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Expression of an *Arabidopsis* cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV-A, and green light

(photoreceptor)

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ABSTRACT **The *Arabidopsis HY4* gene, required for blue-light-induced inhibition of hypocotyl elongation, encodes a 75-kDa flavoprotein (CRY1) with characteristics of a blue-light photoreceptor. To investigate the mechanism by which this photoreceptor mediates blue-light responses *in vivo*, we have expressed the *Arabidopsis HY4* gene in transgenic tobacco. The transgenic plants exhibited a short-hypocotyl phenotype under blue, UV-A, and green light, whereas they showed no difference from the wild-type plant under red/far-red light or in the dark. This phenotype was found to cosegregate with overexpression of the *HY4* transgene and to be fluence dependent. We concluded that the short-hypocotyl phenotype of transgenic tobacco plants was due to hypersensitivity to blue, UV-A, and green light, resulting from overexpression of the photoreceptor. These observations are consistent with the broad action spectrum for responses mediated by this cryptochrome in *Arabidopsis* and indicate that the machinery for signal transduction required by the CRY1 protein is conserved among different plant species. Furthermore, the level of these photoresponses is seen to be determined by the cellular concentration of this photoreceptor.**

Light from the blue and near-UV spectral regions has profound effects on plant growth and development. Some prominent examples of this are the photomorphogenesis and photomovement responses, including phototropism, chloroplast rearrangement, stomatal opening, and inhibition of hypocotyl elongation (1–7). In *Arabidopsis thaliana*, the hypocotyl-elongation response is mediated by at least two photoreceptors: the red/far-red photoreceptor, phytochrome, and the blue/UV-A photoreceptor, cryptochrome (8–10). In spite of the fact that plant responses to blue light were recognized over a century ago (11), our understanding of the blue-light photoreceptor is very limited in comparison with that of phytochrome. We have previously demonstrated that the *Arabidopsis HY4* gene, required for blue-light-dependent inhibition of hypocotyl elongation (8), contained an open reading frame encoding a protein with significant sequence similarity to microbial DNA photolyase (12). As photolyase is a rare class of flavoenzyme that functions as the result of photon absorption, we proposed that the protein encoded by *HY4* was a flavin-type blue-light photoreceptor (12). We have recently demonstrated that the *HY4* gene product was indeed a flavoprotein (13). We refer to this protein as CRY1, after cryptochrome, the name commonly given to plant blue/UV-A light photoreceptors.

Further understanding of CRY1 and its function in blue-light signal transduction will require the development of assays to enable us to explore the relationship between structure and biochemical and physiological properties of this photoreceptor. One such system involves transgenic overexpression studies, which have significantly improved our understanding of the plant photoreceptor phytochrome (14–26). In these studies, it was shown that phytochrome-overexpressing transgenic plants exhibit elevated sensitivity to red or far-red light. These observations extended the previous genetics studies which had demonstrated that the red/far-red light-induced inhibition of hypocotyl elongation is phytochrome gene-dosage dependent, distinguishing the phytochrome apoprotein mutants from the chromophore biosynthetic mutants and indicating that the first step in the red/far-red-light signal transduction pathway is rate limiting. In addition, these transgenic studies have been demonstrated to be extremely valuable in the investigation of the structure/function relationship (20, 21, 25). We report here a transgenic-overexpression study for CRY1 in which we demonstrate a light-hypersensitive response in transgenic plants overexpressing this blue-light photoreceptor.

MATERIALS AND METHODS

Plant Materials. Tobacco plants (*Nicotiana tabacum* cv. SR1) were transformed with plasmid pTCOE3 by using the *Agrobacterium*-mediated leaf disc procedure (27). The plasmid pTCOE3 contains a 2.3-kb *Arabidopsis HY4* cDNA (12) fused to the cauliflower mosaic virus *35S* promoter in Ti vector pKYLX7 (28). Transformants were analyzed for both kanamycin (500–700 mg/l) resistance and CRY1 protein accumulation. The primary transformants were referred to as T_0, the progenies of the selfed T_0 as T_1, and so on. For hypocotyl-length determination, seeds were sown on soil (modified peat-lite mixes, Metromix 200, GraceSierra, Milpitias, CA) or agar plates containing MS salt and 1% sucrose. The seeds were germinated under continuous white light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 3–4 days to facilitate uniform germination and then subjected to different light or dark treatments for 7–8 days, except where otherwise stated. Experimental light sources and filters were as described (12). The fluence responses were determined by illuminating plants with lights at different fluence rates for a fixed time period. The percentage inhibition of seedling growth was calculated as $[(Ld - Lx)/(Ld - Lw)] \times 100$, where Ld is hypocotyl length of seedlings grown in the dark; Lx is hypocotyl length of seedlings grown under blue, UV-A, or green light; and Lw is hypocotyl length of seedlings grown under strong white light (approximately 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The purpose of this formula is to express inhibition observed under a specified light condition relative to that obtained under strong white light. (Hypocotyl length under strong white light ranges from 1 to 3 mm, varying according to conditions of growth. In contrast, hypocotyl length is approximately 22 mm after 7 days of growth in the dark.)

CRY1 Protein Analysis. Polyclonal antibodies against CRY1 protein were obtained by immunizing rabbits with a 17-kDa C-terminal fragment of CRY1 which was expressed and purified from *Escherichia coli* (C.L., unpublished data). Protein samples, prepared by homogenizing plant tissues in 2× SDS/PAGE sample buffer (29) and immediately boiling the homogenate for 3 min, were fractionated by SDS/PAGE (mini-

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format; Hoefer), and blotted onto a nitrocellulose filter. Samples containing equal amounts of protein were analyzed; the equal loading and transfer of proteins were monitored by staining the blot with Ponceau S (Sigma). The blot was probed first with the anti-CRY1C IgG (25 ng/ml) and then with the second antibody (goat anti-rabbit IgG) conjugated to horseradish peroxidase (diluted 1/5000) (Amersham). The signal of the bound antibodies was detected by chemiluminescence (ECL; Amersham) and recorded by exposure of the blot to x-ray film for ≈5–60 s.

RESULTS

Transgenic Tobacco Plants Expressing CRY1 Protein. The Arabidopsis *HY4* cDNA under the control of cauliflower mosaic virus *35S* promoter was introduced into SR1 tobacco plants by using *Agrobacterium*-mediated transformation. Five independent transformants expressing the transgene were obtained and further studied; each of them exhibited phenotypes similar to those described here for the transgenic line Q, which was shown to have the transgene inserted at a single locus (Table 1). The expression of the *Arabidopsis* CRY1 protein in transgenic plants was detected by using antibody specifically recognizing the C-terminal nonphotolyase portion of CRY1 (Fig. 1). Although CRY1 protein was accumulated in both etiolated and light-grown *Arabidopsis* (C.L., unpublished data), the CRY1-related protein in wild-type tobacco was detectable in etiolated plants but not in an extract of light-grown plants (Fig. 1). We previously observed that CRY1-related proteins in pea and broccoli were susceptible to proteolytic degradation during the extraction process (C.L., unpublished data), and it was possible that the difficulty in detecting CRY1-related protein in light-grown tobacco was due to similar proteolytic activity. Irrespective of the cause, we never detected a significant quantity of CRY1-related protein in light-grown wild-type (SR1) tobacco plants, and we therefore concluded that the detectable CRY1 protein in the light-grown transgenic plants was derived from expression of the *HY4* transgene.

Transgenic Tobacco Segregated for the Blue-Light-Induced Short-Hypocotyl Phenotype. The T_1 progenies of the transgenic tobacco showed no apparent phenotypic difference compared with the wild-type parents when they were grown in the dark, under white light, red light (Fig. 2 *A* and *B*), or far-red light (data not shown); in all these cases the seedling height for wild-type and transgenic plants exhibited a uniform size distribution indicative of a single population. However, when the T_1 progenies of the CRY1-overexpressing transformants were grown under dim white light, it was found that a significant proportion of the seedlings were shorter than that of the wild-type plants (data not shown). The major component of white light responsible for this exaggerated inhibition of hypocotyl elongation was further revealed to be blue light. As demonstrated in Fig. 2, under blue light, the transgenic plants clearly segregated into two distinct populations: one population had hypocotyl lengths similar to those of the wild-type plants, whereas the second population exhibited substantially shorter hypocotyls (Fig. 2, blue). Similar segregation for the blue-light-induced short-hypocotyl phenotype was found in other transgenic lines (Table 1). Within each transgenic line, the ratio of short hypocotyl to wild-type hypocotyl and the ratio of kanamycin-resistant to kanamycin-sensitive phenotype were the same (Table 1); namely it was 3:1 for the transgenic lines Q and E and 15:1 for line G. Line B was apparently a nontransgenic line that somehow escaped from the initial kanamycin selection. Its progeny did not segregate for kanamycin resistance, neither did they show the short-hypocotyl phenotype (Table 1). These results are consistent with the notion that both the blue-light-induced short-hypocotyl phenotype and kanamycin resistance resulted from the introduced DNA, which was integrated either at a single locus of the genome for lines Q, E, and P or at two loci of the genome for lines G and H. The blue-light-specific short-hypocotyl phenotype of transgenic tobacco was inherited in the next generation. For example, under blue light, the T_2 progeny of the Q line, which was homozygous for the *HY4* transgene (Q_{16}), was uniformly shorter than that of the segregated sibling (Q_{35}) lacking the *HY4* transgene (Figs. 1 and 3).

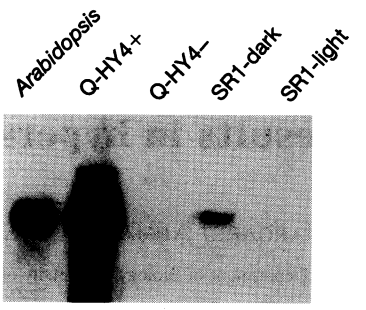

FIG. 1. Expression of CRY1 protein in wild-type and transgenic plants. Total tissue proteins extracted from wild-type seedlings grown for 7 days under white light (*Arabidopsis* and SR1-light) or in the dark (SR1-dark) or extracted from T_2 progeny of transgenic tobacco line Q_{16} (Q-HY4+) and Q_{35} (Q-HY4−) seedlings grown for 7 days under white light were analyzed by immunoblotting with anti-CRY1C antibody.

The Short-Hypocotyl Phenotype of the Transgenic Plants Cosegregated with Overexpression of CRY1 Protein. We then asked if the blue-light-induced short-hypocotyl phenotype of these transgenic tobacco seedlings was associated with overexpression of the CRY1 protein. Twenty seedlings (T_1) exhibiting the short hypocotyl or wild-type tall hypocotyl phenotype under blue light were pooled separately and analyzed for

Table 1. Segregation of kanamycin resistance and the blue-light-induced short-hypocotyl phenotype of transgenic tobacco plants

Plant lines	Kanamycin resistance				Hypocotyl length			
	Resistant, no. of plants	Sensitive, no. of plants	χ^2 (3:1)	χ^2 (15:1)	Short, no. of plants	High, no. of plants	χ^2 (3:1)	χ^2 (15:1)
Q	243	87	0.36	—	251	78	0.19	—
E	52	17	0.12	—	139	48	0.02	—
P	112	28	1.87	—	ND	ND	—	—
G	130	9	—	0.11	135	14	—	1.5
H	106	8	—	0.14	ND	ND	—	—
B	0	114	—	—	0	111	—	—
SRI	0	127	—	—	0	120	—	—

T_1 progenies of six independently regenerated tobacco lines (Q, E, P, G, H, and B) and wild-type tobacco (SRI) plants were germinated under white light and grown either on agar plates containing kanamycin (500 mg/l) for 14 days before being scored for kanamycin resistance or on agar plates containing no kanamycin for 7 days under blue light (5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) before the hypocotyl length was measured. Short hypocotyl length, 1–7 mm; high hypocotyl length, 8–15 mm. ND, not determined.

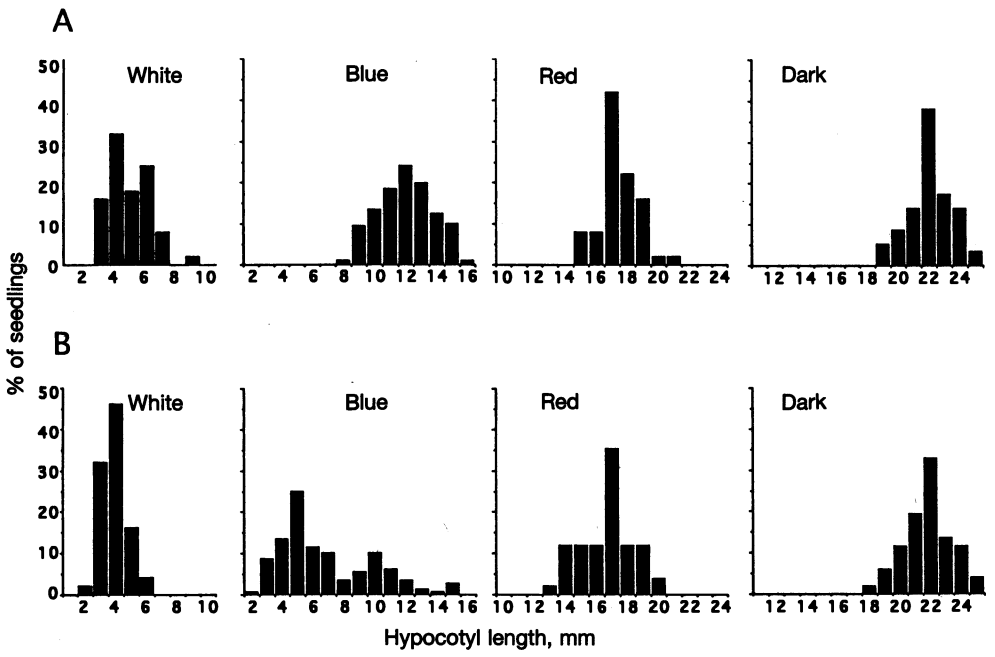

FIG. 2. Transgenic tobacco plants segregated for the blue-light-induced short-hypocotyl phenotype. The seeds of wild-type (*A*) and T_1 transgenic tobacco line Q (*B*) were germinated on soil under white light for 4 days and then grown under blue light (5 μmol·m^{-2}·s^{-1}), red light (19 μmol·m^{-2}·s^{-1}), white light ($\approx$100 μmol·m^{-2}·s^{-1}), or in the dark for 7 days before the hypocotyl length of the seedlings were measured.

CRY1 protein by immunoblot analysis. The CRY1 protein was detected only in seedlings from the short-hypocotyl group but not from the wild-type seedlings (Fig. 4, blue). In spite of the fact that under white light a segregating population was not immediately discernible (Fig. 2*B*, white), when samples of relatively short and tall seedlings were selected from this apparently homogeneous population and assayed for the presence of CRY1, it was found that the protein was clearly present in the short seedlings but barely detectable in the tall seedlings (Fig. 4, white). We concluded that the short-hypocotyl phenotype under white light was caused by overexpression of CRY1 but that the difference in this case was sufficiently small such that segregation was obscured. In contrast, when the transgenic seedlings were grown either in the dark or under red light, no correlation of hypocotyl size and overexpression of CRY1 was observed. This is demonstrated in Fig. 4, where the relatively short and tall seedlings were selected from the apparently homogeneous populations of plants grown in the dark or under red light and their proteins were analyzed as described above. In this case, CRY1 protein was detected in both "short" and "tall" groups (Fig. 4, red and dark), indicating that the differences in hypocotyl size observed for these plants simply reflected the natural variation in height within the population and was not correlated with CRY1 overexpression. In an independent study, the Q line seedlings grown under blue light were measured for hypocotyl length and then transplanted to soil and grown under white light. An immunoblot analysis of these plants 2 weeks later demonstrated that every plant from the short-hypocotyl group accumulated CRY1, while most plants from the wild-type hypocotyl group had no detectable CRY1 (data not shown). These results clearly demonstrated that the blue-light-induced short-

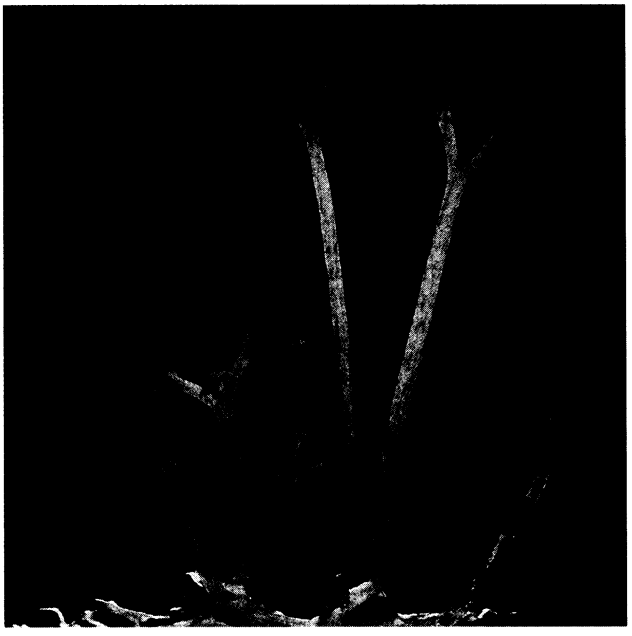

FIG. 3. Blue-light-induced short-hypocotyl phenotype of transgenic tobacco plants overexpressing CRY1. Q_{16} seedlings (the short seedlings) homozygous for the *HY4* transgene and the segregated wild-type Q_{35} seedlings (the tall seedlings) lacking the *HY4* transgene were grown on agar plates under blue light for 7 days before they were removed for photography.

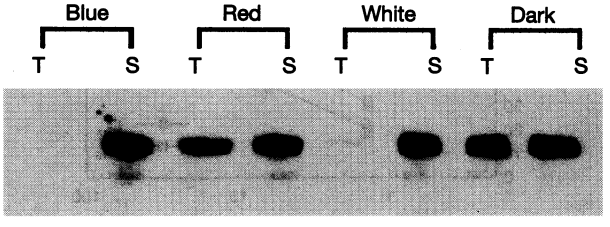

FIG. 4. Cosegregation of the short-hypocotyl phenotype and CRY1 overexpression. T_1 seedlings of transgenic line Q were grown under different light conditions, as described in the legend to Fig. 2. Seedlings from the same sample were divided into two groups with relatively tall [T: longer than 10 mm (blue), 18 mm (red), 5 mm (white), and 23 mm (dark), respectively] or short [S: shorter than 5 mm (blue), 15 mm (red), 3 mm (white), and 18 mm (dark), respectively] hypocotyls. A total of 20 seedlings from each group were pooled together. Protein samples were prepared from each group and analyzed by immunoblotting with anti-CRY1C antibody.

hypocotyl phenotype cosegregated with overexpression of CRY1 protein in transgenic plants.

Transgenic Tobacco Plants Overexpressing CRY1 Were Hypersensitive to Blue, UV-A, and Green Light. Having demonstrated the blue-light effect on tobacco hypocotyl length resulting from overexpression of CRY1, it was of interest for us to determine the sensitivity of these transgenic plants to different blue-light intensities and to compare this fluence response to that obtained with the wild-type plants. In Fig. 5, the phenotype of the CRY1-overexpressing line Q_{16} was compared with the "wild-type" sibling line Q_{35}, when grown under blue light of different fluence rates for 7 days. It was clear that for almost every fluence rate tested Q_{16} seedlings demonstrated stronger inhibition of hypocotyl elongation than the wild-type Q_{35} line (Fig. 5, blue). For example, the fluence rate required for a 50% inhibition of hypocotyl elongation for wild-type Q_{35} seedlings was 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$, whereas less than 20% of that fluence rate (0.75 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was required to confer the same response in the CRY1-overexpressing Q_{16} line (Fig. 5, blue). This difference in the blue-light response of Q_{16} and Q_{35} was most dramatic at a fluence rate of 2.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$; there was almost no difference between the two lines when the fluence rate approached a level that resulted in almost complete inhibition in wild-type plants (Fig. 5, blue). We concluded that the short-hypocotyl phenotype in the transgenic tobacco seedlings resulted from the hypersensitivity to blue light and that this hypersensitivity was the consequence of overexpression of the CRY1 protein.

A similar analysis revealed that CRY1 overexpression increased the sensitivity of transgenic tobacco plants to UV-A light as well (Fig. 5, UV-A). This enhanced UV-A sensitivity observed for these transgenic plants was in keeping with the previous observation that *HY4* mutant plants exhibited reduced sensitivity to UV-A, as well as to blue light (8, 12).

We were also interested in determining whether overexpression of CRY1 altered the sensitivity of the transgenic plants to green light because it had been found that the *Arabidopsis HY4* mutant also had reduced sensitivity to green light in addition to blue and UV-A light (13) (R. Hangarter, personal communication). Indeed, overexpression of CRY1 resulted in hypersensitivity to green light (490–570 nm) in the transgenic tobacco plants (Fig. 5, green). Similar to the hypersensitivity described above for blue light, overexpression of CRY1 resulted in a >5-fold higher sensitivity to green light in the transgenic plants. It was noted, however, that the overall sensitivity of the transgenic plants to green light was lower than that to blue light. For example, under our conditions, the fluence rate required for 50% inhibition of hypocotyl elongation of the CRY1-overexpressing Q_{16} seedlings was 0.75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for blue light compared with 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of green light required for a similar level of inhibition (Fig. 5, blue and green).

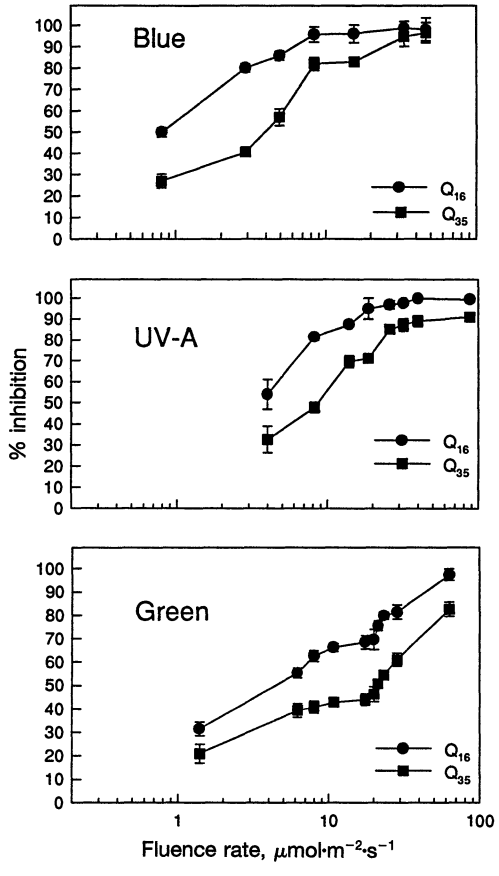

FIG. 5. Transgenic tobacco plants are hypersensitive to blue, UV-A, and green light. Seeds of the T_2 lines Q_{16} and Q_{35} were germinated on agar plates for 4 days under white light and then grown under blue light, UV-A, or green light at different fluence rates for 7 days before the measurement of hypocotyl length. The percentage inhibition of seedling growth was calculated as described in *Materials and Methods*. The data are presented as the mean values ± SEM obtained from three determinations, each with a sample size of more than 30 seedlings.

DISCUSSION

The studies presented here demonstrated that transgenic tobacco plants overexpressing the *Arabidopsis HY4* gene had a much shorter hypocotyl under blue light than that of their nonoverexpressing siblings and this phenotype was due to elevated sensitivity of these plants to blue light. The results that we have presented here concerning CRY1 are reminiscent of related studies with the red/far-red photoreceptor, phytochrome. In both cases the degree of the response to either blue or red/far-red light was influenced by the cellular concentration of the corresponding photoreceptor. Sensitivity to gene dosage—as reflected in a semidominant phenotype—is a distinguishing feature of these photoreceptor mutants (12). Here, once again in a manner similar to that observed for phytochrome, we have demonstrated for transgenic plants phenotypic sensitivity to *HY4* gene dosage and overexpression levels. The light-hypersensitive phenotype that we report here for tobacco plants has also been observed for transgenic *Arabidopsis* plants overexpressing CRY1 (C.L., unpublished data).

This blue-light hypersensitivity resulting from overexpression of CRY1 provides a much desired model system for further characterization of the CRY1 protein. For example, the structure/function relationship of CRY1 protein could be investigated by means of overexpressing different mutant forms of CRY1 in transgenic plants and correlating phenotypic changes with changes in structure. This strategy has been successfully utilized for phytochrome (19, 21). Furthermore, the sensitivity of phenotype to overexpression, as demonstrated for CRY1, may be useful in our efforts to assign function to other cryptochrome family members. Once again, a precedent for this strategy has been established in the phytochrome field where, for example, the observation that PhyB affected hypocotyl elongation in overexpression studies (17, 26) was consistent with the demonstration that the *HY3* locus corresponded to the structural gene for PhyB (30).

The fact that overexpression of CRY1 also resulted in hypersensitivity to green light substantiates the notion that light from this region of the spectrum may also contributed to CRY1 action. The green-light effect that we reported here is in agreement with the previous observation made in this and other laboratories that *Arabidopsis HY4* mutants were im-

paired in their response to green light (ref. 13; R. Hangarter, personal communication) in addition to their deficiency in responding to blue and UV-A light (8, 12). We have recently demonstrated that CRY1 protein contained noncovalently bound flavin adenine dinucleotide (13). Interestingly, in characterizing the redox properties of CRY1, we demonstrated an unexpected stability for the green-light-absorbing semiquinone FADH· (13). This flavosemiquinone, if it exists *in vivo*, could readily explain the sensitivity to green light observed for the CRY1-mediated response.

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