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Evidence That Zeaxanthin Is Not the Photoreceptor for Phototropism in Maize Coleoptiles[1]

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The photoreceptor that mediates blue-light-induced phototropism in dark-grown seedlings of higher plants has not been identified, although the carotenoid zeaxanthin has recently been proposed as the putative chromophore. In the experiments described in this paper, we analyzed phototropism and a blue-light-induced protein phosphorylation that has been genetically and physiologically implicated in phototropism in wild-type maize (*Zea mays* L.) seedlings and compared the results with those from seedlings that are either carotenoid deficient through a genetic lesion or have been chemically treated to block carotenoid biosynthesis. The blue-light-dependent phototropism and phosphorylation responses of seedlings deficient in carotenoids are the same as those of seedlings containing normal levels of carotenoids. These results and those in the literature make it unlikely that zeaxanthin or any other carotenoid is the chromophore of the blue-light photoreceptor for phototropism or the blue-light-induced phosphorylation related to phototropism.

In plants, a diverse array of phenomena are induced by blue light, ranging from changes in growth and development, such as phototropism and the inhibition of hypocotyl elongation, to transitory phenomena, such as solar tracking of leaves and the opening of stomata (Short and Briggs, 1994). These responses occur in a wide variety of tissues and cell types and are induced under widely differing light conditions, an indication that different blue-light photoreceptors may mediate these responses.

Over the years, a variety of different chromophores for blue-light photoreceptors has been proposed, including flavins (Galston, 1949), carotenoids (Wald and DuBuy, 1936), pterins (Galland and Senger, 1988), and retinal (Lorenzi et al., 1994). Recently, Ahmad and Cashmore (1993) described a putative photoreceptor for the suppression of hypocotyl elongation in *Arabidopsis thaliana* (L.) Heyhn. The cloned gene encodes a protein with sequence homology to prokaryotic and eukaryotic DNA photolyases, enzymes known to use a flavin as a chromophore, and the protein itself has recently been shown to be associated with FAD (Lin et al., 1995b). Although this chromoprotein is almost certainly the photoreceptor for inhibition of hypocotyl growth (Lin et al., 1995a), both biochemical and genetic evidence indicate conclusively that a different protein serves the photoreceptor function for phototropism (Liscum and Briggs, 1995).

Quiñones and Zeiger (1994) recently observed several correlations between the level of zeaxanthin, a carotenoid of the xanthophyll cycle, and phototropic sensitivity in maize (*Zea mays* L.). They suggested that zeaxanthin might be the photoreceptor chromophore for phototropism in this species on the basis of these correlations. In this paper we present data that indicate that carotenoids, including zeaxanthin, are unlikely to be the chromophores mediating phototropism in dark-grown maize coleoptiles, because under our conditions, the correlation between zeaxanthin content and phototropism fails completely, as does the correlation between any other carotenoid and phototropism. Since different maize cultivars can vary considerably in various aspects of their phototropic responses (Briggs, 1963), we included the cultivar used by Quiñones and Zeiger (1994) in these studies.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Light Sources

PH seeds were a gift from Pioneer Hi-Bred International (Johnston, IA) and from Dr. E. Zeiger at the University of California at Los Angeles. NK seeds were purchased from Northrop King, Inc. (Fresno, CA). Seeds of *W3* mutants were obtained from Professor J.D. Smith and Dr. Paula Neumann at Texas A & M University (College Station) and from the Illinois Maize Genetic Cooperative (Urbana, IL). Albino homozygous mutants (*w3/w3*) of *W3* were also obtained by self-fertilizing greenhouse-grown heterozygous plants and exposing the developing cobs to 1 mM ABA to prevent premature germination of the mutant seeds. The homozygous recessive seeds can easily be segregated from the heterozygote seeds because they are white, whereas the heterozygotes are a deep yellow. NK, PH, *W3/w3*, and *w3/w3* seeds were soaked in distilled water for 4 h in total darkness and planted under green light on Kimpak paper (Kimberly-Clark, Neenah, WI) soaked in sterile, distilled

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Abbreviations: NF, norflurazon [4-chloro-5-(methylamino)-2-(α,α,α,-trifluoro-*m*-tolyl)-3-(2H) pyridazinone (SAN 9789)]; NK, Northrop King (PX-9540); PH, Pioneer Hi-Bred (3362).

water. Prior to imbibition, *W3/w3* and *w3/w3* seeds were surface sterilized in 1.8% commercial bleach. Plants were allowed to grow either for 3 d (NK, PH) or for 5 d (*W3/w3* and *w3/w3*) in total darkness. Except where specific red- or blue-light treatments are indicated, all manipulations were carried out under a dim green safelight (Short et al., 1992). Red-, blue-, and green-light sources were as described elsewhere (Short et al., 1992).

Herbicide Treatment

NF was solubilized in 500 μL of DMSO and diluted to a final concentration of 100 μM in 500 mL of sterile, distilled water. Seedlings treated with NF were soaked and germinated as described by Bartels and McCullough (1972) but planted on Kimpak paper soaked with 100 μM NF instead of distilled water.

Carotenoid Assays

Tissue extractions and HPLC analyses were as described by Thayer and Björkman (1990). Prior to extraction, 5- to 10-mm-long maize (*Zea mays* L.) coleoptile tips were harvested and lyophilized overnight in darkness. Calculations of pigment content were based on dry weight measurements of each dried sample.

Phosphorylation Assays

Phosphorylations were carried out as described by Palmer et al. (1993b). Phosphorylation reactions contained 35 μg of total membrane protein and 6.7 μCi of [γ-^{32}P]ATP. The reactions were stopped, and samples were denatured in an 8 M urea buffer (8 M urea, 10% SDS, 1.4 M β-mercaptoethanol, 6.25 mM Tris, pH 6.8) and electrophoresed on a 5 to 20% polyacrylamide gradient denaturing gel for 17 h at 5.5 mA of constant current. The intensity of phosphorylation in various gel bands was quantified with a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

We analyzed the possible role of carotenoids on phototropism in three different strains of *Z. mays* L.: NK, PH, and W3 (Lindstrom, 1924). W3 heterozygotes (*W3/w3*-86GN12 and *W3/w3* 86GN16) carry a recessive lesion in the gene encoding phytoene desaturase (Anderson and Robertson, 1960; Bartley et al., 1994), a key enzyme in the biosynthetic pathway of carotenes and xanthophylls. Plants homozygous for the *w3* mutation (*w3/w3*) have undetectable levels of carotenoid pigments. In addition, we produced carotenoid-deficient plants by chemically treating NK, PH, and *W3/w3* seedlings with NF, an inhibitor of phytoene desaturase (Bartels and McCullough, 1972). Mutants and chemically treated seedlings deficient in carotenoids were also tested for blue-light-induced phosphorylation of a plasma membrane protein, a reaction demonstrated by physiological and genetic studies to be involved in phototropism (Short and Briggs, 1994; Liscum and Briggs, 1995).

Seedlings highly deficient in carotenoids for any reason are albino in the light because of their inability to stabilize the light-harvesting complex of PSII (Plumley and Schmidt, 1987; Markgraf and Oelmüller, 1991). Although these albino seedlings will not survive for many hours in the light, they can be germinated and grown in darkness. Separate batches of plants were germinated and grown in the presence or absence of NF. Prior to phototropic induction, harvesting for chemical analysis of carotenoid content, or harvesting for membrane preparation, all seedlings were either kept dark or given light treatments as indicated below.

HPLC was used to determine the content of various xanthophylls and carotenes as described by Thayer and Björkman (1990) in lyophilized 5- to 10-mm coleoptile tips. Figure 1 shows representative HPLC chromatograms of the pigment content in coleoptiles of NF-treated, mutant, and control plants. The area under each peak was used to quantify the amount of pigment (Thayer and Björkman, 1990), and the results for all test and control seedlings are shown in Table I.

NK, PH, and *W3/w3* plants grown in darkness have relatively large peaks of violaxanthin and little (NK, *w3/w3*) or no (PH) zeaxanthin (Fig. 1; Table I). This result is to be expected because in the xanthophyll cycle under conditions of darkness zeaxanthin is largely epoxidized to form violaxanthin. Exposure to high-intensity light causes violaxanthin to be de-epoxidized to zeaxanthin, a process that is correlated with the putative photoprotective function of

Table I. *Quantification of pigment concentration in different maize strains*

The number of picomoles of each pigment per milligram dry weight of tissue was calculated from peak area integration of the curves in the chromatograms as shown in Figure 1 according to the method of Thayer and Björkman (1990). Some plant samples were treated with NF to induce carotenoid deficiency chemically (NK+NF, *W3/w3*+NF, and PH+NF). Pchl region (Pchl) is probably a mixture of Pchl and Pchlide. Limit of detection, 1 pmol; n.d., not detectable.

Pigment	NK	NK+NF	PH	PH+NF	*W3/w3*	*W3/w3*+NF	*w3/w3*
Neoxanthin	9.18	n.d.	5.52	n.d.	5.19	0.99	n.d.
Violaxanthin	58.37	n.d.	23.89	n.d.	20.29	1.26	n.d.
Antheraxanthin	19.72	n.d.	2.62	n.d.	1.66	n.d.	n.d.
Lutein	74.48	n.d.	30.58	<1.00	15.95	1.24	n.d.
Zeaxanthin	12.85	n.d.	n.d.	n.d.	0.94	n.d.	n.d.
Pchl	14.14	9.60	19.99	7.41	9.36	11.56	9.48
α-Carotene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-Carotene	13.58	n.d.	7.95	n.d.	3.59	n.d.	n.d.

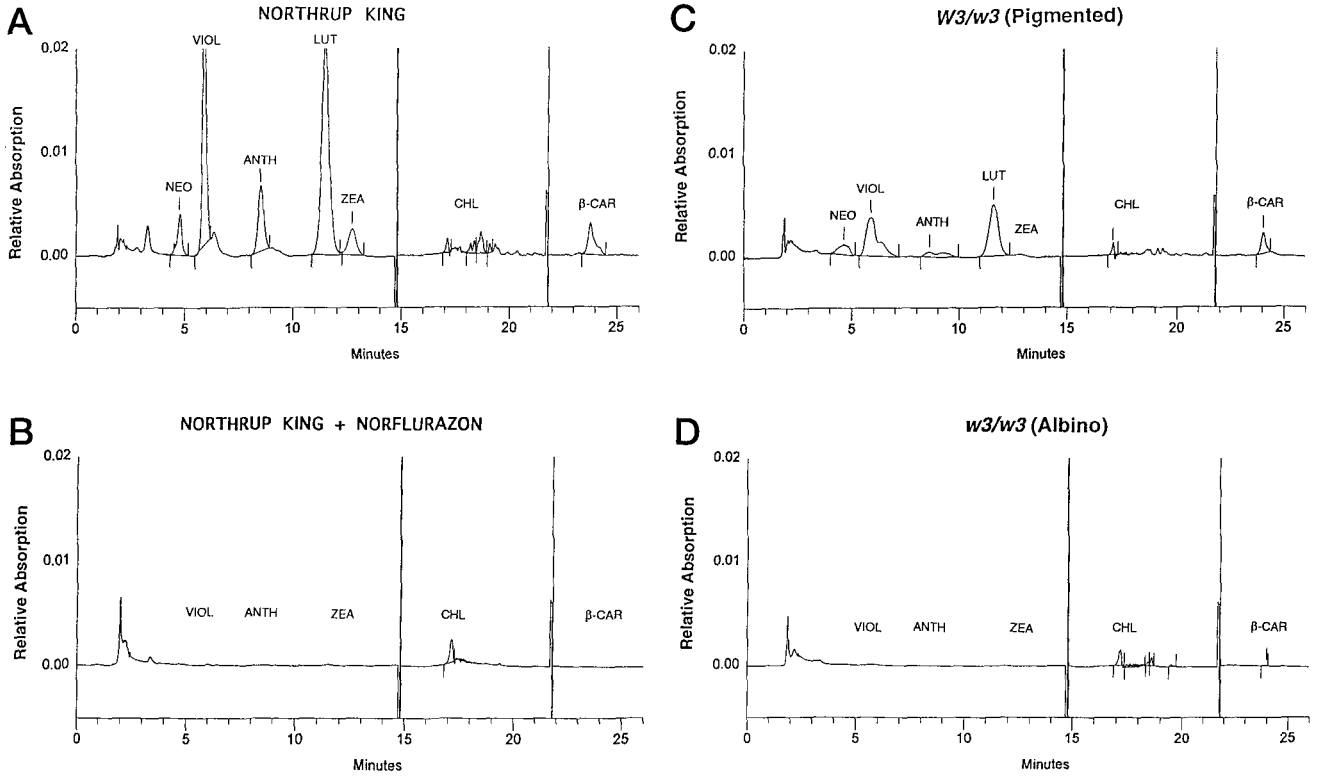

Figure 1. HPLC analysis of pigment concentration in control and test coleoptiles. Coleoptile tips (5–10 mm) were harvested, and pigments were quantified (Thayer and Björkman, 1990). A, NK seedlings grown in the dark. B, NK seedling treated with NF and grown in the dark. C, *W3/w3* (pigmented) seedlings grown in the dark. D, *w3/w3* seedlings grown in the dark. NEO, Neoxanthin; VIOL, violaxanthin; ANTH, antheraxanthin; LUT, lutein; ZEA, zeaxanthin; CHL, Chl region (Pchl plus Pchlide); β-CAR, β-carotene.

carotenoids in the light-harvesting complexes of photosynthesis (Demmig-Adams and Adams, 1992). NK, PH, and *W3/w3* coleoptiles also contain other xanthophylls (lutein, antheraxanthin, and neoxanthin), small amounts of what is probably a mixture of Pchl and Pchlide, and β-carotene. No α-carotene was detected in any of these plants. Treatment of seedlings with 2 h of red light (total fluence 1.4×10^4 μmol m^{-2}) and/or phototropically inductive blue light (3 μmol m^{-2}) did not alter the pattern of extractable carotenoids except for a slight increase in zeaxanthin in some cases (not shown).

Maize plants that had been treated with NF showed dramatically reduced levels of carotenoids. NF treatment of NK seeds resulted in albino seedlings that lack detectable xanthophylls and carotenes. The level of Pchl(ide) was essentially unaffected (Fig. 1B; Table I). Exposure of these carotenoid-deficient seedlings to 2 h of red light and/or inductive blue light did not cause the appearance of any detectable xanthophylls or carotenoids (not shown). Similar results were obtained with NF-treated PH and *W3/w3* seedlings (Fig. 1; Table I). In some cases, the levels of xanthophylls were strongly reduced (e.g. *W3/w3*-NF; Table I) but not entirely eliminated.

In the *w3/w3* strain of maize, xanthophylls and carotenes were undetectable (Fig. 1; Table I). Pchl(ide) was present at levels comparable to those in *W3/w3* plants. Exposure of the albino seedlings to 2 h of red light either just before harvesting or daily during growth and/or inductive blue light did not result in the appearance of any detectable xanthophylls or carotenoids (not shown). These results indicate that mutant and NF-treated maize coleoptiles lack detectable levels of zeaxanthin and show dramatically reduced levels of the other xanthophylls and β-carotene.

Each of the plant samples was tested for its ability to respond phototropically to an inductive pulse of blue light (3 μmol m^{-2}). The plants were germinated as before but were grown in a mixture of 60% Supersoil (Rod McLellan, South San Francisco, CA)/40% vermiculite in individual 50-mL beakers instead of on Kimpak. The soil was watered either with sterile, distilled water or with 100 μM NF. Plants were grown in the beakers in total darkness for 3 to 4 d (NK and PH) or 4 to 6 d (*W3/w3* and *w3/w3*) until the coleoptiles had emerged and begun vertical growth. At this stage, the seedlings were exposed to 2 h of red light once every 24 h (total fluence 1.4×10^4 μmol m^{-2} d^{-1}). Red-light pretreatment during coleoptile growth promotes vertical growth and enhances sensitivity to low-level pulses of blue light (Chon and Briggs, 1966). All plants were tested for their phototropic responsiveness when the coleoptiles were 2 to 4 cm in length by positioning them with the narrow face of the coleoptile oriented toward the blue-light source and exposing them to a 10- to 15-s pulse of blue light (Short

et al., 1992) at a total fluence of 3 μmol m^{-2}. Blue-light exposures were given immediately following the terminal red-light exposure. This amount of blue light has been shown to elicit maximum first-positive curvature in red-light-pretreated maize coleoptiles (Iino, 1987). The plants were then incubated in the dark for 2 h, after which curvature was measured.

Maize coleoptiles, treated as above, responded with 15 to 23° of curvature (Table II). Neither dramatic reduction of carotenoid levels by NF treatment nor genetic disruption of carotenoid biosynthesis affected the phototropic response under the experimental conditions used. Maize plants lacking or severely deficient in carotenoids exhibited the same phototropic response as did plants that contained carotenoids. Indeed, even untreated PH coleoptiles lacking detectable zeaxanthin (Table I) showed a normal phototropic response. (Our results are similar to those of Vierstra and Poff [1981], who obtained fluence response curves for NF-treated and control maize seedlings for their phototropic responses to high-intensity long exposures of light at 450 and 380 nm. The authors used spectrophotometric analysis of crude water extracts [and enclosed primary leaf tips] to estimate the level of bulk carotenoids remaining in the tissues after NF treatment and concluded that bulk carotenoids were not directly involved in sensing phototropic stimuli, because strong phototropic responses were obtained, although measurable carotenoids were estimated to be between 1 and 2% of normal. Because of the method used to estimate carotenoids, they could, of course, not eliminate the possibility that a minor carotenoid such as zeaxanthin remained unchanged in the coleoptile tips while bulk carotenoids were drastically reduced.)

Blue-light-induced phosphorylation of a plasma membrane-associated protein has been shown by physiological and genetic evidence to play a role in phototropism (Short and Briggs, 1994; Liscum and Briggs, 1995). Hence, we tested the various maize strains for their ability to carry out this phosphorylation reaction. In maize coleoptiles, the protein phosphorylated is a plasma membrane protein with a molecular mass of 114 kD and is most abundant in the coleoptile tips, the site of maximum sensitivity to phototropic stimuli (Hager and Brich, 1993; Palmer et al., 1993b). Thus, 5- to 10-mm-long coleoptile tips were harvested from plants grown as described above, and phosphorylation was assayed in total cellular membranes from each strain of maize (Palmer et al., 1993b). Triton X-100-permeabilized membrane samples, containing 35 μg of protein, were either kept in darkness or exposed to blue light at a total fluence of 3×10^4 μmol m^{-2} prior to addition of [γ-^{32}P]ATP. Following ATP addition, the reactions were incubated for 1 min at room temperature in darkness, then terminated, and electrophoresed on a denaturing polyacrylamide gel. The results are shown in Figure 2.

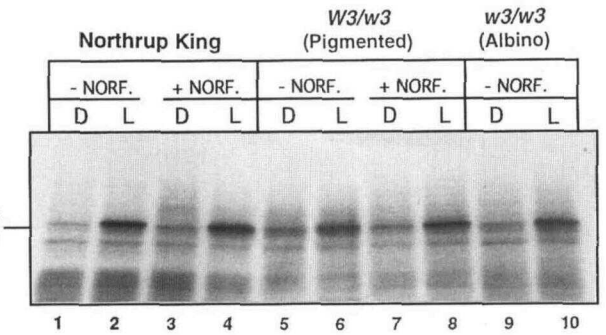

Figure 2. Autoradiograph of blue-light-induced protein phosphorylation in different strains of maize. Maize seedlings were grown either with or without NF (NORF.) added to the growth medium to inhibit carotenoid biosynthesis. A genetic mutant of maize that lacks carotenoids (*w3/w3*) was also tested. Microsomal membranes from coleoptiles were purified, and phosphorylation reactions were prepared as described by Palmer et al. (1993b) with the following modifications. EGTA (2 mM) was included in the phosphorylation reaction buffer to reduce the activity of calcium-activated kinases that co-purify with the membranes. Also, incubations following the light treatments were done at room temperature, and the reactions were stopped with a urea gel-loading buffer. The dark control for each maize membrane sample consisted of membranes that were kept in the dark during incubation with [γ-^{32}P]ATP. Membrane samples exposed to blue light received 3×10^4 μmol m^{-2} immediately prior to incubation with [γ-^{32}P]ATP. Lane 1, NK, dark; lane 2, NK, blue light; lane 3, NK plus NF, dark; lane 4, NK plus NF, blue light; lane 5, *W3/w3*, dark; lane 6, *W3/w3*, blue light; lane 7, *W3/w3* plus NF, dark; lane 8, *W3/w3* plus NF, blue light; lane 9, *w3/w3*, dark; lane 10, *w3/w3*, blue light. D, Dark; L, light.

Both the NK and *W3/w3* strains of maize exhibited blue-light-induced phosphorylation of a 114-kD protein in membrane samples isolated from the coleoptile tips (Fig. 2; Table III). Treatment with NF did not prevent the blue-light-induced protein phosphorylation (Fig. 2, lanes 3 and 4 and 7 and 8). Blue-light-induced phosphorylation was also apparently unaffected in the albino *w3/w3* (Fig. 2, lanes 9 and 10), which lacks detectable xanthophylls, including zeaxanthin (Fig. 1; Table I). The results presented in Table III confirm that treatment with NF did not affect the level of blue-light-induced phosphorylation, nor did a genetic lesion blocking carotenogenesis.

In addition to the Vierstra and Poff (1991) paper mentioned above, several lines of evidence in the literature make it unlikely that a carotenoid is the chromophore for

Table II. *Quantification of the phototropic response in different strains of maize*

Plants that have been treated with 100 μM NF to block carotenoid biosynthesis are indicated. Curvature of coleoptiles toward the light source was measured 2 h after exposure to unilateral blue light. Data represents the means ± SE. Numbers of seedlings are given in parentheses.

Seed	Curvature
	degrees
NK	17.9 ± 2.0 (28)
NK+NF	15.9 ± 2.1 (26)
W3/w3	14.8 ± 2.4 (31)
W3/w3+NF	22.4 ± 2.8 (16)
w3/w3	16.8 ± 2.1 (30)
PH	18.3 ± 1.8 (42)
PH+NF	20.8 ± 1.9 (41)

Table III. *Quantification of phosphorylation intensity in a 114-kD protein from maize coleoptile tip membrane*

The numbers in the columns corresponding to "Dark" and "Light" were obtained by PhosphorImager analysis (Palmer, 1993, #140) of the phosphorylation intensity in the 114-kD band of each lane in the gel of Figure 2. Each number is a point value assigned to the gel band based on integration of the relative phosphorylation intensity in the band normalized against the background level in each lane of the gel. NF treatment, Seedlings were grown in 100 μM NF prior to harvesting membranes and subsequent phosphorylation.

Seed	Light	Dark	Light:Dark
NK	751,153	263,094	2.9
NK+NF	965,980	347,099	2.8
W3/w3	758,701	509,725	1.5
W3/w3+NF	741,874	345,686	2.2
w3/w3	822,009	379,219	2.2

phototropism. First, there is strong evidence that this photoreceptor is associated with the plasma membrane in higher plants (Short and Briggs, 1994; Liscum and Briggs, 1995), not with the chloroplast, where the xanthophyll cycle components and other carotenoids are located. Second, the action spectra for phototropism in higher plants (Shropshire and Withrow, 1958; Thimann and Curry, 1960; Baskin and Iino, 1987) and activity spectra for light-dependent protein phosphorylation (Palmer et al., 1993a) show strong UV-A light dependence, a spectral region not significantly absorbed by carotenoids. Third, two abstracts (Bandurski and Galston, 1951; Labouriau and Galston, 1955; see Galston, 1959) reported that carotenoid-deficient mutants of albino maize and barley, respectively, show normal light sensitivity for phototropism, although no methods or data are presented. (Galston [1959] presented some of the curvature data in his review.) Finally, a carotenoid-less mutant of the fungus *Phycomyces blakesleeanus* shows a normal phototropic response with no more than 0.004% of the wild-type levels of β-carotene (Presti et al., 1977).

It is, of course, not possible to eliminate the possibility that a minute amount of any carotenoid is still present in the carotenoid-deficient tissues studied here. However, the kinds of correlations between phototropism and zeaxanthin content on which the Quiñones and Zeiger (1994) hypothesis was based in proposing zeaxanthin as the chromophore for the photoreceptor for phototropism are completely lacking in the present experiments. Hence, in the absence of any other definitive evidence, given the above results and earlier reports in the literature, we conclude that neither zeaxanthin nor any other carotenoid is a likely candidate to be the chromophore moiety for phototropism in maize.

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