Phytochrome Control of the Development of Ascorbate Oxidase Activity in Mustard (Sinapis alba L.) Cotyledons

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The activity of ascorbate oxidase (AOX) in mustard (Sinapis alba L.) cotyledons was markedly increased by irradiation with continuous far-red light. The involvement of phytochrome in this light-mediated response was demonstrated by red/far-red reversibility experiments. To determine immunochemically the contents of AOX in cotyledons, the antibody against the enzyme was raised in rabbit. However, the antiserum was not monospecific to AOX; it also recognized glycoproteins. To remove antibodies that are specific to a carbohydrate moiety of glycoproteins, the anti-AOX antiserum was applied to a horseradish peroxidase-conjugated Sepharose column. By using the antibodies that were not retained in the column, the changes in the content of AOX were followed. Western immunoblot profiles revealed that the content of AOX protein in cotyledons notably increased after continuous far-red light treatment. Pulse-labeling experiments indicated that the synthesis of AOX protein occurred in the cotyledons. These results are in good agreement with the hypothesis that phytochrome-mediated increase in AOX activity is accompanied by the synthesis of the enzyme.

It is well known that phytochrome mediates the changes in the activities of a large number of enzymes (Schopfer, 1977; Schopfer and Apel, 1983). AOX in mustard cotyledons is one such enzyme (Drumm et al., 1972). In general, the increase in enzyme activities can be explained by: (a) an increase in the rate of the synthesis of the enzyme proteins leading to their accumulation; (b) activation of inactive enzymes; or (c) a combination of the two. With regard to the mechanism by which the activity of mAOX is increased by phytochrome, two different hypotheses have been offered so far. Attridge (1974) and Acton et al. (1974) have concluded from density labeling experiments that the phytochrome-mediated increase in the activity of AOX in mustard (Sinapis alba L.) cotyledons is related to the increase in the rate of the enzyme synthesis. On the other hand, Newbury and Smith (1981) and Leaper and Newbury (1989) have recently determined the immunochemical content of AOX protein in mustard cotyledons and reported that the content remains the same despite a marked, phytochrome-mediated increase in the enzyme activity. From this they claimed that the increase in the activity is due to the activation of a precursor. In our opinion, it is still unclear which hypothesis is valid, and more-detailed studies on the mechanism that increases the activity of AOX are necessary. Newbury and Smith (1981) and Leaper and Newbury (1989) used polyclonal antibodies raised against cAOX for determining mustard enzyme. The accuracy of the results depends on the cross-reactivity of such heterologous antibodies. We began the present study by examining this point.

MATERIALS AND METHODS

Plant Material

Seeds of white mustard (Sinapis alba L.) were placed on a moist paper towel and incubated at 25°C in the dark. After 1 d, the seedlings were either retained further in darkness or transferred to cFR. For the photoreversibility experiments, 3-d-old, dark-grown seedlings were treated with short R or FR as described in Table I.

Irradiation

R was obtained by passing light from fluorescent lamps (Toshiba FL 20SS, Tokyo Shibaura Electric Co., Kawasaki, Japan) through a red, acrylic plastic plate (Acrylite #317, Asahi-kasei Co., Tokyo, Japan); the fluence rate was 3 W m⁻². FR was obtained from a tungsten filament lamp filtered through a heat-absorbing layer of water and an acrylic-resin filter (Delaglass A, Asahi-kasei Co.), giving radiation with wavelengths of >700 nm and a fluence rate of 2 W m⁻². Irradiation was performed at 25°C.

Assay of AOX Activity

Thirty pairs of mustard cotyledons were homogenized in a mortar and pestle with 6 mL of 0.1 M K-phosphate buffer (pH 6.0) and 0.1 g of PVP and centrifuged at 12,000g for 10 min. The resultant supernatant was used for the enzyme assay. An aliquot of the sample was added to 2.5 mL of homogenizing buffer along with 20 μL of 15 mM ascorbate. Enzyme activity was spectrophotometrically measured at 30°C by recording the decrease of A265. The molar extinction coefficient for ascorbate is ε265 = 7000 (McDawson et al., 1986).

Purification of mAOX

The enzyme was extracted from cotyledons (250 g) of mustard seedlings that had been grown in vermiculite for

Abbreviations: AOX, ascorbate oxidase; cAOX, Cucurbita ascorbate oxidase; cFR, continuous far-red light; FR, far-red light; mAOX, mustard ascorbate oxidase; R, red light.
about 4 d under natural light conditions at room temperature (20–25°C). The cotyledons were homogenized in 0.1 M K-phosphate buffer (pH 6.0) containing 0.1% (v/v) 2-mercaptoethanol and an appropriate amount (fresh tissue weight × 0.1) of PVP. The homogenate was filtered through two layers of cheesecloth and centrifuged at 12,000g for 10 min. Low mol wt contaminants such as anthocyanins were removed from the supernatant by gel filtration (Sephadex G-25). Solid (NH₄)₂SO₄ was added to the active filtrate to 30% saturation. The precipitated proteins were dissolved in 10 mM K-phosphate buffer (pH 6.4) containing 0.1% (v/v) Z-mercaptoethanol and applied to a CM-Toyopearl 650M column (1.5 x 20 cm) equilibrated with buffer A and dialyzed against buffer A containing 0.15 M NaCl and 0.02% (w/v) NaN₃. Crude immunoglobulin was separated from serum by (NH₄)₂SO₄ precipitation at 40% saturation and dialyzed against 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.02% (w/v) NaN₃.

To remove those antibodies that recognize the carbohydrate moiety of AOX, the antiserum raised against the purified mustard enzyme was applied to a column of Sepharose 4B to which horseradish peroxidase, a glycoprotein, is covalently linked. The peroxidase-linked Sepharose was prepared from cyanogen bromide-activated Sepharose (Pharmacia) and horseradish peroxidase (Boehringer) according to a manual by the manufacturer. The antiserum not retained in the column was dialyzed against 20 mM Tris-HCl (pH 7.4) containing 0.15 NaCl and 0.02% (w/v) NaN₃ and concentrated using an Amicon concentrator (B-15).

**Western Immunoblot**

Proteins on a gel were electroblotted to a nitrocellulose membrane using a NovaBlot cell (LKB) according to the manufacturer's instructions. Immunodetection was done using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin.

**In Vivo Labeling and Immunoprecipitation**

Cotyledons (30 pairs) isolated from 2-d-old white mustard seedlings that had been exposed to cFR for 1 d or retained in darkness were incubated for 2 h at 28°C under FR or in the dark, respectively, in 1 mL of a mixture of [³⁵S]Met and [³⁵S]-Cys (3.7 MBq, approximately 37 TBq mmol⁻¹, EXPRE³⁵S³⁵S, New England Nuclear). After incubation, cotyledons were washed and homogenized in a mortar and pestle with 50 mM K-phosphate buffer (pH 6.4). The homogenates were centrifuged at 18,000g for 10 min, and the supernatants were used for immunoprecipitation. Immunoprecipitation of the in vivo-labeled proteins was performed as described previously (Sugimoto and Morohashi, 1989).

![Image](https://plantphysiol.org)
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RESULTS AND DISCUSSION

The treatment with cFR brought about a marked increase in the activity of AOX in mustard cotyledons (Fig. 1). With regard to photomorphogenesis, the phenomena caused by cFR are known to be mediated by phytochrome (Mohr, 1972; Drumm et al., 1975). The involvement of phytochrome in this light-mediated response is also known from the observation that short (5 min) irradiation with R caused an increase in AOX activity and that short (10 min) FR given immediately after R reversed the R effect (Table I). Therefore, it is clear that phytochrome is involved in the development of AOX activity in mustard cotyledons. This confirms the results previously reported by Drumm et al. (1972).

To determine the immunochemical contents of AOX in mustard cotyledons, the polyclonal antiserum against cAOX, obtained from Boehringer, was prepared. The antiserum formed a precipitine line with crude extracts from cFR- or dark-treated mustard cotyledons in the Ouchterlony double-diffusion test (data not shown). This indicates that AOX-like protein(s), which can cross-react with the anti-cAOX, is present in mustard cotyledons. It seemed as if the antiserum could be used to determine the contents of mAOX, as was done previously by Newbury and Smith (1981). However, when the crude extracts were applied to the SDS-PAGE column and analyzed with western immunoblot, many signals were detected (Fig. 2A) (no signal was detected by preimmune serum; data not shown). Because AOX is a glycoprotein (D’Andrea et al., 1988), it is probable that a glycan side chain shares antigenic determinants and that most signals detected in immunoblot profiles are due to glycoproteins.

The carbohydrate moiety of cAOX was chemically removed and the antibodies against this deglycosylated protein were prepared. Most of polypeptide bands detected by the antiholoenzyme antiserum were no longer detected by the anti-deglycosylated enzyme antiserum (Fig. 2B). Although the antiserum against the deglycosylated cAOX still recognizes, although weakly, a few polypeptides, this may be due to unspecific reactions. However, it is clear that polyclonal antibodies raised against cAOX are not monospecific to AOX, and hence, this antiserum cannot be used for the determination of AOX contents. Leaper and Newbury (1989) did not seem to encounter such trouble (as mentioned above) in western immunoblot analyses of crude extracts of mustard cotyledons, in spite of the fact that polyclonal antiserum against cAOX (Boehringer) were used. At present we have no explanation for this discrepancy.

If the anti-deglycosylated cAOX antiserum cross-reacts with mustard enzyme, it would be useful, but because the cross-reactivity does not seem to be high, the antibody cannot clearly recognize mAOX (Fig. 2B).

We next tried to prepare anti-mAOX antiserum. Although AOX was purified from mustard cotyledons about 1600-fold over the crude extract through several purification steps, as shown in Table II, it was still contaminated with a few minor polypeptides (Fig. 3A, lane 1). The enzyme protein was further purified by preparative SDS-PAGE and, as a result, the enzyme polypeptide was seen as a single band at 68 kD on SDS-PAGE by silver staining (Fig. 3A, lane 2). We prepared polyclonal antiserum against this purified mAOX. By using this antiserum, the SDS-PAGE-purified polypeptide was analyzed by western immunoblot. As shown in Figure 3B, only one discrete band was detected at the position corresponding to mAOX, indicating that the antigen used for raising the antibodies was practically pure.

When crude extracts from mustard cotyledons were analyzed by immunoblot, a large number of signals were again detected (Fig. 2C). The polypeptide pattern was similar to that observed when anti-Cucurbita holoenzyme antiserum was used (Fig. 2A). It is clear that anti-mAOX antiserum also

![Figure 2. Western immunoblot of SDS-PAGE (7.5% gel) of crude extracts prepared from mustard cotyledons. Mustard seedlings were grown in darkness or transferred to cFR after 1 d in darkness. A, Probed with anti-cAOX (holoenzyme) antiserum; B, probed with anti-deglycosylated cAOX antiserum; C, probed with anti-mAOX antiserum. Lanes 1, mAOX; lanes 2, extract from cotyledons exposed to cFR for 2 d; lanes 3, extract from dark control cotyledons. Arrowheads indicate the mAOX band. The numbers on the right indicate approximate molecular mass in kD.]

| Table II. Purification of AOX from mustard cotyledons |
|----------------|----------------|----------------|----------------|----------------|
| Step           | Total Protein | Total Activity | Specific Activity | Purification |
|                | mg             | µmol min⁻¹      | µmol mg⁻¹ protein min⁻¹ | -fold         |
| Crude extract  | 6820           | 7090            | 1.04              | 1             |
| (NH₄)₂SO₄      | —              | 4310            | —                 | —             |
| CM-Toyopearl   | 32             | 1530            | 48                | 46            |
| Butyl-Toyopearl| 0.54           | 610             | 1130              | 1090          |
| Hydroxyapatite | 0.23           | 390             | 1700              | 1640          |

* Not determined.
recognizes glycoproteins. One important result shown in Figure 2 is that a clear signal of mAOX could be detected only in the extract prepared from FR-treated cotyledons (Fig. 2C, lanes 2 and 3). However, the presence of many bands of glycoproteins hinders detailed analyses of immunoblot profiles. It is preferable to use the antiserum against deglycosylated mustard enzyme, but such a slight amount of the protein as is purified from mustard cotyledons is not fit for chemical deglycosylation. We tried to remove antibodies that specifically recognize the carbohydrate moiety of glycoproteins from the polyclonal anti-mAOX antibodies. The anti-mAOX antiserum was applied to a horseradish peroxidase-linked Sepharose column; the peroxidase is a glycoprotein and, hence, is expected to bind to antibodies that are specific to glycan chains. The antibodies not retained in the column were used for immunoblotting.

As shown in Figure 4, a strong signal of mAOX was detected in the crude extracts from FR-treated cotyledons but not in the extracts from dark control cotyledons. The content of AOX protein was markedly increased by FR treatment. Although several weak signals other than those of AOX are also detected, they may be for glycoproteins; antibodies specific to glycan chains probably were not completely removed by the peroxidase treatment.

Newbury and Smith (1981) and Leaper and Newbury (1989) suggested that an inactive AOX that shows cross-reactivity to anti-cAOX antiserum is present in cotyledons of dark-grown mustard seedlings and that the conversion of an inactive form to an active one is induced by phytochrome action. However, as seen from Figure 4, such a precursor-type protein does not seem to be present in dark, control cotyledons.

It is suggested from the immunoblot profiles (Fig. 4) that the synthesis of AOX may be induced in FR-treated cotyledons. To test this possibility, in vivo pulse-labeling experiments were carried out. Proteins that were immunoprecipitated with the peroxidase-treated antiserum were separated with SDS-PAGE and the gel was prepared for fluorography. Although the antibodies still recognized several radioactive polypeptides (probably glycoproteins), a discrete signal was detected at the position of mAOX in the sample from FR-treated cotyledons (Fig. 5). This indicates that de novo synthesis of AOX protein occurs in mustard cotyledons under the control of phytochrome, and that the AOX synthesis is, at least in part, responsible for the development of the enzyme activity. This contention is in good agreement with the proposal by Attridge (1974) and Acton et al. (1974).

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LITERATURE CITED

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