The phytochrome-deficient aurea mutant of tomato has been widely used for the study of both phytochrome function and the role of other photoreceptors in the control of development in higher plants. To date the exact nature of the aurea mutation has remained unknown, though this information is clearly important for the interpretation of these studies. It has been proposed that aurea and yellow-green-2, another mutant of tomato that has a similar phenotype to aurea, could be deficient in phytochrome chromophore synthesis. We have examined this hypothesis by measuring the activity of the enzymes committed to phytochrome chromophore synthesis in these mutants. The approach takes advantage of a recently developed high pressure liquid chromatography-based assay for the synthesis of the free phytochrome chromophore, phytocromobilin from its immediate precursors biliverdin IXα and heme. Isolated etioplasts from aurea and yellow-green-2 seedlings were specifically unable to convert biliverdin IXα to 3Z-phytochromobilin and heme to biliverdin IXα, respectively. In addition, the level of total noncovalently bound heme in the mutants was the same as in wild-type seedlings. Together, these results identify both aurea and yellow-green-2 as mutants that are deficient in phytochrome chromophore synthesis.

In order to regulate their development, higher plants employ a variety of photoreceptors to gather information about the light environment around them. The most extensively characterized of these photoreceptors are the phytochromes, which regulate many responses throughout the life cycle of the plant, including seed germination, stem elongation, leaf and chloroplast development, and flowering (1). The phytochromes are photoreversible chromoproteins comprised of an apoprotein of approximately 124 kDa that is covalently bound to a linear photoreversible chromoproteins comprised of an apoprotein of plastid development, and flowering (1). The phytochromes are regulatemanyresponsesthroughoutthelifecycleoftheplant,
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strated that neither au nor yg-2 are mutations in PHYA. Moreover, a phyA-deficient tomato mutant, fri, which maps to chromosome 10 has a phenotype that is clearly distinguishable from that of both au and yg-2 (28), confirming that the au and yg-2 phenotypes are not simply a consequence of a deficiency in phyA alone. An alternative hypothesis to explain the au and yg-2 phenotypes is that they are deficient in phytochrome chomophore synthesis. This hypothesis is supported by a considerable amount of indirect evidence. The phenotype of au and yg-2 is similar to that of the chomophore-deficient, phyA alone. An alternative hypothesis to explain the absence of the chomophore synthesis. This hypothesis is supported by a considerable amount of indirect evidence. The phenotype of au and yg-2 is similar to that of the chomophore-deficient, phyA alone. An alternative hypothesis to explain the absence of the phyllogenesis of pea (8). Etio-

The au mutant has been widely used as a tool to investigate phytochrome-mediated responses at both the physiological (24–27, 29–31) and biochemical (32–35) level. Most recently, it has been used to great advantage to study the signal transduction of phytochrome responses using microinjection techniques (36, 37). It is therefore clearly of great importance that questions concerning the biochemical basis of this mutation are resolved as this knowledge will greatly aid the interpretation of such data. We have addressed this problem by measuring the committed steps of phytochrome chomophore synthesis directly in the au and yg-2 mutants of tomato. Such an approach has been made possible by the recent availability of HPLC-based assays for these steps (8, 11). We report here that au and yg-2 are specifically deficient in the conversion of BV IXα to 3Z-PφB and heme to BV IXα, respectively.

EXPERIMENTAL PROCEDURES

Plant Material—The aurea (au) mutant of tomato (Lycopersicon esculentum Mill.) used in this study was isolated by Lesley and Lesley (38) and later bred into cv. Ailsa Craig to make a near isogenic line in the L. esculentum background (39). The yellow-green-2 (yg-2) mutant was originally isolated as an irradiation-induced mutation in Lycopersicon pimpinellifolium (17, 40). It was subsequently transferred into L. esculentum, but the precise genetic background is unknown. The WT plants used for these experiments were Ailsa Craig.

Seeds of both WT and mutants were pre-treated with 1% (v/v) bleach and sown on 0.6% (w/v) agar containing 0.46 g/liter of Murashige-Skoog salts (Life Technologies, Inc.) in plant tissue culture containers (Flow laboratories, McLean, VA). Seedlings were grown for 5 days at 25 °C in complete darkness. For all experiments, the upper half of the hypocotyl and the cotyledons were harvested following the removal of any remaining seed coats. These procedures were performed under dim green safelight.

Reagent Preparation—BV IXα was obtained from Porphyrin Products, Inc. (Logan, UT) and further purified by C18 reverse phase HPLC as described previously (8). Heme was prepared by dissolving hemin chloride (Sigma) in 0.1 n NaOH and adjusting to pH 7.7 with 1 n HCl. 3E-PφB and 3Z-PφB were purified by HPLC following conversion of BV IXα to PφB by isolated pea etioplasts (8) and stored dry at −80 °C. All reagents were dissolved in HPLC solvent immediately prior to injection. The following molar absorption coefficients were used for bilin quantification: 66,200 M−1 cm−1 at 377 nm for BV IXα (41) and 64,570 M−1 cm−1 at 386 nm and 38,020 M−1 cm−1 at 382 nm for 3E-PφB and 3Z-PφB, respectively (42).

Etioplast Isolation—Etioplasts were isolated from tomato seedlings by differential centrifugation using the method of Terry and Lagarias (10) with minor modifications. The homogenization medium used was 20 mM TES/10 mM Hepes/NaOH buffer, pH 7.9, containing 500 mM sorbitol, 0.5% (w/v) PVP, 1 mM MgCl2, 1 mM EDTA (free acid), 1 mM EDTA (sodium salt), and 142 mM 2-mercaptoethanol, 5 mM cysteine and 0.2% (w/v) bovine serum albumin, which were added immediately prior to use. Following purification, the final plastid pellet was washed once with assay buffer stock (20 mM TES/10 mM Hepes/NaOH buffer, pH 7.7, containing 500 mM sorbitol) before use. For the experiments measuring protoporphyrin IX synthesis from ALA, 2-mercaptoethanol was omitted from the homogenization medium, and the pH was 7.7.

Assay for PφB Synthesis—PφB synthesis from heme and BV IXα was assayed in isolated tomato etioplasts as described previously (8, 11), but with the following minor modifications. In the PφB synthesis assays, dithiothreitol was replaced by 5 mM sodium ascorbate, and HPLC analysis of bilins was performed using the mobile phase ethanol/acetone/100 mM formic acid (25:65:10, v/v/v) with a flow rate of 1.5 ml/min. The concentration of plastid protein used was between 0.91 and 1.59 mg/ml for assays with BV IXα and 0.44–0.55 mg/ml for assays with heme.

Protoporphyrin IX Synthesis from ALA—Etioplasts were resuspended in assay buffer (20 mM TES/10 mM Hepes/NaOH buffer, pH 7.7, containing 500 mM sorbitol, 1 mM dithiothreitol, and 0.1% (w/v) bovine serum albumin) and subjected to 10 photographic flashes separated by 15-s intervals to deplete endogenous protoporphyrilide. The reaction mixture contained 1 mM EDTA (sodium salt) in a final volume of 1 ml, and the reaction was initiated by the addition of 10 mM ALA. The reaction mixtures were incubated at 28 °C with shaking (50/min) and kept under dim green safelight. Reactions were stopped by the addition of 3 ml of cold acetone/0.1% NH4OH (90:10, v/v). Following a 10-min incubation, the mixture was centrifuged at 30,000 × g for 10 min. The supernatant was then washed with an equal volume of hexane to remove esterified tetrapyrroles and analyzed using a Hitachi fluorescence spectrophotometer F-3010 (Hitachi Ltd., Tokyo, Japan). Fluorescence from protoporphyrin IX was measured by the emission peak at 632 nm following excitation at 410 nm. The amount of protoporphyrin IX synthesized was determined by subtracting the fluorescence from the 0 h sample and calculating the concentration of protoporphyrin IX in the sample from a standard curve constructed using protoporphyrin IX (Sigma) in the same solvent. The protoporphyrin IX concentration in stock solutions was calculated using a molar absorption coefficient of 158,000 M−1 cm−1 at 404 nm for the dimethyl ester in ether (43). No magnesium porphyrins were synthesized under these assay conditions.

Heme Quantitation—Total noncovalently bound heme was quantitated from 3 g of fresh weight etiolated seedlings as described previously (8).

Protein Quantitation—Protein was determined by the method of Bradford (44) using Bio-Rad protein assay reagent and bovine serum albumin as a standard.

RESULTS

Both au and yg-2 Seedlings Have WT Levels of Heme—The immediate precursor of the committed steps of phytochrome chomophore synthesis is heme (see Fig. 1). Heme is a vital component of the electron transport chains required for both photosynthesis and respiration. Because au and yg-2 plants are relatively healthy and are not impaired in photosynthetic effi-
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Fig. 2. HPLC analysis of PφB synthesis from BV IXα by isolated plastids from WT, au, and yg-2 seedlings. Plastids were incubated for 3 h at 28°C with 8 μM BV IXα and an NADPH regenerating system, and the products were analyzed by reverse phase HPLC. Absorbance was monitored at 380 nm. Trace a, incubation in the absence of plastids (BV IXα only); trace b, incubation in the absence of BV IXα (WT plastids only); trace c, WT plastids + BV IXα; trace d, yg-2 plastids + BV IXα; trace e, au plastids + BV IXα. For clarity the absorbance scale of trace a is 6 times that of traces c–e, and the scale of trace b is half that of traces c–e.

In order to test the hypothesis directly we measured total noncovalently bound heme in 5-day-old etiolated seedlings as an estimate of the total heme level in these mutants. WT seedlings contained 6.11 ± 0.23 nmol/g fresh weight (mean ± S.E., n = 5) while au and yg-2 contained 6.10 ± 0.44 and 6.67 ± 0.36 nmol/g fresh weight (n = 3), respectively. These results demonstrate that there is no significant difference in the level of noncovalently bound heme in WT, au, and yg-2 seedlings. Therefore, a deficiency in phytochrome chromophore synthesis in these mutants is predicted to be the result of a block between heme and PφB, and we examined these steps individually.

Etioplasts from au Seedlings Are Unable to Convert BV IXα to 3Z-PφB—Firstly we addressed the question of whether au and yg-2 are deficient in PφB synthe-sis activity. Isolated etioplasts were incubated with BV IXα and an NADPH regenerating system, and the products were analyzed by reverse phase HPLC (Fig. 2). WT etioplasts converted BV IXα to both 3Z-PφB and 3E-PφB (trace c, Fig. 2), which were identified by co-injection with purified PφB samples (data not shown). These products are identical to those detected previously following the incubation of BV IXα with etioplasts from both oat (11) and pea (8) seedlings. No PφB was synthesized following incubation of BV IXα in the absence of etioplasts or with WT, au, or yg-2 etioplasts in the absence of BV IXα (traces a and b, Fig. 2; data not shown). Incubation of etioplasts isolated from yg-2 seedlings also resulted in the synthesis of 3Z-PφB and 3E-PφB (trace d, Fig. 2). The identity of both products was again confirmed by co-injection with purified PφB samples (data not shown). When BV IXα was incubated with au etioplasts no PφB products were observed (trace e, Fig. 2). To verify that the peak with a retention time of approximately 12 min was not 3Z-PφB, co-injection experiments with authentic, purified 3Z-PφB were performed (Fig. 3). The peak at 12 min elutes as a shoulder of the 3Z-PφB peak, demonstrating clearly that it is not 3Z-PφB and that no 3Z-PφB accumulates following incubation of au etioplasts with BV IXα (Fig. 3). Experiments using an additional au allele, au" (17, 46), resulted in identical results (data not shown). The inability of au to synthesize 3Z-PφB from BV IXα is therefore most likely to be a direct consequence of the au mutation.

Although the data presented suggest that the au mutation results in the loss of the enzyme activity that converts BV IXα to 3Z-PφB, there is a formal possibility that the mutation leads to the degradation of PφB, thereby preventing its accumulation. The au mutation cannot reside in a putative catabolic enzyme itself because the mutation is recessive. However, a mutation in a repressor of such an activity would be expected to result in the increased degradation of PφB or bilins in general. To test this possibility we analyzed PφB synthesis by WT etioplasts in the presence of au etioplasts. Co-incubation of au etioplasts with WT etioplasts did not prevent the accumulation of either 3Z-PφB or 3E-PφB (data not shown). In addition, analysis of BV IXα recoveries during the course of these experiments demonstrated that au etioplasts did not result in increased BV IXα degradation compared with WT or yg-2 etioplasts (data not shown), indicating there is no increase in nonspecific bilin catabolism in au. These results confirm that the absence of 3Z-PφB and 3E-PφB following the incubation of BV IXα with au etioplasts is a consequence of reduced PφB synthesis and not increased degradation.

Etioplasts from yg-2 Seedlings Are Unable to Convert Heme to BV IXα—To test whether au and yg-2 could convert heme to BV IXα, isolated etioplasts were incubated with heme, and the bilin products were analyzed by reverse phase HPLC. Incubation of etioplasts isolated from WT seedlings with heme in the absence of etioplasts or with WT, au, or yg-2 etioplasts resulted in a single major peak (identified by co-injection with purified PφB samples (data not shown)). When etioplasts isolated from yg-2 seedlings were incubated with heme, a small quantity of BV IXα (identified by
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4). The yg-2 mutant is therefore blocked at the step that has been proposed to be accomplished by heme oxygenase and is analogous to the pcd1 mutant of pea (8). Conversely, au is able to synthesize BVIXa from heme (Fig. 4) but cannot synthesize 3Z-PΦB from BVIXa, the reaction accomplished by PΦB synthase (Figs. 2 and 3). Two independent alleles were shown to lack the ability to convert BVIXa to 3Z-PΦB and heme to BVIXa, respectively.

DISCUSSION

In this paper we have examined the hypothesis that au and yg-2 are mutations that affect the biosynthetic pathway of the phytochrome chromophore by measuring the steps in this pathway directly. Since au and yg-2 were not heme-deficient, we focused on the steps committed to the synthesis of PΦB. Analysis of these biosynthetic steps demonstrated that the yg-2 mutant was unable to convert heme to BVIXa (Fig. 4), while the synthesis of 3E- and 3Z-PΦB from BVIXa was normal (Fig. 2). The yg-2 mutant is therefore deficient in the synthesis of BVIXa from heme and is analogous to the pcd1 mutant of pea (8). Conversely, au is able to synthesize BVIXa from heme (Fig. 4) but cannot synthesize 3Z-PΦB from BVIXa, the reaction accomplished by PΦB synthase (Figs. 2 and 3). Two independent alleles were shown to lack the ability to convert BVIXa to 3Z-PΦB, confirming that this deficiency is the biochemical basis of the au phenotype. Whether au is also deficient in the proposed PΦB isomerase activity is unknown. In addition, the additive phenotype of the au, yg-2 double mutant (23) is consistent with the identification of au and yg-2 as mutations affecting sequential steps in this pathway.

We believe that the deficiencies in phytochrome chromophore synthesis in au and yg-2 are most likely to be the result of mutations in the biosynthetic enzymes themselves and are not due to pleiotropic effects on plastid structure and function. Such a conclusion is based on the observation that au and yg-2 are only deficient in a single step of the pathway committed to PΦB synthesis and that etioplasts from the mutants are equally capable of synthesizing protoporphyrin IX in isolated etioplasts, we measured the synthesis of protoporphyrin IX from ALA. Six enzymes are required for the conversion of ALA to protoporphyrin IX, and these are thought to be membrane-associated (47). It might therefore be expected that pleiotropic effects of the mutations on plastid structure would lead to a reduction in the ability of au and yg-2 to synthesize protoporphyrin IX from ALA. However, as shown in Fig. 6, there was no effect of the au and yg-2 mutations on protoporphyrin IX synthesis in isolated etioplasts. This result, together with the demonstration that au and yg-2 are each deficient in a single step between heme and 3Z-PΦB, shows that these mutants are specifically impaired in the conversion of BVIXa to 3Z-PΦB and heme to BVIXa, respectively.

**Fig. 3. Co-injection of 3Z-PΦB with the products of incubating au plastids with BVIXa.** Absorbance was monitored at 380 nm. Trace a, au plastids + BVIXa; trace b, equivalent sample to trace a co-injected with 30 pmol 3Z-PΦB.

**Fig. 4. HPLC analysis of heme metabolism by isolated plastids from WT, au, and yg-2 seedlings.** Plastids were incubated for 3 h at 28°C with 10 μM heme and an NADPH regenerating system, and the products were analyzed by reverse phase HPLC. Absorbance was monitored at 380 nm. Trace a, incubation in the absence of plastids (heme only); trace b, WT plastids + heme; trace c, yg-2 plastids + heme; trace d, au plastids + heme.

**Fig. 5. Identification as BVIXa as the major product following incubation of heme with WT plastids.** Absorbance was monitored at 380 nm. Trace a, WT plastids + heme; trace b, half of equivalent sample to that in trace a co-injected with 50 pmol 3Z-PΦB; trace c, half of equivalent sample to that in trace a co-injected with 7.5 pmol of BVIXa.

**Fig. 6.** Comparison of BVIXa synthesis in isolated etioplasts from au and yg-2 seedlings. Plastids were incubated for 3 h at 28°C with 10 μM heme and an NADPH regenerating system, and the products were analyzed by reverse phase HPLC. Absorbance was monitored at 380 nm. Trace a, incubation in the absence of plastids (heme only); trace b, WT plastids + heme; trace c, yg-2 plastids + heme; trace d, au plastids + heme.

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from ALA as WT etioplasts (Fig. 6). Six enzyme steps are required for protoporphyrin IX synthesis from ALA and such an assay represents a good measure of the integrity of the isolated etioplasts.

The deficiency in phytochrome chromophore biosynthesis can account for the phenotype of au and yg-2 seedlings, since at this developmental stage they would be expected to be reduced in all holophytochrome species. Consistent with this conclusion, both au and yg-2 lack responses that are attributable to multiple phytochrome species (28, 48). For example, hypocotyls of au and yg-2 are elongated in both R and FR (17), responses that are specifically deficient in mutants lacking phyA (28) and a phyB (48), respectively. Similarly, anthocyanin synthesis in response to R and FR is absent in au (17, 49). There is also a well-characterized role for phytochrome in the synthesis of both chlorophyll (33) and chlorophyll-binding proteins (21, 32, 34) in tomato, and phytochrome-deficient tomato seedlings would be expected to display the pale, yellow-green phenotype of au seedlings. Interestingly, both phyA- and phyB-deficient plants are not chlorophyll-deficient at the seedling stage (28, 48). This suggests that additional phytochromes or other photoreceptors are involved in these responses, although redundancy between phytochromes, as noted for chlorophyll synthesis in Arabidopsis (50), cannot be ruled out.

The phenotype of au and yg-2 seedlings is also consistent with the phenotypes observed in seedlings of other phytochrome chromophore-deficient mutants such as the hy1 and hy2 mutants of Arabidopsis (13, 14) and the pcd1 mutant of pea (8). One exception is that in both au and yg-2, PHYA accumulates at least to 20% of the WT level (22, 23), whereas PHYA levels are close to WT in pcd1 (8) and hy1 and hy2 (14, 51). This observation was one reason that au was considered to be specifically deficient in phyA. However, this PHYA instability under conditions of chromophore deficiency appears to be specific for the tomato protein since oat PHYA overexpressed in an au or yg-2 background accumulates to levels equivalent to those detected in WT seedlings overexpressing this protein (23). A second misleading observation, the reported inability to rescue a WT phenotype following growth of au seedlings on BV IXα (23), is not surprising in the light of the data presented here. However, attempts to recover au and yg-2 on phycocyanobilin, an analog of PhyB, and yg-2 on BV IXα were also unsuccessful. A similar observation was noted with the pcd1 mutant of pea (8), and Arabidopsis appears to be the only species tested in which chromophore-deficient mutants can be fully rescued (in terms of elongation growth) by chromophore precursors (15).

The phenotype of mature au plants with respect to elongation growth is considerably less exaggerated than the seedling phenotype. The observation that the end-of-day FR response (25) and shade-avoidance responses are normal (26, 27) or nearly normal (31) in mature au plants is consistent with the presence of more than half the holophytochrome of WT plants (25) including photoreactive phyB (22). The au (and yg-2) mutants are therefore leaky and can synthesize phytochrome chromophore in more mature tissues. In this respect they are similar to the pcd1 mutant of pea, which responds progressively to end-of-day FR with increasing developmental age (8). The ability of these mutants to synthesize some chromophore may be the result of the mutant enzymes retaining some biosynthetic activity. Alternatively, the increased biosynthetic activity may be the result of additional genes encoding these enzymes, which may be more highly expressed at later stages of plant development. One phenotypic characteristic that remains pronounced in mature au and yg-2 plants is the yellow-green foliage. Why such a chlorophyll deficiency remains so pronounced when other phytochrome responses are present is not clear. One possibility is that the lesions in the chromophore pathway lead to an accumulation of free heme, which has been proposed to act as an inhibitor of tetrapyrrole biosynthesis by inhibiting the rate-limiting step of ALA synthesis (52). This hypothesis is currently under investigation but would help explain not only the pale phenotypes of mature au and yg-2 plants but also the observation that photoreversible protochlorophyllide is reduced in etiolated au seedlings (33) and the altered development of au etioplasts (36).

The results in this paper have a number of implications for both the extensive published data using au and also for future studies with au and yg-2. While its use as a phytochrome-deficient mutant is completely justified, it should be borne in mind that the deficiency will not be restricted to the phyA pool but will most likely affect all phytochrome species to some extent. Conversely, such mutants are unlikely to be null for phyA or any other phytochrome. In addition, in experiments where chlorophyll synthesis or chloroplast function are being assessed (32–34, 36, 37) consideration should be given to the possibility that the accumulation of intermediates in the phytochrome chromophore pathway, such as heme, may affect the parameters being studied.

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