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PROTOCHLOROPHYLLIDE b OCCURS IN GREEN BUT NOT IN ETIOLATED PLANTS*

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It has recently been reported that protochlorophyllide (Pchlide) b is an abundant pigment in barley etioplasts but is rather unstable, as it is rapidly converted to Pchlide a by 7-formyl reductase during pigment extraction with conventional 80% acetone (Reinbothe, S., Pollmann, S., and Reinbothe, C. (2003) J. Biol. Chem. 278, 800–806). It has also been claimed that extraction of barley etioplasts with 100% acetone containing 0.1% diethyl pyrocarbonate prevents the conversion of Pchlide b to Pchlide a and leads to the detection of large amounts of Pchlide b in the isolated etioplasts. In this work the extraction protocol of Reinbothe et al. is compared with the more conventional 80% aqueous acetone extraction method. No Pchlide b was detected either in etiolated barley leaves or isolated barley etioplasts irrespective of the extraction protocol. On the other hands, small amounts of Pchlide b were detected in green barley leaves and isolated chloroplasts, extracted either with 80% acetone or 100% acetone containing 0.1% diethyl pyrocarbonate. It is concluded that the proposed occurrence of a light-harvesting POR-Pchlide-b complex in etioplasts is untenable, and its ensuing consequences and implications, for the greening process, are irrelevant.

The occurrence of monovinyl (MV)1 Pchlide b in green plants was reported in 1991, and its chemical structure was ascertained by 1H NMR and fast atom bombardment mass spectrometry as well as by fluorescence spectroscopy (1). Later on, spectrofluorometric techniques were developed for the quantitative determination of MV-Pchlide b under various growth conditions (2). Its concentration in green cucumber cotyledons ranged from 400 to 800 pmol/100 mg of tissue protein. It was not detectable in etiolated tissues but was proposed as a logical precursor of chlorophyll b via two distinct biosynthetic routes in greening and green plant tissues (3).

In 1999, Reinbothe et al. (4) proposed that in barley etioplasts, Pchlide a and Pchlide b formed a supramolecular complex with NADPH and two closely related NADPH-Pchlide oxidoreductases (POR), namely PURA and PORB. The complex was referred to as a light-harvesting POR-Pchlide complex (LHPP). It has also been suggested that LHPP contained five non-photoactive Pchlide b and one photoactive Pchlide a. Reinbothe et al. (4) conjectured that the formation of LHPP is essential for the development of the photosynthetic apparatus and conferred photoprotection upon the greening system.

The above claims were rapidly refuted by two separate research groups that could not find any convincing evidence supporting the occurrence of Pchlide b in etiolated barley (5, 6). In response to the aforementioned criticism, Reinbothe et al. (7) recently refined their claims. Although they reasserted their belief in the ubiquitous occurrence of LHPP in etiolated tissues, they proposed that Pchlide b is metabolically unstable, and during conventional pigment extraction in organic solvents, it is rapidly converted to MV-Pchlide a by 7-formyl reductase, an enzyme that has been shown to be active in the conversion of chlorophyll (Chl) b to Chl a (8).

In this work we evaluate the assertions of Reinbothe et al. (7) and present experimental evidence that rule out (a) the occurrence of Pchlide b in etiolated tissues and (b) its putative conversion to Pchlide a during conventional pigment extraction with 80% acetone. Therefore the proposed occurrence of an LHPP in etioplasts is untenable, and its ensuing consequences and implications (9, 10) for the greening process are irrelevant.

MATERIALS AND METHODS

Plant Material—Barley (Hordeum Vulgare, Hi Barley Brand) seeds were purchased from Illini FS Inc. (Urbana, IL). Germination was carried out in plastic trays containing wet vermiculate either in darkness or in a growth chamber illuminated with 1000-watt metal halide lamps (211 Watt m−2) under a 14-h light/10-h dark photoperiod. At midday, etiolated or green leaves were harvested after 6–7 days of growth at 28 °C.

Preparation of Pchlide b Phytyl Ester—Pchlide b phytyl ester was prepared as described elsewhere (1). Pchlide b and its phytyl ester exhibit identical absorbance and fluorescence properties (1).

Preparation of Etioplasts and Chloroplasts—Etioplasts were prepared from etiolated barley leaves by Percoll density gradient centrifugation as described by Reinbothe et al. (11, 12). Chloroplasts were similarly prepared from green barley leaves.

Protochlorophyllide Extraction from Etiolated and Green Barley Leaves—All centrifuge tubes used for plastid lysis and pigment extraction were autoclaved. To prevent biochemical artifacts, the upper half of etiolated or green barley leaves (0.3 g) were hand ground in mortar filled with liquid nitrogen. The leaf powder, still covered by liquid nitrogen, was transferred to propylene tubes that were prechilled to −20 °C and containing 30 ml of 100% acetone and 0.1% DEP, as recommended by Reinbothe et al. (7), or containing 6 ml of 80% acetone lacking DEP. According to Reinbothe et al. (7) extraction with 80% acetone leads to the conversion of MV-Pchlide b to MV-Pchlide a. The tissue was further homogenized for 30 s, using a Brinkman homogenizer (Westbury, NY), fitted with a PT 10/35 probe.

Pchlide Extraction from Isolated Etioplasts and Chloroplasts—Pigments of either wet etioplast pellets or etioplast suspensions were extracted with a 100-fold excess of 100% acetone containing 0.1% DEP or with 80% acetone lacking DEP. To prevent any enzymatic activity during pigment extraction, etioplast pellets or suspensions were frozen for 30 min at −20 °C.
Protochlorophyllide b Does Not Occur in Etiolated Plants

Results

Experimental Strategy—To resolve the controversy surrounding the occurrence of MV-Pchlide b in etiolated tissues, a two-pronged experimental approach was adopted. First, Pchlide extraction was carried out as described by Reinbothe et al. (7) either under conditions that prevented the putative conversion of MV-Pchlide b to Pchlide a (i.e., by extraction with 100% acetone containing 0.1% DEP) or under conditions that presumably led to the conversion of MV-Pchlide b to Pchlide a (i.e., by extraction with 80% acetone, lacking DEP). Second, determination of the MV-Pchlide a and b content was achieved unambiguously by use of well established 77 K spectrofluorometric techniques (1, 2, 13). Indeed, in diethyl ether at 77 K, MV-Pchlide b exhibits well pronounced emission and excitation maxima at 642–643 and 462–463 nm, respectively (1). On the other hand under identical conditions, pentacoordinated MV-Pchlide a exhibits an emission maximum at 625–626 nm and split Soret excitation maxima at 437–438 and 443–445 nm (13, 15).

Pchlide b Does Not Occur in Etiolated Barley—According to Reinbothe et al. (7), extraction of etiolated barley tissues or barley etioplasts, with 100% acetone containing 0.1% DEP prevents the conversion of Pchlide b to Pchlide a and results in the detection of substantial amounts of Pchlide b. All attempts to repeat these results with etiolated barley leaves or isolated barley etioplasts failed, irrespective of whether the extraction was performed with 80% acetone (Fig. 1A and B, traces a and b) or 100% acetone containing 0.1% DEP (Fig. 1, A and B, trace c). All emission spectra exhibited pentacoordinated Pchlide a.
emission maxima in ether at 77 K, at 625 nm. Pchlide, –
authentic MV-Pchlide 0.23, elicited by excitation at 463 nm, corresponds to that of 438 nm and a Bx (0-0) excitation shoulder at 443 nm. A BY (0-0) MV-Pchlide b emission maximum of Pchlide spectra recorded at 643 nm, which corresponds to the mixed pyrrole pools of green barley leaves extracted with 80% acetone or 100% acetone containing 0.1% DEP were reported previously that small amounts of Pchlide b were formed by green plants (1, 2). To determine whether the amounts of detectable Pchlide b in green barley were affected by the acetone concentration used in pigment extraction, the Pchlide emission profile of green barley leaves extracted with 80% acetone or 100% acetone containing 0.1% DEP were recorded.

As shown in Fig. 2, the ether extract of the carboxylic tetrapyrrrole pools of green barley leaves extracted with 80% acetone or with 100% acetone containing 0.1% DEP and excited at 463 nm (the Soret excitation maximum of Pchlide b) exhibited a considerable increase in the Pchlide b + hexacoordinated Pchlide a emission at 642–643 nm in comparison with the pentacoordinated Pchlide a emission at 626–627 nm. This in turn resulted in an 8.6-fold increase in the 642–643/626–627 nm emission ratio in comparison with authentic Pchlide a, due to the presence of MV-Pchlide b in the green leaf extract. Excitation at 463 nm also elicited emissions at 662 nm and an emission shoulder at 678 nm that correspond to the emission maxima of Chlide a and Chlide b, respectively. The longer wavelength emission of Pchlide b contributes slightly to the emission at 678 nm (Fig. 2, trace c). The amounts of Pchlide a and b accumulation detected in isolated barley chloroplasts suspensions and pellets extracted with 80% acetone or 100% acetone containing 0.1% DEP are reported in Table II. The only observed difference between the two extraction methods was the incomplete pigment extraction achieved with 100% as compared with 80% acetone (Table II).

**DISCUSSION**

Small amounts of MV-Pchlide b are readily detectable in green barley leaves and isolated chloroplasts (Fig. 2 and Table II). However, none was detected in etiolated barley leaves and isolated etioplasts irrespective of the extraction procedure (Fig. 1). The only observed difference between extraction with 80% acetone or 100% acetone containing 0.1% DEP was the incomplete pigment extraction achieved with 100% as compared with 80% acetone.

The confusion surrounding the occurrence of MV Pchlide b in etiolated tissues may have been caused by the identical fluo-

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**Protochlorophyllide b Does Not Occur in Etiolated Plants**

**TABLE I**
Conversion of the Pchlide pool of isolated barley etioplasts to Chlide a by a single actinic white light flash

| Exp. | Light treatment | Extraction method | Tetrapyrroles |
|------|----------------|------------------|---------------|
|      |                |                  | Pchlide b | Pchlide a | Chlide a | Chl a | Total Tets | Percent conversion |
| A    | Dark           | 80% acetone      | ND        | 1751      | ND       | ND   | 1751       | 62                |
| B    | Dark           | 100% acetone     | ND        | 983       | ND       | ND   | 983        |                   |
|      | Light          | 100% acetone     | ND        | 267       | 665      | 29   | 961        | 72                |

**TABLE II**
Effect of solvent used in pigment extraction on the detection of accumulated tetrapyrroles in isolated barley chloroplasts

The 100% acetone contained 0.1% DEP.

| Exp. | Plant material   | Extraction method | Tetrapyrroles |
|------|------------------|------------------|---------------|
|      |                  |                  | Pchlide a | Pchlide b | Chlide a | Chlide b | Chl a | Chl b |
|      |                  |                  | nmol g protein |
| A    | Chloroplast pellet | 80% acetone     | 112      | 31       | 138     | 80      | 89,251 | 52,259 |
|      | Chloroplast suspension | 80% acetone | 104      | 27       | 182     | 94      | 171,667 | 98,148 |
| B    | Chloroplast pellet | 100% acetone    | 19       | 25       | 95      | 91      | 107,401 | 59,266 |
|      | Chloroplast suspension | 100% acetone | 24       | 14       | 62      | 55      | 145,751 | 92,685 |

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Small Amounts of Pchlide a along with Larger Amounts of Pchlide b Are Synthesized in Green Barley Leaves—It was reported previously that small amounts of Pchlide b were formed by green plants (1, 2). To determine whether the amounts of detectable Pchlide b in green barley were affected by the acetone concentration used in pigment extraction, the Pchlide emission profile of green barley leaves extracted with 80% acetone or 100% acetone containing 0.1% DEP were recorded.

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Rescence emission maxima of hexacoordinated MV-Pchlide \textit{a} and the red emission maximum of MV-Pchlide \textit{b} at 642–643 nm, in diethyl ether at 77 K (Fig. 1A) (16). However, the hexacoordinated Pchlide \textit{a} emission maximum at 642–643 nm observed in the Pchlide pools of etiolated barley in diethyl ether at 77 K (Fig. 1A) lacks the Pchlide \textit{b} signature, \textit{i.e.} a corresponding Soret excitation maximum at 462–463 nm (Fig. 1B). Finally, although Reinbothe \textit{et al.} (7) reported tabular \textit{mlz} data for 7-OH-Pchlide \textit{a}, Pchlide \textit{a} and Pchlide \textit{b}, in etiolated barley, the quality of the reported data could not be evaluated in the absence of the authentic mass spectra.

Altogether the above results indicate that although small amounts of Pchlide \textit{b} are detected in chloroplasts isolated from green tissues, none is present in etiolated tissues. It is therefore concluded that Pchlide \textit{b} plays no role in the greening process of etiolated tissues. On the other hands, we have proposed that in green plants MV Pchlide \textit{b} contributes to the formation of Chlide \textit{b} and Chl \textit{b}, via two distinct routes, namely routes 8 and 11, which are responsible for Chl \textit{b} formation in green plants (3). As for the proposed photoprotection imparted by the putative LHPP to etiolated tissues (4), that function is fully assumed by the high concentration of carotenoids present in etiolated tissues.

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