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Hydrogen Peroxide-Dependent Oxidation of 3,4-Dihydroxyphenylalanine in Vacuoles of Mesophyll Cells of *Vicia faba* L. Participation of Peroxidase in the Oxidation

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Peroxidase activity and 3,4-dihydroxyphenylalanine (DOPA) were found in vacuoles isolated from mesophyll protoplasts of *Vicia faba* L. A peroxidase isozyme localized in vacuoles migrated to the cathode during electrophoresis at pH 8.7, indicating that the vacuole peroxidase was a basic isozyme. When isolated vacuoles were treated with 2 mM H_2O_2, dopachrome, a product of oxidation of DOPA, was formed in a reaction that was inhibited by KCN and NaN_3. These results suggest that DOPA can serve as a donor of electrons to the peroxidase in vacuoles.

Key words: 3,4-Dihydroxyphenylalanine (DOPA) — Hydrogen peroxide — Peroxidase — Vacuoles — *Vicia faba*.

Hydrogen peroxide is formed during the metabolism of oxygen in plant cells (Halliwell and Gutteridge 1985). If the rate of formation of H_2O_2 in organelles is in excess of the rate at which it is scavenged, H_2O_2 should diffuse away from the sites at which it is formed. Chloroplasts may be one of the possible sites at which the formation of excess H_2O_2 occurs. Since the vacuole occupies most of each mesophyll cell in mature leaves, it is possible that, if the H_2O_2 generated in H_2O_2-generating organelles is not completely removed by scavenging, the peroxide may diffuse into the vacuole. This possibility can be deduced from the observation (Takahama 1989) that illumination of isolated mesophyll cells of *Vicia faba* in the presence of methyl viologen causes the oxidation of DOPA which may be present in vacuoles. The presence of peroxidase (Boller 1982, Cassab and Varner 1988, Grob and Matile 1980, Lütz 1987, Thomas and Jen 1980) and phenolics (Harborne and Williams 1988), which can serve as donor of electrons to peroxidase (Barz et al. 1985), in vacuoles suggests the probable oxidation of phenolics by H_2O_2 in the organelle. This communication describes a study of peroxidase and DOPA in vacuoles of mesophyll cells of *V. faba* and the H_2O_2-dependent oxidation of DOPA in isolated vacuoles.

Materials and Methods

Plant materials—Seeds of *V. faba* L. (cv. Longreen; from Mikado Seeds, Chiba) were grown in vermiculite for 2–4 months under normal daylight conditions, by a window, and watered once a week with a 0.1% solution of Hyponex.

Preparation of mesophyll protoplasts—Young, fully expanded leaves were harvested in the morning (about 9 a.m.) and used for the preparation of mesophyll protoplasts. Immediately after harvest, about 20 leaves from which the epidermis has been partially removed were floated on a solution of enzymes which consisted of 550 mM sorbitol, 1% cellulase and 0.5% macerozyme in 5 mM MES-KOH (pH 5.8). The solution of enzymes was infiltrated into leaves in vacuo, and the infiltrated leaves were incubated at 25°C for 1 h with gentle shaking under room light. Released protoplasts were collected by passage through 4 layers of gauze and centrifugation at $100 \times g$ for 2 min. Isolated protoplasts were suspended in 550 mM sorbitol in 5 mM MES-KOH (pH 5.8) and kept at 0°C after two washings with the suspension medium.

Preparation of vacuoles—Vacuoles were prepared by the method of Foster et al. (1987) with a slight modification. To release vacuoles from mesophyll protoplasts, suspensions of protoplasts (0.4 ml) equivalent to 800–1,700 μg of Chl were added to 2 ml of a 0.1 M solution of K_2HPO_4 adjusted to pH 8.5 with HCl. After a few minutes at room temperature (15–20°C), each suspension was loaded onto a discontinuous Ficoll gradient which consisted of 1.5 ml each of 1, 3, 5 and 10% (w/v) Ficoll-400 in 50 mM HEPES-KOH (pH 7.8) that contained 0.4 M mannitol, 1 mM $CaCl_2$ and 1 mM EDTA. The gradients were centrifuged at

Abbreviations: DAB, 3,3′-diaminobenzidine; DOPA, 3,4-dihydroxyphenylalanine.

3,700 × *g* for 15 min at 5°C. Vacuoles accumulating at the interfaces between 1 and 3% and between 3 and 5% Ficoll were collected from the gradient with a Pasteur pipet and washed twice by centrifugation (200 × *g*, 2 min) in the buffer solution without Ficoll. The preparation of vacuoles was found to contain a small number of protoplasts and chloroplasts when examined under the light microscope, and the ratio of vacuoles to protoplasts ranged from 9 : 1 to 19 : 1. Isolated vacuoles were kept at 0°C. The concentration of protein was determined with a protein-assay reagent from Bio-Rad. Bovine serum albumin was used as a standard.

Assay of enzymatic activities—Catalase activity was assayed in a reaction mixture (2 ml) of 10 mM H_2O_2 and 50 mM Na-phosphate, pH 7.4, with a Clark-type oxygen electrode, at 25°C. The activity of cytochrome *c* oxidase was estimated by measuring decrease in absorbance at 550 nm in the presence of reduced cytochrome *c* (value of the difference absorbance coefficient of reduced minus oxidized cytochrome *c* at 550 nm was taken as 19.6 $mM^{-1} \cdot cm^{-1}$). The reaction mixture (1 ml) contained 15 μM sodium ascorbate to reduce ferricytochrome *c*, 50 μM ferricytochrome *c* from horse heart and 80 mM Na-phosphate, pH 7.4. The activity of glucose-6-phosphate dehydrogenase was followed at 340 nm (absorbance coefficient, 6.2 $mM^{-1} \cdot cm^{-1}$) in a reaction mixture (1 ml) that contained 1 mM glucose-6-phosphate, 0.2 mM $NADP^+$ and 80 mM Na-phosphate, pH 7.4. Peroxidase activities were measured in the presence of 1 mM H_2O_2, 80 mM Na-phosphate, pH 6.0, and one of the following electron donors: 0.89 mM guaiacol (absorbance coefficient at 470 nm due to tetraguaiacol, 26.6 $mM^{-1} \cdot cm^{-1}$), 1 mM DAB or 1 mM sodium ascorbate (absorbance coefficient at 290 nm, 2.8 $mM^{-1} \cdot cm^{-1}$). α-Mannosidase activity was followed in a 1-ml reaction mixture that contained 0.5 mM *p*-nitrophenol-α-mannopyranoside and 50 mM citrate–sodium citrate, pH 5.0. After incubation for 1 h, 1 ml of 1 M Na_2CO_3 was added and the absorbance was determined at 420 nm. Blank values were taken into account in the calculation of enzymatic activities. All reactions were started by the addition of protoplasts or vacuoles. Spectrophotometric measurements were made with a Hitachi 557 spectrophotometer at room temperature (15–21°C).

Gel electrophoresis—Polyacrylamide gel electrophoresis was performed on 3-mm-thick slabs of 7.5% acrylamide (pH 8.7) at 15°C. Peroxidase activity was detected by soaking gels in 1.3 mM DAB in 50 mM Na-phosphate buffer, pH 6.0, for 10 min and then 5 mM H_2O_2 in 50 mM Na-phosphate, pH 6.0 for 15–20 min. In the absence of H_2O_2, no oxidation of DAB was observed. Thus, it appears that the oxidation was not due to oxidases.

Spectrophotometric studies with a microscope spectrophotometer—H_2O_2-dependent changes in absorption spectra of a single vacuole were followed in a single-beam microscope spectrophotometer (Olympus MMSP-TU) combined with a reflective object lens (MO 30 ×), as described previously (Takahama 1988). Suspensions of vacuoles were placed on quartz slide glasses and covered with a second quartz slide glass. The distance between the two pieces of quartz glass was adjusted to 0.18 mm by insertion of spacers. The diameter of the measuring beam was 6 μm.

Analysis by HPLC—HPLC was performed as described previously (Takahama 1988, 1989) using a Shimadzu CLC-ODS column. Dopachrome was extracted with 80% methanol from H_2O_2-treated epidermal strips of *V. faba*. The mobile phase for the separation of dopachrome was a mixture of methanol, water and acetic acid (7 : 13 : 1, v/v), and the flow rate was 1 ml/min. DOPA was extracted from protoplast and vacuole fractions by the addition of 70 μl of methanol to 30 μl of each fraction. DOPA was separated by use of a mixture of methanol and water (1 : 9, v/v) as mobile phase, at a flow rate of 1 ml/min. These two compounds were detected with a spectrophotometer equipped with a photodiode array (Shimadzu SPD-M1A), which allowed determination of the absorption spectra of the compounds separated by HPLC.

Reagents—Macerozyme R-10 and Cellulase "Onozuka" RS were obtained from Yakult Honsha Co. Ltd. (Tokyo), horseradish peroxidase (grade II) from Boehringer Mannheim GmbH, and Ficoll-400 from Pharmacia (Uppsala, Sweden). DAB was purchased from Dojin (Kumamoto) and L-DOPA from Wako Pure Chem. Ind. (Osaka). Protein-assay reagent was obtained from Bio-Rad (Richmond, CA, U.S.A.).

Results and Discussion

Table 1 shows typical values of the specific activities of various enzymes and the relative values of enzymatic activities in terms of activity of α-mannosidase which is localized mainly in vacuoles (Boller and Kende 1979). In a given vacuole fraction, the relative activities of cytochrome *c* oxidase (mitochondria), catalase (peroxisomes), glucose-6-phosphate dehydrogenase (cytoplasmic matrix) and ascorbate peroxidase (chloroplasts) were lower than those in the corresponding protoplast fraction. The ratios of the relative activities of each enzyme in a vacuole fraction to that in a protoplast fraction ranged from 0.05 to 0.10. Essentially the same results were obtained using different preparations of vacuoles, although specific activities and the ratios of the relative activities of each enzyme changed depending on the particular preparations. These data suggest some contamination of the vacuole fractions by mitochondria, cytoplasmic matrix, peroxisomes and chloroplasts. Ascorbate-specific peroxidase has been reported to be localized in chloroplasts (Nakano and Asada 1981). However, ratios of the relative peroxidase activities for

Table 1 Specific enzymatic activities and levels of DOPA of protoplast and vacuole fractions

	Protoplasts (I)[d] [mol·(mg protein)$^{-1}$·min^{-1}]	Vacuoles (II)[d] [mol·(mg protein)$^{-1}$·min^{-1}]	(II)/(I)
α-Mannosidase[a]	1.00 (100.0)	18.86 (100.0)	1.00
Peroxidase (guaiacol)	0.165 (16.5)	3.95 (20.9)	1.27
Peroxidase (DAB)[b]	1.2 (120)	31.1 (167.2)	1.39
Peroxidase (ascorbate)	0.13 (13)	0.24 (1.3)	0.10
Catalase	1.96 (196)	1.93 (10.2)	0.05
Cytochrome *c* oxidase	0.056 (5.6)	0.084 (0.4)	0.07
Glucose-6-P dehydrogenase	0.026 (2.6)	0.042 (0.2)	0.08
DOPA[c]	0.007 (0.7)	0.141 (0.7)	1.00

[a] Relative rates of increase in absorbance at 420 nm per mg protein.
[b] Relative rates of increase in absorbance at 460 nm per mg protein.
[c] mol·(mg protein)$^{-1}$.
[d] Values in parentheses are enzymatic activities in protoplast and vacuole fractions relative to the mannosidase activity in each fraction.

guaiacol and DAB in vacuole and protoplast fractions were much greater than the analogous ratios of other enzymes, suggesting the presence of the guaiacol- and DAB-specific peroxidase activities in vacuoles. One of reasons for the relative values being greater than 1 may be the contamination by peroxidase that is present in cytoplasmic fractions other than the vacuole fraction (cf. Fig. 1). The presence of peroxidase activity in isolated vacuoles has been reported in other plants (Boller 1982, Grob and Matile 1980).

The presence of peroxidase activity in vacuoles was confirmed by cytochemical observations made under the light microscope. The addition of DAB alone (1.3 mM), which is used for cytochemical detection of peroxidase activity (Gahan 1984), to vacuoles suspended in 50 mM HEPES-KOH (pH 7.8) that contained 0.4 M mannitol, 1 mM $CaCl_2$ and 1 mM EDTA did not result in its oxidation, suggesting the absence of oxidases for DAB in vacuoles. However, after addition of both DAB (1.3 mM) and H_2O_2 (6.7 mM), the vacuoles turned brown. The oxidation of DAB was inhibited by KCN (1 mM). These data suggest that DAB can be oxidized by peroxidase in vacuoles. It has been shown that peroxidase activity in vacuoles is prominent at the inner surface of the tonoplast in the cells of young tomato fruit (Thomas and Jen 1980).

Figure 1 shows the results of analysis by polyacrylamide gel electrophoresis of peroxidase isozymes. Two major (bands A and B) and two minor bands were detected using DAB as the electron donor in protoplasts. The major bands were observed in every preparation of protoplasts. However, the activity of minor bands was dependent on the season during which protoplasts were prepared: in leaves harvested in autumn, the minor bands were very faint or undetectable; in leaves obtained in winter, the minor bands were distinct. Bands A and B were also observed when the electrophoretic analysis of peroxidase activity was performed using leaves of *V. faba* (data not shwon), suggesting that the peroxidases were not derived from the enzymes used to prepare protoplasts but from the leaves themselves.

In vacuole fractions, two isozymes of peroxidase were detected: isozymes A and B. No minor bands were detected, even when vacuoles were prepared from leaves harvested in winter. Isozyme A, which migrated to the cathode, had much greater peroxidase activity than isozyme B, which migrated to the anode. The data suggest

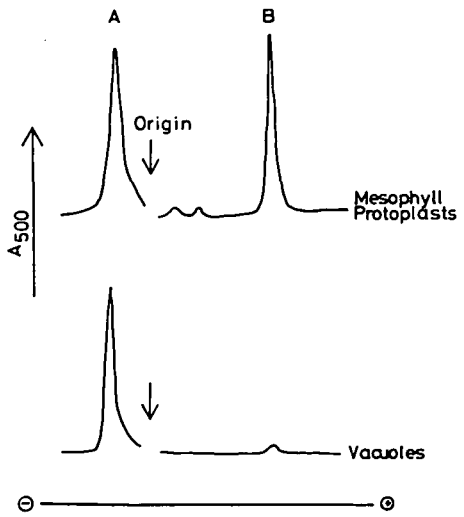

Fig. 1 Densitometric tracings of gels, after electrophoresis, in which peroxidase activity was visualized with DAB. Gels were scanned at 500 nm. Upper curve, a protoplast fraction equivalent to 25 μg protein; lower curve, a vacuole fraction equivalent to 1.6 μg protein.

that at least one basic peroxidase is localized in vacuoles. The presence of isozyme B in vacuole fractions may be the result of contamination by cytoplasmic components other than vacuoles. The basic isozyme, which was separated from other proteins by electrophoresis of vacuole fractions, was able to oxidize DOPA (2.5 mM) in addition to DAB, when still in the gel. However, the oxidation of DOPA was very slow compared to the oxidation of DAB and the product was gray in color, indicative perhaps of formation of a melanin-like substance. During the oxidation of DOPA on the gel, no dopachrome, visible as a red product was observed. Dopachrome is an intermediate in the synthesis of melanin from DOPA (Young et al. 1974). The absence of visible dopachrome may be due to a low steady-state concentration of this compound.

The peroxidase activity of the vacuole fractions might be due to catalase activity resulting from contamination by peroxisomes (Table 1). However, this possibility can be excluded since the molecular weight of the basic peroxidase, as estimated by sucrose density gradient centrifugation, was about 49,000 and the peroxidase had three absorption maxima at 404, 510 and 635 nm in its oxidized form (data not shown). These data were obtained with a partially purified preparation of peroxidase, prepared by ammonium sulfate precipitation, ion-exchange chromatography and gel filtration. In general, the molecular weight of catalase is about 240,000 and, in its oxidized form, it has absorption maxima at 405, 500, 540 and 629 nm.

Figure 2 (upper panel) shows the profile after HPLC of a 70% methanol extract of the vacuole fraction. Two peaks (A and B) were detected. Peak B had a retention time of 4.3 min and an absorption maximum at 282 nm. Authentic DOPA had a retention time of 4.3 min and its absoprtion spectrum coincided with that of peak B (Fig. 2, lower panel), indicating that peak B contains DOPA. The compound was also detected in protoplasts. The amounts of DOPA relative to amounts of protein in protoplast and vacuole fractions are shown in Table 1. The data suggest the presence of DOPA, in addition to peroxidase, in vacuoles of mesophyll cells of *V. faba*.

Figure 3A shows a typical absorption spectrum of a single vacuole with an absorption maximum at 275 nm and a shoulder at 345 nm. The maximum at 275 nm may be partially due to DOPA since DOPA has an absorption maximum at 279 nm in 50 mM Na-phosphate buffer at pH 6.0.

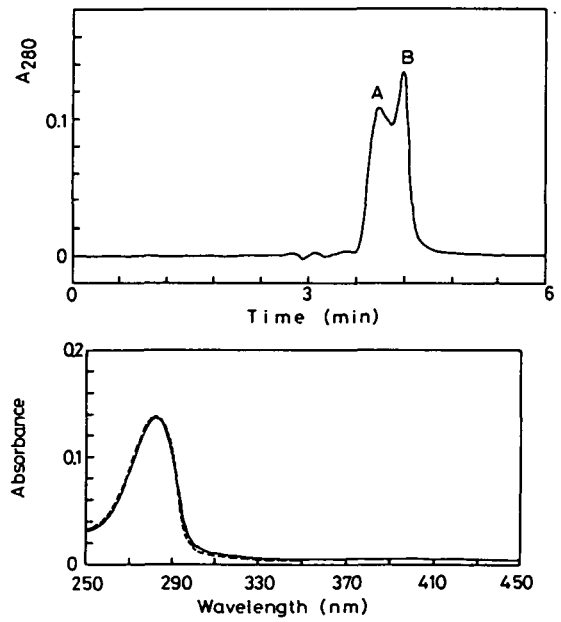

Fig. 2 Profile after elution by HPLC of an extract of vacuoles (upper panel) and the absorption spectra (lower panel) of authentic DOPA and the material indicated as B in the upper panel. Upper panel: 70% methanol extract of a vacuole fraction. Retention time of peak B was the same as that of authentic DOPA. Lower panel: solid line, material in peak B; dashed line, authentic DOPA. The spectra were recorded in the mobile phase used for HPLC, with a spectrophotometer equipped with a photodiode array and normalized at 282 nm.

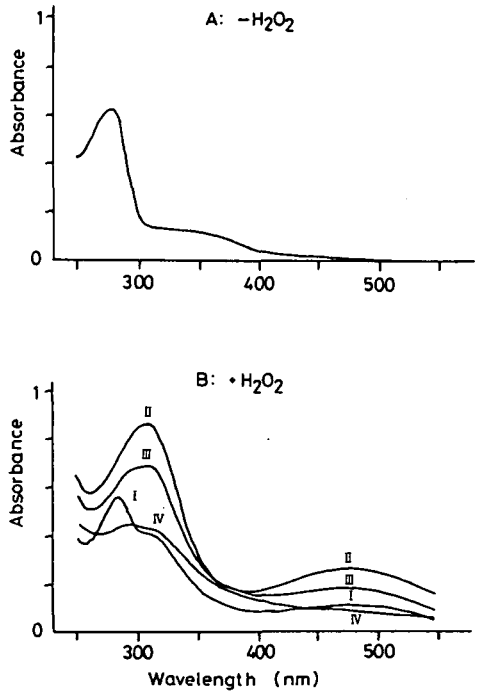

Fig. 3 Absorption spectra of single vacuoles obtained with a single-beam microscope spectrophotometer. (A), Absorption spectrum of an untreated vacuole; (B), absorption spectra of a vacuole treated with 2 mM H_2O_2 for 2.5 (I), 10 (II), 20 (III) and 40 (IV) min. The single vacuole used for (A) was different from that used for (B).

The shoulder at 345 nm may be due to flavonols. It has been reported that the epidermis (Weissenböck et al. 1984, Vierstra et al. 1982) and protoplasts of mesophyll cells (Takahama 1989) of *V. faba* contain kaempferol glycosides.

When 2 mM H_2O_2 was added to the vacuole fraction, its color was seen to turn red under the light microscope. The absorption spectrum of a single vacuole after incubation for 2.5 min had absorption maxima at 285, 310 (shoulder) and 470–480 nm (Fig. 3B, trace I). After incubation for 10 min, the peak 285 nm disappeared and absorption intensities at 310 and 470–480 nm increased (Fig. 3B, trace II), with maximal intensities attained 10–15 min after the addition of H_2O_2. The increase in absorbance at 310 and at 470–480 nm may be due to the formation of dopachrome (see below). Such increases in absorbance were observed even when only 0.1 mM H_2O_2 was added to the vacuole fraction.

If vacuoles were incubated for more than 20 min, the absorbance at 310 and 470–480 decreased (Fig. 3B, trace III). Ultimately, the peak at 470–480 nm disappeared and a dark precipitate appeared (Fig. 3B, trace IV), indicative of the transformation of the red compound to a melanin-like compound, the final product of the oxidation of DOPA. It is known that melanin is formed from DOPA via a red compound, dopachrome (Young et al. 1974). The H_2O_2-dependent formation of the red and melanin like compounds was inhibited by more than 90% by 1 mM KCN and by 70% by 20 mM NaN_3. These data suggest the participation of peroxidase activity in vacuoles in the production of the red compound. The peroxidase isozyme A (Fig. 1) may be the active enzyme in this reaction. Partially purified isozyme A was able to oxidize DOPA (1 mM), producing the red compound in the presence of 1 mM H_2O_2 (data not shown). An H_2O_2-dependent increase in absorbance with a maximum at 470–480 nm and the oxidation of DOPA have been reported in lower epidermis stripped from (Takahama 1988) and mesophyll protoplasts isolated from (Takahama 1989) leaves of *V. faba*, respectively.

To obtain larger amounts of the red compound, lower epidermis prepared from leaves of *V. faba* was incubated in 1 mM H_2O_2 for 10 min at room temperature (about 20°C). The red compound was extracted with 80% methanol and its absorption spectrum was determined after separation by HPLC. A red compound with absorption maxima at 304 and 480 nm was obtained with a retention time of 2.9 min (Fig. 4, solid line). Incubation of 0.5 mM DOPA in 50 mM Na-phosphate buffer, pH 6.0, in the presence of 0.25 mM H_2O_2 and 10 μg/ml of horseradish peroxidase, resulted in the formation of red solution. The absorption spectrum of this solution had a maximum at 480 nm. The product could also be separated by HPLC. The retention time and absorption spectrum of the product (Fig. 4, dashed line) were the same to those of the compound extracted from

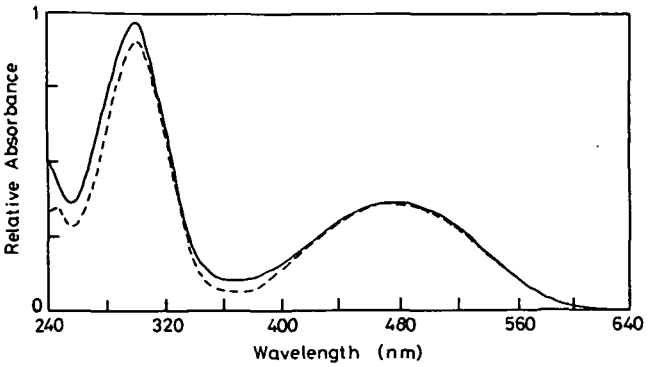

Fig. 4 Absorption spectra of the red product formed in epidermal strips. Absorption spectra were determined in the mobile phase used for HPLC with a spectrophotometer equipped with a photodiode array, after separation of the compound by HPLC. Solid line, product extracted from H_2O_2-treated epidermal strips; dashed line, product obtained by the oxidation of DOPA (0.5 mM) with H_2O_2 (0.25 mM) in the presence of horseradish peroxidase (10 μg/ml) in 50 mM Na-phophate, pH 6.0. The spectra were normalized at 480 nm.

H_2O_2-treated epidermal strips. One of reasons for the difference in wavelengths of the peaks of absorbance of the red compound in Figures 3 and 4 in the UV region may be due to the difference in the solvents in which the red compound was dissolved. In Figure 3, the absorption spectra were measured in a single vacuole and in Figure 4, spectra were determined in the mobile phase used for HPLC.

The data in Figures 3 and 4 suggest that the red compound in vacuoles is a product of oxidation of DOPA. Since DOPA is converted to a red oxidation product, dopachrome, with an absorption maximum at 475 nm (Horowitz et al. 1970), the product shown in Figure 4 is identified as dopachrome. It has been reported that peroxidases, in general, can oxidize DOPA to dopachrome in the presence of H_2O_2 (Gahan 1984).

In this study, both the presence of peroxidase activity and DOPA in vacuoles and the oxidation of DOPA by H_2O_2 have been demonstrated. These data suggest that if H_2O_2 diffuses into vacuoles, the peroxidase in vacuoles can oxidize DOPA. The light-dependent oxidation of DOPA and the formation of a red compound, which is very likely dopachrome, in mesophyll cells and the stimulation of the oxidation reaction by methyl viologen (Takahama 1989) suggest that H_2O_2 generated in chloroplasts can diffuse into vacuoles to oxidize DOPA. In the light, methyl viologen can accept electrons on the reducing side of PS I, producing methyl viologen monocationic radicals that donate electrons to molecular oxygen producing O_2^- radicals (Halliwell and Gutteridge 1985, Nakano and Asada 1980). The O_2^- radicals are dispropotionated to produce H_2O_2 and

O_2 in a reaction catalyzed by superoxide dismutase. In the presence of methyl viologen, all reducing equivalents in PS I are trapped by the reagent and no donor of electrons to ascorbate peroxidase for the scavenging of H_2O_2 is available, with the resultant accumulation of H_2O_2 (Nakano and Asada 1980). Diffusion of H_2O_2 from the sites where it is formed has also been reported in algae (Patterson and Myers 1973, Takahama et al. 1985). Flavonols, which are present in vacuoles (Harborne and Williams 1988, Weissenböck et al. 1984), can also be oxidized by H_2O_2 (Takahama et al. 1989) in mesophyll and epidermal cells of *V. faba*. These data and the data obtained in the present study together suggest the participation of H_2O_2 in the metabolism of phenolics in the vacuoles and/or the participation of phenolics in the metabolism of H_2O_2. The turnover of phenolics has been reported in some plants (Harborne 1972).

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