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PHYTOCHROME CONTROLLED PHENYLALANINE AMMONIA LYASE IN *HORDEUM VULGARE* PLASTIDS*

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Abstract—Chloroplasts isolated from 5-day-old barley shoots by differential centrifugation have L-phenylalanine ammonia lyase (PAL) activity. Based on chlorophyll/PAL ratios, the chloroplasts have about 29% of the PAL of the whole shoot. When plastid preparations are fractionated by discontinuous sucrose gradient centrifugation, highest PAL activity is found at the interface containing predominantly intact plastids. Continuous sucrose gradient centrifugation shows a correlation between chlorophyll content and PAL levels. PAL activity in 5-day-old etioplasts is increased *ca* 40% by giving the plants 4 min of red light 5 hr before harvest. Far-red light reverses the red light effect and demonstrates phytochrome control of barley plastid PAL.

INTRODUCTION

L-Phenylalanine ammonia lyase (PAL) was first isolated from shoots of young light-grown barley [1]. When etiolated barley seedlings are given 10 hr of continuous white light, PAL levels are increased about 40% [2]. Phytochrome control of barley PAL levels in whole-shoot homogenates has been shown by time-course red far-red photoreversibility studies [3] and supported by low-energy action spectra for the response [4].

At the subcellular level most workers find highest levels of PAL in the soluble fraction obtained during organelle isolation [5], yet the contribution of disrupted organelles to the soluble fraction is often not determined. High PAL activity is also associated with glyoxysomes of castor bean endosperm [6], peroxisomes of spinach [7], and in various microsome fractions prepared from *Quercus* [8], buckwheat [9] or sorghum [10] seedlings. In potato tubers, PAL activity is found in all particulate fractions except the microbodies (peroxisomes) [11].

Hachtel [12] reports that plastid inheritance determines the degree to which PAL levels in various *Oenothera* hybrids are influenced by light, and Löffelhardt *et al.* [13] have found PAL activity in intact plastids and in thylakoid fragments prepared from several types of plants including the monocotyledons corn and wheat. There are several reports of the presence and probable synthesis of flavonoids and other phenolic compounds in chloroplasts [14–17], and both PAL [3, 4] and flavonoids [18] are phytochrome-controlled in barley. Accordingly, we investigated PAL activity in chloroplasts isolated from white-light grown barley shoots and in etioplasts given various light treatments 5 hr [3] before harvest.

RESULTS

Preliminary experiments showed that most of the PAL activity was present in the supernatant fraction from 2000 *g* differential centrifugation of filtered homogenates. However, quantitative recovery of organelles by such techniques is usually quite low and recovery of less than 4% of the plastids is to be expected [19]. When we measured the chlorophyll content of whole shoots, and of chloroplasts isolated from an equivalent weight of tissue, only *ca* 3% of the chlorophyll (and thus the

* Part 5 in the series "Phenolic Biosynthesis in Barley Seedlings". For Part 4 see Ref. [4].

plastids) was recovered in the centrifugation pellet. This led us to measure the ratio of chlorophyll to PAL activity in whole shoots and in chloroplasts. We find that either 1 g of whole shoots, of $5{\cdot}88 \times 10^9$ chloroplasts (determined by plastid counting), contain 0·45 mg total chlorophyll. Assuming only that chlorophyll is restricted to the chloroplasts, then this gives us the number of chloroplasts per gram of tissue.

The supernatant from a 30900 *g* whole-shoot homogenate produces 173·7 nmol cinnamic acid/min/g fr. wt and $5{\cdot}88 \times 10^9$ chloroplasts produce 50·1 nmol cinnamic acid/min. Thus the chloroplast fraction obtained by differential centrifugation contains *ca* 29% as much PAL activity as one finds in the 30900 *g* supernatant of whole-shoot homogenates prepared from a corresponding weight of tissue. These results are in line with those of Löffelhardt *et al.* [13] who found that chloroplasts of *Dunaliella marina* contain *ca* 17% of the PAL of the whole cell.

Discontinuous sucrose gradient centrifugation was performed by layering a resuspended 2000 *g* pellet on the surface of a four-step (13·7, 25, 45 and 55% w/v) sucrose gradient and centrifuging for 20 min at 13200 *g*. As determined by phase and Nomarski microscopy, *ca* 95% of the particles accumulating at the boundary between 45 and 55% sucrose were class I plastids. A mixture of class I and II plastids, other cytosomes, and membrane fragments accumulated between 25 and 45% sucrose. Microbodies should pellet in this gradient [20] and typical microsome preparations should be in the upper part of the gradient away from the chloroplasts [21]. When PAL and chlorophyll were assayed in the rather diffuse bands accumulating at the gradient interfaces, the highest PAL activity was associated with the fraction richest in chloroplast (Table 1).

Continuous sucrose gradient fractionation was carried out by layering a resuspended 2000 *g* plastid preparation on the surface of a gradient ranging from 25·3 to 43% (w/v) sucrose. After centrifugation for 20 min at 13200 *g*, the tubes were punctured from below and the contents collected as 1 ml samples for either chlorophyll or PAL determination. The results of a typical experiment are shown in Fig. 1. From all of these experiments we conclude that PAL is closely associated with barley chloroplasts.

Phytochrome control of PAL in etioplasts isolated from dark-grown seedlings

As barley chloroplast preparations have PAL activity (Table 1, Fig. 1) and since PAL levels in the supernatants from whole tissue homogenates are controlled by phytochrome [3, 4], we investigated phytochrome control of PAL levels in etioplasts isolated by discontinuous centrifugation. Four minutes of red light given 5 hr before harvest increases PAL levels in both whole tissue homogenates and in etioplasts (Table 2). The effects of red light are reversed by a subsequent far-red treatment and from this we conclude that plastid PAL is phytochrome controlled in barley.

Chloroplasts isolated from shoots grown in continuous white light have PAL levels not significantly different from those of etioplasts (Table 2). This low PAL level in continuous light is probably explained by an initial increase in response to illumination, reaching a maximum at *ca* 5 hr, followed by an eventual decrease as illumination continues for prolonged periods [3].

DISCUSSION

Much evidence has accumulated for the association of PAL with particulate fractions [6–11]. For example, purified microsomes from buckwheat retain *ca* 3·5% of the PAL activity asso-

Table 1. PAL activity and chlorophyll content of a barley chloroplasts preparation after gradient centrifugation

Interface sampled (% sucrose)	Chlorophyll (μg)	Cinnamic acid (cpm) per 1 ml sample	Cinnamic acid (cpm) per μg chlorophyll
25/45	75	930	12·4
45/55	97	1600	16·5

Plastids from 5-day-old light-grown barley shoot homogenates were pelleted at 2000 *g*. This preparation was layered on the surface of a four-step (13·7, 25, 45 and 55%) discontinuous sucrose gradient and centrifuged at 13200 *g* for 20 min. Samples (1 ml) were collected from the interfaces and assayed as described in the Experimental.

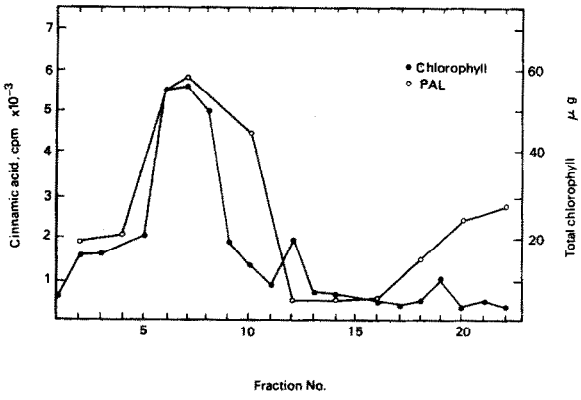

Fig. 1. Distribution of PAL and of chlorophyll after continuous sucrose gradient centrifugation of a barley chloroplast preparation. Chloroplasts were isolated from 5-day-old light-grown barley shoots by differential centrifugation at 2000 *g*. This preparation was layered on the surface of a continuous sucrose gradient ranging from 43% (Fraction 1) to 25·3% (Fraction 22) sucrose. After centrifugation for 20 min at 13200 *g* the tubes were punctured from below and the contents collected as 1 ml fractions for either chlorophyll or PAL determination by techniques given in the Experimental.

ciated with a 12000 *g* supernatant after repeated washing [2] and an organelle pellet (10000 *g*) from potato tubers retains its PAL activity after washing [11].

Many of these studies have been carried out in tissues such as potato tuber [11], castor bean endosperm [6, 7] or the roots of *Quercus* seedlings [8] which have neither chloroplasts nor well developed etioplasts. These tissues do, however, have proplastids and in the castor bean endosperm the proplasts have about two-thirds as much specific activity for PAL as do the glyoxysomes [6]. When Stafford [10] examined first internodes of sorghum she found that about one-third of the total PAL was in a "high speed" fraction that pelleted between 500 and 100000 *g*. About 40% of the activity of this pellet was present in a fraction sedimenting at 18000 *g* and she concluded that some of the PAL activity might be associated with an organelle such as a plastid. Löffelhardt *et al.* [13] have reported that PAL is thylakoid bound in several plants and in this paper we present evidence that PAL activity in barley etioplasts is under phytochrome control.

None of the data so far unequivocably pinpoints the intracellular localization of PAL or of any other enzyme of the C_6–C_3 sequence to a single subcellular organelle or fraction [22], and the association of these enzymes with such a variety of particles and the cytosol may be, in part, an artifact of random absorption during the traumatic isolation required for preparation of the fractions. Still, we do not consider it likely that all of the PAL in plastid preparations is an artifact of isolation. We find a reasonable correlation between PAL and chlorophyll in chloroplasts fractionated by continuous sucrose gradient centrifugation (Fig. 1) and Löffelhardt *et al.* [13] report a near-constant ratio of chlorophyll to PAL in whole plastids, thylakoids, or sub-thylakoid fractions prepared by ultrasonic disruption. This extensive purification and washing would surely remove gross contamination.

In our preliminary experiments we attempted to free PAL from our chloroplast preparations by osmotic shock in sugar-free solution or by 1 min of *ca* 15 W sonication. Low levels of enzyme activity

Table 2. PAL levels in plastids and in whole tissue homogenates from Atlas 68 barley

Treatment	Cinnamic acid/min/ 2×10^{10} plastids (nmol)	Cinnamic acid/ min/g fr. wt in supernatant from whole shoot homogenate (nmol)
White light	163·8 (6·6)*	173·7 (2·7)
Dark control	143·4 (20·4)	146·4 (1·8)
4 min R	213·0† (45·0)	192·6† (5·4)
4 min R + 2 min FR	133·2 (7·8)	161·7 (4·5)

* Mean deviation.

† Significantly different from dark controls at 99% confidence limits.

White light treated plants were grown under 8800 lux continuous cool-white fluorescent light for 4 days. Etiolated plants were grown for 4 days in a darkroom and, when appropriate, given red (1·1 kerg cm^{-2} sec^{-1}) or far-red (34 kerg cm^{-2} sec^{-1}) light 5 hr before harvest. Plastids were isolated from filtered homogenates by differential centrifugation at 2000 *g*. Whole shoot samples were homogenized and the 30900 *g* supernatant used as an enzyme source. The spectral PAL assay is described in the experimental section.

were obtained by these techniques and increasing the sonication energy to 45 W increased the level of PAL in the supernatant about five-fold. In the marine alga *Dunaliella marina*, which has a single plastid with large flattened thylakoids, such rigorous treatments do not appear to be necessary [13]. It is probably relevant that such large lamellae are more sensitive to sonication than are the numerous small grana that one finds in barley [23].

Subcellular organelles are not static, independent, structures within the living plant cell. In particular, the outer portion of the chloroplasts is maintained in a highly mobile state [24]. Membrane continuities have been observed between the outer membrane of the chloroplasts and the rough endoplasmic reticulum, the plasma membrane, the outer membrane of mitochondria, the tonoplast, the dictyosome vesicles, and various microbodies [24]. During organelle isolation the outer plastid membrane may give rise to microsomes [24]. Membrane continuities of this sort, and the extensive organelle damage associated with large scale isolation of subcellular fractions, probably relate to some of the reports of PAL compartmentalization.

It is likely that we have only approximated the distribution of PAL between plastids and other cytoplasmic pools in barley. For example, we find it necessary to apply rigorous ultrasonic treatment to liberate high levels of PAL from our plastid preparations and such harsh treatments may damage enzymes and alter enzyme subunit structure [25]. Also, we have not differentiated between increases in PAL synthesis and the cessation of PAL degradation nor have we attempted to measure active and inactive pools of PAL [26]. Until more is known about these later aspects it will be difficult to explain why 4 min of red light increases PAL *ca* 40% in either etioplasts or in supernatants from whole shoot homogenates (Table 1), or why 1×10^{-5} M cyclohexamide, but not chloramphenicol, blocks the stimulatory effects of red light on PAL levels in whole shoot homogenates (McClure, unpublished).

Chloroplasts have been reported to have cinnamic acid hydroxylase activity [27, 28], to synthesize 6-methylsalicylic acid [14], and to contain flavonoids [15–17]. Genetic inference [12] and direct measurement (Ref. [13] and this paper) of PAL in plastids further supports their role in polyphenolic biosynthesis. Phytochrome control of plastid responses is well known and includes such phenomena as; chloroplast orientation [29], control of chloroplast development and of chlorophyll biosynthesis [30], and heterotrophic carbohydrate metabolism of plastids [31]. Accordingly, if PAL is associated with plastids, then it is not surprising that its level is phytochrome controlled. We are currently working to improve our techniques of organelle isolation and enzyme recovery and will extend our investigations into other aspects of plastid phenolic biochemistry.

EXPERIMENTAL

Plant material and light treatments. Culture conditions and light treatments for etiolated Atlas 68 barley (a gift of Dr. Burt Ray, Agronomy Department, Univ. of California, Davis) have been described [3]. White-light grown seedlings were maintained in a plant growth chamber under 8800 lx continuous cool-white fluorescent light at 23°.

Plastid isolation. Chloroplasts were isolated under laboratory lighting conditions. All manipulations of etiolated plants or etioplasts were carried out in the dark or under a dim green safelight [3]. Plastids were isolated from *ca* 35 g of 5-day-old shoots. The shoots were excised at the point of emergence from the caryopsis, cut into 1 cm segments, and ground in a Virtis model 23 homogenizer by three successive 2-sec treatments at max. velocity. The isolating medium was 100 ml of cold 25 mM borate buffer (pH 8·8) containing 0·33 M sorbitol, 2 mM disodium EDTA, 1 mM $MgCl_2$, and 0·4 ml mercaptoethanol. The homogenate was filtered through two layers of cheesecloth and two layers of Miracloth. All purification steps were carried out at 4° in a Sorvall RC2-B centrifuge. The filtrate was centrifuged for 60 sec at 200 *g*, the pellet discarded, and plastids pelleted by centrifugation at 2000 *g* for 60 sec. The pellet was resuspended in 45 ml of homogenizing solution and 20 μl removed for plastid number determination with an AO Spencer Bright Line hemacytometer. Occasionally a Coulter particle counter was used with similar results. The plastids were then repelleted by centrifugation at 2000 *g* for 2 min. This pellet served as the differential centrifugation preparation or as starting material for gradient centrifugation. Discontinuous sucrose gradient centrifugation was carried out by layering the 2000 *g* plastid preparation on the surface of a four-step (13·7, 25, 45 and 55% w/v) discontinuous sucrose gradient prepared in pH 8·8 25 mM borate buffer. This was centrifuged for 20 min at 13200 *g*. The cellulose acetate tubes were punctured from below and a Beckman fraction recovery system used to collect 1 ml fractions near the interfaces. Continuous sucrose gradients were prepared with a Beckman density gradient former between the range of 43% (1·192 g/cm^3) and 25·3% (1·104 g/cm^3) sucrose in borate buffer. Linearity of the gradient was verified with a B & L Abbe refractometer. The 2000 *g* plastid preparation was layered on the surface of the gradient and centrifuged for 20 min at 13200 *g* in a Sorvall H-34 swinging bucket head. The gradient was collected as 1 ml fractions with a Beckman fraction recovery system.

Enzyme assays. Whole tissue PAL was assayed by a spectrophotometric technique modified from Zucker [32]. Four shoots

were weighed and thoroughly ground in a chilled Ten Broeck homogenizer containing 8 ml of cold 25 mM borate buffer (pH 8·8), 23 μl mercaptoethanol, and 0·3 g Polyclar AT which had been soaked overnight in buffer and mercaptoethanol before use. The homogenate was centrifuged at 4° for 30 min at 30900 *g* and the supernatant used as a source of enzyme. The assay system consisted of 1 ml of supernatant, 1 ml of buffer, and 1 ml of 50 mM L-phenylalanine in buffer. Controls contained buffer in place of L-phenylalanine. The change in absorptivity at 290 nm was monitored in 1 cm light-path cells at 10–15 min intervals for 1 hr or more at 40°. Under these conditions, a change in absorbance of 0·01 was found to be equivalent to the production of 3·09 nmol of cinnamic acid. We have recently shown that side reactions involving transamination from phenylalanine onto α-keto acids, producing phenylpyruvate which tautomerizes with borate and absorbs at 290 nm [33], do not interfere with the spectrophotometric assay of PAL in barley seedlings [34]. Each data point is the mean of at least five determinations, each done in triplicate. PAL activity for whole shoot assays is corrected for the negative correlation coefficient between fr. wt and PAL content per g [3,4]. Plastids isolated by differential centrifugation at 2000 *g* were resuspended in 4·5 ml of buffer with mercaptoethanol and subjected to ultrasonic disruption in an ice bath for 1 min at 45 W (see Discussion) with a model W-185 Branson Sonifier. The suspension of disrupted plastids was centrifuged at 30900 *g* for 30 min and the supernatant used as a source of enzyme for assay by the spectrophotometric technique. Discontinuous and continuous sucrose gradient fractions (1 ml) were similarly sonicated and centrifuged. The supernatant was incubated with 1 μ Ci of L-phenylalanine [^{14}C-U] with sp. act. 464 m Ci/mM (New England Nuclear). After 45 min (discontinuous) or 1 hr (continuous) at 40° the reaction was stopped by the addition of 1 ml of methanol and 1 ml 1 N HCl. The samples were centrifuged at 2000 *g* for 10 min, the supernatant reduced to a vol. of about 1 ml, and phased with 5 × 10 ml diethylether. The ether fraction plus *ca* 200 μmol of unlabeled cinnamic acid was chromatographed on Whatman No. 1 paper in 2:1:2 (*t*-BuOH–HOAc–H_2O). Cinnamic acid was identified under short wave uv light and an elongated ellipse containing the compound cut from the chromatogram. Cinnamic acid was eluted from the paper by methanolic irrigation for 12 hr directly into a scintillation vial. The samples were taken to dryness and resuspended in 0·7 ml methanol. Aquasol scintillation fluid (19·3 ml) was added and radioactivity determined with a Packard Tri-Carb scintillation counter. Total chlorophyll was determined by the method of Arnon [35].

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REFERENCES

1. Koukol, J. and Conn, E. C. (1961) *J. Biol. Chem.* **236,** 269.
2. Amrhein, N. and Zenk, M. H. (1971) *Z. Pflanzenphysiol.* **64,** 145.
3. McClure, J. W. (1974) *Phytochemistry* **13,** 1065.
4. McClure, J. W. (1974) *Phytochemistry* **13,** 1071.
5. Camm, E. L. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 961.
6. Ruis, H. and Kindl, H. (1970) *Z. Physiol. Chem.* **351,** 1425.
7. Ruis, H. and Kindl, H. (1971) *Phytochemistry* **10,** 2627.
8. Alibert, G., Ranjeva, R. and Boudet, A. (1972) *Biochim. Biophys. Acta* **279,** 282.
9. Amrhein, N. and Zenk, M. H. (1970) *Naturwissenschaften* **57,** 312.
10. Stafford, H. A. (1969) *Phytochemistry* **8,** 743.
11. Camm, E. and Towers, G. H. N. (1972) *Phytochemistry* **12,** 1575.
12. Hachtel, W. (1972) *Planta* **102,** 247.
13. Löffelhardt, W., Ludwig, B. and Kindl, H. (1973) *Z. Physiol. Chem.* **354,** 1006.
14. Kannangra, C. G., Henningsen, K. W., Stumpf, P. K. and Von Wettstein, D. (1971) *European J. Biochem.* **21,** 334.
15. Oettmeier, W. and Heupel, A. (1972) *Z. Naturforsch.* **27,** 177.
16. Weissenbock, G. (1973) *Ber. Deut. Botan. Ges.* **86,** 351.
17. Saunders, J. A. and McClure, J. W. (1972) *Am. J. Botany* **59(S),** 673.
18. Carlin, R. M. and McClure, J. W. (1973) *Phytochemistry* **12,** 1009.
19. Gibbs, M. Personal communication.
20. Tolbert, N. E. (1971) *Ann. Rev. Plant Physiol.* **22,** 45.
21. DeDuve, C., Berthet, J. and Beaufey, H. (1959) *Prog. Biophys. Biophys. Chem.* **9,** 325.
22. Stafford, H. A. Forthcoming publication.
23. Jacobi, G. (1967) *Z. Pflanzenphysiol.* **57,** 255.
24. Crotty, W. J. and Ledbetter, M. C. (1973) *Science* **182,** 839.
25. Hanson, K. R. and Havir, E. A. (1972) in *Recent Advances in Phytochemistry* (Runeckles, V. C. and Watkins, J. E., eds), p. 45. Appleton-Century-Crofts, New York.
26. Attridge, T. H. and Smith, H. (1973) *Phytochemistry* **12,** 1569.
27. Bartlett, D. J., Boulton, J. E. and Butt, V. S. (1972) *FEBS Letters* **23,** 265.
28. Parish, R. W. (1972) *European J. Biochem.* **31,** 446.
29. Haupt, W. (1968) *Z. Pflanzenphysiol.* **58,** 331.
30. Virgin, H. I. (1961) *Physiol. Plantarum* **14,** 439.
31. Klein, W. H., Price, L. and Mitrakos, K. (1963) *Photochem. Photobiol.* **2,** 233.
32. Zucker, M. H. (1968) *Plant Physiol.* **43,** 365.
33. Erez, A. (1973) *Plant Physiol.* **51,** 409.
34. Saunders, J. A. and McClure, J. W. (1974) *Plant Physiol.* **54,** 412.
35. Arnon, D. I. (1949) *Plant Physiol.* **24,** 1.