The Signaling State of Arabidopsis Cryptochrome 2 Contains Flavin Semiquinone*  

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Cryptochrome (Cry) photoreceptors share high sequence and structural similarity with DNA repair enzyme DNA-photolyase and carry the same flavin cofactor. Accordingly, DNA-photolyase was considered a model system for the light activation process of cryptochromes. In line with this view were recent spectroscopic studies on cryptochromes of the CryDASH subfamily that showed photoreduction of the flavin adenine dinucleotide (FAD) cofactor to its fully reduced form. However, CryDASH members were recently shown to have photolyase activity for cyclobutane pyrimidine dimers in single-stranded DNA, which is absent for other members of the cryptochrome/photolyase family. Thus, CryDASH may have functions different from cryptochromes. The photocycle of other members of the cryptochrome family, such as Arabidopsis Cry1 and Cry2, which lack DNA repair activity but control photomorphogenesis and flowering time, remained elusive. Here we have shown that Arabidopsis Cry2 undergoes a photocycle in which semireduced flavin (FADH⁺) accumulates upon blue light irradiation. Green light irradiation of Cry2 causes a change in the equilibrium of flavin oxidation states and attenuates Cry2-controlled responses such as flowering. These results demonstrate that the active form of Cry2 contains FADH⁺ (whereas catalytically active photolyase requires fully reduced flavin (FADH⁻)) and suggest that cryptochromes could represent photoreceptors using flavin redox states for signaling differently from DNA-photolyase for photorepair.

Blue light signaling has been historically of great interest in plants, because distinct blue light receptors are involved in such key processes as photomorphogenesis, phototropism, and initiation of flowering (1). Cryptochromes (Crys)² are UV-A/blue light photoreceptors (also found in animals and bacteria (2)) and are implicated in several of these responses. Because of their high sequence and structural similarity (3–6) and identical flavin cofactor content as DNA-photolyase (7, 8), it was hypothesized that cryptochromes might use the same mechanism for signaling as does photolyase for catalysis, which is light-driven electron transfer from the fully reduced flavin FADH⁻ (2, 4, 7–9). Indeed, spectroscopic studies on Vibrio cholerae Cry1 (10) and Arabidopsis Cry3 (11) have shown that blue light illumination results in the formation of fully reduced flavin similar to the photoreduction process of DNA-photolyase (2). However, CryDASH members were recently shown to have photolyase activity for cyclobutane pyrimidine dimers in single-stranded DNA (12), which is absent for other members of the cryptochrome/photolyase family.

The photoreduction of flavin in DNA-photolyase involves several aromatic amino acid residues (mostly tryptophans) that transfer electrons from the protein surface to the flavin adenine dinucleotide (FAD), which is buried in a U-shaped conformation in the α-helical domain of the protein (13–16). The cryptochrome structures solved so far, namely Synecocystis CryDASH (3), the photolyase-related domain of Arabidopsis Cry1 (4), and Arabidopsis Cry3 (5, 6) show a protein fold similar to DNA-photolyase with FAD in the U-shaped conformation and with the conserved tryptophans in the right distances to mediate electron transfer. Indeed, time-resolved ultrafast absorption spectroscopy on Arabidopsis Cry1 showed the transient formation of tryptophan (and tyrosine) radicals (17) consistent with a photoreduction process similar to DNA-photolyase. In contrast to DNA-photolyase, Arabidopsis Cry1 accumulated semireduced FAD under blue light illumination, an observation that had been noted previously when Cry1 was expressed and purified from insect cells (8). These results suggested that cryptochromes in their signaling state could carry the flavin cofactor in another redox state than DNA-photolyase in its catalytically active form.

To elucidate this possibility, we analyzed the photocycle of Arabidopsis Cry2 in vitro and observed the accumulation of the flavin semiquinone radical under blue light irradiation. This...
flavin species absorbs green light, in contrast to fully oxidized and fully reduced FAD (18). Green light irradiation of Cry2 in vitro caused a change in the equilibrium of the flavin oxidation states associated with a decrease in the level of semireduced FAD and attenuated Cry2-controlled responses such as flowering and the induction of the flowering gene FT. Together these results strongly suggest that the signaling state of Arabidopsis Cry2 contains flavin semiquinone.

**EXPERIMENTAL PROCEDURES**

Plant Material, Light Treatments, and Flowering Experiments—Wild type and cry2 (jha-1) (19) mutant Arabidopsis plants were of the Landsberg erecta ecotype. They were grown on soil under short day conditions (8 h of light/16 h of darkness) for 14 days (flowering experiments) or 7 days before the treatments with monochromatic light following established procedures (20, 21). Monochromatic light treatments were for 72 h for flowering experiments or 8 h for gene expression and immunoblot analysis. All mono- and dichromatic light treatments (20, 21) were done with a fluence rate of 2 μmol m⁻² s⁻¹ applied through double interference filters (Schott, Mainz, Germany).

For the determination of the flowering time, plants were shifted back to short day after the treatment with monochromatic light or kept continuously under short day as a control. Flowering was measured as the number of days from sowing until the first flower of each plant had opened.

**Cry2 Expression and Spectroscopy**—Full-length Arabidopsis Cry2 was expressed as an N-terminal His-tagged fusion in Sf21 insect cells using the baculovirus expression system (Clontech Laboratories/BD Biosciences, Mountain View, CA). Purification was carried out on nickel-nitrilotriacetic acid (Qiagen, Hilden, Germany). All purification steps were performed under dim red light.

Samples containing Cry2 in 20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 10% (v/v) glycerol, and 10 mM β-mercaptoethanol or 10 mM EDTA (instead of β-mercaptoethanol) at 10°C, were illuminated with blue light (450 ± 12 nm; 16 or 50 μmol m⁻² s⁻¹ as indicated) and absorption spectra measured every 5 min on a Shimadzu UV-visible spectrophotometer (UV/2401 PC, Kyoto, Japan). Green light (559 ± 11 nm, fluence rate 160 μmol m⁻² s⁻¹) was applied for the indicated periods of time. During all light treatments, heat emitted from the lamp was blocked by a heat-absorbing filter (KG1, 5 mm, Schott, Mainz, Germany) positioned in a water column with a 5-cm path length. In addition, the temperature of the probe was measured before and after light treatments, and the increase in temperature was found to be <0.1°C. All experiments were performed under aerobic conditions. Energy fluence rates were determined using a P-2000 optometer combined with a calibrated PD-9306-2 detector (Gigahertz, Puchheim, Germany), and photon fluence rates were calculated from these measurements.

**EPR Spectroscopy**—X-band continuous wave EPR spectra were recorded using a pulsed EPR spectrometer (Bruker Elexsys E580) with a cavity resonator (Bruker SHQE-4122-W1) and helium Cryostat (Oxford CF-910). X-band-pulsed electron nuclear double resonance (ENDOR) spectra were recorded on the same spectrometer using an ENDOR accessory (Bruker E560-DA), a radio frequency amplifier (Amplifier Research 250A250A), and a dielectric ring ENDOR resonator (Bruker EN4118X-MD-4W1) immersed in a helium gas flow Cryostat (Oxford CF-935). The temperature was regulated to ±0.1 K by a temperature controller (Oxford ITC-503S). The continuous wave EPR spectra were recorded at 120 K with a microwave power of 5.0 μW at a 9.38-GHz microwave frequency with a field modulation amplitude of 0.3 millitesla (at 100 kHz modulation frequency). For Davies-type ENDOR spectroscopy, a microwave pulse sequence π- T'-π/2-π with 64- and 128-ns π/2 and π pulses, respectively, and a radio frequency pulse of 10 μs duration starting 1 μs after the first microwave pulse were used. The pulse separations T and T' between the microwave pulses were selected to be 13 μs and 500 ns, respectively. To avoid saturation effects due to long relaxation times, the entire pulse pattern was repeated with a low repetition frequency of 200 Hz. Spectra were taken at a magnetic field of 345.7 milliteslas and a microwave frequency of 9.71 GHz. Sf21 insect cells expressing N-terminal His-tagged Cry2 and control Sf21 cells were resuspended in phosphate-buffered saline supplemented with 50% (v/v) glycerol in the dark. Aliquots were transferred into EPR quartz tubes (3 mm inner diameter) and illuminated for different times at 290 K with blue light (HaloLuz 30HL, Streppel, Wermelskirchen-Tente, Germany) using a 420-470-nm band filter (Schott, Mainz, Germany). Samples were then frozen rapidly under illumination in liquid nitrogen and stored therein.

**Gene Expression Studies**—Total RNA was extracted from the aerial part of seedlings using RNeasy kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with superscript RNase H-reverse transcriptase and oligo(dT) (Invitrogen). Real time PCR was performed using SYBR green I (Roche Applied Science), Platinum TaqDNA polymerase (Invitrogen) with an icycler machine (Bio-Rad). Fragments of FT and UBQ10 were amplified with the following primers: FT (5′-CCCTGCTATC-CAACTGGAACAACC-3′, 5′-CTGTTCCTGCTCCAGCT-GTCC-3′); and UBQ (5′-GCAAGAGTTCTGCCATCCTCC-3′, 5′-CGGGAAAGACGATTACTCTTGAGG-3′).

Data treatment was done as described previously (22). Gene expression data were represented relative to the maximum value among all data sets after normalization to the UBQ control.

**Immunoblot Analysis**—Total protein extracts from 7-day-old Arabidopsis wild type or cry2 mutant seedlings were isolated and used for ECL immunoblot analysis as described previously (23). Membranes were incubated with CRY2 antibodies and reprobed with an antibody against HSC70 as a control for equal loading and transfer. The density of the bands was scanned to obtain quantitative data.

**Statistical Analysis**—Data from flowering time were treated with analysis of variance and post hoc tests (Tukey’s honestly significant difference test) using the program Statistica 6.0.

**RESULTS**

To investigate light effects on the redox state of FAD in Cry2, we expressed full-length protein in Sf21 insect cells, purified the protein close to homogeneity (Fig. 1A), and confirmed its identity by MALDI-TOF MS. Approximately 80% of the purified Cry2 contained the FAD chromophore, and from our isolation.
FIGURE 1. Photocycle of Cry2. Recombinant Arabidopsis Cry2 was light-treated in vitro and absorption spectra recorded thereafter as described under “Experimental Procedures.” A, Coomassie-stained SDS-PAGE of Cry2 protein expressed in Sf21 insect cells and affinity-purified by nickel-nitrilotriacetic acid chromatography. The band in lane E indicated by the thick arrow corresponds to full-length Cry2, whereas the smaller band (thin arrow) represents a minor Cry2 degradation product as identified by Western blot analysis (data not shown); also shown is the SDS-7B marker (lane M). B, dark-adapted sample (black spectrum) and spectra of samples, which received blue light (450 nm, 50 μmol m⁻² s⁻¹) for 5 min (red spectrum), 10 min (yellow spectrum), 25 min (green spectrum), and 30 min (blue spectrum). C, absorption spectra of dark sample (black line) treated for 20 min with blue light (450 nm) with fluence rates of 50 (blue spectrum) or 16 μmol m⁻² s⁻¹ (green spectrum), respectively. D, absorption spectra of sample treated with blue (450 nm, 50 μmol m⁻² s⁻¹) light for 30 min (blue spectrum) followed by dark incubation for 5 min (yellow spectrum), 10 min (green spectrum), and 15 min (red spectrum); the spectrum of the dark sample is shown in black. E, absorption spectra of Cry2 treated for 15 min with blue light (50 μmol m⁻² s⁻¹, light blue spectrum) followed by simultaneous treatment with blue (450 nm, 16 μmol m⁻² s⁻¹) and green light (559 nm, 160 μmol m⁻² s⁻¹) for 5 min (yellow spectrum), 25 min (green spectrum), and 30 min (red spectrum). As the control, the spectrum of the dark sample (dark blue spectrum) is shown.
procedure, we conclude that most, if not all, of the expressed Cry2 protein is soluble (data not shown). Continuous treatment of the sample with monochromatic (450 nm) blue light under aerobic conditions led to a light-induced absorbance change with a strong decrease in the blue absorption and a concurrent increase of the absorption at wavelengths >500 nm (Fig. 1B), indicative for the formation of semireduced FADH⁺. Blue light-induced formation of semireduced flavin was described previously also for *Arabidopsis* Cry1 (8, 17). Cry1, however, requires anaerobic conditions for efficient photoreduction. Significant FADH⁺ accumulation can be observed within minutes after the onset of blue irradiation, and the reaction was nearly completed with little fully oxidized FAD left after 5 min (Fig. 1B). The half-life of oxidized FAD in Cry2 under these conditions is 30 ± 10 s (supplemental Fig. 1A). Reducing the fluence rate from 50 to 16 μmol m⁻² s⁻¹ had only a small effect on the final level of formed semireduced FADH⁺ (Fig. 1C), indicating that these fluorescence rates were already saturating for photoconversion. Subsequent incubation of the photo-reduced sample in darkness led to dark recovery of the fully oxidized form within ~15 min (Fig. 1D) with a half-life of the semiquinone form of 250 ± 40 s (supplemental Fig. 1B), indicating that semireduced Cry2 is maintained *in vitro* only under continuous blue light irradiation. In contrast to fully oxidized FAD, semireduced FADH⁺ absorbs at wavelengths >500 nm (18). We therefore evaluated whether the ratio between the fully oxidized and semireduced flavin in Cry2 is altered when blue (450 nm) and green light (559 nm) are applied simultaneously. Comparison of Fig. 1, panels C and E, shows that there is a shift in the equilibrium of the flavin species with a decrease in the level of semireduced flavin under green light illumination (supplemental Fig. 2). This effect was also observed by Gindt et al. (24) in *Escherichia coli* DNA-photolyase. Because in the double beam experiment the total fluorescence change applied was higher than in those experiments where the sample was irradiated only with blue or green light, we performed a control double beam experiment in which the Cry2 sample was irradiated simultaneously with blue (450 nm) and red light (682 nm) with the same fluence rates as for the blue plus green light treatment. Red light had no effect on the equilibrium of the flavin species as expected, because neither the fully oxidized nor the semiquinone form of FAD absorb in this wavelength region (supplemental Fig. 2).

Decomposition of the UV-visible spectra into components of all three flavin redox states allowed a reliable determination of only those species contributing >10% of the total flavin content. The amount of fully reduced flavin upon blue or green light treatment in Cry2 remained below this threshold. Because of the small extinction coefficient in the visible range, however, some formation of fully reduced FAD in Cry2 cannot be excluded (extinction coefficient >400 nm is below 3000 m⁻¹ cm⁻¹). To confirm that the observed light-induced absorbance change is caused by the reduction of FAD, fluorescence emission spectra were recorded. The peak emission at 520 nm and its decrease after blue light treatment clearly shows that only FAD and no other cofactor produces this light-induced absorbance change and that the fully oxidized FAD is reduced by blue light treatment (supplemental Fig. 3). Furthermore, EPR measurements were performed using SF21 cells expressing Cry2. The spectra (see Fig. 2A) show the formation of a paramagnetic species with an EPR signal centered at g=2 and a peak-to-peak line width of 1.75 milliteslas. This signal reaches its maximum intensity after ~12 min of blue light illumination and completely decays overnight in the dark at 277 K. To characterize this signal, X-band-pulsed ENDOR spectroscopy was applied (Fig. 2B). The observed spectrum is very similar to that of the flavin semiquinone radical observed in DNA-photolyase (25) and, in particular, found for purified Cry1 and in whole Cry1-expressing insect cells (26). Together with our companion study on Cry1 (26), these experiments are the first ENDOR characterization of a protein cofactor in whole cells of an overexpression system to arise from the formation of FADH⁺ and identify the absorbance changes in isolated Cry2 and the EPR signal in intact SF21 cells expressing Cry2 upon blue light illumination. In summary, the UV-visible and EPR measurements show that Cry2 undergoes a blue light-induced photoreduction in which the formation of fully reduced flavin is, in contrast to DNA-photolyases and other DASH cryptochromes, dramatically reduced.

To determine whether our spectroscopic data are of biological relevance, we analyzed the effects of blue and green light on responses that are controlled by Cry2. Flowering time of *Arabidopsis* is regulated by day length. Long day conditions promote flowering of wild type plants, whereas the *cry2* mutant flowers late both under long day and short day conditions (27). *Arabidopsis* wild type and *cry2* mutant plants were grown for 14 days under short day followed by continuous monochromatic light treatments for 72 h and shifted back afterward to short day conditions. Fluence rates of monochromatic lights used in these experiments did not induce strong Cry2 degradation (28). Wild type plants kept continuously under short day started
flowering after 45.2 ± 2.5 days (Fig. 3). Blue light-treated wild type plants flowered ~10 days earlier (35.1 ± 2.6 days), whereas treatment with green light alone had essentially no effect on flowering time (46.5 ± 2.1 days), as already described previously, based on night break experiments with *Sinapis alba* (29). Dichromatic light treatments with blue plus green light led to a significant delay in flowering (41.5 ± 2.3) compared with plants that received blue light alone (Fig. 3). Because the cry2 mutant plants did not show any significant response to the same light treatments (Fig. 3), we conclude that the observed promotion of flowering by blue light is mediated by Cry2 and that this effect can be reverted, at least in part, by simultaneous green light treatment.

Cry2 plays a key role in transmitting the blue light signal to flowering by its effect on stabilizing the product of the flowering gene *CONSTANS*. High levels of *CONSTANS* induce the expression of the downstream gene *FLOWERING LOCUS T* (*FT*) only in the presence of light through the action of Cry2 (30–33). We therefore analyzed how the observed promotion of flowering by blue light and the inhibitory effect of green light affect *FT* expression. Plants grown under short day conditions were given monochromatic light for 8 h at the end of the short day (day extension), and FT transcript levels were quantified by real time PCR. As shown in Fig. 4, blue light promotes *FT* expression in wild type plants. Similarly to flowering time, green light alone had no effect on *FT* transcript levels, but given simultaneously with blue light, it reduces the strong blue light effect. To confirm that the effects of blue and green light on the transcript level of *FT* are indeed caused by Cry2, we quantified *FT* transcripts in the cry2 mutant under the same light conditions (Fig. 4). Indeed, the *FT* transcript level remained low in the cry2 mutant under all light treatments, indicating that Cry2 (but no other photoreceptor) is the blue/green photoreversible pigment that induces *FT* expression.

Cry2 is degraded *in vivo* when irradiated with blue light, most likely resulting from direct absorption of light by Cry2 followed by proteasomal degradation (28, 34). To determine whether the above described photoreversible responses were also reflected in the stability of Cry2, we analyzed cry2 protein levels under the same conditions as for the quantification of *FT* transcripts. Irradiation with weak monochromatic blue light (2 μmol m⁻² s⁻¹) caused only a minor reduction in the Cry2 protein level to 73 ± 8% of the dark control, as expected (Fig. 5), whereas green light and blue plus green light given together caused no reduction effect in Cry2 protein levels (Fig. 5). Repetitive 3-min pulses of blue light (447 nm, 10 μmol m⁻² s⁻¹) applied to etiolated Arabidopsis seedlings followed by 3-min dark intervals given over 48 min caused a strong degradation of the Cry2 protein. When the dark periods were replaced by green light (559 nm, 10 μmol m⁻² s⁻¹), Cry2 degradation was essentially abolished (26), demonstrating again that Cry2 itself is affected by green light.
and that no other photoreceptor is involved in the responses analyzed here.

**DISCUSSION**

Action spectroscopy on Cry-regulated processes in plants shows a broad maximum in the blue region resembling the absorption spectrum of a fully oxidized flavin in Cry (35) that represents most likely the ground state of these photoreceptors. However, it remained unknown which flavin oxidation state is present in the signaling state of cryptochromes. In analogy to the structurally related DNA-photolyase enzymes, it was hypothesized that cryptochromes could also use the fully reduced FAD cofactor for signaling either through intramolecular electron transfer inducing conformational changes within the protein moiety of the photoreceptor or by transfer of an electron to a substrate or signaling partner (4, 7, 9). Indeed, irradiation of *V. cholerae* Cry1 and *Arabidopsis* Cry3 with blue light leads to the accumulation of fully reduced flavin in *vitro* (10, 11). However, *V. cholerae* Cry1 and *Arabidopsis* Cry3 belong to a separate cryptochrome group, the CryDASH subfamily (which was shown most recently to have photolyase activity for single-stranded DNA (12)), and has evolved independently from the previously identified plant and animal cryptochromes (36).

Our results from *in vitro* and *in vivo* studies suggest that Cry2 undergoes a photocycle in which fully oxidized FAD represents the ground state and semireduced FADH, the signaling state of this photoreceptor. This conclusion is based on our spectroscopic studies, which show significant accumulation of flavin semiquinone upon blue light treatment. Green light, which is efficiently absorbed by the semireduced flavin (18), reverses this process likely by the formation of a fully reduced intermediate that is rapidly reoxidized. The long known reversion of blue light-induced responses by green light, such as the leaflet closure in *Oxalis* (37), can now be explained by the action of a single flavin receptor. However, our results do not preclude the existence of a unique green light-sensing photoreceptor, and the more recently described enhancement of the hypocotyl growth rate and repression of chloroplast gene expression by a pulse of green light (38, 39) seem not to be caused by the inactivation of the cryptochrome signaling state, because these responses were the same in wild type and cry mutant plants.

The inhibitory effect of green light on the responses analyzed here (flower induction, *FT* expression, Cry2 degradation) is seen, however, only when Cry2 is present (no effect in the cry2 mutant) and when Cry2 is activated by simultaneous blue light irradiation or by a pulse of blue light that is applied directly before the pulse of green light. Together these data strongly suggest that green light inactivates Cry2 directly.

Studies on stomatal blue light responses have shown green light reversion of the blue light-stimulated opening of stomata (40). In this case, the *npq1* mutant that is not synthesizing the putative blue light receptor zeaxanthin does neither respond to blue nor to green light in contrast to the phot1/phot2 double mutant. It is therefore possible that blue light photoreceptors, which have no flavin chromophore, are also inactivated by green light as proposed previously by Frechilla et al. (41). Because cryptochromes also regulate stomatal opening (42), several blue light photoreceptors involved in the same response could be affected by green light.

The inhibitory effect of green light on Cry2 activity seems to conflict with data showing an enhanced hypocotyl inhibition response to the green light of tobacco seedlings that overexpress *Arabidopsis* Cry1 (43). However, broadband (490–570 nm) green light was used in the prior study that extends into the region of Cry absorption in its fully oxidized state (see Fig. 1), in contrast to the narrow bandwidth green light (559 ± 11 nm) applied here to excite specifically the semireduced flavin in Cry2.

There are many examples for co-action of photoreceptors in plants. For example, phytochrome B acts antagonistically to Cry2 in flower induction (33, 44), raising the question as to whether the inhibitory effect of green light on the flower and *FT* induction that is described here could be mediated by phytochrome B. We consider this possibility unlikely, because the continuous blue light irradiation with $\lambda_{max}$ at 470 nm and a fluence rate of 2 $\mu$mol m$^{-2}$ s$^{-1}$ already establishes a calculated $P_{fr}/P_{tot}$ ratio of 45% (45). Analysis of floral induction in the phyB mutant plants grown under the identical light conditions that we used for wild type and the cry2 mutant (see “Experimental Procedures”) showed an early flowering phenotype under long day and short day conditions as well as under short days interrupted by continuous blue, green, or blue plus green light (data not shown). This result is in accordance with the published data (44) showing early flowering of the phyB mutant under short day and long day conditions and the resulting model showing that Cry2 suppresses the inhibitory effect of phytochrome B on flower induction. In addition, we consider the fact that a pulse of green light given after a pulse of blue light prevents the blue light-induced Cry2 degradation to the same extent as when blue and green light were given simultaneously to be a strong argument against the participation of another photoreceptor in the green light responses described here. This behavior resembles the photoconversion of the physiologically active $P_{fr}$ form of phytochrome by a pulse of far red light to the *P* form (46).

The findings presented here, in conjunction with our companion study on Cry1 (26), strongly indicate that the photochemistry of the plant cryptochromes and that of photolyase are different, because photolyase requires fully reduced FAD$^-$ for electron transfer to its substrate (2). In addition, photolyase can photoreduce oxidized FAD in a process called photoactivation (2). It seems that cryptochromes have conserved the photoactivation part of the photolyase photochemistry but accumulate the flavin radical instead of fully reduced flavin and use this process for signaling, most likely by conformational changes in their C-terminal output domains that are sufficient for Cry signaling (47, 48). This work, together with studies on light-driven electron transfer (17), phosphorylation (34, 49, 50), and dimerization (48) of cryptochromes provides a foundation for analyzing in detail the early events in the light activation and signal transduction of plant cryptochromes.
Cryptochrome 2 Photocycle

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