the semiquinone flavin redox state of cry1[14] (around 6 minutes). Also, it must be considered that additional time may be required for the return of the activated conformational state to its inactive conformation even subsequent to flavin reoxidation (*i.e.* for dephosphorylation to occur, or for dissociation of Cry from a substrate partner protein). Therefore, the active state would not have time to undergo sufficient decay to the ground state for magnetic sensitivity to be measurable. As a consequence, magnetic sensitivity resulting during the flavin reoxidation step would be expected to occur primarily during the prolonged dark interval, which is indeed what is observed (Fig. 2 and 3).

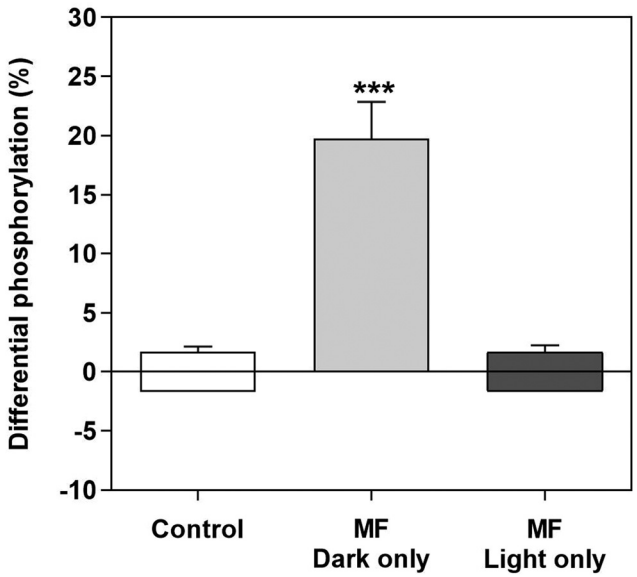

Fig. 2 Phosphorylation of Cry1 in response to a 500 μT magnetic field under pulsed light conditions. 4-day old dark-grown *Arabidopsis* seedlings were exposed to pulsed light conditions of 5 min at 60 μmoles m^{-2} s^{-1}, followed by 10 minutes of darkness for a total of 90 minutes and analysed for C-terminal phosphorylation. Seedlings were exposed to the magnetic field either during the dark interval exclusively (dark only)[33] or during the light interval exclusively (light only), and compared to a control group that had been sham-exposed to the magnetic field. +ve and −ve values indicate increased or decreased phosphorylation of the test group relative to the control group (see Methods). Details of methods and statistical analysis are as described.[33] Statistically significant increase in phosphorylation ($p < 0.001$) were only observed in the case of dark exposure to the magnetic field.

Mechanism for cryptochrome-dependent magnetosensing

Our results show that a 500 μT magnetic field enhances the biological response of plant cryptochrome at a given blue light intensity. This effect is consistent with a change in the reaction rate of the reverse (reoxidation) reaction from FADH° to FADox, which inactivates the receptor by reducing the concentration of the biological signaling state (FADH°) (summarized in Fig. 4). We note that studies with isolated proteins that have investigated magnetic field effects on isolated cryptochromes have used very high magnetic field intensities (milliT range) under various non-physiological conditions of temperature, pH, solvent, *etc.*[46] Since we have now shown that the forward reaction (flavin photoreduction) does not contribute to the magnetic effect *in vivo*, the rate constant of light-driven flavin radical formation remains unchanged by the magnetic field *in vivo*, and only the rate of reoxidation can be affected. We have further shown that biological activity is increased by the magnetic field.

We conclude that the flavin reoxidation rate must be slowed down to give an enhanced concentration of the biologically

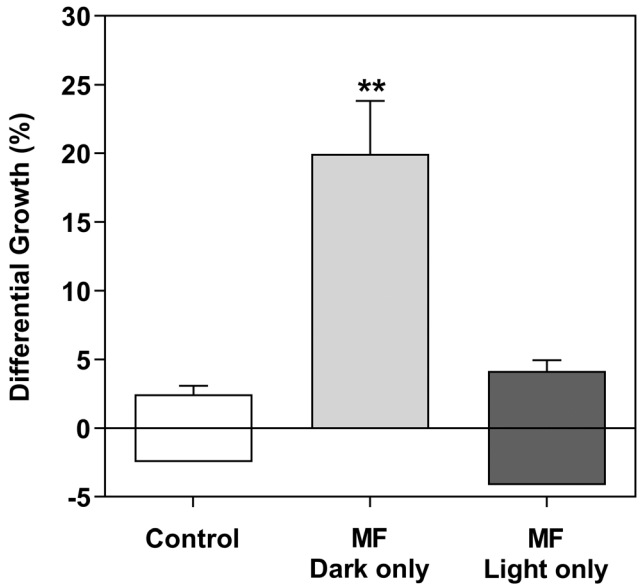

Fig. 3 Cry1 – dependent seedling growth in response to 500 μT magnetic field under pulsed light conditions. *Arabidopsis* seedlings were exposed to pulsed light conditions of 5 min at 60 μmoles m^{-2} s^{-1}, followed by 10 minutes of darkness for a total of 5 days subsequent to germination. Seedling hypocotyl lengths were measured to evaluate growth inhibition as described.[33] Seedlings were exposed to the magnetic field either during the dark interval exclusively (dark only)[33] or during the light interval exclusively (light only), and compared to a control group that had been sham-exposed to the magnetic field. +ve and −ve values indicate increase or decrease in seedling growth for the test group relative to the control group (see Methods). The magnetic field effect was to decrease plant length consistent with improved Cry1 activity.[33] Details of methods and statistical analysis are as described.[33] Statistically significant alteration in plant growth ($p < 0.01$) was only observed in the case of dark exposure to the magnetic field.

active signaling state (FADH°) *in vivo* in a 500 μT static field (Fig. 4). Since the phosphorylation state of the CRY receptor is itself directly modified *in vivo*, we further conclude that the magnetic field is likely acting directly on radical pairs generated by the cryptochrome protein itself. However, our study does not specifically identify the magnetosensitive radical pair.

The original suggestion of a flavin-superoxide radical pair[41] was criticized because it is expected that strong spin–orbit coupling in superoxide will lead to very short spin-relaxation times, precluding effects of a 50 μT magnetic field *via* the standard radical-pair mechanism.[42] However, this does not conclusively rule out a role of superoxide perhaps *via* alternate forms of the radical pair mechanism.[43–45] Theoretical arguments suggest that superoxide radicals are unlikely to participate in mediating magnetic sensitivity *via* the radical pair model, essentially due to their very short lifetimes[37] although alternate viewpoints have emerged.[42] It also cannot be excluded that amino acid residues in the protein may participate either in stabilizing the oxygen radicals in some manner, or themselves form transitory radicals in the course of oxidation. It can furthermore not be ruled out that additional, unknown molecules from the cellular environment participate in formation of radicals during flavin reoxidation, as it is known that diverse small metabolites can affect cryptochrome reoxidation rates.[13] Finally, it can not be ruled out that these effects, although involving cryptochrome at a very central level, are not themselves mediated by the cryptochrome photoreceptor itself. Although there is currently no evidence for this case, it is still possible that an unknown magnetosensor is the actual receiver of the magnetic signal and somehow rapidly and specifically transmits the information to cryptochrome.

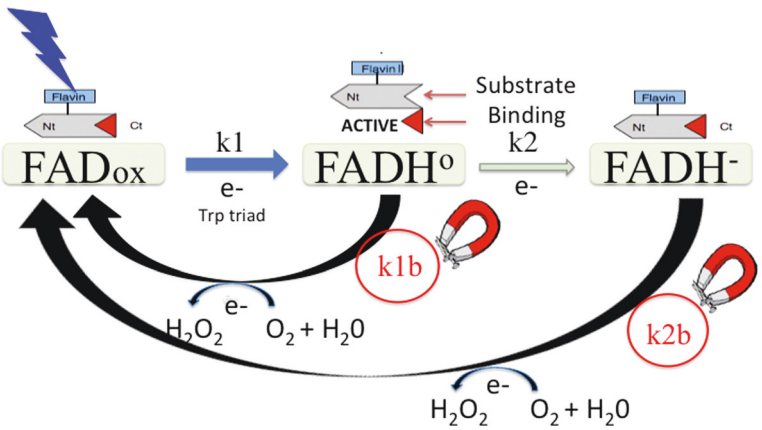

Fig. 4 Effect of static magnetic field on cryptochrome photocycle. In the dark, cryptochrome flavin occurs in the *INACTIVE* (oxidized) redox state (see flavin at left). Upon illumination by blue light, electron is transferred to the flavin through a chain of conserved Trp or Tyr residues in the protein to form the *ACTIVE* signaling state. Proton transfer to the flavin occurs during this step to yield the neutral radical FADH° redox state. Further illumination by both blue and green light causes a second electron transfer event to the flavin to produce the fully reduced FADH-redox state which is also *INACTIVE*. The system is restored to the *INACTIVE* resting state (FADox) *via* a dark reversion reaction from FADH- or FADH° to FADox, occurs independently of light and generates ROS (reactive oxygen species). Biological activity is determined by the equilibrium concentration of the *ACTIVE* redox state FADH° under continuous illumination conditions. Magnetic sensitivity occurs exclusively during the dark reversion reaction. The 500 microT magnetic field increases CRY biological activity. This is consistent with the magnetic field reducing the rate of flavin reoxidation and thereby rate constants k1b and k2b (circled).

Experimental methods

Plant materials and growth conditions

Arabidopsis thaliana seeds and growth conditions were as previously described.[33] Phytochrome A, B deficient lines (for cry1 phosphorylation experiments) and transgenic cry1 overexpressing lines (for hypocotyl growth tests) were surface sterilized by incubation with 25% bleach solution for 30 min, washed 3× with sterile water, then plated on Petri-dishes containing 2% (w/v) sucrose, 0.5× MS Murashige and Skoog Basal Salt Mixture pH 6.0 (MP biomedicals, INC, Illkirch, France), 0.9% (w/v) agar. Plates were maintained at 4 °C in the darkness for 48 h before illumination with red light (633 nm LED) for 24 h to induce germination. For the phosphorylation assay seedlings were maintained in darkness for 4 days at 22 °C before the start of the assay. For the hypocotyl growth inhibition, seedlings were transferred from red light to the test blue light and magnetic field condition for 5 days growth.

Magnetic field and blue light exposure conditions

Magnetic field conditions were as previously described,[33] using double wound Helmholtz coils. The test conditions (parallell currents) generated a static magnetic field of 500 μT intensity at the plant seedling position. For each test condition, a corresponding control field condition was generated by cancelling the magnetic field by opposing currents of the same intensity as the test conditions. As a result, parameters such as electrical current, vibration, *etc*. were the same in the control condition as compared to the test condition. The geomagnetic field was 40 μT, which corresponded to the local field. Temperature was monitored electronically by thermocouple attached to the computer during the entire course of the experiment.

Blue-light exposure was applied through 40 mm diameter round pre-mounted arrays of 7 high intensity 'Royal Blue' (peak 448 nm) blue light LEDS (https://www.luxeonstar.com/royal-blue-sinkpad-ii-40mm-7-uP-led-modules). LEDs were placed 4.5 cm above the seedlings at the center of the magnetic coils.

The magnetic coils and LEDs were controlled by custom – built automated programmable switches as described previously[33] In this way, the current to the LEDs as well as to the magnetic field coils was automatically and continuously set to generate the alternating pulses of blue light and magnetic field exposure throughout the duration of the experiment. Photon fluence of light intensity for the experiment was detected by a Quantum light meter (LI-185B, LI-COR, Inc., USA).

Phosphorylation assay TEST exposure conditions

Arabidopsis phyAphyB double mutant etiolated seedlings that had been previously germinated in darkness for 4 days were exposed to the TEST condition (magnetic field, and/or blue-light exposure) as indicated in the 'Results' section. *In cases where the seedlings were exposed to magnetic fields only in the dark interval* between light pulses, an interval of 10 s after the end of the light period was inserted before the application of the magnetic field. This resulted in the following programmed exposure condition: (i) 5 min blue light (60 μmol m^{-2} s^{-1}) at local geomagnetic field; (ii) 10 s darkness at local geomagnetic field; (iii) 9 min + 50 s darkness at 500 μT magnetic field. This cycle was repeated six times in succession for this experiment. *In cases where the seedlings were exposed to magnetic fields only in the light interval* the programmed exposure condition was as follows: (i) 5 min blue light (60 μmol m^{-2} s^{-1}) at 500 μT magnetic field; (ii) 10 s darkness at local geomagnetic field; (iii) 9 min + 50 s darkness at local magnetic field. This cycle was repeated six times in succession for this experiment. For each experiment, a reference was included in which a comparable plate which was exposed to the identical program except that the 500 μT magnetic field applied in the MF condition was replaced by a cancelled field set at 40 μT (the local field).

Subsequently, protein was extracted from plant seedlings and subjected to western blot analysis as described.[33] For the imaging analysis, the intensity of the upper, phosphorylated band from the western blotting (cry1(Pi)) was determined using imaging software and expressed as a percentage of the intensity of the total cry1 protein (sum of phosphorylated plus unphosphorylated cry1) in the same lane. The formula is thereby [cry1(Pi)]/[cry1 (total)] × 100 yielding the percentage of phosphorylated cryptochrome per lane. Three triplicate lanes per individual experiment were averaged to yield the percentage of phosphorylation at the respective condition ('MF' or 'local field'). The percent phosphorylation of the MF was then subtracted from that of the corresponding local field condition to yield the difference in phosphorylation between the test and local field (MF *vs*. Local Field) condition for a given experimental repeat. Thus, a single percentage value of differential phosphorylation was obtained for each biological repeat (MF *vs*. Geomagnetic Field). At least five independent biological repeats were performed for each condition involving both a test (MF) and geomagnetic field comparison, and each involving three replicate harvested samples per experimental plate.

To obtain the experimental test data values plotted in Fig. 2 the percentage difference in values (MF *vs.* Geomagnetic Field) originating from the five independent biological repeat experiments were averaged. The values from these five independent experiments were further subjected to statistical analysis as described below to obtain the *P* values plotted in Fig. 2.

Phosphorylation assay CONTROL experiment

The TEST experiments (presented as 'MF dark only' and 'MF light only' in Fig. 2) represent the effect of MF as compared to the local geomagnetic field condition. In order to control for any possible variations induced by our experimental setup, known or unknown we performed a separate series of five 'CONTROL' experiments which are shown in Fig. 2 as 'control'. In this series of experiments, we compared seedlings maintained under the local field to those that had been SHAM exposed (*i.e.* SHAM MF *vs*. geomagnetic field). In these experiments, the identical LEDs, magnetic coils, position of the plates, *etc*. were used as in the TEST condition, except that in

this case the magnetic fields in the double wound coil was also *canceled* to provide a 'Sham Test' condition. Thus, the CONTROL condition represents the percentage difference in phosphorylation obtained by comparison of a SHAM MF exposed sample to another cancelled – field geomagnetic field control sample. In other words, all plates in the CONTROL experiment were treated under the identical conditions. Accordingly, the control group would reveal any variability or artifact inherent in our experimental setup, no matter the cause.

This data is presented in Fig. 2 as the 'control' experiment, and represents the percentage difference in phosphorylation between the SHAM magnetic field (canceled field) and local geomagnetic field (also cancelled field) conditions and thereby provides a true baseline for the inherent variation in our experimental setup. Statistical differences were insignificant.

Western blotting

All phosphorylation experiments were analyzed by western blotting as previously described.[33] Triplicate samples of seedlings were harvested from each plate per condition and homogenized in SDS-gel electrophoresis sample buffer (2% SDS, 0.5 M Tris-HCl pH 6.8, 20% glycerol, 100 mM β-mercaptoethanol and 0.01% bromophenol blue), boiled for 10 min. Gel electrophoresis was carried out in a 10% SDS-PAGE separation gel: 30% bis/acrylamide stock (30% stock solution from Sigma), 75 M Tris pH 8.8, 1% SDS, and resolved on a mini-gel apparatus (Amersham, USA) for up to 4 h at 20 mA. Resolved proteins were transferred to a nitrocellulose membrane (Amersham). The efficiency of protein transfer was verified by staining of blots with 2% ponceau S prior to detection by antibody. Anti-cry1 antibody and detection procedure was as described previously.[33]

Each experimental sample plate was harvested in triplicate to obtain three separate readings per sample plate per experimental condition, with imaging analysis performed as described previously.[33] The experimental value obtained per condition for a single experiment was the mean of the three triplicate measurements. For each magnetic field condition, duplicate plates were prepared such that one plate was used for the test applied magnetic field condition (either MF or SHAM MF) and the other maintained under the cancelled field (geomagnetic) field condition, at the same position in the coil and with identical illumination. The magnetic field effect is expressed as a percentage of the response to the magnetic field in the test condition in comparison to the response to the local (cancelled) field condition. Each such comparison experiment was repeated for at least five independent biological repeats performed on different days and using different batches of seeds.

Hypocotyl growth TEST exposure conditions

Subsequent to germination, cry1-overexpressing *Arabidopsis* seedlings were exposed to the following programmed TEST condition (magnetic field, and/or blue-light exposure) as indicated in the 'Results' section: *in cases where the seedlings were exposed to magnetic fields only in the dark interval* between light pulses, an interval of 10 s after the end of the light period was inserted before the application of the magnetic field. This resulted in the following programmed exposure condition: (i) 5 min blue light (60 μmol m^{-2} s^{-1}) at local geomagnetic field; (ii) 10 s darkness at local geomagnetic field; (iii) 9 min + 50 s darkness at 500 μT magnetic field. This cycle was repeated over a period of 5 days for each experiment. *In cases where the seedlings were exposed to magnetic fields only in the light interval* the programmed exposure condition was as follows: (i) 5 min blue light (60 μmol m^{-2} s^{-1}) at 500 μT magnetic field; (ii) 10 s darkness at local geomagnetic field; (iii) 9 min + 50 s darkness at local magnetic field. This cycle was repeated over a period of five days for this experiment.

Concomitantly with the test condition, in a separate coil, a duplicate plate of seedlings was exposed to the identical program except that the 500 μT magnetic field applied in the test MF condition was replaced by a cancelled field set at 40 μT (the local field). The magnetic field was accordingly cancelled by opposing currents running through the double wound coils at the same voltage as for the TEST conditions. As a result, the seedlings in the cancelled field condition were never exposed to a 500 μT magnetic field but in all other respects received the identical experimental treatment. During the experiment, the temperature was monitored continuously and did not vary significantly between both coils. Hypocotyl lengths of 15 seedlings were measured per plate using ImageJ 1.50i software (NIH). For each experiment, the mean seedling length in the test condition (+MF) was expressed as a percentage of the mean seedling length of the local field (−MF) condition. Five independent biological repeats were performed under each condition to generate the average values presented in Fig. 3 (MF dark only and MF light only). These values were used for statistical analysis presented in the figure.

Hypocotyl growth CONTROL exposure conditions

The TEST experiments (presented as 'MF dark only' and 'MF light only' in Fig. 2) represent the effect of MF as compared to the local geomagnetic field condition. In order to control for any possible variations induced by our experimental setup, known or unknown we performed a separate series of five 'CONTROL' experiments which are shown in Fig. 3 as 'control'. In this series of experiments, we compared seedlings maintained under the local field to those that had been SHAM exposed (*i.e.* SHAM MF *vs.* geomagnetic field). In these experiments, the identical LEDs, magnetic coils, position of the plates, *etc.* were used as in the TEST condition, except that in this case the magnetic fields in the double wound coil was also *canceled* to provide a 'Sham Test' condition. Thus, the CONTROL condition represents the percentage difference in seedling growth obtained by comparison of a SHAM MF exposed sample to *another* cancelled – field geomagnetic field control sample. In other words, all plates in the CONTROL experiment were treated under the identical conditions, placed under the identical coils, under the identical illumination,

except that neither received an MF stimulus. Accordingly, the control experiment would reveal any variability or artifact inherent in our experimental setup, no matter the cause.

This data is presented in Fig. 3 as the 'control' experiment, and represents the percentage difference in growth between the SHAM magnetic field (canceled field) and local geomagnetic field (also cancelled field) conditions. It thereby provides a true baseline for the inherent variation in our experimental setup. Statistical differences were insignificant.

Double blind conditions

Since selecting the seedlings to measure from a plate of seedlings of varying lengths may be subject to experimenter bias, all experiments were performed double blind. Firstly, several dozen plates were prepared before each experiment, such that plates for the experiment (test and control condition) were selected randomly from the stack and could not be known in advance. Secondly, fifteen ungerminated seeds were marked by a felt tip underneath the plate before the beginning of the experiment, such that the seedlings to be measured had been pre-selected' in advance, before the start of the experiment. Thirdly, the plates were placed within the experimental setup by a different person than the one who removed them. Fourthly, the person who measured the seedlings was different to the one who removed the plates, and who had not been in the room. In this way, the person measuring the seedlings did not know from under which condition they were taken.

Data and statistical analysis

As described above, all experiments involved five or six independent biological repeats comparing the effect of application of a 500 μT magnetic field to the identical untreated control seedlings. The magnetic field effect was expressed as a differential percentage (treated *vs.* untreated sample) for each individual experiment in MF group. In addition to the test experiments performed in this manner, a control group experiment was performed for each condition. In this case, all plates in a given experiment were treated with the identical light but with the magnetic field was cancelled. Therefore, no magnetic field was applied to either test or control plates and the effect of the 'mock' magnetic field condition expressed as a differential percentage of the 'local field' condition (control *vs.* control sample), which were in fact identical. The data obtained from both the MF group and control group were subjected to the same analysis. This is the data plotted on the experimental figures (Fig. 2 and 3).

For statistical analysis, all data was analyzed by SPSS Statistics program software IBM SPSS Statistics 23 (IBM). Data were analyzed for normality with the Shapiro–Wilk test ($P < 0.05$) and Homogeneity of Variances with Levene ($P < 0.05$). Results were expressed as means ± standard error of the mean (SE). The different between the MF group and the control group were compared by using Anova analysis. Differences were considered statistically significant with a P-value < 0.05 (*), <0.01 (**) and <0.001 (***).

In summary, the work described in this study is an extension of experimental observations published in a prior study on effects of magnetic fields on *Arabidopsis*.[33] All of the experimental methods used in this study including details of experimental setup and light sources, plant growth methods, assay procedures for western blot analysis and imaging, and details of statistical analysis and setup of sham and test experimental conditions are fully and exhaustively documented in previously published work.[33]

Conclusions

Our data show that the requirement for illumination can be completely separated from the magnetically sensitive step during cry-dependent magnetoresponses. This eliminates the possibility that any rapid, short-lived radical pairs generated as a direct result of illumination could underly magnetic sensitivity in *Arabidopsis* and, by extension, in other living systems such as birds where similar phenomena appear to hold. As a result, our findings rule out the prevailing hypothesis of forward electron transfer involving the Trp triad as the basis for cryptochrome-dependent magnetosensing, at least in the case of plant cry (see *e.g.* ref. 40 and 46–48).

Our findings set several clear constraints on the radical pair mechanism that could mediate magnetic sensitivity involving *Arabidopsis* cryptochrome. The crucial radical pairs must be formed from light-induced intermediates of the Cry photocycle which are relatively stable *in vivo*, lasting for a period of many minutes after the cessation of the triggering light pulse. The actual magnetically sensitive radical pairs must furthermore be generated by a light-independent process likely involving the consumption of molecular oxygen. Finally, the end effect on the spins of these radical pairs by a 500 μT magnetic field should be to result in a decrease in quantum efficiency of the flavin reoxidation reaction in the course of the Cry photocycle (Fig. 4). Our results should help to focus and refine efforts towards the ultimate identification of the components responsible for the radical pair mechanism of magnetosensing involving plant cryptochromes, and, by extension, cryptochromes from other organisms.

Abbreviations

cry	Cryptochrome
FAD	Flavin adenine dinucleotide
GMF	Geomagnetic fields
MF	Magnetic fields
phy	Phytochrome
Trp	Tryptophan

Conflicts of interest

There are no conflicts of interest.

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