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Research

Blue-light dependent reactive oxygen species formation by *Arabidopsis* cryptochrome may define a novel evolutionarily conserved signaling mechanism

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Summary

- Cryptochromes are widespread blue-light absorbing flavoproteins with important signaling roles. In plants they mediate de-etiolation, developmental and stress responses resulting from interaction with downstream signaling partners such as transcription factors and components of the proteasome. Recently, it has been shown that *Arabidopsis* cry1 activation by blue light also results in direct enzymatic conversion of molecular oxygen (O_2) to reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) *in vitro*. Here we explored whether direct enzymatic synthesis of ROS by *Arabidopsis* cry1 can play a physiological role *in vivo*.
- ROS formation resulting from cry1 expression was measured by fluorescence assay in insect cell cultures and in *Arabidopsis* protoplasts from cryptochrome mutant seedlings. Cell death was determined by colorimetric assay.
- We found that ROS formation results from cry1 activation and induces cell death in insect cell cultures. In plant protoplasts, cryptochrome activation results in rapid increase in ROS formation and cell death.
- We conclude that ROS formation by cryptochromes may indeed be of physiological relevance and could represent a novel paradigm for cryptochrome signaling.

Introduction

Cryptochromes are blue-light absorbing photoreceptors found in plants and animals that have multiple signaling roles. In plants, cryptochromes are involved in de-etiolation, elongation growth and development, entrainment of the circadian clock and the photoperiodic initiation of flowering (Chaves *et al.*, 2011). Cryptochromes have also been implicated in responsivity to stress including pathogen resistance (Jeong *et al.*, 2010; Wu & Yang, 2010), high light stress (Danon *et al.*, 2006; Kleine *et al.*, 2007; Lopez *et al.*, 2012; Kim & Apel, 2013; Sharma *et al.*, 2014), and temperature and osmotic stress (Xu & Ma, 2009; Xu *et al.*, 2009; Sanchez *et al.*, 2011; Sharma *et al.*, 2014). Cryptochromes undergo blue-light dependent redox reactions involving flavin photoreduction *in vivo* which are thought to induce conformational change in the receptor leading to interaction with the biological signaling partners (reviewed in Chaves *et al.*, 2011). In fact, several proteins that bind to plant cryptochromes in a light-dependent manner have been identified and linked to cryptochrome signaling, including bHLH transcription factors such as Cib1 and elements of the proteasome including Cop1 and Spa1 (Liu *et al.*, 2008; Zuo *et al.*, 2011). Therefore, current paradigms for cryptochrome activation presume that light-induced conformational change within the protein is followed by interaction with protein substrates, leading to downstream signaling events.

Recently, in the course of elucidating the cryptochrome photocycle, we have shown that activation of cry1 by blue light results in formation of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) under continuous blue light irradiation *in vitro* (Müller & Ahmad, 2011). Absorption of a photon of light energy by a cryptochrome leads to transfer

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of an electron to excited state flavin from a nearby Trp residue in the protein. This Trp residue is in turn reduced through a chain of additional Trp or Tyr residues leading to the protein surface. The reduced (FADH° or $FADH^-$) flavin is then reoxidized to the resting (oxidized) state via cleavage of molecular oxygen (O_2) to form superoxide and subsequently H_2O_2. At this point, the entire cryptochrome photocycle (light-induced flavin reduction followed by flavin reoxidation) can repeat itself, for as many times as photon energy is available. As a result, under continuous blue light illumination, cryptochrome activation catalyzes the enzymatic accumulation of ROS and H_2O_2. Cryptochromes in this way serve as light-activated generators of ROS.

ROS in themselves are important signaling molecules in most organisms, including in induction and/or responsivity to stress (Mittler *et al.*, 2011). In plants, ROS are implicated in many processes including in seed germination, development, senescence, heat or cold stress, pathogen defense, and responsivity to high light stress (Orozco-Cardenas & Ryan, 1999; Apel & Hirt, 2004; Mittler *et al.*, 2004; Gechev *et al.*, 2006; Bailly *et al.*, 2008; Miller *et al.*, 2008; Quan *et al.*, 2008; Barrero *et al.*, 2014). Given this plethora of signaling roles for ROS, it is striking that a number of the signaling pathways in which cryptochromes have been implicated, including pathogen defense, osmotic stress and response to high light stress, in fact intersect with known pathways in which ROS are important regulators. The intriguing possibility therefore suggests itself that cryptochromes may directly alter cellular concentrations of ROS, particularly under high blue-light illumination, which induces known stress responses. Given the highly evolutionarily conserved nature of ROS sensitivity and implication in responsivity to stress (Mittler *et al.*, 2011), perhaps one of the mechanisms of cryptochrome signaling may be by directly altering concentrations of ROS found in specific cellular compartments. We therefore wondered whether production of ROS by cryptochromes may have physiological consequences to the cell.

In order to test this possibility, we have investigated the effect of *Arabidopsis* cryptochrome-1 (Atcry1) on formation of reactive oxygen *in vivo* using insect cell culture overexpressing Atcry1. We reasoned that insect cells are unlikely to have downstream signaling intermediates and protein partners involved in the classic signaling pathways of plant cryptochromes. We have used transgenic cry1-expressing insect cell cultures, which produce high concentrations of plant cryptochromes in the cells and which respond to light in an *in vivo* context (Bouly *et al.*, 2007; Burney *et al.*, 2012). We found that elevated concentrations of ROS were formed by Atcry1-expressing insect cell cultures in response to blue light, followed by rapid cell death. Taken together with immunolocalization studies verifying the colocalization of the site of ROS formation with Atcry1 localization in these insect cultures, we conclude that ROS formation by cryptochromes indeed occurs in a physiological context. We further obtained intriguing results using *Arabidopsis* protoplasts which implicated cryptochrome activation in ROS formation in the plant system. We conclude that cryptochrome production of ROS upon illumination may be a novel feature of cryptochrome signaling.

Materials and Methods

Insect cell cultures and cell viability assays

We infected SF21 insect cells with baculovirus constructs of Atcry1 as previously described (Bouly *et al.*, 2007). Cells were harvested 3 d post infection for analysis. For assays of cell viability, insect cell cultures were divided into 2-ml aliquots and incubated in 10-ml flat bottom glass vials under red and blue light or in darkness at 21°C with gentle agitation (70 ***g***). For Trypan blue colorimetric assays, 20 µl of cells were harvested in culture medium at the appropriate time point and added directly to 20 µl of dye (Trypan blue at a concentration of 1 mg ml^{-1} in phosphate buffered saline (PBS) at pH 7.4). Aliquots were applied to a Kova® Glasstic® slide (Hycor Biomedical Inc., Indianapolis, IN, USA) with demarcated grids (volume = 6.6 µl) and were counted using a Zeiss Apotome fluorescent microscope, with Axio Vision imaging software. A minimum of six independent aliquots were counted per time point with between 100 and 200 cells counted per aliquot. High-intensity red (600–700 nm) and blue (400–500 nm) lighting at 100 µmol m^{-2} s^{-1} was achieved using OSRAM Lumilux fluorescent tubes (Lumilux blau and rot) ref. L36W/67 and L36W/60. Light intensities expressed in W m^{-2} are 26.58 W m^{-2} in blue light (wavelength range 400–500 nm) and 19.2 W m^{-2} in red light (wavelength range 600–700 nm).

Arabidopsis thaliana protoplasts and plant cell viability assays

Sterilized seeds of *Arabidopsis thaliana* (L.) Heynh. (wild-type (WT) and *cry1cry2* in Wassilewskija ecotype; see Bouly *et al.*, 2007) were seeded in petri dishes on Murashige and Skoog (MS) solid media and grown under low-intensity red light (5 µmol m^{-2} s^{-1}) for 15 d. Illumination was from red light LEDS (Ledgalaxy, Hamburg, Germany; JDR-1438R E14). For making protoplasts, 5–7 g of whole seedlings were incubated with 90 mg of cellulase (Onozuka, Duchefa Biochimie, Haarlem, the Netherlands; 16 000 U g^{-1}), 30 mg of pectolyase (Y-23, Duchefa Biochimie, 1000 U g^{-1}) and mannitol (0.6 M) in 20 ml of MS medium. Tubes were incubated for 2 h in the dark under agitation (70 ***g***), and then filtered through bluttex cloth (100-µm mesh) and centrifuged at 300 ***g*** for 5 min. The pellets containing protoplasts were gently resuspended in 15 ml of MS media containing 0.6 M mannitol. Two millilitres of this final protoplast suspension were placed in glass vials and incubated under the same high-intensity light conditions (red or blue) as for the insect cell cultures (see earlier) with gentle shaking (70 ***g***) for cell viability counting and ROS quantification. Protoplast cell viability was determined colorimetrically by mixing 20 µl of cells from the culture medium at the appropriate time point and adding it directly to 20 µl of dye (Trypan Blue at a concentration of 1 mg ml^{-1} in 50 mM PBS at pH 7.4; 0.6 M mannitol). Counting and imaging were performed as for insect cell cultures (see earlier). Six independent aliquots containing a minimum of 100 protoplasts were counted per sample.

ROS analysis

For both plant and insect cell cultures, H_2O_2 concentrations in cell culture medium were assayed by a modification of published methods (Martino & Castello, 2011). One milliliter of incubation medium was harvested for each datum used for ROS analysis. Ten micromoles of Amplex UltraRED (Invitrogen) and 0.2 U of Horse Radish Peroxidase (Sigma) in 50 mM sodium phosphate buffer at pH 7.4 was added directly to each sample. After 30 min of incubation time in the dark, fluorescence was read in triplicate from each sample (100-µl volume for each reading) in 96 well plates (Greiner Bio-One, Monroe, NC, USA) with a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA) at excitation 560 nm, emission 590 nm. Fluorescence units were converted to concentration of peroxide using a standard curve of H_2O_2 concentration vs fluorescence units as described previously (Martino & Castello, 2011). These values were then normalized to number of living cells in the culture volume.

Protein isolation and analysis

Arabidopsis cry1 protein was isolated from expressing insect cells by histidine (His) tag affinity purification as described (Bouly *et al.*, 2007). For determination of ROS, Atcry1 protein at a concentration of 10 µM in PBS pH 7.4 and in the absence of added reducing agent was illuminated in strong white light (3000 µmol m^{-2} s^{-1}) for 30 min. Aliquots were taken at the indicated times (see later Fig. 5) and diluted 10-fold into 1 ml of 50 mM of sodium phosphate buffer at pH 7.4. Amplex UltraRED detection of ROS was then completed as for cell cultures (see earlier). Final values of peroxide concentration were normalized to the number of cells in the cell culture volume which produced the concentration of protein used in the assay.

Oxygen concentration experiments

For assays of oxygen concentration dependence, insect cell cultures were divided into 2-ml aliquots in glass vials. The atmospheric O_2 content was modulated using the procedure of Côme & Tissaoui (1968); gas mixtures containing 3% and 21% oxygen were obtained through capillary tubes connected to sources of compressed air and nitrogen. The gaseous atmospheres were passed continuously at a flow rate of 2 l h^{-1}, for 2–5 min through the incubation flasks, by Neolus needles (Terumo, Tokyo, Japan) through serum caps. The purge was conducted for 10 min, and samples were subsequently sealed and transferred to light conditions for cell viability counting and fluorescence assay. Each time point corresponded to a separate sample vial to avoid artifacts resulting from multiple sampling/breaking of the seal of the vials. Cell viability assay and fluorescence detection of ROS was as indicated earlier.

Immunofluorescence labeling of cry1

After incubation for 2 h on glass coverslips, Sf21 cells were exposed to dark or blue light for 10 min and fixed with 2% paraformaldehyde for 10 min at room temperature (RT). To stain the intracellular compartments or the cell surface, cells were permeabilized with 0.1% Triton X100 or not permeabilized, respectively, and then incubated with an anti-CRY1 rabbit polyclonal antibody and an Alexa 488-conjugated anti-rabbit secondary antibody. Coverslips were mounted in Fluoroshield with 4′,6′-diamino-2-phenylindole (DAPI) and viewed using a Leica upright SP5 confocal microscope with a ×40 objective or a ×63 objective for higher magnification images. DAPI and Alexa 488 were excited at a 405- and 488-nm wavelengths, respectively, and the emission fluorescence intensities were detected by using a photomultiplicator atbetween 410 and 480 nm, 495 and 550 nm, respectively. Two channels were recorded sequentially at each z-step. Z series projections, merge images and three-dimensional (3D) visualizations were performed using ImageJ software (W. S. Rasband, ImageJ, US National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij, 1997–2009). Nuclear 3D analysis was performed using Tools for Analysis of Nuclear Genome Organization (TANGO) (Ollion *et al.*, 2013) and visualized with 3D viewer on ImageJ.

Intracellular localization of ROS

After incubation for 2 h in cell observation chambers, Sf21 cells were exposed to were exposed to dark or blue light for 10 min and incubated in 20 mM potassium phosphate buffer (pH 6.4) containing 50 µM DCFH-DA (Molecular Probes, Life Technologies, Grand Island, NY, USA) for 15 min at RT in the dark. Cells were rinsed for 15 min in the potassium phosphate buffer solution and observed with an inverted Leica TCS SP5 microscope using a ×20 or a ×63 objectives. Green fluorescence from DCFH-DA and differential interference contrast (DIC) were excited at 488- and 561-nm wavelengths, respectively. Emission fluorescence intensities and DIC were detected by using a photomultiplicator between 410 and 480 nm, and a transmission photomultiplicator, respectively. Two channels were recorded sequentially. Z series projections were performed using ImageJ software (W. S. Rasband, ImageJ).

Results

Activation of Atcry1 by blue light leads to cell death in recombinant expressing insect cell cultures

Prior experiments with purified protein revealed that cryptochrome activation *in vitro* leads to formation of ROS and H_2O_2, which is toxic to cells in high concentrations. To test whether cryptochrome activation results in ROS accumulation *in vivo*, we used a heterologous insect cell expression system in which *Arabidopsis* cryptochromes can be expressed to high levels and show a robust photocycle in response to blue light illumination (Bouly *et al.*, 2007; Burney *et al.*, 2012). Cultures expressing Atcry1 were accordingly illuminated in blue (400–500 nm) or red (600–700 nm) light at an intensity of 100 µmol m^{-2} s^{-1} for several hours. Cell cultures were

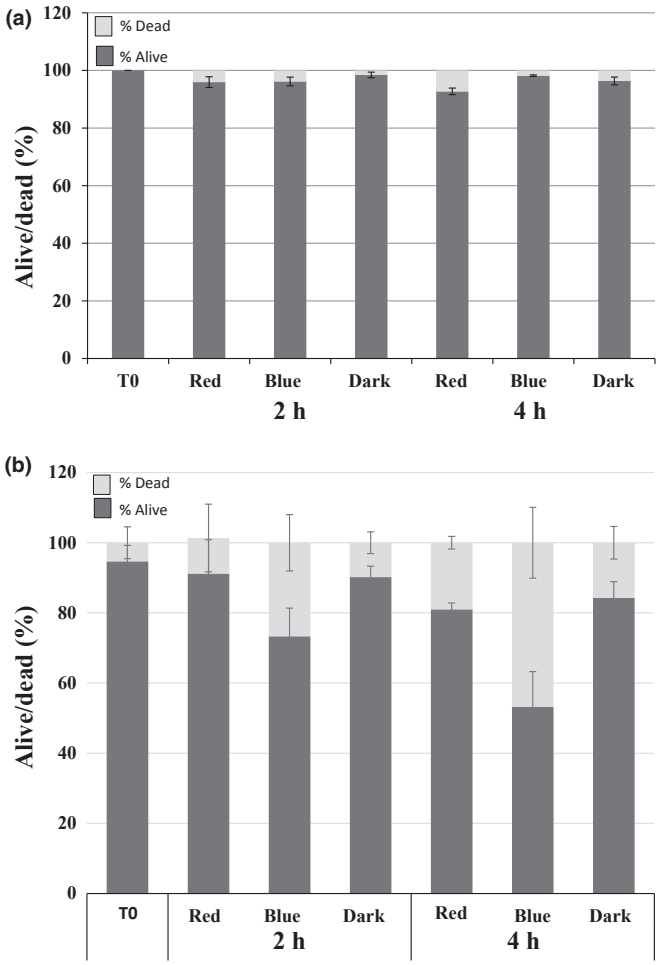

Fig. 1 (a) Cell viability of *Spodoptera frugiperda* Sf21 insect cell control cultures in response to high light stress. Control cell cultures that do not express *Arabidopsis thaliana* Atcry1 were evaluated for percentage of cell death (dead) or survival (alive) subsequent to illumination in high (100 μmol m^{-2} s^{-1}) light. Light conditions (dark, red, blue) and illumination time in hours are indicated. T0, culture cell counts at the beginning of the experiment. Error bars represent ± SD of six independent measurements. (b) Cell viability in insect cell culture in response to cryptochrome activation. *Spodoptera frugiperda* Sf21 insect cells expressing *Arabidopsis thaliana* Atcry1 were evaluated for percentage of cell death (dead) or survival (alive) subsequent to illumination with red or blue light (100 μmol m^{-2} s^{-1}). Light conditions (dark, red, blue) and illumination time in hours are indicated. T0, time point at the beginning of the experiment. Error bars represent ± SD of six independent measurements.

evaluated for cell death using Trypan Blue vital stain which provides a colorimetric assay effectively distinguishing living from dead insect cells.

First, we verified the resistance of control SF21 cell cultures which had not been infected with Atcry1 to light stress (Fig. 1a). Under the indicated conditions of illumination, there was no significant cell death in response to blue or red light indicating that cell cultures are resistant to this light stress. We then tested cell cultures expressing Atcry1. After only 2 h of blue light illumination, Atcry1-expressing cells showed a significant increase in cell death as compared with uninfected control cells (Fig. 1a) reaching up to 46% cell death after a 4 h period. By contrast, cell death in cultures maintained under high-intensity red light or in darkness was not dramatically increased.

From these data it can be concluded that cryptochrome expression was deleterious to these cell cultures under conditions where the photoreceptor is activated by blue light.

Activation of Atcry1 by blue light leads to ROS formation in insect cell cultures

We next determined whether this effect of Atcry1 activation on cell death in insect cell cultures could be explained by the production of ROS. A sensitive and rapid test for production of H_2O_2 is the Amplex Red bioassay, which reacts with H_2O_2 with a 1 : 1 stoichiometry to produce highly fluorescent resorufin that can be readily detected by spectroscopic means. This assay has been successfully used with cell cultures, where H_2O_2 can be assayed in the culture medium (Martino & Castello, 2011), and was performed with insect cell cultures expressing Atcry1 under the illumination conditions used in Fig. 1.

The results of this analysis show that insect cells expressing Atcry1 show significantly elevated concentrations of H_2O_2 in the culture media in blue light compared with unilluminated (dark) cells or cells maintained under red light (Fig. 2). This increase in ROS production was not observed in control nonexpressing insect cells incubated under blue light. ROS production occurs rapidly (within 2 h) and under conditions that activate cryptochromes (blue light), and therefore is specific to the biological activity of Atcry1. We note that a small (relative to blue light) increase in cell death and ROS production also occured under red light in Atcry1-expressing insect cells. This could be explained by a small concentration of flavin in the neutral radical form bound to Atcry1 in insect cells. The neutral radical form of flavin absorbs somewhat in red light (Beel *et al.*, 2012) and thereby produces ROS (Müller & Ahmad, 2011). In either event, increased ROS formation as a result of cryptochrome activation

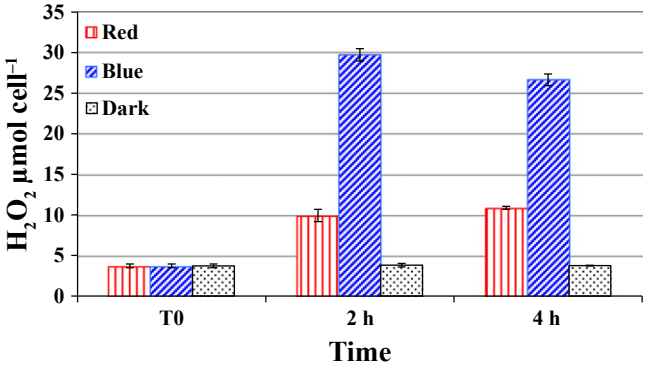

Fig. 2 Accumulation of reactive oxygen species (ROS) in *Spodoptera frugiperda* Sf21 cell cultures expressing *Arabidopsis thaliana* Atcry1. Concentrations of H_2O_2 were determined by AMPLEX RED fluorescence detection kit (see the Materials and Methods section) measured by fluorimetry. The concentration of peroxide detected was normalized to the number of surviving cells in the cultures as previously described (Martino & Castello, 2011). Error bars represent ± SD of six independent measurements.

can explain the increased cell death observed in blue light in these cell cultures (Fig. 1b).

Oxygen concentration dependence of the Atcry1 response in insect cell cultures

In the case of isolated cryptochrome, the amount of ROS formation and oxygen consumption of the protein is exquisitely sensitive to the concentration of ambient oxygen, in addition to the intensity of blue light (Müller & Ahmad, 2011). We therefore explored the effect of lowering the ambient oxygen concentration (to 3%) on the evolution of ROS in the insect cell cultures. Lowering the oxygen concentration in the cell cultures was achieved by purging culture flasks with defined oxygen concentrations as described by Côme & Tissaoui (1968) and sealing the vials before placing them under the test light conditions for determination of cell viability and ROS accumulation.

We initially expected that cell cultures maintained at low oxygen concentration would show enhanced cellular survival in blue light due to decreased ROS synthesis by the cryptochrome. Instead, we observed that after 2 h, the situation was similar for cells maintained at 3% oxygen environment (Fig. 3a) and those at atmospheric oxygen (21%). In both cases, there was an increase in the levels of cell death after 2 h under blue light illumination as compared with red light illumination or darkness (compare with Fig. 1b). Therefore, after 2 h an effect of lowering the oxygen concentration was not apparent. However, at longer illumination times (4 h) the situation was reversed. The cell cultures illuminated in red light or maintained in darkness actually showed markedly reduced cell viability as compared with blue-light-illuminated cells where cryptochrome is activated (Fig. 3a). This observation is in marked contrast to cellular viability in atmospheric oxygen conditions, where there was no decrease in viability in dark or red light (Fig. 1b). Evidently, we had not taken into consideration the fact that 3% oxygen is by itself toxic to cell cultures, consistent with decreased metabolic activity and the onset of hypoxia. Therefore, this experiment led to the unexpected observation that cryptochrome activation actually protected cell cultures and enhanced their resistance to the stress of hypoxia and low oxygen concentration (Fig. 3a – compare cell death in red light and dark vs blue light).

We next evaluated the production of ROS under low oxygen concentration (Fig. 3b). As for previous experiments (Fig. 2), it can be seen clearly that cryptochrome activation by blue light resulted in a significant increase in ROS formation. However, in cells kept at atmospheric (21%) oxygen (Fig. 2 – see 'dark' values) ROS formation does not increase in dark-grown cell cultures over time, whereas there was a significant increase in ROS formation in the dark after even 2 h incubation at low 3% oxygen concentration (Fig. 3b – see 'dark' values). This response necessarily occurs independently of cryptochrome activation, because cryptochromes are not activated in the dark. The increased ROS formation in the dark at low oxygen concentration therefore appears to be an endogenous cellular response to the stress of hypoxia.

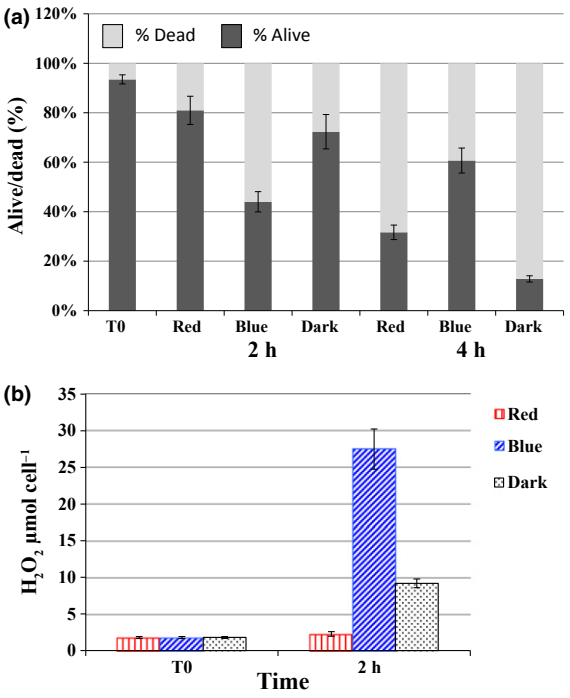

Fig. 3 (a) Cell death at 3% oxygen concentration. *Spodoptera frugiperda* Sf21 insect cells expressing *Arabidopsis thaliana* Atcry1 were evaluated for percentage of cell death (dead) or survival (alive) subsequent to illumination with red or blue light for the indicated times (2 or 4 h). Error bars represent ± SD of six independent measurements. (b) Accumulation of reactive oxygen species (ROS) at 3% oxygen concentration. *Spodoptera frugiperda* Sf21 insect cells expressing *Arabidopsis thaliana* cry1 were evaluated for concentrations of H_2O_2 in the extracellular medium using the AMPLEX RED fluorescence detection kit (see the Materials and Methods section) measured by fluorimetry. The concentration of peroxide detected was normalized to the number of surviving cells in the cultures as previously described (Martino & Castello, 2011) Error bars represent ± SD of six independent measurements.

We further evaluated the effect of blue light illumination after a shorter 1-h time period, which does not appear to induce cell death resulting from low oxygen concentration. In this case (3% oxygen), cells do not show cell death in the absence of cryptochrome action (Fig. 4 – see dark and red light samples). Under these conditions there is little detectable cell death resulting from cryptochrome activity (Fig. 4 – see blue) under low oxygen (3%), whereas cells from the identical culture incubated at atmospheric (21%) oxygen show significantly enhanced cell death (Fig. 4). These results are consistent with the direct conversion of oxygen to peroxide through cryptochrome flavin reoxidation as previously described (Müller & Ahmad, 2011).

In summary, we find that lowering the oxygen concentration alters the cryptochrome response in cell cultures. At shorter illumination times (1 h) cells are more resistant to blue light (and cryptochromes) likely because concentrations of ROS are not as high under this lower oxygen concentration (Fig. 4). However, after longer time periods the effect of a cryptochrome is reversed – even though ROS formation likely continues under high blue light, the cell cultures are protected from the severe effects of hypoxia as a result of cryptochrome activation.

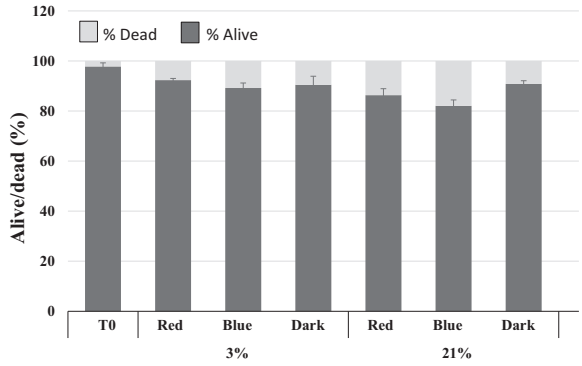

Fig. 4 Cell death of cryptochrome-expressing cell cultures after 1 h incubation. *Spodoptera frugiperda* Sf21 insect cells expressing *Arabidopsis thalinana* Atcry1 were evaluated for percentage of cell death (dead) or survival (alive) subsequent to illumination with red or blue light at the indicated (3% or 21%) oxygen concentrations. Light conditions and illumination time in hours are indicated. Error bars represent ± SD of six independent measurements.

The simplest explanation for these results is that conditions of stress resulting from hypoxia by themselves lead to increased ROS formation in insect cell cultures, independently of any function of cryptochromes. The much more rapid and high spike in ROS concentration resulting from cryptochrome activation (Fig. 3b) may thereby jump-start the endogenous cellular defensive response and lead to greater resistance to low oxygen concentration.

Illumination of isolated Atcry1 protein produces ROS at concentrations consistent with that of Atcry1-expressing cell cultures

From the data described earlier, it is not clear whether concentrations of ROS observed in cell culture media correlate with direct enzymatic action of the expressed Atcry1. In other words, it must be verified whether there is enough Atcry1 produced in these cell cultures to explain the observed concentrations of ROS formation. In order to address this question, we first determined the amount of Atcry1 protein that can be isolated from a given number of insect cells. From 100 ml of cell culture (*c.* 10^8 cells), we isolated a preparation of 1 ml of purified Atcry1 protein at a 100 μM concentration. In the cell culture assays, we used a sample of 1 ml undiluted cell culture (see the Materials and Methods section). Therefore, to obtain comparable values for ROS formation from the purified protein as for the cryptochrome expressed in the cell cultures, the purified protein sample was diluted 100-fold before H_2O_2 detection (see the Materials and Methods section). In this way, the concentration of purified protein analyzed *in vitro* was comparable to the concentration of protein present in 1 ml of expressing cell cultures (i.e. *c.* 1 μM). No reducing agent such as βME or DTT was added to samples to enhance photoreduction (Bouly *et al.*, 2007) as this perturbed the fluorescence assay. Nonetheless, even in these suboptimal conditions, concentrations of ROS visibly rose over time in purified Atcry1 samples (Fig. 2). Furthermore, when normalized to cell number in the cultures from which the proteins were isolated, the concentrations of ROS produced *in vitro* by purified Atcry1 protein – although somewhat increased – are not dramatically distinct from concentrations of ROS produced by Atcry1 insect cell cultures in blue light (Fig. 5a).

All of these experiments (Figs 1–5a) assume that photochemical conversion of molecular oxygen to ROS occurs through Atcry1 flavin photochemistry by the previously deduced mechanism (Müller & Ahmad, 2011). We therefore directly verified that flavin reduction indeed occurred both in isolated proteins and in whole cell cultures under the illumination conditions used in this study. Purified isolated Atcry1 protein was illuminated at the high-intensity (3000 μmol m^{-2} s^{-1}) white light used for the ROS assay in Fig. 5(a). Oxidized flavin (Fig. 5b, left panel, solid line) showed clear evidence of radical formation upon illumination (dotted line), with decrease in absorbance at 450 nm and increase between 500 and 600 nm. This occurred even though no reducing agent was present, indicating that under our experimental conditions (Fig. 5a), ROS synthesis follows from Atcry1 photoreduction. Atcry1 flavin photoreduction

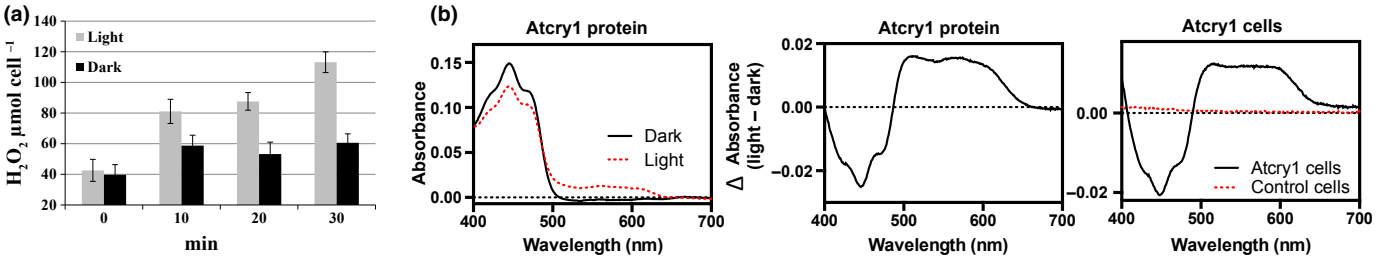

Fig. 5 (a) Reactive oxygen species (ROS) production *in vitro* by purified isolated *Arabidopsis thaliana* Atcry1 protein. *x*-axis, illumination time (min) under white light (3000 μmol m^{-2} s^{-1}). Concentration of peroxide was normalized to number of expressing cells used in protein sample preparation. (b) *Arabidopsis thaliana* Atcry1 flavin photoreduction in isolated proteins and in whole expressing *Spodoptera frugiperda* Sf21 cells. Left panel, spectra of purified Atcry1 protein before (dark) and after (light) illumination with 3000 μmol m^{-2} s^{-1} white light. Illumination and buffer conditions are identical with those for ROS assay (a). Middle panel, light–dark difference spectrum of traces from the left panel (isolated protein). Right panel, cell culture pellets were diluted five-fold into lysis buffer of 50 mM Tris pH 7.5, 1% Triton and centrifuged at 14 000 ***g*** for 30 min at 4°C to obtain clear lysates. Lysates were placed into a spectrophotometer and spectra taken before (dark) or after (light) illumination at 100 μmol m^{-2} s^{-1} blue light. Light–dark difference spectra from Atcry1-expressing (solid line) and control nonexpressing (control) cells (dotted red line) are shown.

is known to occur in whole expressing insect cells using a variety of spectroscopic approaches (Bouly *et al.*, 2007; Burney *et al.*, 2012). To confirm that this also occurs under our illumination conditions, we examined extracts from whole cells in which Atcry1 protein can be directly visualized optically (Engelhard *et al.*, 2014). Whole cell lysates from both Atcry1-expressing and control cells were taken either before (dark) or after (light) illumination with 100 $\mu mol\, m^{-2}\, s^{-1}$ blue light, as used in Figs 1–4. The 'light' scan was subtracted from the 'dark' scan to provide a so-called 'difference spectrum' (light–dark) that detects any absorbance change in the sample after illumination and therefore any photochemical reaction. As can be seen (Fig. 5b, right panel, solid line), there is clear evidence of flavin radical formation in Atcry1-expressing cells but none in control cultures (Fig. 5b, right panel, red dotted line). The difference spectrum obtained from Atcry1-expressing cell cultures is moreover essentially identical to that obtained from the isolated purified protein (Fig. 5b, middle panel). It can therefore be concluded that increased concentrations of ROS in cry1-expressing cell cultures is consistent with direct enzymatic conversion of O_2 by cryptochromes (Müller & Ahmad, 2011).

Colocalization of cryptochromes with sites of ROS production in Atcry1-expressing insect cell cultures

In order to obtain definitive proof linking ROS accumulating in insect cell cultures to the enzymatic activity of cryptochromes, it is necessary that a cryptochrome colocalizes with the site of ROS formation in these cell cultures. To test this prediction, we first performed immunolocalization studies of cryptochromes in expressing insect cells (Fig. 6), which were stained with DAPI in order to identify the nucleus. To unequivocally assess the subcellular localization of Atcry1, we used a classical method to label cells before or after permeabilization (Mottola *et al.*, 2000). Without permeabilization, the antibody crosses the plasma membrane very poorly and recognizes mainly the Atcry1 protein present at or near the cell surface. After permeabilization of cells with 0.1% Triton, antibodies can cross the cell membrane and label protein within the entire cell including the nucleus.

Labeling with a polyclonal anti-Atcry1 antibody showed no signal in control uninfected cells. In Atcry1-expressing cells, immunolabeled without permeabilization, there was clear protein signal outside the nucleus, preferentially at the cellular membrane in large concentrated zones (Fig. 6e). This is clearly seen in cells observed at higher magnification on the optical section across the nucleus (Fig. 6h,i). Images performed with permeabilized cells, showed the cryptochrome within the entire cytoplasm and also within the nucleus itself (Fig. 6k,l). Here it is found mainly within the interstitial spaces within the chromatin and not apparently associated with the chromatin itself (see arrows on Fig. 6k,l). This distribution is consistent with that reported in plants (Yang *et al.*, 2000), where cryptochromes are localized in both cytosolic and nuclear compartments. A 3D image of the immunolocalization of the cryptochrome in insect cells is further included in Supporting Information Video S1 (nonpermeabilized cells) and Video S2 (permeabilized cells).

In order to better visualize Atcry1 in subnuclear structures, we performed a 3D fluorescence imaging analysis of a cry1 positive nucleus by using TANGO, a powerful image analysis tool dedicated to the study of nuclear architecture (Ollion *et al.*, 2013). With this tool we analyzed Atcry1 staining in the entire nucleus independently from the cytoplasmic Atcry1 staining. First nuclei are segmented from the DAPI stained images, then green channels corresponding to Atcry1 staining are cropped around the segmented nuclei, and finally cry1 staining contained in subnuclear structures can be segmented. A representative result can be visualized by a 3D representation movie in Video S3 where a cry1-positive structure can be clearly seen throw the nucleus by transparency.

In order to evaluate the site of formation of ROS in these cells, we used a fluorescent stain (hydroxyphenyl fluorescein) that can directly visualize H_2O_2 within living cells (Lariguet *et al.*, 2013). Living insect cells were treated with fluorescent substrate and then either kept in the dark or illuminated with blue light (Fig. 7). Under these conditions, no ROS was detected in uninfected SF21 control cell cultures, and very little in SF21 expressing insect cells kept in darkness (Fig. 7, top panels). However, illumination with just 10 min of blue light resulted in accumulation of ROS within cells. Furthermore, the localization of the accumulated ROS was in both cytoplasmic and nuclear compartments, consistent with the immunolocalization studies (Fig. 6). ROS formation was particularly abundant in vesicular structures surrounding the nucleus that perfectly match endoplasmic reticulum observed by DIC (Fig. 7g,h).

In summary, ROS formation occurs on a rapid timescale consistent with cryptochrome activation and is colocalized within the same cellular compartments.

Cryptochromes and ROS formation in plant protoplasts

The role of cryptochromes in plant pathogen response, high light stress and programmed cell death (PCD) has been documented in numerous studies (Chaves *et al.*, 2011). However, no study has analyzed whether cryptochromes by themselves directly synthesize ROS in living plant cells. This question has been difficult to address, because most forms of oxidative stress resulting from high light have been linked to formation of ROS in the chloroplast. A direct involvement of cryptochrome in production of reactive oxygen is therefore unlikely, because cryptochromes with known signaling roles such as Atcry1 and Atcry2 are not localized to the chloroplast. The involvement of cryptochrome in responses to ROS is thought to be indirect, either through regulating the biosynthesis of the photosynthetic apparatus (including light-absorbing antenna and photoprotective pigments such as anthocyanins), or else by interacting with defense and stress response signaling pathways through effects on transcriptional activators in the nucleus such as hyh, hy5 and so on (Chaves *et al.*, 2011).

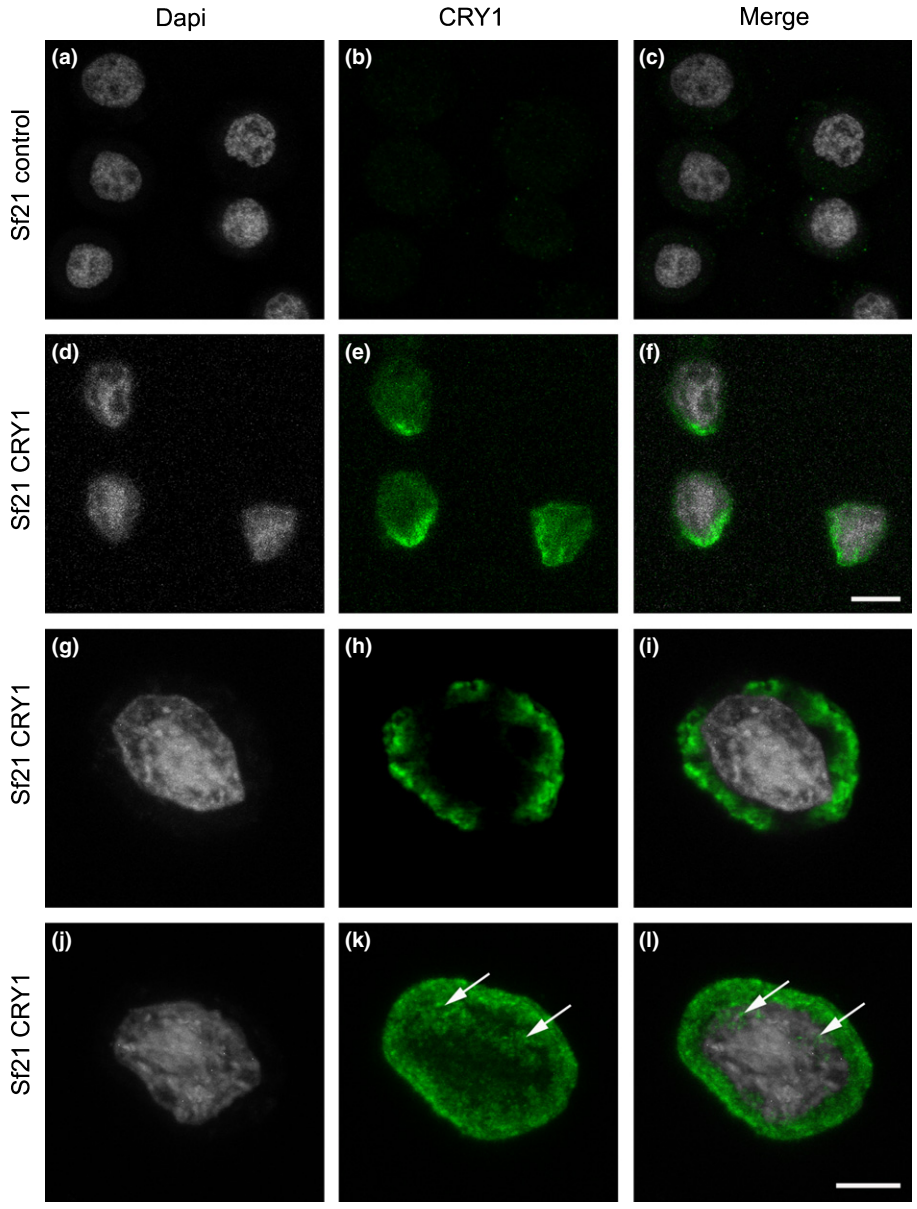

Fig. 6 Subcellular localization of *Arabidopsis thaliana* Atcry1 in expressing Sf21 *Spodoptera frugiperda* insect cells by immunofluorescence and confocal microscopy. Sf21 control cells and cells stably expressing Atcry1 were fixed with paraformaldehyde, permeabilized with Triton X100 (j–l) or not (a–i), incubated with an anti-cry1 rabbit polyclonal antibody and an Alexa 488-conjugated anti-rabbit secondary antibody, DNA were stained with 4′,6′-diamino-2-phenylindole (DAPI). Cells were observed with a Leica TCS SP5 confocal microscope. Images (a–f) show projections of optical sections (bar, 10 μm). Images (g–l) show single confocal z-section (bar, 5 μm). CRY 1 is clearly localized on cell membrane of nonpermeabilized cells (g–i) and in the entire cytoplasm as well as in the nucleoplasm (arrows) of TX-100 permeabilized cells (j–l).

Nonetheless, in light of the results of our insect cell culture experiments, we wondered whether similar effects might be observable in plants. In order to keep conditions as comparable as possible with the insect cell culture experiments, we addressed the question of cryptochrome and ROS formation using cell cultures of plant protoplasts, which can be readily assayed for cell death and ROS production in response to high light stress as were insect cells. To determine the role of cryptochromes, WT and *cry1cry2* double mutant *Arabidopsis* lines were compared. The *cry1cry2* double mutant lacks both Atcry1 and Atcry2 and therefore retains no cryptochrome responsivity. Because cryptochrome function has profound indirect effects on plant growth and morphology, *Arabidopsis* seedlings for this study were germinated and grown under continuous low-intensity (5 $\mu mol\ m^{-2}\ s^{-1}$) red light for 2 wk before protoplasting. This wavelength and intensity of light does not activate cryptochromes, so that seedlings grown under these conditions should not have undergone any indirect developmental or morphological effects resulting from action of a cryptochrome. In other words, both WT and *cry1cry2* double mutant protoplasts should be in all respects physiologically identical at the beginning of blue light illumination.

We first examined the effect of high light illumination on WT *Arabidopsis* seedlings (Fig. 8). Protoplasts were isolated (see the Materials and Methods section) and illuminated with high-intensity blue light (at 100 $\mu mol\ m^{-2}\ s^{-1}$) or else kept in darkness. Cell death was monitored by staining with Trypan blue to distinguish living from dead cells. We found that a significant (as determined by standard deviation) increase in number of cells staining blue could be observed after even a 2 h illumination period, consistent with cell death caused by high light stress (Fig. 8a). Interestingly, cell death occurred fairly randomly among cell types within the sample and not just within photosynthetic cells. Therefore, the phenomena we are observing is not exclusively due to classic high light effects on plants

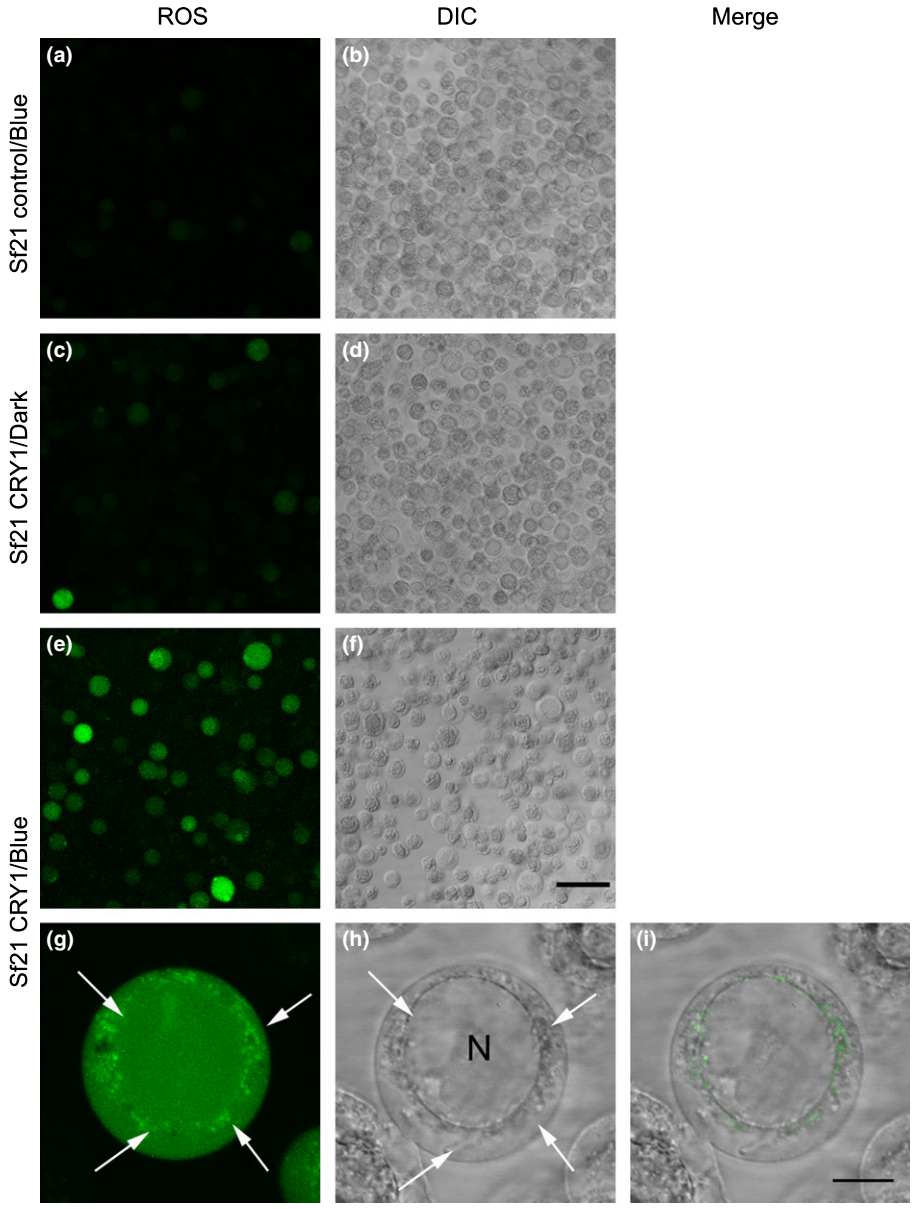

Fig. 7 Production and subcellular localization of reactive oxygen species (ROS) by *Spodoptera frugiperda* Sf21 cells expressing *Arabidopsis thaliana* Atcry1 exposed to blue light. Living Sf21 control cells and stably expressing CRY1 were exposed to dark or blue light, treated with DCFH-DA (5-(and-6)-chloromethyl-2′,7′-dichlorofluorecein diacetate) and viewed by an inverted Leica TCS SP5 microscope. Images (a–f) show maximal projection of optical sections (bar, 100 μm). Images (g–i) show single confocal *z* section and higher magnitude from images (e, f). Diffused fluorescent ROS staining can be seen in nucleus (N) and cytoplasm. Punctuate and intense fluorescent ROS staining colocalizes perfectly with ER (arrows) observed all around nucleus by differential interference contrast (DIC). Bar, 10 μm.

resulting from ROS induced through the photosynthetic apparatus (Apel & Hirt, 2004). We next determined the concentrations of ROS formed in response to light stress by a fluorescence-based assay of protoplast cell cultures. The results showed significant induction of ROS in blue light (Fig. 8b), consistent with the increase in cell death under these conditions (Fig. 8a).

We next determined whether cryptochromes have an effect on high light sensitivity in such plant protoplasts. Accordingly, protoplasts were made from the *cry1cry2* double mutant, which lacks both Atcry1 and Atcry2, and were evaluated for cell death under blue light illumination conditions (Fig. 9a). We found that blue light illumination for short time periods was significantly less effective in inducing cell death (Fig. 9a) in these *cry1cry2* mutants than in wild-type seedlings (compare with Fig. 8a). In particular, after 2 h illumination cell death in protoplasts was not significantly increased as compared with levels in darkness. Therefore, the lack of a functional cryptochrome appears to contribute somewhat to resistance to blue light stress in plant protoplasts.

Formation of ROS under blue light illumination was also altered in the *cry1cry2* double mutant protoplasts as compared with WT seedlings (Fig. 9b). Under high blue light conditions these *cry1cry2* double mutant seedlings did not show concentrations of ROS accumulation significantly above

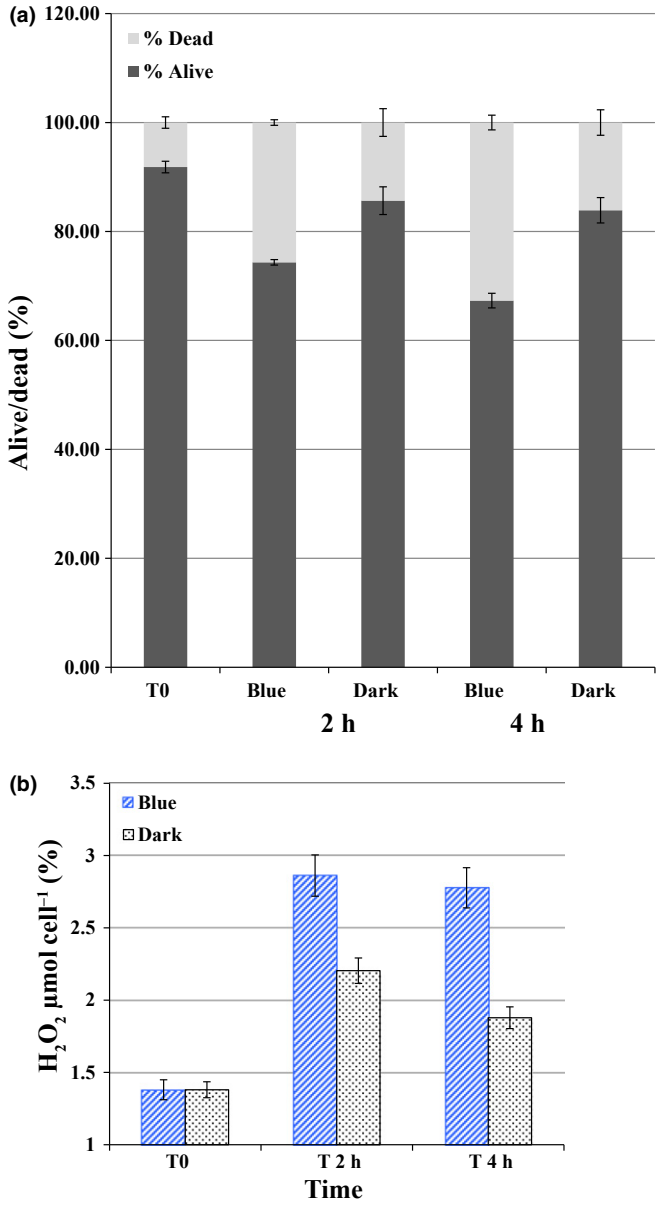

Fig. 8 (a) Percentage of cell survival in protoplasts of wild-type (WT) *Arabidopsis* seedlings in blue light. Times (2 h or 4 h) are indicated. T0, time point at the beginning of the experiment. Error bars represent ± SD of six independent measurements. (b) Accumulation of reactive oxygen species (ROS) in cell cultures of WT *Arabidopsis* protoplasts. Concentrations of hydrogen peroxide (H_2O_2) were determined by AMPLEX RED fluorescence detection kit (see the Materials and Methods section) measured by fluorimetry. The concentration of peroxide detected was normalized to the number of surviving cells in the cultures as previously described (Martino & Castello, 2011). Error bars represent ± SD of six independent measurements.

dark controls (Fig. 9b, see 2 and 4 h time points). This is in contrast to the response of WT protoplasts, which show significantly elevated concentrations of ROS produced as a result of blue light illumination after already 2 h (Fig. 8b). As a result, these data suggest a role for cryptochromes in ROS formation in plant protoplasts in response to high blue light stress.

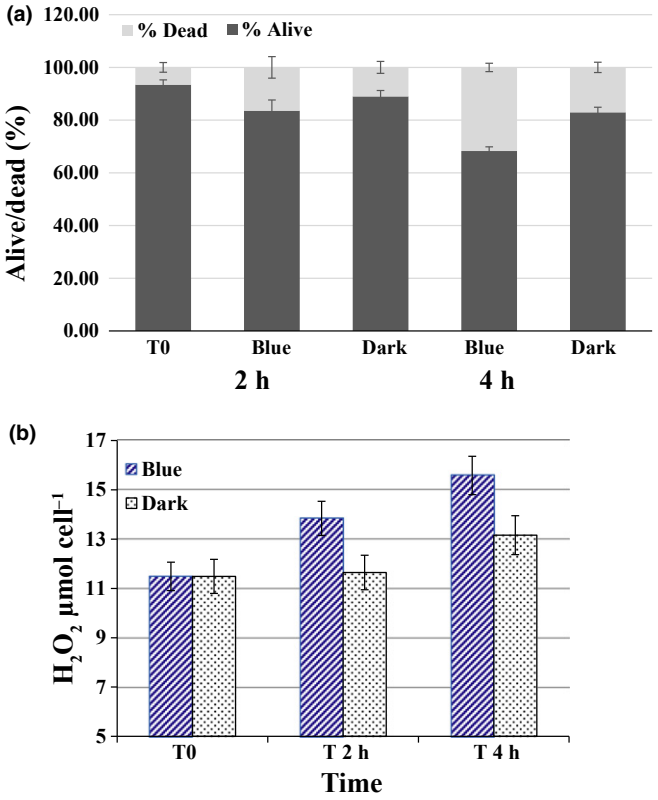

Fig. 9 (a) Percentage of cell survival of protoplasts of *cry1cry2 Arabidopsis* seedlings in blue light. Times (2 h or 4 h) are indicated. T0, time point at the beginning of the experiment. Error bars represent ± SD of six independent measurements. (b) Accumulation of reactive oxygen species (ROS) in cell cultures of *cry1cry2 Arabidopsis* protoplasts. Concentrations of hydrogen peroxide (H_2O_2) were determined by AMPLEX RED fluorescence detection kit (see the Materials and Methods section) measured by fluorimetry. The concentration of peroxide detected was normalized to the number of surviving cells in the cultures as previously described (Martino & Castello, 2011). Error bars represent ± SD of six independent measurements.

Discussion

The goal of this study is to explore whether a direct enzymatic conversion of O_2 to ROS by cryptochromes may occur in living systems and have physiological consequences. Our results provide conclusive evidence that indeed, in a heterologous system such as SF21 insect cells, ROS formation can be linked directly to activation of cryptochromes and this induction has physiological consequences including cell death. Because it is highly unlikely that signaling components specific to any kind of PCD response in insect cell cultures could recognize a heterologous plant cryptochrome, we conclude that cell death almost certainly arises as a direct consequence of ROS formation in the course of the cryptochrome photocycle (Müller & Ahmad, 2011). Therefore, this is the first reported instance of the physiological effect of a cryptochrome that depends exclusively on its enzymatic properties in the formation of reactive oxygen and peroxide *in vivo*.

An additional feature of the cryptochrome-dependent ROS formation in insect cell cultures was their effect on cellular

survival under conditions of hypoxia (low oxygen concentration). These experiments were initially undertaken in order to determine whether low oxygen concentration results in reduced ROS formation by cryptochromes and thereby reduced cell death. This prediction arises from the enzymatic properties of cryptochromes and their (reduced) activity at low oxygen concentration (Müller & Ahmad, 2011). In keeping with this prediction, after a short illumination time (1 h) there was indeed reduced cell death under low oxygen concentration (3%) as compared with atmospheric oxygen (21%) (Fig. 4). A direct enzymatic role for cryptochromes in cell death by means of formation of H_2O_2 and ROS could therefore be further strengthened.

However, an unexpected finding of the effect of low oxygen concentration was that at longer illumination times (4 h), cryptochromes actually played a protective role. Under conditions of low oxygen, cell cultures die as a result of hypoxia, as can be seen from the high percentage of cell death after 4 h continuous darkness (Fig. 3a). Under blue light illumination (and therefore activation of Atcry1) there was significantly less cell death after 4 h incubation in low oxygen than in dark controls. This indicates that that activation of the cryptochrome resulted in induction of resistance (defensive mechanisms) to hypoxia. Consistent with this interpretation, we noted elevated concentrations of ROS in cell cultures subjected to low oxygen concentrations, even in the dark (Fig. 3b). This indicates that hypoxia leads to the onset of oxidative stress and ROS formation independently of any action of cryptochromes; however, the concentrations of ROS are lower than under blue light illumination. The likely explanation of the protective effect of Atcry1 on insect cell cultures under conditions of hypoxia is to provide a large early burst of ROS compared with cell cultures maintained in darkness. This burst of ROS then releases downstream signaling pathways that lead to protective mechanisms. Examples of both protective or toxic effects of ROS abound in the literature (Mittler *et al.*, 2011) and in the case of insect cells ROS signaling may play a part in the endogenous response to hypoxia. These results are therefore further consistent with a physiological role resulting from enzymatic ROS formation by cryptochrome photoreceptors.

Production of ROS in animal cells is thought to occur primarily as a result of metabolic activity in the mitochondria (Holmström & Finkel, 2014). By contrast, plant cryptochromes have been reported to be localized to cytosolic and nuclear cellular compartments (Guo *et al.*, 1999; Kleiner *et al.*, 1999; Yang *et al.*, 2000). Consistent with this data, immunolocalization studies for Atcry1 performed in insect cell cultures show that Atcry is localized to both nuclear and cytosolic compartments. Fluorescent staining showed that H_2O_2 is localized to the same compartments as the Atcry1 protein. Moreover, that formation of ROS is clearly apparent only 10 min after the onset of illumination, a timescale too short for significant diffusion across membrane compartments. These results suggest that increased ROS formation in insect cells is most likely to result directly from enzymatic action of Atcry1, and not involve any endogenous signaling mechanisms.

Amounts of cryptochromes in plant cells are at least 100-fold less concentrated than in insect cells (M. Ahmad, unpublished) and therefore plant cells should not form as large concentrations of ROS as in insect cell cultures in response to Atcry enzymatic conversion of O_2. Nonetheless, illumination of plant cell protoplasts obtained from *Arabidopsis* seedlings revealed increased cell death and ROS formation induced after even 2 h illumination in high-intensity blue light. Significantly, both cell death and ROS formation in blue light was somewhat reduced in cryptochrome double mutant seedlings (*cry1cry2* mutants). Furthermore, cell death resulting from the action of cryptochromes occurred in both photosynthetic and nonphotosynthetic protoplasts at apparently equal efficiency, and also in protoplasts from dark grown etiolated seedlings (not shown), suggesting that evolution of ROS did not require the photosynthetic apparatus. These effects, although significant, are rather weak, and need to be confirmed by additional studies and also in other systems.

It cannot be concluded that the elevated ROS concentrations measured in the cell cultures result directly from enzymatic conversion of oxygen to ROS by cryptochromes in this material. The concentrations of cryptochrome are simply too low (almost 100× lower than in transfected insect cell cultures – M. Ahmad, unpublished) to account for the concentrations of ROS being synthesized in plant protoplasts, unless enzymatic conversion is in some way dramatically enhanced. It is nevertheless possible that an initial burst of ROS formation resulting from the cryptochrome – which occurs within both cytosolic and nuclear cellular compartments – provides sufficient stress to the plant protoplasts to release further ROS signaling and/or synthetic cascades.

This role for cryptochromes in plant cell protoplasts appears to occur by a mechanism that is distinct from the well-studied PCD and apoptosis response (Danon *et al.*, 2006; Kim & Apel, 2013). In these prior studies, cryptochromes did not affect ROS formation *per se* (singlet oxygen accumulates in the plastids as a result of the *flu* sensitizer mutation). Instead, the role of Atcry1 was determined to be as a downstream transcriptional activator for expression of singlet-oxygen induced genes required for the PCD response. In fact, no effect of Atcry1 on PCD was documented in the absence of the *flu* mutation, which results in increased production of singlet oxygen in the plastid and is required for the subsequent effect of Atcry1 (Danon *et al.*, 2006; Kim & Apel, 2013). By contrast, under the much higher blue light illumination used in our present study (100 $\mu mol\ m^{-2}\ s^{-1}$), the cryptochrome effect on ROS accumulation occurs in the absence of the *flu* mutation and appears to be modulated by Atcry1 by some other means. A more recent study has shown reduced ROS accumulation and cell death in response to high light stress in cry1 and cry2 mutants after a shorter illumination in high white light (Chen *et al.*, 2013), more consistent with our present results. These authors proposed that the downstream transcriptional effects of cryptochrome on induction of stress-regulated genes were responsible for the reduced ROS production and cell death they observed in cryptochrome mutants. However, in our findings we see effects on cell viability already after 2 h, which do not appear readily explainable by mechanisms that require *de novo* transcription/protein synthesis. Further experiments will be needed to resolve what effects a direct enzymatic conversion of

molecular oxygen to ROS by cryptochromes may have on these responses.

In conclusion, cryptochromess are involved in numerous cellular processes in multiple organisms that implicate formation of ROS. In flies, circadian clock function has been linked to bursts of oxidative stress; whereas blue-light dependent seed germination, cell death and de-etiolation is known to be under the control both of ROS and cryptochromes (Chen *et al.*, 2013; Barrero *et al.*, 2014; Hoang *et al.*, 2014). A role for ROS in photoprotective and stress responses in mammalian cell cultures has also been extensively documented (Holmström & Finkel, 2014). The current widely accepted paradigm for cryptochrome activation involves light-induced conformational change followed by interaction with downstream signaling proteins to provide a versatile light activated switch with many different output functions. Here we present an additional possibility of cryptochromes playing a direct enzymatic role in the generation of ROS signals which may have impacted on responsivity to stress in multiple systems through the course of evolution.

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References

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55**: 373–399.

Bailly C, El-Maarouf-Bouteau H, Corbineau F. 2008. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Comptes Rendus Biologies* **331**: 806–814.

Barrero JM, Downie AB, Xu Q, Gubler F. 2014. A role for barley CRYPTOCHROME1 in light regulation of grain dormancy and germination. *Plant Cell* **26**: 1094–1104.

Beel B, Prager K, Spexard M, Sasso S, Weiss D, Müller N, Heinnickel M, Dewez D, Ikoma D, Grossman AR *et al.* 2012. A flavin binding cryptochrome photoreceptor responds to both blue and red light in *Chlamydomonas reinhardtii*. *Plant Cell* **24**: 2992–3008.

Bouly JP, Schleicher E, Dionisio-Sese M, Vandenbussche F, Van Der Straeten D, Bakrim N, Meier S, Batschauer A, Galland P, Bittl R *et al.* 2007. Cryptochrome blue light photoreceptors are activated through interconversion of flavin redox states. *Journal of Biological Chemistry* **282**: 9383–9391.

Burney S, Wenzel R, Kottke T, Roussel T, Hoang N, Bouly JP, Bittl R, Heberle J, Ahmad M. 2012. Single amino acid substitution reveals latent photolyase activity in *Arabidopsis* cry1. *Angewandte Chemie International Edition* **51**: 9356–9360.

Chaves I, Pokorny R, Byrdin M, Hoang N, Ritz T, Brettel K, Essen LO, van der Horst GT, Batschauer A, Ahmad M. 2011. The cryptochromes: blue light photoreceptors in plants and animals. *Annual Review of Plant Biology* **62**: 335–364.

Chen D, Xu G, Tang W, Jing Y, Ji Q, Fei Z, Lin R. 2013. Antagonistic basic helix-loop-helix/bZIP transcription factors form transcriptional modules that integrate light and reactive oxygen species signaling in *Arabidopsis*. *Plant Cell* **25**: 1657–1673.

Côme D, Tissaoui T. 1968. Induction d'une dormance embryonnaire secondaire chez le pommier (*Pirus malus* L.) par des atmospheres très appauvries en oxygene. *Comptes Rendus de l'Académie des Sciences, Paris* **266**: 444–479.

Danon A, Coll NS, Apel K. 2006. Cryptochrome-1-dependent execution of programmed cell death induced by singlet oxygen in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **103**: 17 036–17 041.

Engelhard C, Wang X, Robles D, Moldt J, Essen L-O, Batschauer A, Bittl R, Ahmad M. 2014. Cellular metabolites enhance light sensitivity through alternate electron transfer pathways in *Arabidopsis* cryptochrome. *Plant Cell* **26**: 4519–4531.

Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C. 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays* **28**: 1091–1101.

Guo H, Duong H, Ma N, Lin C. 1999. The Arabidopsis blue light receptor cryptochrome 2 is a nuclear protein regulated by a blue light-dependent post-transcriptional mechanism. *Plant Journal* **19**: 279–287.

Hoang HH, Sechet J, Bailly C, Leymarie J, Corbineau F. 2014. Inhibition of germination of dormant barley (*Hordeum vulgare* L.) grains by blue light as related to oxygen and hormonal regulation. *Plant, Cell & Environment* **37**: 1393–1403.

Holmström KM, Finkel T. 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nature Reviews Molecular Cell Biology* **15**: 411–421.

Jeong RD, Chandra-Shekara AC, Barman SR, Navarre D, Klessig DF, Kachroo A, Kachroo P. 2010. Cryptochrome 2 and phototropin 2 regulate resistance protein-mediated viral defense by negatively regulating an E3 ubiquitin ligase. *Proceedings of the National Academy of Sciences, USA* **107**: 13 538–13 543.

Kim C, Apel K. 2013. Singlet oxygen-mediated signaling in plants: moving from *flu* to wild type reveals an increasing complexity. *Photosynthesis Research* **116**: 455–464.

Kleine T, Kindgren P, Benedict C, Hendrickson L, Strand A. 2007. Genome-wide gene expression analysis reveals a critical role for *CRYPTOCHROME1* in the response of Arabidopsis to high irradiance. *Plant Physiology* **144**: 1391–1406.

Kleiner O, Kircher S, Harter K, Batschauer A. 1999. Nuclear localization of the Arabidopsis blue light receptor cryptochrome 2. *Plant Journal* **19**: 289–296.

Lariguet P, Ranocha P, De Meyer M, Barbier O, Penel C, Dunand C. 2013. Identification of a hydrogen peroxide signalling pathway in the control of light-dependent germination in *Arabidopsis*. *Planta* **238**: 381–395.

Liu H, Yu X, Li K, Klejnot J, Yang H, Lisiero D, Lin C. 2008. Photoexcited *CRY2* interacts with CIB1 to regulate transcription and floral initiation in *Arabidopsis*. *Science* **322**: 1535–1539.

Lopez L, Carbone F, Bianco L, Giuliano G, Facella P, Perrotta G. 2012. Tomato plants overexpressing cryptochrome 2 reveal altered expression of energy and stress-related gene products in response to diurnal cues. *Plant, Cell & Environment* **35**: 994–1012.

Martino CF, Castello P. 2011. Modulation of hydrogen peroxide production in cellular systems by low level magnetic fields. *PLoS ONE* **6**: 1.

Miller G, Shulaev V, Mittler R. 2008. Reactive oxygen signaling and abiotic stress. *Physiologia Plantarum* **133**: 481–489.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science* **9**: 490–498.

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F. 2011. ROS signaling: the new wave? *Trends in Plant Science* **16**: 300–309.

Mottola G, Jourdan N, Castaldo G, Malagolini N, Lahm A, Serafini-Cessi F, Migliaccio G, Bonatti S. 2000. A new determinant of endoplasmic reticulum localization is contained in the juxtamembrane region of the ectodomain of hepatitis C virus glycoprotein E1. *Journal of Biological Chemistry* **275**: 24 070–24 079.

Müller P, Ahmad M. 2011. Light-activated cryptochrome reacts with molecular oxygen to form a flavin-superoxide radical pair consistent with magnetoreception. *Journal of Biological Chemistry* **286**: 21 033–21 040.

Ollion J, Cochennec J, Loll F, Escudé C, Boudier T. 2013. TANGO: a generic tool for high throughput 3D image analysis for studying nuclear organization. *Bioinformatics* **29**: 1840–1841.

Orozco-Cardenas M, Ryan CA. 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proceedings of the National Academy of Sciences, USA* **96**: 6553–6557.

Quan LJ, Zhang B, Shi WW, Li HY. 2008. Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *Journal of Integrative Plant Biology* **50**: 2–18.

Sanchez A, Shin J, Davis SJ. 2011. Abiotic stress and the plant circadian clock. *Plant Signaling & Behavior* **6**: 223–231.

Sharma P, Chatterjee M, Burman N, Khurana JP. 2014. Cryptochrome 1 regulates growth and development in *Brassica* through alteration in the expression of genes involved in light, phytohormone and stress signalling. *Plant, Cell & Environment* **37**: 961–977.

Wu L, Yang HQ. 2010. CRYPTOCHROME 1 is implicated in promoting R protein-mediated plant resistance to *Pseudomonas syringae* in *Arabidopsis*. *Molecular Plant* **3**: 539–548.

Xu P, Ma Z. 2009. Plant cryptochromes employ complicated mechanisms for subcellular localization and are involved in pathways apart from photomorphogenesis. *Plant Signaling & Behavior* **4**: 200–201.

Xu P, Xiang Y, Zhu HL, Xu HB, Zhang ZZ, Zhang CQ, Zhang L, Ma Z. 2009. Wheat cryptochromes: subcellular localization and involvement in photomorphogenesis and osmotic stress responses. *Plant Physiology* **149**: 760–774.

Yang HQ, Wu YJ, Tang RH, Liu D, Liu Y, Cashmore AR. 2000. The C-termini of *Arabidopsis* cryptochromes mediate a constitutive light response. *Cell* **103**: 815–827.

Zuo Z, Liu H, Liu B, Liu X, Lin C. 2011. Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in *Arabidopsis*. *Current Biology* **21**: 841–847.

Supporting Information

Additional supporting information may be found in the online version of this article.

Video S1 Three-dimensional (3D) localization of cell membrane CRY1.

Video S2 Three-dimensional (3D) localization of intracellular CRY1.

Video S3 Three-dimensional (3D) subnuclear localization of CRY1.

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