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Control of the appearance of ascorbate peroxidase (EC 1.11.1.11) in mustard seedling cotyledons by phytochrome and photooxidative treatments

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Abstract. In photosynthetic cells the plastidic ascorbate-glutathione pathway is considered the major sequence involved in the elimination of active oxygen species. Ascorbate peroxidase (APO; EC 1.11.1.11) is an essential constituent of this pathway. In the present paper control of the appearance of APO was studied in the cotyledons of mustard (*Sinapis alba* L.) seedlings with the following results: (i) Two isoforms of APO (APO I, APO II) could be separated by anion-exchange chromatography; APO I is a plastidic protein, while APO II is extraplastidic, very probably cytosolic. (ii) The appearance of APO is regulated by light via phytochrome. This control is observed with both isoforms. Moreover, a strong positive control over APO II appearance (very probably over APO II synthesis) is exerted by photooxidative treatment of the plastids. (iii) Additional synthesis of extraplastidic APO II is induced by a signal created by intraplastidic pigment-photosensitized oxidative stress. The response is obligatorily oxygen-dependent and abolished by quenchers of singlet oxygen such as α-tocopherol and *p*-benzoquinone. (iv) A short-term (4 h) photooxidative treatment suffices to saturate the signal. Signal transduction cannot be abolished or diminished by replacing the plants in non-photooxidizing conditions. Several observations indicate that control of APO synthesis by active oxygen is not an experimental artifact but a natural phenomenon.

Key words: Active oxygen – Ascorbate peroxidase (isoforms) – Peroxidase, ascorbate specific – Photooxidation – Phytochrome – *Sinapis*

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Abbreviations: APO = ascorbate-specific peroxidase (EC 1.11.1.11); D = darkness; FPLC = fast protein liquid chromatography; FR = far-red light (3.5 $W \cdot m^{-2}$); NF = Norflurazon; R = red light (6.8 $W \cdot m^{-2}$)

Introduction

Production of various species of toxic oxygen from ground-state (triplet) dioxygen is a characteristic feature of normal aerobic metabolism (Simon 1974). Toxic (or "active") oxygen species are produced chemically by a series of univalent reductions of triplet oxygen leading to the formation of the superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the particularly toxic hydroxyl radical ($\cdot OH$). These processes are mediated by several substances occurring normally in living cells, e.g. ferredoxin or Fe-ions (Haber and Weiss 1934; Jennings and Forti 1975; Steiger et al. 1977; Halliwell 1979). Dioxygen can also be activated enzymatically by several oxygenases (Elstner 1990) or physically by pigment-photosensitized reactions, both yielding singlet oxygen ($^1O_2$), and eventually, among other products, H_2O_2 (e.g. Szigeti and Vág-ujfalvi 1984; Elstner 1990).

Because of the high reactivity of active oxygen with cellular components, its immediate scavenging is indispensable, and the cells suffer from photooxidative damage unless scavenging systems operate at a sufficient rate (Asada and Takahashi 1987). As a consequence, all aerobic organisms have evolved mechanisms to remove toxic oxygen species.

In photosynthetic cells the plastidic ascorbate-glutathione pathway was suggested as the major sequence for the elimination of active oxygen species. This chain of enzymatic reactions starts with a very fast disproportionate reduction of $\cdot O_2^-$ to H_2O_2 by the enzyme superoxide dismutase (SOD; McCord and Fridovich 1969; Elstner and Heupel 1974; Jackson et al. 1978; van Ginkel and Brown 1978). In chloroplasts, lacking catalase, the resulting H_2O_2 is removed by a peroxidase highly specific for ascorbate (APO; Groden and Beck 1979; Kelly and Latzko 1979; Nakano and Asada 1981; Jablonski and Anderson 1982). The ascorbate is regenerated by a cooperative action of dehydroascorbate reductase, monodehydroascorbate reductase, and an NADPH-dependent glutathione reductase (GR; Foyer and Halliwell 1976, 1977; Halliwell and Foyer

1978; Nakano and Asada 1980; Jablonski and Anderson 1981, 1982; Anderson et al. 1983; Hossain et al. 1984; Kalt-Torres et al. 1984; Winkler 1987). Thus, the removal of excited oxygen is eventually accomplished by the consumption of NADPH.

Surprisingly little is known about the regulation of the enzymes of the ascorbate-glutathione pathway. Glutathione reductase was previously shown in mustard cotyledons to be regulated by light via phytochrome (Drumm and Mohr 1973). Mehlhorn et al. (1987) observed coincident increases in both APO and GR activities in plants exposed to air pollutants and suggested – like other investigators (Tanaka et al. 1985; Shaaltiel et al. 1988) – that APO may protect plant cells against the build-up of toxic concentrations of H_2O_2 when exposed to oxidative stress. Because formation of active oxygen species takes place wherever biological redox reactions with oxygen occur (Marx 1987), detoxifying pathways must be expected to exist outside the chloroplasts as well.

The present paper reports studies of the regulation of APO in the cotyledons of the mustard (*Sinapis alba* L.) seedling. Since extraplastidic isoforms were previously described in the cases of GR (Drumm-Herrel et al. 1989) and SOD (Palma et al. 1986), and recently in the case of APO in tea and spinach leaves (Chen and Asada 1989; Tanaka et al. 1991), the possibility of differential regulation of APO isoforms had to be considered.

Photooxidative stress inside the chloroplasts can be brought about by irradiating carotenoid-free seedlings with light strongly absorbed by chlorophyll. Normally, chlorophyll, which can be oxidized by singlet oxygen, is protected against photooxidation by carotenoids which are capable of quenching $^1O_2$ (Foote 1976; Takahama 1978; Krinsky 1979). As with other seedlings, accumulation of coloured carotenoids is prevented in the mustard seedling in the presence of the herbizide Norflurazon (NF; Frosch et al. 1979). When carotenoid-deficient seedlings are grown in strong white or red light (R), photooxidative destruction of many plastidic components results because photoprotective carotenoids are absent (Frosch et al. 1979; Reiss et al. 1983). Far-red light (FR), however, is only weakly absorbed by protochlorophyll (-ide) or chlorophyll, and no photooxidative stress is observed even in the complete absence of carotenoids (Frosch et al. 1979). Since FR strongly activates phytochrome, FR-grown seedlings develop normally, i.e. photomorphogenesis occurs, even in the absence of photosynthesis (see Mohr 1972) as long as storage material is available (Reiss et al. 1983). With appropriate fluence rates of R and FR the same time course of development can be achieved in mustard seedlings irrespective of whether they contain intact chloroplasts (R, no NF), photooxidized plastids (R/NF) or superetioplasts (FR), i.e. plastids of normal size with a small amount of chlorophyll a and a large prolamellar body. Appearance of the FR-superetioplasts is not affected by the presence of NF (see Reiss et al. 1983, for details).

This fortunate situation offers the possibility of turning on and off photooxidation within the plastids without affecting the course of development of the seedling.

The following questions will be addressed: (i) Can isoforms of APO be distinguished and assigned to different cell compartments? (ii) What factors regulate the appearance of APO isoforms? (iii) In particular, is the appearance of APO affected by pigment-photosensitized oxidative stress?

Material and methods

Growth conditions. Seeds of white mustard (*Sinapis alba* L., harvest 1986) were produced by a local grower from our original seed stock (Mohr 1957). They were selected and grown at 25° C as described previously (Mohr 1966). The herbizide Norflurazon (NF, 4-chloro-S-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone, 10 μM in the medium) was used to specifically inhibit carotenoid biosynthesis (Frosch et al. 1979; Reiss et al. 1983).

Seedlings were grown in standardized light fields (Mohr and Drumm-Herrel 1981): red light (R, 6.8 $W \cdot m^{-2}$) and far-red light (FR, 3.5 $W \cdot m^{-2}$. As indicated in the *Introduction,* these light sources were chosen because they allow strong phytochrome action and normal photomorphogenesis. In contrast to R, which leads to normal greening of the cotyledons, illumination with FR causes only a small rate of photoconversion of protochlorophyllide to chlorophyllide. In NF-treated seedlings, R produces strong photooxidative stress because of the absence of carotenoids, while in FR no photooxidative stress occurs. To decrease the amount of toxic oxygen species the seedlings were grown in some experiments in the presence of the singlet-oxygen-quenching substances *p*-benzoquinone (10 mM in the medium) or α-tocopherol, according to Feierabend and Winkelhüsener (1982). To raise seedlings in the presence of α-tocopherol, 100 μmol of D,L-α-tocopherol acetate per box was dissolved in ethanol and the solution applied to four layers of germination paper (8 · 8 cm^2, for details see Mohr 1966). After evaporation of the solvent the four germination papers per box were wetted as usual.

Enzyme assay. Because APO is inactivated in vitro instantaneously by a deficiency of ascorbate (Hossain and Asada 1984; Nakano and Asada 1987), all buffers must contain ascorbate in amounts above a threshold value, (20 μM; Miyake et al. 1991). A crude extract of 20 pairs of cotyledons was prepared on ice using mortar and pestle with 5 ml extraction buffer (50 mM Na-phosphate, 1 mM EDTA, 1 mM ascorbic acid), 2 g quartz sand, 100 mg Dowex 1 × 2–400 (equilibrated with extraction buffer), and 1 g sorbitol. After centrifugation (20 min, 18 000 rpm, SM–24-rotor; Sorvall, Wilmington, Del., USA), APO activity was determined in the clear supernatant according to Nakano and Asada (1981) with minor modifications. Using a 1-ml quartz cuvette with 0.5-cm pathway a concentration of 1 mM ascorbic acid could be used without too much UV absorbance. The assay was carried out at 25° C in a final volume of 1 ml, containing test buffer (20 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol (Bistris), pH 6.0), 1 mM ascorbic acid, and 20–100 μl enzyme extract. After recording any decrease in UV absorption – probably caused by ascorbate oxidase – for 30 s, the APO reaction was started by the addition of 20 μl H_2O_2 (15 mM). The initial slope of the curve was used to calculate APO activity after correcting for ascorbate-oxidase activity. The basis of the calculations was: APO [nkat/assay] = 11.9 · $\Delta A_{290} \cdot min^{-1}$

Experiments at different O_2 concentrations. The influence of a change in O_2 concentration in the atmosphere around the seedlings was investigated by growing the seedlings in gas-tight glass vessels and flushing them with N_2 or O_2.

Anion-exchange chromatography. The proteins of the crude extract were separated using a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) with a Mono Q HR 5/5-col-

umn equilibrated with 20 ml buffer A (20 mM triethanolamine, pH 7.5; 2 mM ascorbate; 2 mM $Na_4P_2O_7$). After gel filtration with Sephadex G–25, equilibrated with buffer A, 2 ml extract were filtered through a 0.22-µm Millipore filter and layered onto the column. After rinsing the loaded column with 15 ml buffer A the elution of bound proteins was carried out with an increasing gradient of NaCl (0–400 mM) in 20 ml buffer A. Fractions of 0.5 ml were collected for APO determination. This procedure led to a recovery ≥90% of the APO layered onto the column. Thus, under our conditions the APO was sufficiently stable. Because of the strong UV-absorbance of the ascorbate-containing buffer a direct continuous recording of the protein concentration of the eluate (A_{280}) was not possible. Instead the absorbance at 405 nm of the eluted material was measured to estimate the amount of heme-containing proteins, among them APO, in the different fractions.

Isolation of chloroplasts. The seedlings were grown in plastic boxes ($20 \cdot 30 \cdot 9\ cm^3$) under the above-mentioned light conditions. The procedure is based on a method described in Schwitzguebel and Siegenthaler (1984) with some modifications. Eighty grams of cotyledons were homogenised in 300 ml ice-cold isolation buffer (50 mM Na_2HPO_4, pH 7.6; 2 mM $MgCl_2$; 300 mM sorbitol; 1 mM Na-ascorbate; 0.6% (w/v) polyvinylpolypyrrolidone (PVPP, Polyclar AT)) with a blender (3 × 5 s at highest speed). After filtration through two layers of Miracloth (Calbiochem, La Jolla, Cal., USA) the extract was centrifuged to remove particles (2 min, 1100 rpm, HB–4-rotor; Sorvall). Crude chloroplasts were pelleted by a second centrifugation of the supernatant (2 min, 3300 rpm, HB–4-rotor). The pellets were resuspended each in 1 ml isolation buffer (without PVPP) and pooled. The resuspended pellets (2 ml) were layered onto a Percoll density-gradient (2, 4, 14, 8, 8 ml with 70, 60, 45, 27 and 21% Percoll, respectively) in isolation buffer (without PVPP). After centrifugation (30 min, 10 000 rpm, SS–34-rotor; Sorvall) the gradients were partitioned into fractions of 2 ml. In the case of the carotenoid-free, light-susceptible FR/NF-seedlings the whole isolation procedure was carried out under dim green safelight. For in vitro photooxidation of these chloroplasts the centrifuged gradients were kept on ice for 4 h in R before fractioning.

For chromatographic separation of plastidic proteins the fractions with the intact plastids were pooled and washed three times with 30 ml isolation buffer (without PVPP). After lysis of the plastids in 40 ml buffer A the extract was concentrated to 10 ml by ultrafiltration (Centriprep, Amicon), gel-filtrated with Sephadex G–25 (equilibrated with buffer A), and chromatographed as described above.

Chlorophyll was determined according to Lichtenthaler (1987).

Statistics. All given values are means of three to ten independent experiments with at least three parallels each. The estimates of the standard error are below 6%. The FPLC chromatograms are based on at least three independent experiments. The results were always identical in principle. Thus, the elution profiles selected for publication are representative of those obtained in several independent runs.

Results

Time course of appearance of APO in mustard cotyledons. No difference in APO levels could be detected between seedlings grown in darkness or light until 48 h after sowing (Fig. 1). Later on, however, there was a marked increase of APO in plants grown in R as well as in FR compared with plants grown in continuous darkness (D). In plants grown on water only slight differences between R (strong phytochrome action, chlorophyll accumulation and photosynthesis) and FR (strong phytochrome

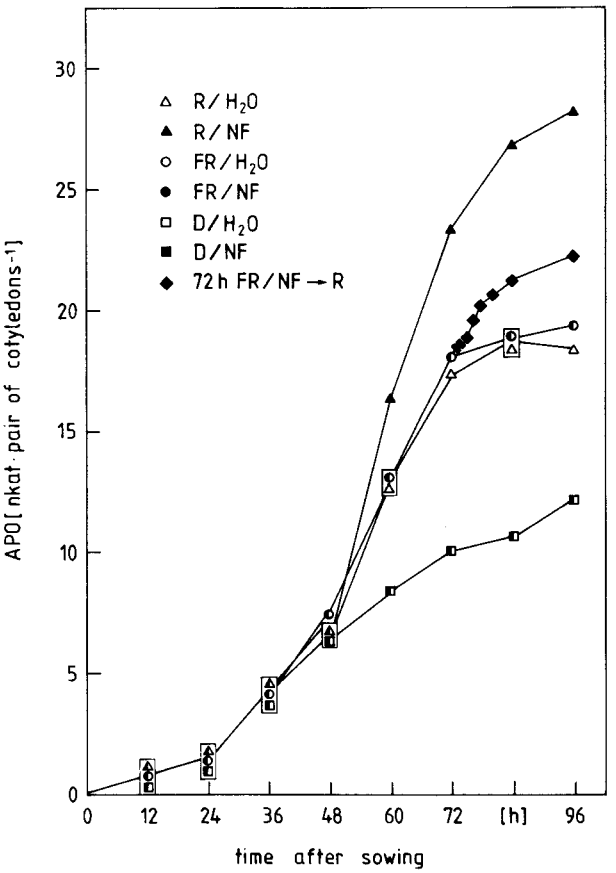

Fig. 1. Time course of APO activity isolated from the cotyledons of mustard seedlings. The seedlings were grown from sowing onwards in darkness (*D*), in red (*R*) or far-red (*FR*) light in the absence or presence of Norflurazon (*NF*) in the medium. ◆—◆, seedlings grown in FR in the presence of NF (10 µM) were transferred to R 72 h after sowing. Several values within the same frame means that the values are not significantly different. The APO activity which could be isolated from the seed (after 5 h imbibition) was 0.03 nkat · pair of cotyledons^{-1}

action, but only a small rate of chlorophyll accumulation and no photosynthesis) could be detected, FR values sometimes being a little higher. Thus, the light-induced increase of APO levels appears to be independent of ongoing photosynthesis. The participation of phytochrome in the regulation of APO levels could be demonstrated in experiments where short light pulses were applied, either with R (2 min) to bring about a high ratio of the far-red-absorbing form of phytochrome to total phytochrome (P_{fr}/P_{tot}) ($\varphi_R \leq 0.8$) or with long-wavelength FR (RG 9-light, 5 min) to revert P_{fr} formation ($\varphi_{RG\ 9} \leq 0.01$) (Table 1). The effect of R pulses was weak compared with continuous R but it could be reverted to the RG 9 level, which was the same as the D level, by subsequent RG 9-light pulses.

As pointed out in the *Introduction*, accumulation of coloured carotenoids in mustard seedling cotyledons is prevented in the presence of the herbicide NF without detectable side effects. In continuous FR, which strongly activates phytochrome, but is only weakly absorbed by

Table 1. Effect of pulses of red light (R, 6.8 W · m^{-2}, 2 min) or long-wavelength far-red light (RG 9-light, 10 W · m^{-2}, 5 min) on the APO activity isolated from the cotyledons of mustard seedlings. Seedlings were grown in darkness (D), light pulses were given at 48 h and 60 h after sowing. The seedlings were harvested for enzyme assay 72 h after sowing

Treatment	APO activity (nkat · pair of cotyledons^{-1})
R	13.4 ± 0.3
RG 9-light	10.5 ± 0.2
R + RG 9-light	10.3 ± 0.1
Controls:	
72 h D	10.5 ± 0.1
72 h FR	18.4 ± 0.2
72 h R	17.4 ± 0.3

chlorophyll, no photooxidation occurs even in the absence of carotenoids. As expected, we found no difference in APO levels between seedlings grown in FR with or without NF. However, with seedlings grown under photodestructive conditions (R/NF), the APO level was strongly increased (Fig. 1). This finding was unexpected since APO was considered to be a plastidic protein. Such proteins are usually quickly destroyed under these conditions. The following experiments therefore served to answer the question of whether there is more than one isoform of APO in the cells of mustard cotyledons.

Separation of isoforms by FPLC. The extracts were separated by anion-exchange chromatography using a Mono Q column connected to a Pharmacia FPLC system. In all extracts examined, two isoforms of APO (APO I, APO II) could be detected independently of age and light conditions (Fig. 2). In darkness, with increasing age of the seedlings the isoform APO II was found to increase strongly while APO I appeared to be almost constitutive (Fig. 3). Thus, the changes in APO II essentially account for the changes in total APO levels in darkness. Light certainly affects both isoforms, but the stimulation of APO II dominates by far (Figs. 2, 4).

Treatments with NF were without influence on the elution profiles when the plants were grown in FR or D. Photooxidative destruction of plastids, achieved by a treatment of FR/NF-seedlings with 4 h R did not adversely affect APO, neither the total activity nor the elution profile of the isoforms (Fig. 4). However, when the plants were grown under photooxidative conditions for longer times, the isoform APO II drastically increased (Fig. 4). This increase of APO II activity was correlated with an increase in A_{405} of the corresponding fractions as would be expected for a heme-containing protein (data not shown).

The stimulation of the APO II level by a destructive treatment of the plastids indicates an extraplastidic localization of this isoform (as previously observed with the cytosolic isoform of nitrite reductase, Schuster and Mohr 1990), whereas the localization of APO I remains un-

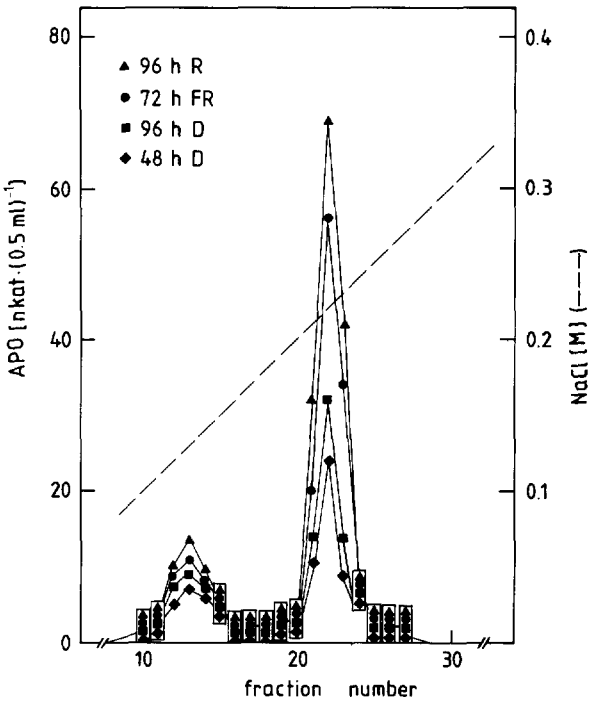

Fig. 2. Elution profiles of APO activity in equal amounts of mustard cotyledon extracts. The seedlings were grown on water in darkness (*D*), or in red (*R*) or far-red (*FR*) light as indicated. Separation of proteins was carried out by anion-exchange chromatography as described in *Material and methods*. No APO activity was detected below fraction 10 and beyond fraction 27

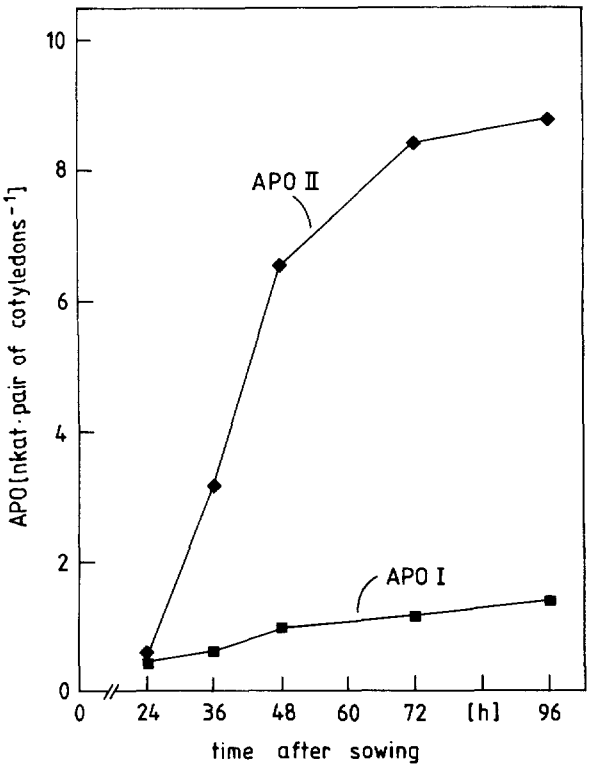

Fig. 3. Time courses of APO I and APO II activities in equal amounts of extracts from mustard seedling cotyledons. The seedlings were grown in darkness. The activity values represent the integrated activities based on the elution profiles of the corresponding isoforms

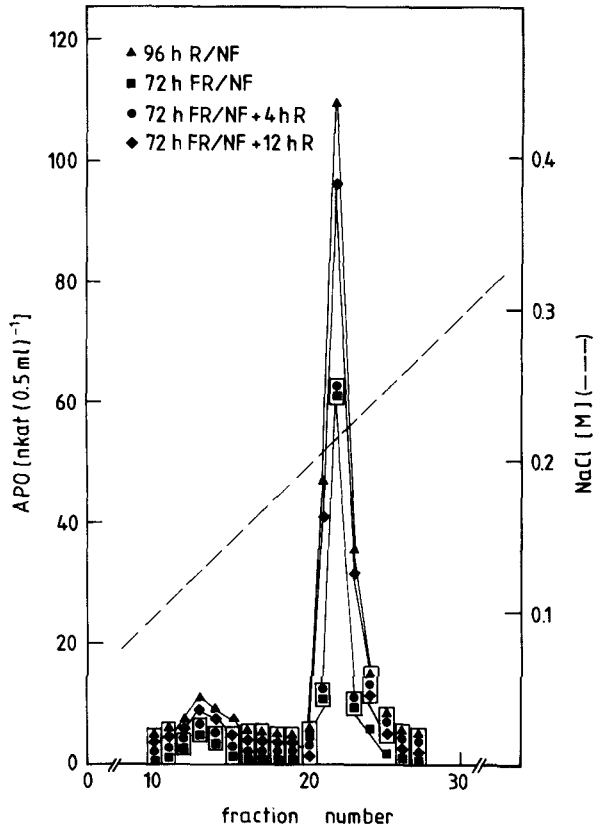

Fig. 4. Effect of photooxidative treatments with red light (*R*) on the elution profile of APO activity from equal amounts of extracts of mustard seedling cotyledons. Seedlings were grown from sowing onwards for the time indicated in red (*R*) or far-red (*FR*) light on a medium containing 10 µM Norflurazon (*NF*)

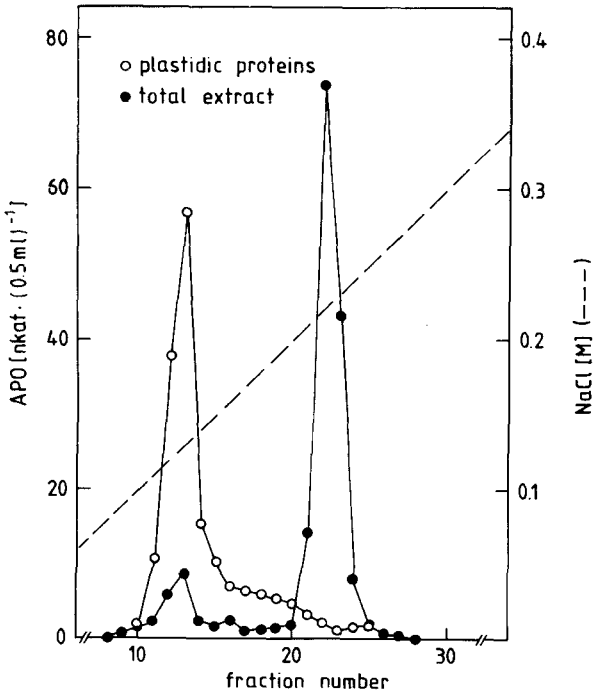

Fig. 5. Elution profile of APO activity from chloroplasts isolated from mustard seedling cotyledons (○—○) and from a crude extract of mustard seedling cotyledons (●—●). All seedlings were grown in red light for 96 h

Table 2. Results of in-vitro-photooxidation of plastids. Mustard seedlings were grown for 96 h in FR in the presence of NF and harvested in green safelight. The carotenoid-free superetioplasts were isolated by density-gradient centrifugation. After centrifugation one of the gradients containing the intact superetioplasts was exposed to R for 4 h while the other remained in darkness. After this differential treatment the gradients were fractionated and the amounts of APO activity and chlorophyll determined

Treatment	APO (nkat)	Chlorophyll (µg)
96 h FR/NF	120	65
96 h FR/NF+4 h R	121	28
96 h FR/NF+4 h D	120	62

clear. This enzyme, if localized in the plastids, must be resistant to oxyradicals. This is not necessarily a characteristic of enzymes involved in oxygen detoxification since the plastidic glutathione reductase (GR) exhibited high sensitivity to photooxidative treatments of the chloroplasts (Drumm-Herrel et al. 1989).

Isolation of chloroplasts. Because of the surprising resistance of APO I to photooxidative treatments the question of whether or not this isoform is localized inside the chloroplasts had to be answered. The isolation of chloroplasts from mustard seedling cotyledons showed that some APO activity was associated with the intact plastids. Anion-exchange chromatography of the plastidic proteins showed that this plastidic APO activity can be attributed exclusively to APO I (Fig. 5). This confirms our suggestion that APO II is localized outside the plastids.

To check the presumed high stability of the plastidic APO I and its resistance to photooxidation, plastids from 96-h-old FR/NR-seedlings (superetioplasts, see *Introduction*) were isolated in green safelight. A 4-h treatment of these highly light-sensitive plastids with R in vitro led to a drastic reduction of chlorophyll while the plastidic APO I remained unaffected (Table 2). These results corroborate the conclusion that APO I is a plastidic enzyme highly resistant to photooxidation.

Transfer experiments. As pointed out above, the extraplastidic isoform APO II is sensitive to photooxidative stress induced inside the plastid. Since extraplastidic enzymes are not normally directly affected by a photooxidative treatment of the plastids (Reiss et al. 1983), this indicates the involvement of a signal. The following experiments support this notion. When 72-h-old FR/NF-seedlings were kept in R for 4 h only and then transferred back to FR, the same stimulation of the APO level was observed 24 h later as occurred with the plants which had remained in continuous R after the transfer (Table 3). Corresponding increases in the APO level, induced by 4 h R, were observed when the seedlings were transferred at other developmental stages. The maximum value occurred when the 4 h R were given at the age of 48 h after sowing. In this case the APO activity at the age of 96 h

Table 3. Effect of a short-term photooxidative treatment on the APO level of mustard seedling cotyledons. Norflurazon-treated seedlings were grown for 72 h in FR (3.5 W · m^{-2}), then transferred to R (6.8 W · m^{-2}) for 1, 2, or 4 h and then returned to FR until the age of 96 h (lines 2–4). A photooxidative treatment with 4 h R (line 4) results in the same amount of APO as if the plants had been in R for 24 h (line 5). Without NF, APO levels are comparable to those in continuous FR (lines 6–8). Controls show that the R-mediated increase of APO activity in NF-seedlings is not yet detectable after 4 h R

Treatment	APO activity (nkat · pair of cotyledons^{-1})
1. 72 h FR/NF + 24 h FR	19.5 ± 0.2
2. 72 h FR/NF + 1 h R + 23 h FR	21.6 ± 0.2
3. 72 h FR/NF + 2 h R + 22 h FR	22.1 ± 0.2
4. 72 h FR/NF + 4 h R + 20 h FR	23.2 ± 0.1
5. 72 h FR/NF + 24 h R	22.3 ± 0.1
6. 72 h FR + 24 h FR	19.4 ± 0.2
7. 72 h FR + 4 h R + 20 h FR	19.3 ± 0.2
8. 72 h FR + 24 h R	19.2 ± 0.1
Controls:	
72 h FR	18.4 ± 0.1
72 h FR + 4 h R	18.0 ± 0.1
72 h FR/NF	17.8 ± 0.2
72 h FR/NF + 4 h R	18.1 ± 0.2

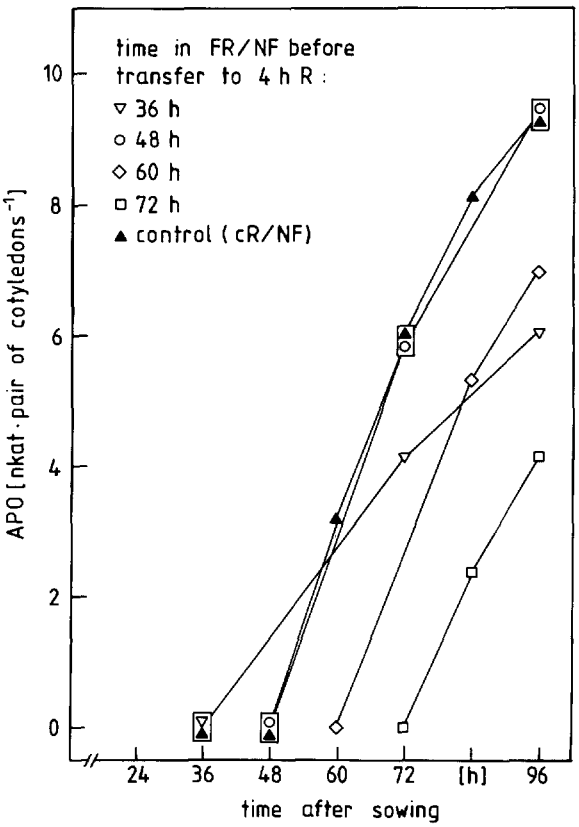

Fig. 6. Long-term effect of short-term photooxidative treatments on the APO level in mustard seedling cotyledons. Seedlings were grown for 36, 48, 60 or 72 h in FR in the presence of 10 μM NF. These FR/NF-seedlings were then transferred to R for 4 h and then returned to FR. The seedlings were harvested and assayed at the time points indicated. The original data were corrected for the action of phytochrome, operationally, by subtracting the corresponding FR/NF values (see Fig. 1). Thus, the values on the ordinate represent the stimulation of the APO level by the photooxidative treatment only. As a control for comparison, the corrected data for seedlings grown in continuous R/NF are given

was the same as if the seedlings were grown under photooxidative conditions from sowing onwards. If one subtracts the APO level which results from phytochrome action, the increases due to the action of R turn out to be approximately the same (Fig. 6). The slope was less only if the inducing 4-h R period was given before 48 h after sowing.

These results support the idea that a signal is released by the action of R on FR/NF-grown seedlings. Presumably the signal is exerted by a transient high production of singlet oxygen following the transfer of an FR/NF-seedling to R. The signal is fully saturated after 4 h and the signal transduction cannot be held up by a transfer to non-photooxidative conditions in FR.

Experiments with varying oxygen concentrations. The data so far obtained indicate that singlet oxygen or other toxic oxygen species turn on a signal-transduction chain which eventually leads to the regulation of the APO level. Further evidence in support of this hypothesis came from experiments where the seedlings were kept in an atmosphere with an elevated oxygen level. These conditions led to a considerable increase of the APO level in R. Even in the absence of NF there was a slight but significant increase (line 2 in Table 4a), probably resulting from a higher production of toxic oxygen species. The FR controls show that this cannot be attributed to an nonspecific stimulation of the seedlings' metabolism by oxygen.

The reverse effect occurred when the ability to form toxic oxygen species was reduced by keeping the seedling in a nitrogen atmosphere (N_2, Table 4b): a 4-h R treatment applied to 72-h-old FR/NF-seedlings, which normally results in increased APO levels 24 h later (see Table 3), was without any effect when the R was given in a pure nitrogen atmosphere. The controls show that it was not the anoxia itself that inhibited the accumulation of APO.

Experiments with quenchers. Quenchers of singlet oxygen, presumably the first active oxygen species produced in chloroplasts by an R/NF treatment, were applied through the growth medium. In the presence of α-tocopherol, as well as of *p*-benzoquinone, the increase of the APO level, induced in NF-treated seedlings by continuous R, was markedly reduced (Table 5). The significant effect of the quencher treatment in R-grown seedlings even in the absence of NF confirms the above finding that even in the normal green seedling R might lead to some production of oxyradicals, while in FR controls there is no decrease in the APO level in the presence of either quencher.

Thus, the stimulating effect of R on the APO level in NF-grown seedlings depends on the presence of O_2 and

Table 4a, b. Effects of different ambient oxygen concentrations on the APO activity isolated from the cotyledons of 96-h-old mustard seedlings. **a** Seedlings kept in air or in an oxygen atmosphere were grown in continuous R or FR with or without NF (10 μM). Oxygen atmosphere means that the seedlings – kept in air-tight vessels – were flushed with pure O_2 for 10 min per 24 h. **b** Seedlings kept in air were grown in R or FR in the presence of NF (10 μM). Line 5: after 72 h in FR the seedlings were kept in air or in a nitrogen atmosphere for 4 h, simultaneously being treated with 4 h R. After return to FR, nitrogen was replaced by air again. Lines 6 and 7: seedlings were kept in a nitrogen atmosphere or in air between 72 and 76 h after sowing (controls). All seedlings were harvested and assayed 96 h after sowing

Light treatment	O_2-atmosphere	Air
	APO (nkat · pair of cotyledons^{-1})	
a.		
1. 96 h R/NF	34.5 ± 0.5	27.9 ± 0.4
2. 96 h R	20.8 ± 0.3	18.7 ± 0.3
3. 96 h FR/NF	19.2 ± 0.2	19.2 ± 0.3
4. 96 h FR	19.1 ± 0.3	19.3 ± 0.3
	N_2-atmosphere	Air
	APO (nkat · pair of cotyledons^{-1})	
b.		
5. 72 h FR/NF + 4 h R + 20 h FR	19.3 ± 0.3	23.2 ± 0.4
6. 96 h R/NF	28.0 ± 0.5	27.8 ± 0.5
7. 96 h FR/NF	19.3 ± 0.2	19.5 ± 0.3

Table 5. Effects of quenchers of singlet oxygen on the APO activity isolated from mustard cotyledons. Seedlings were grown for 96 h in R in the presence of α-tocopherol or *p*-benzoquinone (see *Material and methods* for details)

Treatment	α-tocopherol	*p*-benzoquinone	No quencher
	APO activity (nkat · pair of cotyledons^{-1})		
96 h R/NF	20.2 ± 0.2	19.8 ± 0.2	27.8 ± 0.4
96 h R	17.5 ± 0.1	17.8 ± 0.1	18.5 ± 0.2
96 h FR/NF	19.1 ± 0.4	19.2 ± 0.3	18.9 ± 0.3
96 h FR	19.3 ± 0.4	18.9 ± 0.3	19.0 ± 0.4

is reduced by substances interfering with singlet oxygen. We therefore conclude that the release of oxyradicals inside the chloroplasts leads to a strong increase in the level of the extraplastidic isoform of APO.

Discussion

The questions we have addressed in this study (see *Introduction*) can now be answered as follows:

(i) Two isoforms (APO I and APO II) could be distinguished in extracts from mustard seedling cotyledons using anion-exchange chromatography (Mono Q–FPLC). The isoform APO I is localized in the plastids as we have demonstrated by its occurrence in isolated chloroplasts, while APO II must be considered to be an extraplastidic, presumably cytosolic, protein. The high resistance of the plastidic isoform to photooxidative treatments indicates than even NF-bleached chloroplasts contain ascorbate in concentrations sufficient to stabilize APO. Thus, the steady contact of the enzyme with its reducing co-substrate is probably the reason for its resistance to photooxidation. Normally, in chloroplasts ascorbate is found in concentrations up to 50 mM (Gerhardt 1964; Halliwell 1978).

(ii) The appearance of APO is regulated by light via phytochrome. This control is exerted over both isoforms. Mehlhorn (1990) reported that application of ethylene promotes ascorbate-peroxidase activity. However, ethylene cannot be considered a link in the phytochrome-mediated signal-transduction chain since it was shown repeatedly in the past (e.g. Bühler et al. 1978) that the actions of phytochrome and ethylene are independent of each other. The strong positive control of APO appearance by photooxidative treatments only affects the extraplastidic isoform. The increased level of APO activity may be attributed to synthesis de novo of this hemoprotein, since we found (data not shown) that the increased enzyme activity coincides with the increase of heme absorption. An increase of the plastidic APO level could not be expected even though the isoform appeared to be stable when subjected to a photooxidative treatment. It is well known from previous studies (see Oelmüller 1989, for a review) that expression of those nuclear genes whose protein products are destined for the plastid is arrested as soon as the plastids become damaged by photooxidation.

(iii) Additional synthesis of extraplastidic APO II under conditions where the interior of the plastid suffers from photooxidation is related to pigment-photosensitized oxidative stress. That this response involves activated oxygen species is demonstrated by the obligatory oxygen requirement and the protective role of quenchers of activated oxygen such as α-tocopherol or *p*-benzoquinone. Thus it is inferred that the pigment-sensitized production of toxic oxygen species inside the plastids leads to a dramatic increase in the level of an extraplastidic enzyme involved in the reduction of a toxic oxygen species.

Our experiments regarding the properties of the signal-transduction chain have so far only led to the conclusion that a 4-h photooxidation suffices to saturate the signal and that signal transduction cannot be arrested or diminished by placing the plants in non-photooxidizing conditions.

Recent studies with microorganisms have shown that active oxygen species can positively control the transcription of genes involved in oxygen detoxification. Greenberg et al. (1990), and Storz et al. (1990) have characterized a regulatory protein that in an oxidized form activates transcription of several stress-inducible genes. Work with higher plants has so far remained at a descriptive level. In earlier work, it was repeatedly found that an increase in peroxidase activities is a consistent feature

of a plant's response to exogenous stresses (see e.g. Gaspar et al. 1982, for a review). More recently, e.g. Mehlhorn et al. (1987) reported that fumigation of pea leaves results in an increase in the reaction of an unspecified peroxidase with ascorbate.

Khan and Malhotra (1982) and Grill et al. (1985) reported that oxidative stress induced an increase in many forms of peroxidase. Ozone-tolerant soybean cultivars, on the other hand, responded with a specific increase in only a few peroxidase isoforms when they were treated with ozone (Curtis and Howell 1971; Curtis et al. 1976). Fortunately, there is very little "unspecific" peroxidase activity in the mustard seedling until the age of 4–5 d (Schopfer and Plachy 1973). Thus, the APO activities identified in the present study could be discriminated from other kinds of peroxidases.

In contrast to the experiments performed in the present study, fumigation of plants with ozone leads to the release of oxyradicals in the cell wall, i.e. outside the cell (Tingey and Taylor 1982; Elstner 1984). In addition Tanaka et al. (1985) measured an increasing APO activity in chloroplasts as a response to ozone treatment of whole spinach plants. The only reports which can be directly compared to our study are by Gillham and Dodge (1985, 1987). These authors could trigger an increase of ascorbate peroxidase by a sudden illumination of etiolated or shade-adapted pea plants with strong white light. In this case, however, the localization of the implied enzyme was not achieved. Thus, it remains open whether the effect observed concerned an extraplastidic protein.

In teleological terms, the present findings appear plausible. The appearance of (potentially damaging) light is detected by the plant via phytochrome. The resulting increase in the APO level must be considered a normal feature of photomorphogenesis. However, actual photodynamic damage, which requires a sudden and strong increased level of APO, is sensed by the cell via the appearance of activated oxygen (whatever type of O_2 radical). Obviously, this two-step strategy – anticipation of potential damage as well as response to actual damage – allows a subtle adaptation of gene expression to the environmental conditions in the case of enzymes required for the removal of toxic oxygen species. While at present we cannot prove that photodynamic action in the presence of NF can be considered an amplification of the normal situation in the cell, several observations (oxygen atmosphere, quenchers) indicate that control of APO synthesis by active oxygen is not an experimental artifact but a natural phenomenon.

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