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PHYTOCHROME CONTROL OF AMINO ACID SYNTHESIS IN COTYLEDONS OF *SINAPIS ALBA*

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Abstract—Continuous far red light, acting through phytochrome, stimulates the rate of incorporation of density label into amino acids in the cotyledons of *Sinapis alba*. It is shown that such stimulation leads to increased incorporation of label into proteins. This has important consequences for experiments in which rates of enzyme synthesis in light treated and dark grown plants are compared by labelling methods. The results of some such experiments are re-evaluated.

INTRODUCTION

Profound changes in the development of seedlings are controlled by the proteinaceous photoreceptor, phytochrome. The role of phytochrome in the control of gene expression in seedlings is clearly of great developmental importance and the attempted elucidation of the mechanisms involved has been the subject of much research effort. Despite this, these mechanisms are still a matter of controversy [1–5]. It is known [6] that an increase in the overall rate of protein synthesis occurs in mustard cotyledons irradiated with continuous far red light, which is known to operate exclusively through phytochrome [7]. It has hitherto been assumed that this increase occurs at the expense of amino acids released from the breakdown of storage protein, since the cotyledons contain large reserves of such protein, whose degradation is stimulated by far red light [8].

We present here evidence, obtained by a novel technique involving the measurement by density labelling of the incorporation of ^2H_2O into mustard cotyledon amino acid pools, that phytochrome also increases the rate of amino acid synthesis. Since the results show directly that far red irradiation stimulates the uptake of ^2H_2O into the amino acid pools, the results of some comparative density labelling experiments, in which the effect of phytochrome on the rate of incorporation of ^2H_2O into specific enzymes has been estimated [1–5, 9, 10] need to be scrutinized. A re-evaluation of some of these data, taking into account the results presented here, is also given.

Rationale of the method employed

In essence, the total amino acids are extracted from the experimental plant material after various times of incubation in ^2H_2O and are presented as sole nitrogen source to an inoculum of *Escherichia coli*. After growth of the culture, bacterial proteins are extracted and the incorporation of ^2H_2O into these is measured, as in any density labelling experiment, by isopycnic ultracentrifugation. In this case, bacterial β-galactosidase was selected as a convenient marker enzyme and the extent of incorporation of label into β-galactosidase taken as a measure of the proportion of ^2H_2O originally incorporated into the plant amino acid pools.

RESULTS AND DISCUSSION

The data obtained by the method outlined above, using experimental conditions identical to those employed by the Freiburg group ([11]; full details given in the Experimental) are shown in Fig. 1. It can be seen that, within 1.5 hr, far red light causes *ca* 85% stimulation of incorporation of density label into the amino acid pools, compared with the dark control. The results show also that the proportion of ^2H_2O in the pools declines after 3 hr in the light. This is expected in view of the known effect of light on storage protein breakdown [8]. Two explanations are possible for the initial stimulation of label uptake promoted by phytochrome. One possibility is that the amino acid pool is depleted in far red light treated cotyledons, leading to a greater percentage of newly synthesised amino acids in the pool. The other is that phytochrome action stimulates the rate of amino acid synthesis in the cotyledons. The latter conclusion is supported by the finding [12] that the enzyme nitrate reductase, whose activity is normally the rate limiting step in amino acid synthesis [13], is under rapid phytochrome control in mustard cotyledons. Furthermore, there were no significant differences in the quantity of amino acids extractable from the cotyledons of far red treated and dark grown plants throughout the time course of the experiment (250 ± 25 μg/g fr. wt). Thus, the most likely explanation of the data presented here is that phytochrome regulates amino acid synthesis in the mustard cotyledon.

Implications for density labelling studies

The fact that the extent of labelling of the amino acid pool is different in far red treated plants than in the dark controls is of great importance in the context of com-

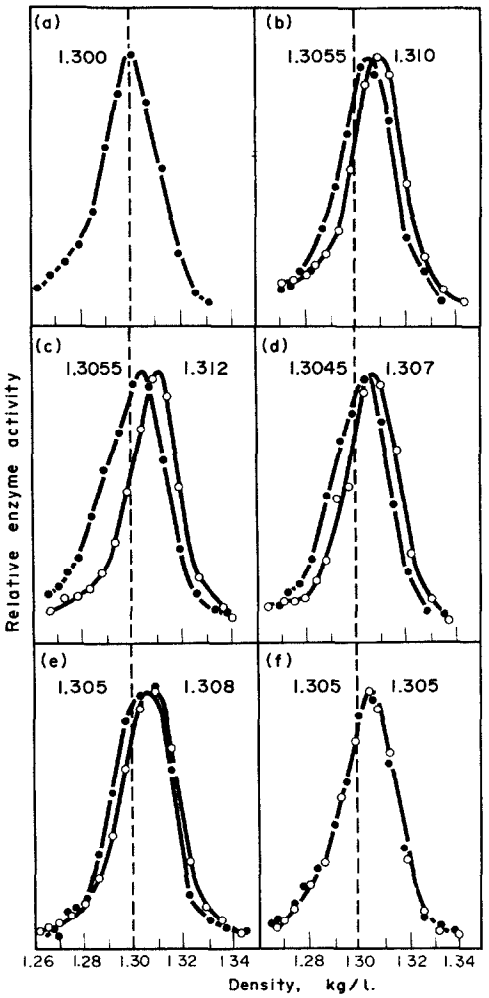

Fig. 1. Incorporation of density label into amino acids extracted from the cotyledons of mustard seedlings. Seedlings were grown in the dark on H_2O for 36 hr and then transferred to 80% ^2H_2O (and, where appropriate, to continuous far red light) for (a) 0 hr, (b) 1.5 hr, (c) 3 hr, (d) 6 hr, (e) 12 hr and (f) 24 hr. Amino acids were extracted from the cotyledons and incorporated into *E. coli* protein. The labelling of bacterial β-galactosidase was measured by isopycnic ultracentrifugation in CsCl The dotted line indicates the density of native β-galactosidase. Symbols: open circles, far red treated plants; closed circles, dark grown plants.

parative density labelling investigations into phytochrome mediated enzyme responses. The increased incorporation of ^2H_2O into amino acids in far red treated tissues should be reflected in increased labelling of proteins, especially those having short half lives. Phenylalanine ammonia-lyase (PAL) is such an enzyme, its activity in the dark having a half life of between 3 and 4 hr in mustard [3]. Furthermore, its activity is under phytochrome control and two groups of workers have obtained apparently conflicting results concerning the level at which this control is exerted. Attridge *et al.* [1] found that, despite a large increase in PAL activity, the proportion of the enzyme population incorporating density label was less in far red light than in the dark control. They argued that this ruled out phytochrome-controlled stimulation of synthesis as the main cause of the increased activity. On the basis of comparisons with similar results obtained with gherkin [14] tissue, where inactive PAL [15] and a PAL inactivator [16, 17] are known to exist, they concluded that phytochrome action led to PAL activation.

These results were obtained using 100% ^2H_2O as density label. A consequence of this was a very slow time course of PAL increase (maximum activity at 44 hr compared with 20 hr in H_2O). Acton and Schopfer [2] criticized this work, primarily because the half life of PAL activity in the dark, which they showed to be *ca* 4 hr, had been substantially exceeded before any increase in activity could be detected. Whilst this criticism is valid, it in no way explains the results of Attridge *et al.*, which could not, in any case, be accounted for by an increased rate of phytochrome-promoted enzyme synthesis. Their other principal criticism was that the internal marker enzyme which had been employed would not, by virtue of its comparatively long half life, reflect sufficiently rapidly, any differences in the rates of incorporation of ^2H_2O into proteins between far red treated and dark grown plants. They suggested that such differences could have been the cause of the reduced incorporation of label in the light treated plants. Strangely, they did not themselves include a suitable marker. The experiments described here, which were carried out using precisely the conditions employed by Acton and Schopfer and their Freiburg colleagues, strikingly demonstrate the importance of such a marker,

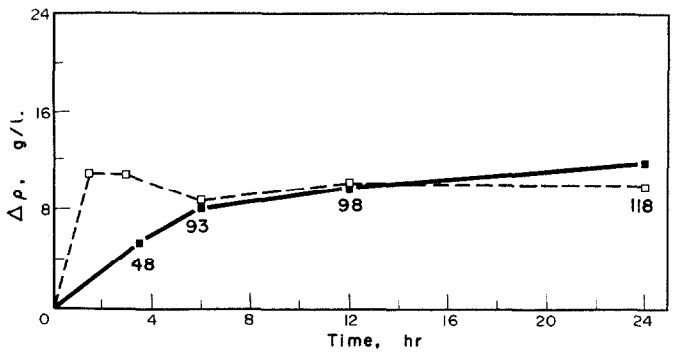

Fig. 2. Incorporation of density label into PAL (closed symbols, solid line Data from ref [2]) compared with the incorporation into total amino acids (open symbols, dotted line, data from Fig. 1) in the cotyledons of dark grown mustard seedlings. The seedlings were transferred to 80% ^2H_2O 36 hr after sowing. The figures indicate the percentage labelling of the lyase compared with that of the amino acid pool at the same time.

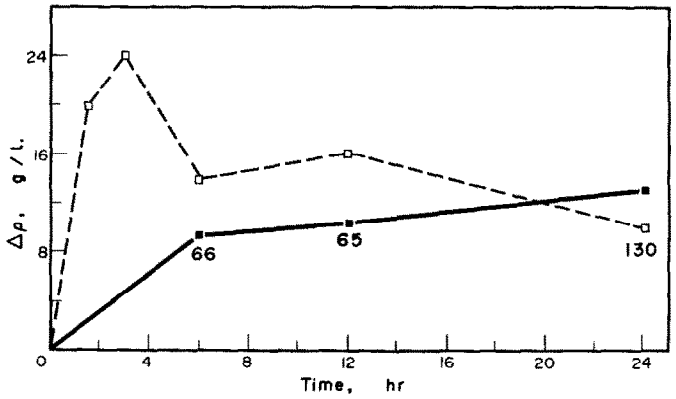

Fig. 3. Incorporation of density label into PAL (closed symbols, solid line. Data from ref. [2]) compared with the incorporation into total amino acids (open symbols, dotted line) in the cotyledons of mustard seedlings transferred to 80% ^2H_2O and continuous far red light 36 hr after sowing. The figures indicate the percentage labelling of the lyase compared with that of the amino acid pool at the same time.

as suggested by Acton and Schopfer, but, ironically, enable conclusions opposite from those drawn by those workers to be reached using their own data.

In Fig. 2 the incorporation of ^2H_2O into the total amino acid pool in the dark is compared with the incorporation into PAL observed by Acton and Schopfer. Compared with the amino acid pool, 50% labelling of PAL is attained after *ca* 3–3.5 hr which is in close agreement with their estimate of 4 hr for the half life of the enzyme. In far red irradiated seedlings the situation is remarkably different (Fig. 3). Whereas the incorporation into PAL (again taken from the data of Acton and Schopfer) is similar to that in the dark, it is far less, when expressed as a proportion of the maximum labelling obtainable, as measured by incorporation of ^2H_2O into the total amino acid pool. When the data of Acton and Schopfer are recalculated to take account of the more extensive labelling possible in far red light, both they and the data of Attridge *et al.* [1] indicate that the phytochrome action leads to a reduction in the proportion of labelled PAL in the mustard cotyledone. Such a result is not possible if the predominant effect of phytochrome on PAL is the stimulation of its rate of synthesis.

Partitioning of amino acid pools

The results presented above do not eliminate the possibility that the phytochrome mediated stimulation of ^2H_2O uptake into the total amino acid pool does not reflect changes occurring in the pool from which PAL is synthesized. To investigate this possibility, nitrate treatment of dark grown plants was used as a means of stimulating amino acid biosynthesis. Nitrate and far red light each independently cause increases in nitrate reductase activity in the mustard cotyledon [12], whereas far red light, but not nitrate, stimulates PAL activity. Four hr after the addition of nitrate in ^2H_2O to dark grown mustard seedlings the activity of PAL is the same as in the control treated with ^2H_2O only. However, the increase in density of the lyase in the former treatment is twice that of the latter (Fig. 4) because of the increased incorporation of ^2H_2O into the amino acid pool resulting from nitrate promoted stimulation of amino acid synthesis. Thus, enhanced amino acid synthesis is reflected in increased density labelling of PAL.

Conclusions

A situation in which a stimulus, in this case far red light, leads to a change in the rate of incorporation of precursor molecules into proteins is obviously an undesirable basis for the study of such changes in individual enzymes. Nevertheless, we believe that in the respect of the crucial question "Does phytochrome stimulate PAL synthesis in the mustard cotyledon?", the

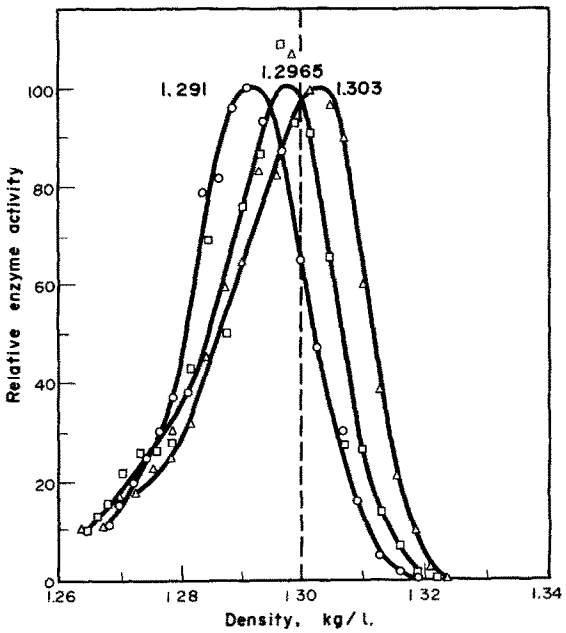

Fig. 4. Density labelling of PAL from the cotyledons of dark grown mustard seedlings. After 36 hr in the dark, seedlings were treated as follows; (a) left in the dark on H_2O for a further 4 hr (circles), (b) transferred to 100% ^2H_2O and left in the dark for a further 4 hr (squares), (c) transferred to 100% ^2H_2O and 100 mM KNO_3 and left in the dark for 4 hr (triangles). After these treatments, PAL was extracted from the cotyledons and centrifuged to equilibrium in RbCl gradients. β-Galactosidase was added to each sample as a control and its density is indicated by the dotted line. The activity of PAL was not affected by nitrate treatment. (Activity in (b) was 225 nmol cinnamate/hr/g and in (c) 207 nmol cinnamate/hr/g).

data of Attridge *et al.* [1] and of Acton and Schopfer [2] seem to be in agreement in suggesting that the answer is no.

EXPERIMENTAL

Growth of plants. Seeds of *Sinapis alba* L. (Asmer, Leicester, UK) were germinated in covered containers in the dark on Whatman No 1 filter paper at 25°. After 36 hr seedlings for far red irradiation were transferred to a light source consisting of 4 × 300W single coil tungsten bulbs filtered through 10 cm of running H_2O, 1 layer of No 5A Deep Orange and 1 layer of No 20 Deep Blue Primary Cinemoid (Rank Strand Electric, Kingsway, London, UK). The cabinet temp. was kept at 25°. ^2H_2O was added simultaneously with transfer to light and to the dark control plants.

Extraction of amino acids. Mustard cotyledon tissue (1 g) was frozen in liquid N_2 and ground in a mortar and pestle. A small vol of MeOH–$CHCl_3$–H_2O (12:5:3) was added with more grinding. The resultant paste was transferred to a centrifuge tube together with washings. The mixture was centrifuged at 0° for 5 min at 3000*g*. The supernatant was retained, and the pellet re-extracted with grinding medium and centrifuged × 5. For each 2 ml of bulked extract 1 ml of $CHCl_3$ and 1.5 ml of H_2O were added and the extract centrifuged at 3000*g* for 15 min. The aq. phase was collected and evapd to dryness. The sediment was taken up in 1 ml of H_2O.

Growth of Escherichia coli. Cells of a K12 strain of *E. coli* were grown on M9 medium with glycerol as carbon source and the extracted amino acids as sole nitrogen source. Isopropyl thiogalactoside (Sigma, UK) was added to induce β-galactosidase in the culture. The size of the initial bacterial inoculum was negligible in comparison with the final yield of bacteria.

Extraction of β-galactosidase from E. coli. The total culture grown on the extracted amino acids was harvested by centrifugation at 10000 *g* for 5 min and resuspended in 0.5 ml of 0.1 M NaPi buffer pH 7. The suspension was homogenized using a steel hammer press after freezing with liquid N_2. The homogenate obtained in this way was centrifuged at 38000 *g* for 20 min at 1° and an aliquot of the supernatant assayed for protein by the method of ref. [18]. Between 30 and 50 μg of protein dissolved in 0.1 ml buffer was used for ultracentrifugation.

Density gradient centrifugation. Enzyme extract was layered over 5 ml of a soln of CsCl in 0.1M Pi buffer pH 7 of buoyant density 1.31 kg/l. The tubes were filled to the top with paraffin oil. Ultracentrifugation was carried out in a Beckman Type 75Ti fixed angle rotor (19) using a Beckman L2-65B ultracentrifuge. After centrifugation for 20–24 hr at 50000 rpm (165000*g*) and 1°, fractions of 3 drops were collected by means of a narrow gauge needle lowered to the bottom of the tube and connected to a peristaltic pump Alternate fractions were used for the measurement of enzyme activity and for the determination of the buoyant density of the soln. These values were calculated from refractive index measurements (Abbé 60 refractometer) using the equation of ref. [20].

Assay of β-galactosidase. To each fraction to the assayed 0.5 mg of *o*-nitrophenyl β,D-pyranogalactoside, dissolved in 1 ml of 0.1 M NaPi buffer pH 7 was added at 0°. After incubation for 5–30 min at 30° the reaction was stopped by the addition of 2 ml of 10% Na_2CO_3 soln. The A_{410} of the reaction product, *o*-nitrophenol, was measured.

Extraction and assay of PAL. Enzyme was extracted from 2 g of mustard cotyledons by grinding in a mortar and pestle with 14 ml of 0.1 M NaPi buffer (pH 7) and 2 g insoluble PVP. The extract was centrifuged at 27000 *g* for 15 min and the supernatant desalted by passage through a Sephadex G-25 column (25 × 1.1 cm). The desalted extract was dissolved in RbCl in 0.1 M NaPi buffer (pH 7) containing 10 mM mercaptoethanol and 40 units of pure β-galactosidase (Sigma). Buffer or RbCl was added to give a final vol. of 5 ml and a density of 1.3 kg/l. Centrifugation and collection of fractions were carried out as described above except that the centrifugation speed was 55000 rpm (200000 *g*). Alternate fractions of 2 and 3 drops were assayed for β-galactosidase and PAL respectively and every eighth fraction was used for the determination of the density of the soln. PAL was assayed by the radioassay described previously [1].

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