Comparison of Three Phytochrome-mediated Processes in the Hypocotyl of Mustard

Received for publication January 5, 1976 and in revised form July 20, 1976

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ABSTRACT

Anthocyanin synthesis, hair formation, and the synthesis of ascorbic acid oxidase are all phytochrome-mediated reactions occurring in the hypocotyl of mustard (Sinapis alba L.), controlled by phytochrome actually located in the hypocotyl. A comparison of these three reactions showed that in certain respects they differ greatly in their response to light. The ability of the seedling to respond to light by showing the three responses was strongly influenced by the state of development of the seedling. White light given very early after seed imbibition was unable to evoke any of the three responses. By 50 hours after imbibition, all systems were fully inducible by light. The addition of actinomycin D to a fully competent seedling coincident with illumination strongly inhibited the development of all three responses. In contrast, the addition of cordycepin at this time inhibited the synthesis of anthocyanin and ascorbic acid oxidase but had no effect on hair formation. Cycloheximide inhibited all three responses when given up to several hours after light. This suggests the necessity for RNA and protein synthesis for light-induced expression of these reactions, and that the RNA species involved in the three reactions may have differing degrees of polyadenylation. The lag period between the onset of light and the first display of the response was 3 hours for anthocyanin and ascorbic acid oxidase synthesis, and about 5 hours for hair formation. Amounts of light sufficient to give large increases in the levels of ascorbic acid oxidase and hair formation gave a much smaller increase in anthocyanin synthesis. Hair formation and ascorbic acid oxidase synthesis showed a much greater sensitivity to induction at early stages of seedling development than did anthocyanin synthesis. Following an inductive light period, anthocyanin synthesis was sensitive to far red light inhibition for a period twice as long as the other two reactions. The differences in the response of the three reactions to light suggest that the phytochrome-mediated reactions which control their development also differ.

There has been much speculation on the mode of action of phytochrome (6, 23, 25-27, 32) particularly in regard to the nature of the primary reaction (25-27). Although various aspects of different phytochrome-controlled reactions have been compared (8, 23, 25-27), no previous investigation has examined the influence of all of the parameters described in this study, on three different responses, occurring at the same time in the same organ. It is essential that such a comparison be conducted on reactions which occur in the same organ since different organs of a seedling respond differently to the formation of Pr (23). Therefore, variations in the phytochrome control of various reactions in different organs could well reflect the nature of the organs involved or the stage of seedling development rather than the inductive processes per se.

In the present study, the induction and development of three different reactions in the hypocotyl of mustard, controlled by phytochrome located in the hypocotyl, were compared at the same stage of seedling development. The three reactions examined were hair formation, anthocyanin synthesis, and the synthesis of AAO.1 Hair formation occurs in the epidermal cell layer of the hypocotyl (24) and anthocyanin synthesis in the subepidermal cell layer (24). AAO synthesis occurs in all organs of the seedlings (9) and so probably also takes place through all tissues of the hypocotyl. All three reactions show red/far red reversibility in the classical test for a phytochrome-controlled reaction (21, 22, 34). In the experiments described below, the three reactions were compared with regard to their induction and development, the effectiveness of ACT.D, cordycepin, and cycloheximide in blocking their development, and the degree to which the reactions are reversible by far red light treatment.

MATERIALS AND METHODS

Seeds of Sinapis alba L. were treated with 2.5% sodium hypochlorite solution for 5 min to remove fungal spore contamination, washed three times with water, and left to imibe for 15 min. This treatment resulted in no promotion of the three reactions when compared with seeds imbibed in the dark. Following imbibition, seedlings were dark-grown at 25 C in 10-cm Petri dishes containing vermiculite, or three layers of filter paper for the experiments with ACT.D, cordycepin, and cycloheximide. At the appropriate time, seedlings were exposed to incandescent white light of irradiance 30 mw/cm² containing 1800 μw/cm² red light and 1920 μw/cm² far red light, measured at 648 to 672 nm and 718 to 742 nm, respectively, on a model SR spectroradiometer (Instrumentation Specialties Co., Lincoln, Neb.). They were then returned to darkness at 25 C prior to measurement at times noted in "Results."

In the reversal experiment, far red light was obtained by filtering incandescent white light through 2.5 cm of water, two layers of Roscolene No. 863 medium blue cellophane, and two layers of Roscolene No. 823 medium red cellophane (Kiegl Bros., Long Island City, N. Y.). This gave a total of 40 mw/cm² containing 210 μw/cm² red light and 2620 μw/cm² far red light measured as above.

In the inhibitor experiments, cycloheximide, ACT.D, and cordycepin (Sigma Chemical Co.) were supplied to the roots of whole seedlings at concentrations of 40 μg/ml, 75 μg/ml, 100 μg/ml, respectively, with 3 ml of each solution added to each Petri dish.

Anthocyanin was assayed by immersing 20 hypocotyls, following removal of the cotyledons and radicle, in 2 ml of extraction solution (methyl alcohol 99%-hydrochloric acid 1%). Hypocotyls remained in the extraction solution for 1 hr at 25 C when

1 Abbreviations: ACT.D: actinomycin D.; AAO: ascorbic acid oxidase; PAL: phenylalanine ammonia-lyase.
hair index is the summation of the 20 individual seedlings.

AAO was assayed essentially by the method of Drumm et al. (9) but using different dilutions. Ten ml of buffer were used in the extraction, and following centrifugation, 1 ml of supernatant was diluted five times with buffer and 1 ml of this added to the reaction mixture (3 ml buffer + 10 μl of 10 mm ascorbic acid). The disappearance of ascorbic acid over 1 hr was measured at 265 nm corrected for any change occurring in a mixture without enzyme.

Hair formation was determined by comparing each of the 20 seedlings used in the anthocyanin assay using a hair index of 1 to 5 as shown in Table I. This index reflects the fact that hairs increase in both size and density as the light exposure increases. Seedlings graded 5 show a hair length and density characteristic of seedlings completely light-grown and were not seen in these experiments. The reported hair index is the summation of 20 individual results.

A high degree of variation was found in any sample of seedlings, ranging from seedlings showing a large photosresponse to seedlings showing no photosresponse. In order to eliminate this variability, seedlings were selected for assays on the basis of the amount of anthocyanin they showed. Thus, each assay on hair formation, AAO, or anthocyanin synthesis was performed on 20 seedlings which showed the most anthocyanin. Such seedlings also showed the most hair formation and AAO synthesis. At least two replicates were used for each measurement. Differences between replicates were never more than 6% for anthocyanin, 8% for AAO, and 10% for hairs. Experiments were performed a minimum of three times with similar results.

RESULTS AND DISCUSSION

Role of the Cotyledons. It has been shown that the synthesis of lipoxygenase in mustard cotyledons is controlled by phytochrome located in the hypocotyl hook (29). To determine if mustard cotyledons have any effect on hair formation, anthocyanin, or AAO synthesis occurring in the hypocotyl, these responses were compared in whole seedlings and seedlings without cotyledons. Removal of cotyledons had no effect on the light promotion of hair formation or AAO synthesis in the hypocotyl. Light-promoted anthocyanin synthesis was, however, abolished in seedlings without cotyledons, and such seedlings showed levels of anthocyanin comparable to dark-grown seedlings. When seedlings without cotyledons were placed on 0.8% agar with 25 g/l sucrose, the ability to synthesize anthocyanins in response to light was restored. Sucrose, fructose, glucose, galactose, and ribose were all found to be effective. Since the biosynthesis of anthocyanin in mustard involves glycosylation of the aglycon cyanidin, it is understandable that the need for sugar would be so much greater for anthocyanin synthesis than for hair formation or synthesis of AAO. Because these latter two reactions show no difference in the hypocotyls of seedlings with or without the cotyledons, and because the cotyledons appear to function only as a source of sugar in anthocyanin synthesis, it is concluded that the control of all three reactions in the hypocotyl is effected by phytochrome located in the hypocotyl.

Effect of the State of Development. Light induction of the various responses was strongly influenced by the state of development of the seedlings (Fig. 1). There was a high level of AAO present in dark-grown seedlings. A 2-hr light exposure of imbibed seeds was necessary before an increase in enzyme level could be detected in seedlings subsequently grown in the dark. A maximum increase in enzyme synthesis in response to light was not reached until 50 hr following seed imbibition (Fig. 2). Anthocyanin synthesis could not be induced by light until 4 hr following imbibition, and reached a maximum level 40 hr after imbibition. A small degree of hair formation occurred in the dark (never more than 6 on the hair index). This hair formation was strongly dependent upon temperature since below 15 C no hair formation occurred in the dark, but above 15 C slight hair formation occurred which increased with increasing temperature to 30 C. Since in these experiments the seedlings were grown at 25 C, some hair formation occurred in the dark. A maximal capacity to produce hairs in response to light was not reached until 50 hr after imbibition. Hsiao and Vidaver (13) have found that hydration of phytochrome is an essential prerequisite for light-promoted lettuce seed germination, and that the germination rate increased up to 2 hr after seeds were placed in a water-

![Fig. 1. Promotion of hairs, anthocyanin, and AAO synthesis by light in early development. Seedlings were grown under continuous light and at different times placed in darkness. Assays for anthocyanin, AAO, or hairs were made 80 hr after imbibition.](https://www.plantphysiol.org)

![Fig. 2. Effect of time of development at which seedlings are first exposed to light on the formation of hairs, anthocyanin, or AAO. Seedlings were grown in darkness and at different times in development exposed to 1 hr light, then returned to darkness. Assays for anthocyanin, AAO, and hairs were made 80 hr after imbibition.](https://www.plantphysiol.org)
saturated atmosphere. It seems that in the present case, hydration of phytocrome during the first 2 hr after seed imbibition was sufficient to promote hair formation and AAO synthesis without having a corresponding effect on anthocyanin synthesis. In spite of the late onset of inducibility for anthocyanin synthesis, maximum induction occurs 10 hr earlier than for the other two reactions, suggesting that the factors governing the onset of inducibility and the attainment of maximum induction may be different. In addition to these two factors, it has recently been shown by Steinitz et al. (33) that factors other than the phytochrome system independently delay the development of competence for anthocyanin synthesis until relatively late in development.

**Relationship between Response and Light Duration.** AAO synthesis and hair formation show a much greater sensitivity to light than does anthocyanin synthesis. A 0.5-sec flash of light given to 50-hr-old seedlings was sufficient to give 53% of the AAO and 44% of the hair formation, but only 9% of the anthocyanin levels that were evoked by a 1-hr light exposure. One-min and 5-minute light exposures were sufficient to evoke half of the maximal response for hair formation and AAO synthesis, whereas a 1-hr light exposure was required to give half of the maximal anthocyanin response (Fig. 3). It has been shown that under continuous far red light, the total amount of detectable phytochrome present in mustard cotyledons is a function of the irradiance (31). The importance of the Pfr level on the synthesis of anthocyanin has been clearly shown by Mancinelli et al. (19). In the present case, amounts of light capable of causing considerable promotion of hair formation and AAO synthesis have a much smaller effect on levels of anthocyanin. This suggests a greater requirement for the presence of Pfr in order to induce anthocyanin synthesis, and possibly reflects the fact that the presence of Pfr is required for a longer time period in order to induce anthocyanin synthesis than is necessary for the induction of the other two photoreponses.

**Effect of Actinomycin D.** When ACT.D was added to seedlings at different times in development, the effectiveness of the inhibitor was found to be dependent on the age of the seedling (Fig. 4). Up to 30 hr after imbibition, the addition of the antibiotic caused a total inhibition of all of the reactions. By 40 hr after imbibition, addition of the ACT.D allowed a small degree of hair formation and AAO synthesis to occur in response to light given 53 hr after imbibition. Anthocyanin synthesis showed no cessation of inhibition by ACT.D unless the antibiotic was added at 50 hr or later following imbibition. When ACT.D was added at the same time as light, it caused an 80% inhibition of anthocyanin synthesis, and 71 and 60% inhibition of hair formation and AAO synthesis, respectively. These results are in agreement with the early work of Mohr (17, 23), who showed that RNA synthesis is necessary for certain phytochrome-controlled reactions to occur. In the present study, it appears that part of the RNA transcription occurs in darkness prior to the exposure of seedlings to light. The fact that ACT.D failed to inhibit any of the reactions if it was added 4 hr or later after the onset of light indicates that transcription is probably complete by this time.

**Effect of Cordycepin.** Treatment of the seedlings with cordycepin at different stages of development showed very different degrees of inhibition of the three reactions (Fig. 5). When cordycepin was added at the same time as light, anthocyanin synthesis showed a 69% inhibition, AAO synthesis a 32% inhibition, while hair formation was uninhibited. Cordycepin is thought to block the addition of poly(A) to mRNA after transcription has been completed (7) although it may also produce other effects on RNA synthesis. Although early work suggested that all mRNA except histone mRNA contains poly(A) sequences (3, 11), later work has indicated that up to 30 or 40% of mRNA in different systems is not polyadenylated (12, 20, 28). In soybean, it has recently been shown that only 50% of the mRNA is polyadenylated (15). The results appear to suggest that any mRNA involved in hair formation is unadenylated or adenylated prior to the addition of the cordycepin. Hair formation involves a considerable increase in the size of the nucleolus (see Fig. 7), suggesting an increase in rRNA synthesis. This synthesis could be a major reaction in hair formation, and since rRNA does not contain poly(A) (11), it should not be inhibited by cordycepin. The smaller inhibition of AAO synthesis than of anthocyanin synthesis by cordycepin might possibly reflect differences in the degree of adenylation of the respective mRNA or differing times of polyadenylation in relation to the action of phytochrome.

The inhibition of hair formation caused when seeds were imbibed in cordycepin or when the inhibitor was added during 10 hr after imbibition may not reflect a specific inhibition of hair formation per se. Cordycepin caused a 64% inhibition of germination and a 72% inhibition of hypocotyl length at the time of exposure to light when seeds were imbibed in the inhibitor. When added at 10 hr, it caused a 24% inhibition of germination and a 60% inhibition of hypocotyl length. This inhibition may affect hair formation adversely.
seeds following a 1-hr light exposure showed that all three reactions were strongly inhibited (Fig. 6), indicating that protein synthesis is required for the full expression of the phytochrome system in each reaction. It has been shown by density labeling that the light-mediated increase in AAO activity in mustard cotyledons is due to de novo enzyme synthesis (2, 4). Similar studies have recently indicated that light-induced increases in PAL activity leading to anthocyanin synthesis are likewise the result of de novo enzyme synthesis (1), so that inhibition of both enzymes by cycloheximide is to be expected, although the increase in PAL activity has also been ascribed to enzyme activation (5).

Hair formation showed an extreme sensitivity to cycloheximide and was inhibited up to 30 hr after light. Since 30 hr is about the time period required for the completion of hair development, it appears that continuous protein synthesis is needed for hair formation. AAO escaped from inhibition by cycloheximide most rapidly, synthesis almost being complete by 10 hr after light. The data for inhibition of anthocyanin showed that application of the antibiotic actually caused an increase in anthocyanin synthesis when added for 15 hr after light treatment. This suggests the production of a degradative enzyme about this time, which could well be a degradative enzyme for PAL. The presence of such an enzyme has been indicated in gherkin (10), pea (14), radish (16), and Xanthium (35). In all of these systems, the application of cycloheximide following a light exposure prevents the destruction of PAL, indicating that PAL inactivation requires protein synthesis.

**Lag Period.** The lag period between the onset of light and the first appearance of anthocyanin has been found to be about 3 hr (18). We have confirmed this finding and have also found that the lag period for the synthesis of AAO is identical. One of the earliest signs of hair formation is a characteristic enlargement of the nucleus in the hair-forming cell. After a 5-hr light exposure, some cells in the epidermal cell layer had enlarged nuclei, and the nucleus in these cells was more apparent. In some cases, these changes were accompanied by the beginnings of an outgrowth of the cell wall (Fig. 7A). By 6.5 hr, a definite outgrowth of the cell wall was apparent, accompanied by increases in size of both nucleus and nucleolus (Fig. 7B). By 10 hr after light, the length of the cell outgrowth was often two or more times the cell width (Fig. 7, C and D). Complete hair formation took about 30 hr. The increase in size of the nucleus and nucleolus during hair formation may reflect the occurrence of endopolyploidy in these cells. Autoradiographic studies have shown a marked incorporation of labeled thymidine into the nucleus by 10 hr after light (manuscript in preparation). Since it is very difficult to define precisely the time at which hair formation commences, the process may begin earlier than 5 hr after light, in which case, the lag phase for all three reactions in fully competent cells could be quite similar.

**Reversal by Far Red Light.** Anthocyanin synthesis takes twice as long (6 hr) to escape from phytochrome control as does the synthesis of ascorbic acid oxidase or hair formation (Fig. 8). Far red light was ineffective in inhibiting these two reactions if given 3 hr or more after the onset of white light. If far red light was given immediately following 1 hr white light, the three reactions showed differing degrees of inhibition: anthocyanin synthesis was inhibited by 94%, whereas AAO and hair formation were inhibited by 73 and 68%, respectively, compared to a treatment not given far red light. A 1-hr exposure is apparently not a sufficient time for phytochrome to cause more than a small promotion of anthocyanin synthesis compared to the other two responses. The time period for which the presence of Pfr is necessary in order to promote a response apparently differs among the three reactions.

**Implications for the Mode of Action of Phytochrome.** From the experiments described in this paper, it can be seen that in three ways AAO and hair formation react quite similarly to light and differently from anthocyanin synthesis. In 50-hr-old dark-grown seedlings, increasing light exposures gave a very rapid increase in the levels of AAO and hair formation, whereas the...
increase in anthocyanin synthesis was more gradual. Within 2 h after seed imbibition, light was able to evoke a significant increase in AAO and hair formation, but caused no increase in anthocyanin synthesis in seedlings subsequently grown in darkness. The time period for which light is required to promote AAO and hair formation appears to be less than for anthocyanin. If at the end of a 1-hr light exposure, Pfr was reverted to Fr by far red light, this gave levels of hair formation and AAO that were much higher than those of anthocyanin relative to the control not given far red light. A possible explanation of these three observations is that the induction of anthocyanin synthesis requires the presence of Pfr for a longer period of time than does the induction of the other two photoresponses. Thus, a greater amount of light would be necessary in order to induce anthocyanin synthesis than would be needed for the other two photoresponses. Hydration of only a small amount of phytocrome very early after seed imbibition would then be able to have a significant effect on hair formation and AAO synthesis, but would not be sufficient to induce anthocyanin synthesis. Reversal of a light induction after a short time period with far red light would result in a much greater promotion of AAO and hair formation than of anthocyanin synthesis.

If the presence of Pfr was required for a longer period to induce anthocyanin synthesis than for the other two photoresponses, this would imply that the primary action of phytocrome in inducing the three reactions was different. It has been suggested by Mohr (25-27) that there must exist a multiplicity of primary reactions. An examination of this question is possible by considering the inhibition of all three processes by far red light. The results of this experiment (Fig. 8) showed that anthocyanin synthesis took twice as long to escape from phytocrome control as did the synthesis of AAO and hair formation. Clearly, the presence of Pfr is still necessary for anthocyanin synthesis long after it has ceased to function in the other two processes. This further supports the idea that the presence of Pfr is required for different lengths of time to induce different reactions. In addition, it supports Mohr’s view that the primary reaction of phytocrome must differ among different photoresponses.

Acknowledgments – We thank H. Mohr for constructive criticism and for providing manuscripts prior to publication, the Cornell Chapter of the Society of Sigma Xi for financial assistance with supplies, and Merk and Co., Inc. for a gift of some actinomycin D.

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