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Science, New Series, Volume 269, Issue 5226 (Aug. 18, 1995), 968-970.

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Association of Flavin Adenine Dinucleotide with the *Arabidopsis* Blue Light Receptor CRY1

Chentao Lin, Dan E. Robertson,* Margaret Ahmad, Andrei A. Raibekas, Marilyn Schuman Jorns, P. Leslie Dutton, Anthony R. Cashmore†

The *Arabidopsis thaliana* HY4 gene encodes CRY1, a 75-kilodalton flavoprotein mediating blue light-dependent regulation of seedling development. CRY1 is demonstrated here to noncovalently bind stoichiometric amounts of flavin adenine dinucleotide (FAD). The redox properties of FAD bound by CRY1 include an unexpected stability of the neutral radical flavosemiquinone (FADH•). The absorption properties of this flavosemiquinone provide a likely explanation for the additional sensitivity exhibited by CRY1-mediated responses in the green region of the visible spectrum. Despite the sequence homology to microbial DNA photolyases, CRY1 was found to have no detectable photolyase activity.

Responses to blue light in living organisms are among the earliest characterized, but least understood phenomena of photobiology (1–6). Despite the elusive nature of such responses, there is evidence that the photoreceptors mediating many of these responses may be flavoproteins, as first suggested by Galston (7). Mutants of *Arabidopsis thaliana* containing lesions at the HY4 locus fail to show inhibition of hypocotyl elongation in response to blue light (8). The HY4 gene has been isolated and found to encode a 75-kD protein with sequence homology to microbial DNA photolyases catalyzing a DNA-repairing reaction dependent on blue and ultraviolet (UV-A) light (9). Because photolyases are flavoenzymes whose activity is absolutely dependent on the absorption of blue-UV-A light (10, 11), we propose that HY4 encoded a flavin-type photoreceptor mediating blue light-dependent inhibition of hypocotyl elongation in *Arabidopsis* (9); we refer to this photoreceptor as CRY1 (12), after cryptochrome, the name commonly given to plant blue-UV-A photoreceptors (13).

To characterize the molecular properties of CRY1, we expressed and purified CRY1 from Sf9 cells transfected with recombinant baculovirus containing the HY4 coding sequence (14). CRY1 was expressed as a soluble protein and purified to near homogeneity (Fig. 1A). Purified CRY1 was a yellow protein with an absorption spectrum resembling that of a flavoprotein (Fig. 2A). The

chromophore was found to be noncovalently bound, being completely released by heat or acid denaturation of CRY1. The absorption spectrum of the free chromophore was identical to that of fully oxidized flavin adenine dinucleotide (FAD) (Fig. 2A). The identity of this chromophore as FAD was confirmed by thin-layer chromatography (14) and electron paramagnetic resonance (EPR) analysis (15). The molar ratio of FAD to the CRY1 apoprotein appeared to be stoichiometric (14). Despite the sequence homology between CRY1 and

microbial DNA photolyases (9), CRY1 demonstrated no photolyase activity in vitro (Fig. 1B), and the expression of CRY1 could not rescue a photolyase-deficient *Escherichia coli* mutant (16). For both of these assays, photolyase showed enzymatic activity regardless of the presence of the second chromophore (17, 18). These experimental findings are consistent with our earlier suggestion that CRY1 is a nonphotolyase, flavin-type photoreceptor (9).

The absorption spectrum of flavoproteins is dependent on the redox status of the bound flavin, which in turn is influenced by both the apoprotein to which the flavin is bound and the redox environment (19). CRY1 purified from the insect cells apparently contained FAD in the fully oxidized state (Fig. 2A). By contrast isolated photolyase usually contains the flavin in a less oxidized state (10, 11, 18, 20, 21). We further explored the redox properties of CRY1 by a photoreduction assay. Under anaerobic conditions, the *Escherichia coli* photolyase containing oxidized FAD and lacking the second chromophore is photoreduced to the fully reduced form without apparent intermediates (18). In contrast, photoreduction of CRY1 under the same conditions resulted in a redox intermediate that absorbed green light (500 to 600 nm) (Fig. 2B), similar to that observed for the neutral radical flavosemiquinone (FADH•)

Fig. 1. (A) Expression and purification of CRY1. A Coomassie blue-stained SDS-polyacrylamide electrophoresis gel (10%) is shown for the total protein of Sf9 cells with (Sf9-HY4) or without (Sf9) virus infection and purified CRY1 protein (HY4, 2 μ g). MW, molecular weight marker. Molecular size markers are shown on the left in kilodaltons. **(B)** CRY1 has no detectable photolyase activity. CRY1 (HY4, 3 μ M) or *E. coli* photolyase (PHR, 0.1 μ M) was assayed for photolyase activity in the presence of 1.7 μ M dT₁₈ (7.5 μ M dimer) as a substrate (14, 27). ΔA_{260} , change in absorbance at 260 nm.

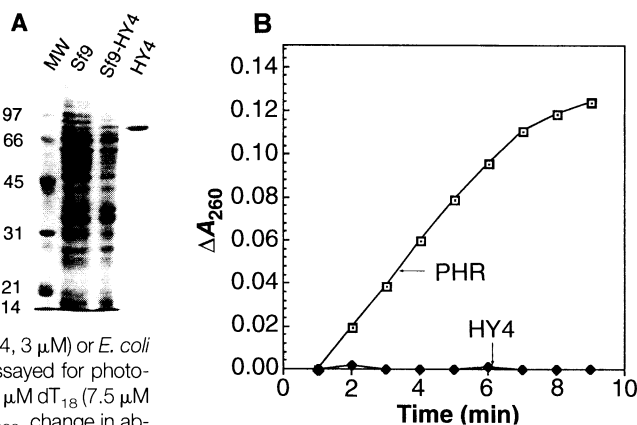
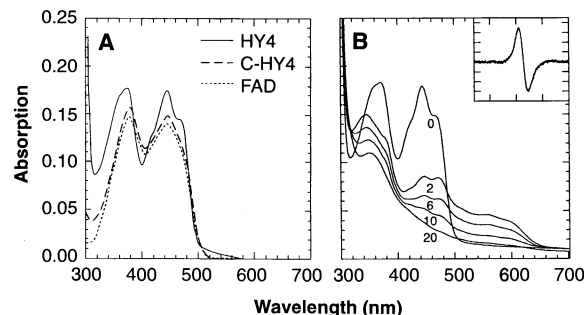


Fig. 2. (A) Absorption spectra of native CRY1 (HY4) purified from Sf9 cells, the chromophore of CRY1 released in 10% trichloroacetic acid (C-HY4), and fully oxidized FAD in 10% trichloroacetic acid (FAD). **(B)** Photoreduction of CRY1 under anaerobic conditions (18). The absorption spectra shown were recorded after the sample was photoreduced for 0, 2, 6, 10, and 20 min, respectively. The inset shows the EPR spectrum (15) of CRY1 photoreduced for 4 min (abscissa: 3270 to 3450 G; ordinate: arbitrary intensity units).



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bound to other flavoproteins (20, 22). The identity of the flavosemiquinone FADH• was confirmed by its unique EPR spectrum, which exhibited a prominent signal at a value of the g factor (g) equal to 2.005 with a bandwidth of about 19 G (Fig. 2B) (15, 22). We determined the redox potentials of CRY1 using conventional redox potentiometry (Fig. 3) (15, 23), which indicated that the flavin bound by CRY1 was primarily the flavosemiquinone (FADH•) under equilibrium conditions of redox potential from -143 to -181 mV.

Given the redox potential that we characterized for the flavin bound to CRY1, it occurred to us that this flavin could exist in vivo in the form of the flavosemiquinone and could mediate green light-induced inhibition of hypocotyl elongation in addition to the response induced by blue and UV-A light. Indeed, the inhibition of *Arabidopsis* hypocotyl elongation under green light was dependent on the fluence rate over a range of 6 to 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and throughout this range the *hy4* mutant was substantially impaired (Fig. 4A). This reduced sensitivity of the *hy4* mutant to green light was noted earlier (24). Furthermore, transgenic tobacco (12) and *Arabidopsis* plants (16) overexpressing CRY1 exhibited exaggerated responses to green light in addition to blue and UV-A light. Two lines of evidence suggest that

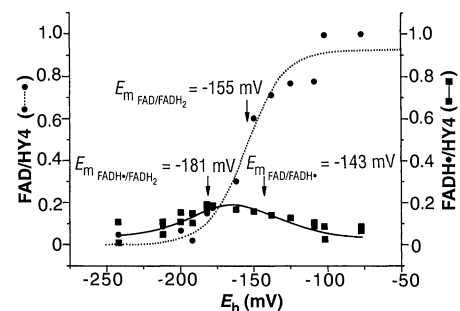
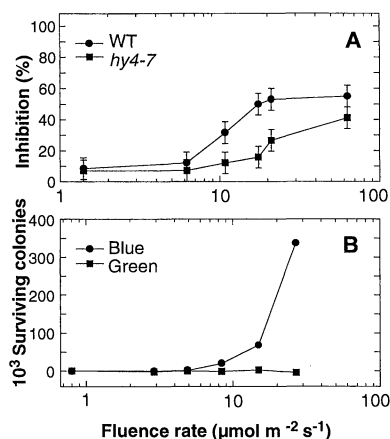


Fig. 3. Optical redox titration of CRY1 (15) showing the midpoint redox potentials (E_m) determined from the redox potentials E_n (15, 23).

Fig. 4. (A) Fluence response of the inhibition of hypocotyl elongation under green light for *Arabidopsis* wild-type (WT) and *hy4* (*hy4-7*) seedlings. *Arabidopsis* seeds were germinated (4 days at 4°C in the dark, followed by 1 day at 21°C under white light) and grown on soil under green light (12) for 3 days. The percentage inhibition was calculated as $[1 - (L_x/L_d)] \times 100\%$, where L_x was the hypocotyl length of seedlings grown under green light and L_d was that of seedlings grown in the dark. The data presented means of three repeats with standard errors shown. **(B)** Photoreactivation response of *E. coli* to green or blue light. *Escherichia coli* cells (KY1056) plated on Luria broth were exposed to a pulse of germicidal UV light (peak at 250 nm) for 10 s and then treated with green or blue light (9) for 30 min and grown overnight in the dark; the surviving colonies were counted and presented as means of three repeats (standard errors were within the symbols).

contamination of our green light source by blue light was not a significant factor. First, the spectrum of the light used for these experiments shows a minimal contribution by photons of wavelength less than 500 nm (9). Second, *E. coli* photolyase showed no activity in response to our green light source (Fig. 4B). These results demonstrated that the CRY1-mediated green light response reflects a feature of this flavoprotein that contrasts with the properties of *E. coli* photolyase. It seems likely that the sensitivity to green light observed for the CRY1-mediated response may reflect the absorption properties of the bound flavosemiquinone. Phototropism—a classical cryptochrome response—can also be induced in *Arabidopsis* by green as well as by blue light (25). It remains to be determined if other responses in plants to blue-UV-A light can also be induced by green light and whether the photoreceptors mediating these responses share sequence relatedness to CRY1.

We have demonstrated that the CRY1 photoreceptor mediates responses to blue, UV-A, and green light (12). The photon absorption properties required to explain these responses could be accommodated by the CRY1-bound flavin oscillating between its different redox states. Such a model would have the intriguing consequence that the plant's relative sensitivity to UV-A, blue, and green light could be influenced by the redox state of the cell. However, it is unlikely that the CRY1-bound flavin is the sole chromophore responsible for all of this activity. Whereas no chromophore other than FAD was detected for CRY1 purified from Sf9 cells (Fig. 2), and attempted reconstitution of CRY1 with either deazaflavins or pterins (the second chromophores associated with photolyases) was unsuccessful (14, 16), preliminary studies of CRY1 protein purified from transgenic plants that overexpressed the photoreceptor indicate the presence of a second chromophore. A complete understanding of this second chromophore and the mechanism of action



of CRY1 will await further characterization of this protein from *Arabidopsis*.

Note added in proof: Malhotra et al. (26) have reported the expression of a fusion protein containing the maltose binding protein and the photolyase homologous region of CRY1 in *E. coli*; this fusion protein was found to have no photolyase activity and to bind FAD and methyltetrahydrofolate.

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14. The coding sequence of *HY4* complementary DNA was inserted at the *Xho*I site following the histidine tag of the baculovirus vector pAC-SG-His NT-A (PharMingen, San Diego, CA). Protein expression [in Sf9 insect (*Spodoptera frugiperda*) cells], purification, and removal of the histidine tag were according to the instructions of PharMingen. Spectroscopic properties of CRY1 (~20 μM) were determined with a Perkin-Elmer Lambda 25 spectrophotometer. The chromophore of CRY1 was released in 10% trichloroacetic acid and found to comigrate with FAD with the use of thin-layer chromatography as described [A. A. Raibekas, *J. Biol. Chem.* **6**, 169 (1991)]. The molar ratio of FAD [extinction coefficient at 450 nm ($\epsilon_{450 \text{ nm}}$) = $11,300 \text{ M}^{-1} \text{ cm}^{-1}$] released from CRY1 relative to the CRY1 apoprotein ($\epsilon_{278 \text{ nm}}$ = $141,750 \text{ M}^{-1} \text{ cm}^{-1}$) varied from 0.6:1 to 0.9:1, depending on the preparation. CRY1 was digested with thrombin to remove the histidine tag and assayed for photolyase activity under anaerobic conditions as described (26), by following the increase in absorbance at 260 nm that accompanied monomerization of pyrimidine dimers of dT₁₈. The in vitro reconstitution of CRY1 with deazaflavins (coenzyme F₄₂₀ and 5-deaza-FAD) and pterin (5,10-CH⁺-H₄ folate) were as described (18).
15. EPR spectra were obtained as described [D. Robertson et al., *J. Biol. Chem.* **259**, 1758 (1984)]; microwave power was 50 μW ; modulation amplitude was 10 G. Optical redox titrations of CRY1 (46 μM) were performed anaerobically (23) at pH 8, with the following redox mediators (5 mM each): 1,4-anthraquinone disulfonate, pyocyanine, *N*-ethylidibenzopyridine ethosulfate, *N*-methylidibenzopyridine methosulfate, and 1,4-hydroxynaphthoquinone.
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 28. We thank colleagues in the Plant Science Institute for critical reading of the manuscript; J. Geisberg, R. Ricciardi, and G. Cohen for assistance with the bac-

ulovirus expression system; and R. Lipman and R. Wolfe for materials and assistance in chromophore binding studies. Supported by grants to A.R.C. from the U.S. Department of Energy (DE-FG02-87ER13680), NIH (GM38409 and GM51956), and the U.S. Department of Agriculture (92-37304-7937); to M.S.J. from NIH (GM31704); to P.L.D. from NIH (GM 27309); and to C.L. from NIH (post-doctoral fellowship).

10 January 1995; accepted 19 June 1995

A Familial Alzheimer's Disease Locus on Chromosome 1

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The Volga German kindreds are a group of seven related families with autosomal dominant early-onset Alzheimer's disease (AD). Linkage to known AD-related loci on chromosomes 21 and 14 has been excluded. Significant evidence for linkage to AD in these families was obtained with D1S479 and there was also positive evidence for linkage with other markers in the region. A 112-base pair allele of D1S479 co-segregated with the disease in five of seven families, which is consistent with a common genetic founder. This study demonstrates the presence of an AD locus on chromosome 1q31–42.

Alzheimer's disease (AD) is genetically heterogeneous and complex. As AD is common in the elderly (1), the clustering of cases in a family may occur by chance, representing either non-allelic, genetic heterogeneity or etiologic heterogeneity (with genetic and non-genetic cases co-existing in the same kindred). In addition, the clinical diagnosis of AD is confounded by other dementing diseases, particularly those common in the elderly. Mutations in the amyloid precursor protein (APP) gene on chromosome 21 cause early-onset (<65 years) autosomal dominant AD (2). Mutations in a gene AD3 on chromosome 14 also result in early-onset autosomal dominant AD (3). For late-onset AD, the APOE $\epsilon 4$ allele elevates risk for AD, possibly by reducing the age of onset, whereas the $\epsilon 2$ allele may be protective (4–7). Gene-gene interactions may also occur in AD; the age of onset of AD caused by the APP Val⁷¹⁷ mutation may be modified by APOE genotypes (8).

The known AD loci do not account for

all cases of AD. In the Volga German (VG) kindreds (9), as in several other families (10) in which AD appears to be inherited as an autosomal dominant trait, the known AD loci have been excluded (3, 10–14). The VG families are a group of related kindreds with AD onset age means ranging from 50.2 to 64.8 years (Table 1). German immigrants in Russia, they remained culturally distinct and did not intermarry with the surrounding population (9). Numerous affected subjects in these families have been characterized, both clinically and neuropathologically (9) and at least one affected subject from each family has had autopsy confirmation of the diagnosis of AD. Except for the relatively early age of onset, AD in the VG is clinically and pathologically indistinguishable from typical AD.

The locus responsible for AD in the VG kindreds does not cosegregate with markers at the APP locus, and no mutations have been detected in the APP gene (11). The AD3 locus on chromosome 14 has been excluded by linkage analysis (3, 12, 13). APOE is unlikely to be the major locus for AD in these families as linkage analysis with a highly informative short tandem repeat polymorphism (STRP) in the APOCII locus, located within 30 kb of ApoE, yields negative linkage results under a number of different models (14). Although APOE $\epsilon 4$ allele frequency in affected VG subjects is elevated relative to Caucasian controls (0.33 versus 0.15), the $\epsilon 4$ frequency in VG spouses is also elevated (0.28) suggesting that the frequency of $\epsilon 4$ may be high in the VG population (14, 15). The APOE genotype does not appear to influence the age of onset in these kindreds (15).

DNA was prepared from lymphoblastoid cell lines from 139 individuals in the VG families, including 37 affected subjects. When suggestive evidence for linkage was found, autopsy-derived tissue, either frozen or embedded in paraffin, was used to prepare DNA from eight additional affected subjects for whom no other tissue was available. Markers on all chromosomes were genotyped (16) and analyzed by the logarithm of the likelihood ratio for linkage (lod score) method (17). For the genome screen, evidence for linkage was evaluated under the assumption of autosomal dominant inheritance with age-dependent penetrance and a 0% sporadic rate. Lod scores were also computed by a low (1%) penetrance model, which makes no assumption about the disease status of at-risk individuals and thus serves as a check that information about linkage was based primarily from the affected individuals (in whom the genotype at the disease locus is more accurately known compared to that of at-risk subjects). Published marker allele frequencies (18) were used unless critical allele frequencies were significantly lower than those estimated in the VG, in which case frequencies based on

Table 1. VG kindreds used for linkage analysis. Families were evaluated as described (9)

Family	Affected (n)	Autopsied (n)	Sampled (n)†		Mean age of onset \pm SD, (n), range
			Affecteds	Total	
H	8	3	4 (2)	4	59.5 \pm 3.9 (6) 56–68
HB	23	5*	6 (2)	30	60.8 \pm 7.1 (22) 54–75
HD	19	2	6	20	59.6 \pm 10.3 (17) 46–82
KS	14	3	8 (1)	29	64.8 \pm 5.4 (13) 55–71
R	20	5	9 (3)	37	50.2 \pm 7.3 (17) 40–67
W	4	2	2	4	52.8 \pm 4.5 (4) 48–58
WFL	6	2	2	15	63.8 \pm 7.6 (6) 55–76
Totals	94	22	37 (8)	139	58.7 \pm 8.9 (85) 40–82

*Includes one unaffected subject autopsied. †Numbers in parentheses indicates DNA samples obtained from autopsy material, either as paraffin blocks or frozen brain.

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