

Control by Phytochrome of Glutathione Reductase Levels in the Mustard Seedling

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The time-courses of the level of glutathione reductase (GR; EC 1.6.4.2) were followed in cotyledons and hypocotyl of the developing mustard seedling in the dark and under the influence of light (phytochrome). Localization of GR on polyacrylamide disc electrophoresis columns indicate that 2 isoenzymes of GR are present in both organs under all experimental conditions. GR can be induced by light (phytochrome) in the cotyledons; however, there is no influence of light (phytochrome) on the time-course of the level of GR in the hypocotyl. While the actual lag-phase of the response in the cotyledons is less than 6 hours (onset of light at 48 hours) there is no detectable effect of light on apparent GR synthesis before approx. 42 hours after sowing. Irrespective of the onset of light (at 24 or 36 hours after sowing) the time-course of the enzyme level remains precisely the same. A red pulse given at 24 hours after sowing leads to an increase of the enzyme level as determined 60 hours later. The effect of the red pulse is fully reversed by a far-red light pulse given at 24 hours but not 12 hours later. Facts of this kind have led to the concept of a *stable* intermediate in the chain of regulatory events between phytochrome (P_{fr}) and GR increase. No indications of a coordinated, simultaneous induction by phytochrome of GR and ascorbate oxidase (AO) could be found. This result does not support the concept that a respiratory chain exists in which AO functions as a terminal oxidase.

We became interested in glutathione reductase* for two completely different reasons. 1. It has been suggested¹ that GR serves as an enzyme in a respiratory chain in which ascorbate oxidase* functions as a terminal oxidase. Since AO can be induced by phytochrome (P_{fr})* in the mustard seedling and since the induction kinetics have been determined with high precision² it was hoped that the investigation of GR would offer the opportunity to demonstrate a simultaneous, coordinated induction by phytochrome of two functionally related enzymes. So far evidence for this phenomenon is very limited. A simultaneous induction of functionally related enzymes by phytochrome was suggested in the case of marker enzymes of the peroxisomes, glycolate oxidase and glyoxylate reductase³. Since "molecular" models to describe developmental genetics in higher organisms⁴ require the assumption that several enzymes can be induced (or repressed) simultaneously and coordinately experimental evidence which justifies this assumption is urgently needed.

2. It was found in preliminary experiments that a phytochrome-mediated increase of the GR level by P_{fr} can only be observed at a relatively late stage of the mustard seedlings development (after approxi-

mately 42 hours after sowing). The question arises of whether or not this increase of the GR level can be induced by P_{fr} (operationally, by a brief irradiation with red light) at an earlier stage of development, *e. g.* 24 hours after sowing. Since P_{fr} disappears rapidly in the dark (with a half life of the order of 45 min in the mustard seedling)⁵ an induction effect of a red light pulse given at 24 hours after sowing would require the concept of a *stable* "transmitter" between the primary action of P_{fr} and the events on the enzyme level. The "transmitter concept" has already been advanced by Schopfer and Plachy⁶ in connection with phytochrome-mediated peroxidase induction in the mustard seedling. However, phytochrome-induced peroxidase appears only late in the development of the seedling (after 72 hours after sowing) when most of the enzymes studied so far can hardly be induced by phytochrome any more. It was hoped that phytochrome-mediated GR induction would offer an opportunity to study the "transmitter concept" within the period of development which has normally been used for studies on phytochrome-mediated control of enzyme levels in the mustard seedling (up to 72 hours (84 hours) after sowing).

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* **Abbreviations:** Glutathione reductase, GR (E.C. 1.6.4.2); ascorbate oxidase, AO (E.C. 1.10.3.3); physiologically active phytochrome, P_{fr} .



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Terminology¹²

Originally, the terms "induction" and "repression" were used operationally to designate the appearance or lack of appearance (or disappearance) of an enzyme (*cf.* ⁷). In the present paper the terms will be used in this original meaning, *i. e.* without *a priori* implications about the actual control mechanism(s). Thus, "enzyme induction by phytochrome" means an increase of extractable enzyme activity (or content) caused by phytochrome.

Since no general answer can be given to the question of whether a phytochrome-mediated difference in observed enzyme activity is due to a difference in the number of enzyme molecules, the term "synthesis" must still be used with caution in the field of photoregulation of enzyme levels. While the use of the term "synthesis" seems to be justified by the results of density or radioactive labelling^{8,9} and of the usual inhibitor experiments^{10,11}, it must be realized that there is no *rigorous* proof so far that a phytochrome-mediated increase of extractable enzyme activity is due to a corresponding synthesis of enzyme molecules (*cf.* ¹²).

Material and Methods

Standard techniques for photomorphogenic research with mustard seedlings (*Sinapis alba* L.) were used¹³. The mustard seeds were purchased in 1969 from Asgrow Company (Freiburg-Ebnat). The seedlings were grown at $25 \pm 0.2^\circ\text{C}$ in the dark and experiments were started at 24, 36 or 48 hours after sowing as indicated in the text.

The standard far-red source¹³ which maintains a low $P_{\text{fr}}/P_{\text{total}}$ ratio in the seedling¹⁴ was used at an irradiance of $3.500 \text{ mW} \cdot \text{m}^{-2}$. The standard red source¹³, which maintains a $P_{\text{fr}}/P_{\text{total}}$ ratio of about 0.8¹⁴, was used at an irradiance of $675 \text{ mW} \cdot \text{m}^{-2}$. Twenty seedlings, 20 pairs of cotyledons or 20 hypocotyls were ground for 5 min at $0 - 2^\circ\text{C}$ with 1.5 g quartz sand and 2 ml of 0.1 M sodiumphosphate buffer, pH 7.4. The homogenate was diluted with 8 ml of the buffer and centrifuged at $18.000 \times g$ for 20 min (0°C). The supernatant was used for the enzyme assay, which was performed at 25°C and pH 8.0 in 0.1 M Tris-HCl buffer (1 mM with EDTA) essentially after Bergmeyer¹⁵. The decrease of the absorbance at 340 nm due to the oxidation of NADPH₂ was used as a gauge for GR activity. The reference cuvette (pathway, 1 cm; volume, 1 ml) contained 0.96 ml Tris-HCl buffer, 0.02 ml enzyme

extract and 0.02 ml NADPH₂ (8.5 mM). In the sample cuvette 0.1 ml of the buffer was replaced by oxidised glutathione (GSSG, 44 mM in Tris-HCl buffer). The enzyme reaction was always started by adding NADPH₂. After thoroughly mixing the content of both cuvettes the reaction was recorded in a dual-wavelength spectrophotometer (Beckman, Type DB-G). The reaction kinetics were always linear after 2–5 min, until the NADPH₂ was nearly used up. Enzyme activity was based on linear reaction kinetics over at least 30 min. It is expressed as $-\text{nmol NADPH}_2/\text{min} \cdot \text{pair of cotyledons or hypocotyl}$. The molar extinction coefficient for NADPH₂ is $\epsilon_{340} = 6.22 \text{ cm}^2/\mu\text{mole}$ ¹⁵.

The use of the biological unit (cotyledon; hypocotyl) as a system of reference was justified previously (*cf.* ²¹).

The average values given in Tables and in Fig. 3 are based on 8–12 independent parallels. The standard errors in the Tables also represent the standard errors of the values in Fig. 3.

The following experiments were obtained in methodological experiments: 1. Enzyme activity was proportional to the concentration of extract in the assay mixture. 2. The light treatment of the seedlings did not influence the enzyme activity in the extracts through the formation of inhibitors or activators. This could be demonstrated in mixing experiments (*cf.* ¹⁶). If purified yeast GR (from Boehringer, Mannheim) was added during extraction of dark-grown or far-red-grown seedlings the enzyme activity was quantitatively recovered in the assay. Several purification procedures of the extracts were tested¹⁶: a. Purification with Sephadex G-25 in order to eliminate low molecular weight substances, b. purification with Divergan, which removes phenolic substances. None of these procedures changed the activity which was present in the crude extracts. 3. GR activity in extracts from both dark grown and irradiated seedlings had the same pH optimum (pH 8.0, Fig. 1) irrespective of seedling age. 4. The enzyme activity of the extracts diminished at a very low but linear rate during the first two hours at 0°C (about 2.5% during the first hour). The extracts were always measured during the first hour after centrifugation. 5. Disc gel electrophoresis. Crude extracts from cotyledons of dark-grown and far-red treated seedlings of various age were purified with Sephadex G-25 and transferred into 0.05 M Tris buffer (pH 6.8). Using system No. 1 after Maurer¹⁷ (7.5% polyacrylamide gels) the extracts were tested for the presence of isoenzymes. The size of the samples was chosen to contain equal enzyme activity irrespective of the age of the seedling and the irradiation regime. Electrophoresis was run in a cold room

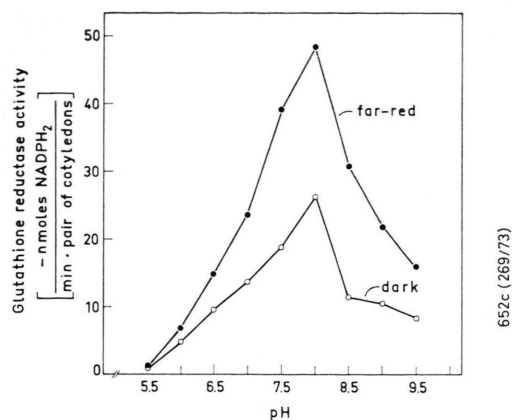


Fig. 1. pH optima of the enzymatic action of glutathione reductase. Extracts from cotyledons of dark-grown and far-red treated seedlings have the same optimum (pH 8.0). Far-red: 36 hours dark + 36 hours far-red; dark: 72 hours darkness. The following buffer systems were used: pH 5.5 to 7.0: 0.1 M citric acid – 0.2 M Na_2HPO_4 ; pH 7.5 to 8.5: 0.1 M Tris-HCl; pH 9 to 9.5: 0.1 M borate buffer.

(0–4 °C) using 2 mA per gel. After the run (1.5–2 hours) the gels were incubated at room temperature in 10 ml Tris-HCl buffer, pH 8.0, containing 0.289 g EDTA; 5.6 mg NADPH_2 ; 0.6 g GSSG and 3 mg DTNB* (modified after Shaw and Prasad¹⁸). DTNB reacts with the anion GS^- that develops during the enzyme reaction resulting in the formation of a yellow pigment which has an absorption maximum at 405 nm¹⁹. The gels were transferred into a densitometer cuvette ($d=4$ mm) and the absorbance at 405 nm was scanned in a Cary-14 spectrophotometer at 25 °C²⁰.

Fig. 2 shows two representative densitograms of extracts of cotyledons of dark- and far-red treated

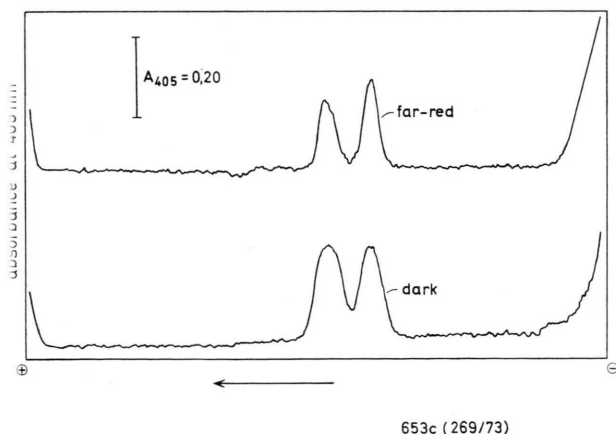


Fig. 2. Extracts from cotyledons of dark-grown (72 hours) and far-red treated (36 hours dark + 36 hours far-red) mustard seedlings were used. Length of the gels was 6 cm.

* Abbreviation: 5,5'-dithiobis-(2-nitrobenzoic acid), DTNB.

seedlings. There are always two distinct peaks which have the R_F -values of 0.35 and 0.43. The area under the two peaks does not represent enzyme activity quantitatively, since diffusion of the colored molecule takes place very rapidly. Variation of the irradiation regime and difference in age of the seedlings had no influence on the electrophoretic pattern. Extracts of hypocotyls contain the same two isoenzymes. Even extracts of individual pairs of cotyledons lead to the same results. In conclusion, P_{fr} alters the enzyme activity only in a quantitative and not in a qualitative way.

Results

1. Operational criteria for the involvement of phytochrome (P_{fr} in the ground state)²¹. The data of Table I show that the increase of GR in the coty-

Table I. The influence of short pulses of standard and far-red light on the increase of glutathione reductase (GR) in the cotyledons of the mustard seedlings. The operational criteria for the involvement of phytochrome are fulfilled.

Treatment after sowing	GR-Activity — nmol NADPH_2 min · pair of cotyledons
84 h d *	32.0 ± 1.0
24 h d + 60 h fr *	53.1 ± 1.2
36 h d + 48 h fr	53.2 ± 1.3
24 h d + 5 min red + 60 h d	40.4 ± 1.6
24 h d + 5 min fr + 60 h d	34.5 ± 1.4
24 h d + 5 min red + 5 min fr + 60 h d	35.4 ± 0.8
36 h d + 5 min red + 48 h d	40.6 ± 1.0
36 h d + 5 min fr + 48 h d	36.2 ± 0.8
36 h d + 5 min red + 5 min fr + 48 h d	36.3 ± 0.6
24 h d + 5 min red + 12 h d + 5 min red + 48 h d	39.8 ± 0.8
24 h d + 5 min fr + 12 h d + 5 min fr + 48 h d	33.9 ± 0.4
24 h d + 5 min red + 5 min fr + 12 h d + 5 min red + 5 min fr + 48 h d	32.5 ± 0.6

* d, Dark; fr, standard far-red light.

ledons of the mustard seedling is enhanced by brief irradiations of a dark-grown seedling with red light and that the effect of the red light treatment can be reversed by a subsequent brief far-red light treatment. This type of response is generally accepted as proof of phytochrome involvement in a light mediated response. However it is obvious that the effect of red light pulses (P_{fr} acting in the dark; cf. ²²) is only small (38%) as compared to the ef-

fect of continuous far-red light ($= 100\%$). This latter effect has been attributed to P_{fr} in some "activated state" ^{22, 23}. Since under standard far-red light chlorophyll *a* is formed very slowly and only in traces, any interaction of photosynthesis with the far-red mediated effect is excluded ²⁴. An interesting feature in Table I is that brief irradiations will lead to the same result (end point determination at 84 hours after sowing) whether the treatment is given at 24 or 36 hours after sowing. Repeated treatments (at 24 and 36 hours after sowing) do not increase the effect as measured at 84 hours after sowing.

2. Kinetics of GR levels in cotyledons and hypocotyl under continuous far-red light. Fig. 3 shows that far-red light enhances the rate of GR appearance in the cotyledons while no significant effect of the

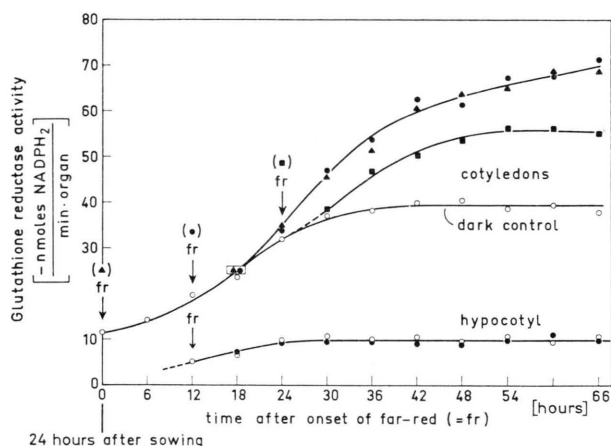


Fig. 3. Time-courses of glutathione reductase levels in cotyledons and hypocotyl of the mustard seedling in the dark and under continuous far-red light. While the actual lag-phase is not longer than 6 hours (onset of light at 48 hours), far-red light does not exert any significant influence before 48 hours after sowing, irrespective of the onset of light (24 or 36 hours after sowing). Whereas the enzyme is induced by far-red light in the cotyledons, no control by light of the enzyme level can be detected in the hypocotyl.

light treatment could be detected in the hypocotyl. The time-courses of GR levels in the cotyledons are identical whether the onset of continuous far-red light is at 24 or 36 hours after sowing. If the onset of far-red light is at 48 hours after sowing the lag-phase (*i.e.* duration of time between onset of light and detectability of the response) is less than 6 hours. Using a terminology developed previously ²⁵ the situation (Fig. 3) can be described as follows: The inductive action of phytochrome (operationally, continuous far-red light) on the increase of the GR

level in the cotyledons is a function of the specific state of responsivity (or competence) of the cells and tissues. The process of differentiation which determines the changing pattern of competence is called "primary differentiation (P_{fr})". The time-course of primary differentiation (P_{fr}) whose causalities are unknown, is largely independent of phytochrome. This latter statement is based on many facts ²¹, including the data in Fig. 3 which show that the time-course of the GR level remains the same whether the onset of continuous far-red light is at 24 or 36 hours after sowing (Fig. 3).

Discussion

1. At first sight Fig. 3 suggests that there is no detectable competence for phytochrome with respect to an increase of the GR level before approx. 42 hours after sowing. While the actual lag-phase is not longer than 6 hours (onset of light at 48 hours), far-red light does not exert any significant influence before 48 hours after sowing, irrespective of the onset of light. However, Table I shows that a red light pulse given at 24 hours after sowing leads to an increase of the enzyme level as measured 60 hours later. Since P_{fr} disappears rapidly from the system in the dark (half-life 45 min at 36 hours after sowing) and since the effect of a red light pulse given at 24 hours can apparently no more be reversed by a far-red light pulse given at 36 hours after sowing (Table II) we need at least one *stable* intermediate in the chain of regulatory events between P_{fr} and the changes of the GR level which acts as a connecting link or "transmitter" between the primary effect of P_{fr} and the events on the enzyme level. Apparently the transmitter can be formed in

Table II. The influence of short pulses of standard red and far-red light on the increase of the glutathione reductase (GR) level in the cotyledons of the mustard seedling. End point determination of GR was carried out 84 hours after sowing. The data show that the inductive effect of a red light pulse applied at 24 hours after sowing can apparently no more be reversed by a far-red light pulse applied at 36 hours after sowing.

Treatment	GR-Activity — nmol NADPH ₂ min · pair of cotyledons
84 h dark	32.0 ± 1.0
5 min red (at 24 h)	42.5 ± 1.3
5 min far-red (at 24 h)	31.7 ± 1.1
5 min far-red (at 36 h)	36.2 ± 1.1
5 min red (at 24 h) plus 5 min far-red (at 36 h)	41.0 ± 1.3

the presence of P_{fr} at an early stage of "primary differentiation" and can act only at a later stage resulting eventually in an increase of the GR level. While the molecular nature of the transmitter is unknown at present, the formal interpretation requires that we keep two "competences" separate: a. The competence of the seedling for P_{fr} with respect to transmitter formation (already there at 24 hours after sowing), and b. the competence of the seedling for transmitter with respect to an increase of the GR level (which comes into play in the cotyledons only at approximately 42 hours after sowing). Facts of a similar kind have already led to the concept of a "transmitter" in connection with phytochrome-mediated peroxidase induction in the mustard seedling⁶.

2. A comparison of Fig. 3 with Fig. 1 in the previous paper on apparent AO synthesis in the mustard seedling² leads to the conclusion that there is no indication of a coordinated, simultaneous induction by phytochrome of GR and AO. While this result does not rule out the existence of a respiratory chain in which AO functions as a terminal oxidase, it certainly does not support this concept.

3. A significant result of the present paper is the finding of an enzyme whose level is increased by

phytochrome in the cotyledons whereas no control by light can be detected in the hypocotyl. The fact that the time course of GR levels in the hypocotyl is not influenced by light is amazing insofar as a hypocotyl grown for 24 or more hours under far-red light differs a great deal from a dark-grown hypocotyl of the same age. The difference in hypocotyl length is at least 5 fold. We conclude that the occurrence of phytochrome-mediated photomorphogenesis (including enzyme induction) is a specific phenomenon and does not automatically affect every aspect of metabolism and integration within the plant. We have found previously that in a given plant some enzymes are induced or repressed by phytochrome while the time-courses of other enzyme levels are not affected²⁶. In the present paper it is shown that the time-course of an enzyme level which is controlled by phytochrome in the cotyledons is not affected in the neighbouring organ (hypocotyl). Since phytochrome is present in the mustard hypocotyl⁵ and since the organ does respond to phytochrome in many respects²¹ it must be concluded that it depends on "primary differentiation" (*cf.*^{21, 25}) whether or not the appearance of a particular enzyme in a particular cell or tissue is accessible to the control by phytochrome.

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