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ENHANCEMENT OF THE PEROXIDASE-DEPENDENT OXIDATION OF DOPA BY COMPONENTS OF *VICIA* LEAVES*

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Key Word Index—*Vicia faba*; Leguminosae; apoplast; cinnamic acids; dopa (3,4-dihydroxyphenylalanine) oxidation; peroxidase.

Abstract—In broad bean (*Vicia faba*) leaves 3,4-dihydroxyphenylalanine (dopa) was localized in the apoplast as well as the symplast. Apoplastic peroxidase oxidized cinnamic acids (4-coumaric, ferulic, caffeic and chlorogenic acids) rapidly and dopa more slowly. The cinnamic acids enhanced the apoplastic peroxidase-dependent oxidation of dopa. Small and heat-stable components that were present in the apoplast of the leaves also enhanced the peroxidase-dependent oxidation of dopa. Furthermore, the cinnamic acids and the apoplastic components enhanced horseradish peroxidase-dependent oxidation of dopa. These results suggest that radicals of the cinnamic acids and small heat-stable apoplastic components can oxidize dopa. The radical-dependent oxidation may play an important role in the formation of melanin when cells are injured. © 1997 Elsevier Science Ltd

INTRODUCTION

Plants normally contain peroxidases (POX, EC 1. 11. 1. 7) and phenolic compounds in apoplast and vacuoles. POX in the apoplast may participate in the biosynthesis of lignins from hydroxycinnamyl alcohols [1, 2] and the formation of cross-links between two molecules of phenolic esters [3]. In the vacuoles of *Vicia faba*, POX can catalyse the formation of melanin-like compounds by the oxidation of dopa [4, 5]. If ascorbic acid (AA) coexists with dopa, AA can readily reduce the phenoxyl radicals and *o*-quinones, which are the oxidation products of dopa or other phenolics, to the original compounds [6–8]. Therefore, the oxidation of dopa in vacuoles can be observed only after almost all of AA has been oxidized to dehydroascorbic acid (DHA) [6]. Under normal conditions, the oxidation products of AA, monodehydroascorbic acid and DHA are rapidly reduced to AA by monodehydroascorbic acid reductase and DHA reductase. The accumulation of melanins is not observed if the formation of H_2O_2 in plant cells is not rapid. If one takes the presence of AA and dopa in the vacuole [6] into account, a POX/dopa/AA system can function to scavenge H_2O_2. Hydrogen peroxide scavenging seems to be also possible for POX/phenolics/AA systems in the apoplast [9–11] because in addition to POX and phenolics, AA is present there also [12–14].

The substrate specificities of POX isoenzymes depend on their sources. For example, apoplastic POX isoenzymes from tobacco stems rapidly oxidize cinnamic acids and cinnamyl alcohols with a 4-hydroxyphenyl, a guaiacyl or a syringyl group, while POX isoenzymes from epicotyls of *Vigna angularis* slowly oxidize sinapic acid and sinapyl alcohol [15]. Horseradish peroxidase (HRP) isoenzymes also slowly oxidize the syringyl compounds [16]. A vacuolar POX isoenzyme isolated from leaves of broad bean (*V. faba*) slowly oxidizes dopa, a major phenolic compound of leaves of this plant [6], but rapidly oxidizes the flavonol aglycones kaempferol and quercetin [17]. However, H_2O_2-induced rapid oxidation of dopa producing dark substances such as melanins has been observed in protoplasts prepared from broad bean leaves and in vacuoles prepared from the protoplasts [18]. In this plant, the oxidation of dopa to the dark substances is possible in infected or wounded cells because the production of H_2O_2 is stimulated when cells are damaged [19].

To understand the discrepancy between the slow oxidation of dopa by isolated POX and the rapid oxidation *in situ*, the oxidation of dopa by POX in the apoplastic fraction was studied because dopa was present not only in the symplast but also in the apoplast (see Results). In addition, apoplastic POX- and HRP-dependent oxidation of dopa was also studied in the presence of various cinnamic acids which are

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normally present in cell walls of plants as esters [3]. The results obtained indicate that cinnamic acids and some components in the apoplast could enhance apoplastic POX- and HRP-dependent oxidation of dopa.

RESULTS AND DISCUSSION

Presence of dopa in the apoplast

Table 1 shows levels of AA plus DHA ([AA + DHA]) and dopa in the apoplastic (intercellular washing fluid, IWF) and symplastic fractions of young and aged leaves. The levels of [AA + DHA] were decreased on ageing in both the apoplast and the symplast, and the redox levels were more oxidizing in the apoplast than in the symplast. The levels of dopa also increased on ageing in both the symplast and the apoplast, and its levels were several times higher than the levels of [AA + DHA] in the symplast, but in the apoplast, the levels of dopa were comparable with those of [AA + DHA]. The average concentrations of [AA + DHA] and dopa in the apoplast were estimated to be 1.4 and 1.5 mM in young leaves and 0.5 and 0.9 mM in aged leaves using the apoplastic volume (11% of fresh weight) reported by Luwe and Heber [20]. The values were smaller than the average concentrations of [AA + DHA] (4–8 mM) and dopa (20–50 mM) in the symplast. The relative amounts of [AA + DHA] and dopa in the apoplast were 1–2% and 0.24–0.76% of the amounts of the respective compounds in the symplast. The relative activities of glucose-6-phosphate dehydrogenase were $0.006 \pm 0.003\%$ ($n = 3$). Since it has been reported that [AA + DHA] is an apoplastic component in leaves of spinach [14, 21], *Sedum album* [12], Norway spruce [13] and *Phaseolus vulgaris* [21], the data in Table 1 suggest that in addition to [AA + DHA], dopa is also present in the apoplast.

The levels of dopa in the symplast in Table 1 were comparable to those in whole leaves (15–27 μmol g^{-1} fr. wt) which had been determined by HPLC [5], suggesting that the spectrophotometric method used in this study was also a convenient method to determine the levels of dopa. The levels of [AA + DHA] in the symplast in this study were also similar to the levels in a previous report (2.5–4 mmol g^{-1} fresh weight) [20]. However, the apoplastic levels of [AA + DHA] were 2-fold larger in this study than in a previous report [20]. This discrepancy may be due to the difference in centrifugal force (80 *g* [20]; 200 *g* in this study) used for the preparation of IWF.

Enhancement of dopa oxidation by cinnamic acids

POX is normally present in the apoplast of plant cells and part of the POX is extracted as IWF [22]. POX in IWF oxidized dopa (0.4 mM) at a rate of 0.01 μmol g^{-1} fr. wt min^{-1}. 4-Coumaric, caffeic, ferulic and chlorogenic acids (0.09 mM) increased the rate *ca* 27-, 35-, 47- and 56-fold, respectively. The oxidation product was orange and the absorption spectra had a maximum at 480 nm even in the presence of cinnamic acids (data not shown), indicating that the major oxidation product was dopachrome. Stimulation of dopa oxidation by cinnamic acids was also observed when dopa oxidation was catalysed by HRP.

Figure 1 shows double-reciprocal plots of rates of dopa oxidation as a function of concentration of cinnamic acids. The dopa oxidation catalysed by POX in the dialysed IWF and HRP was stimulated in order chlorogenic acid > ferulic acid > caffeic acid > 4-coumaric acid [Fig. 1(A)], and chlorogenic acid > 4-coumaric acid > caffeic acid > ferulic acid [Fig. 1(B)], respectively, in the concentration ranges of cinnamic acids used. Figure 2 shows semi-logarithmic plots of POX-dependent oxidation of 30 μM cinnamic acids. The first-order rate constant of each cinnamic acid was in order chlorogenic acid > ferulic acid > caffeic acid > 4-coumaric acid [Fig. 2(A)], and chlorogenic acid > 4-coumaric acid = caffeic acid > ferulic acid [Fig. 2(B)] when catalysed by POX in dialysed IWF and HRP, respectively.

Presence of dopa-oxidation-enhancing components in non-dialysed IWF

When rates of oxidation of dopa (0.4 mM) were measured in the presence of various amounts of non-dialysed IWF, the rates increased as the amounts of non-dialysed IWF added to the reaction mixtures were increased. However, the increases in rates were not proportional to the amounts of the IWF, but accelerated. Namely, the rate of dopa oxidation in the presence of 0.15 ml of IWF (28 nmol min^{-1}) was *ca* 10-fold of that of dopa oxidation in the presence of 0.025 ml of IWF. This increase could not be explained by the increases in POX activity (maximum; 6-fold)

Table 1. Levels of [AA + DHA] and dopa in leaves of *V. faba*

	Symplast (μmol g^{-1} fr. wt)		Apoplast (nmol g^{-1} fr. wt)		% in the apoplast	
	Young	Aged	Young	Aged	Young	Aged
[AA + DHA]	7.8 ± 0.9	4.4 ± 0.3	153 ± 32	55 ± 7	1.9 ± 0.3	1.3 ± 0.2
dopa	47 ± 11	23 ± 7	165 ± 59	104 ± 11	0.34 ± 0.12	0.50 ± 0.26
[AA]/[AA + DHA] (%)	94 ± 1	95 ± 1	63 ± 8	61 ± 10		

Values show means ± SDs ($n = 4$).

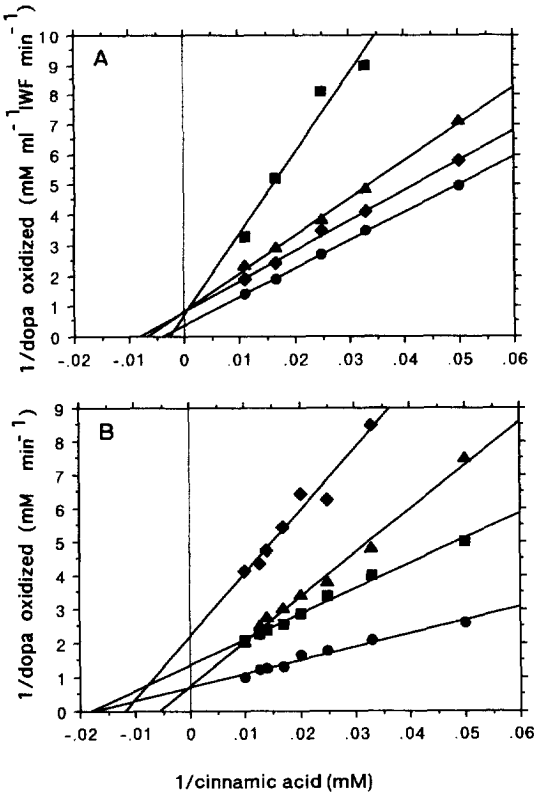

Fig. 1. Double-reciprocal plots of POX-dependent oxidation of dopa. The reaction mixture (1 ml) contained 0.4 mM dopa, 1 mM H_2O_2, 50 μl of dialysed IWF (panel A) or 0.5 μg HRP (panel B) and various amounts of cinnamic acids in 100 mM Na-Pi (pH 6). Reactions were started by adding H_2O_2. ●, chlorogenic acid; ■, 4-coumaric acid; ▲, caffeic acid; ◆, ferulic acid. Oxidation rates of dopa were plotted as a function of concentrations of cinnamic acids.

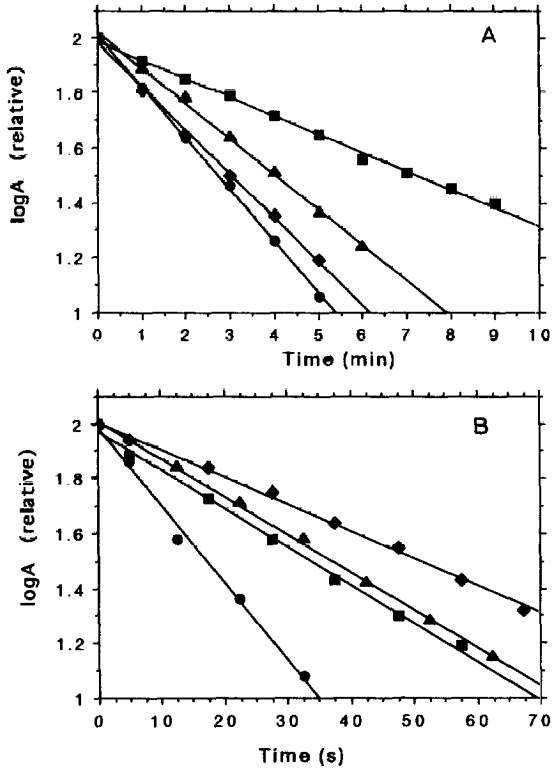

Fig. 2. Semilogarithmic plots of POX-dependent oxidation of cinnamic acids. The reaction mixture (1 ml) contained 1 mM H_2O_2, 30 μM cinnamic acids and 50 μl of dialysed IWF (panel A) or 0.5 μg HRP (panel B) in 100 mM Na-Pi (pH 6). ●, Chlorogenic acid; ■, 4-coumaric acid; ▲, caffeic acid; ◆, ferulic acid.

and concentration of dopa (maximum; 1.05-fold) by the addition of IWF, suggesting the presence of some components in IWF which enhanced the oxidation of dopa.

Figure 3 shows effects of non-dialysed IWF on HRP-dependent oxidation of dopa. Trace 1 is a control experiment; oxidation of dopa that was present in IWF by the POX in IWF. Because of the low POX activity and the low level of dopa in IWF, the rate was very slow. By the addition of 5 μg HRP to trace 1, dopachrome formation was enhanced greatly and attained a maximal level within 10 min (trace 2) indicating formation of 7.3 μM dopachrome. The addition of 1.5 μM dopa to trace 2 caused the small stimulation of dopachrome formation (trace 4) and the dopachrome formed was 8.8 μM. However, in the absence of IWF, HRP (5 μg ml^{-1}) oxidized 7 μM dopa not so rapidly (trace 3).

If there are the components like cinnamic acids in non-dialysed IWF that can stimulate the POX-dependent oxidation of dopa, the components can be released from the dialysis tube during dialysis. The oxidation of 0.4 mM dopa by POX in dialysed IWF (0.5 ml) was, in fact, enhanced *ca* 2-fold by the components of low M_r that moved through the dialysis tube. Heat-stable components that were obtained by heating non-dialysed IWF for 1 min in boiling water, enhanced the oxidation of dopa *ca* 4-fold. Treatment of the small and the heat-stable components by POX plus H_2O_2 caused a decrease in the stimulating activities indicating that the reduced but not oxidized forms are active. There were dose-responses for the stimulating effects. Here again the enhancements could not be explained by the increase in the concentration of dopa when the low M_r components or heated IWF was added to the reaction mixtures because the maximal increase in dopa concentration by the additions was 0.075 mM.

Effects of dopa concentration of dopa oxidation

Degrees of stimulation of HRP-dependent oxidation of dopa by the components in IWF and cinnamic acids depended on the concentration of dopa. A maximal rate of dopa oxidation at an infinite concentration of dopa was calculated to be *ca* 4 μM μg^{-1} HRP min^{-1} and the half-maximal rate was obtained at *ca* 6 mM dopa. Non-dialysed IWF (0.1 ml) did not significantly affect the maximal rate of the dopa oxidation but decreased the concentration of dopa required for the half-maximal rate to *ca* 0.06 mM.

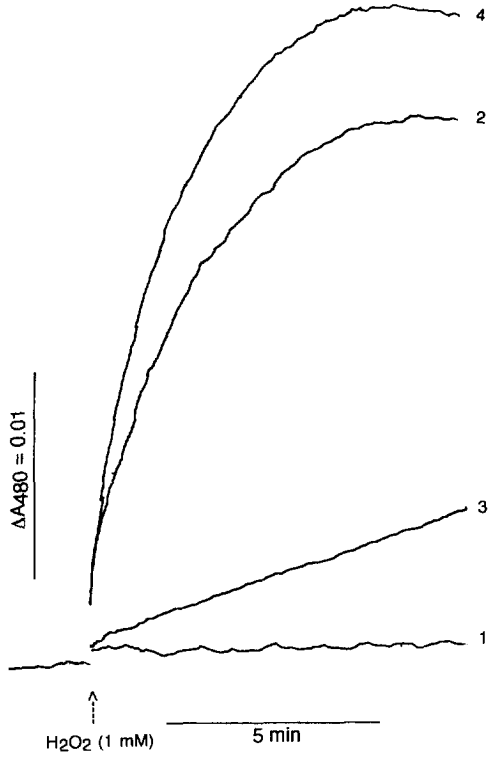

Fig. 3. Stimulation of HRP-dependent oxidation of dopa by non-dialysed IWF. The basic reaction mixture (1 ml) was 0.06 unit of AA oxidase and 1 mM H_2O_2 in 0.1 M Na-Pi (pH 6). Trace 1, 50 μl of IWF; trace 2, 50 μl of IWF plus 5 μg HRP; trace 3, 5 μg HRP plus 7 μM dopa; trace 4, 50 μl of IWF plus 5 μg HRP plus 1.5 μM dopa.

4-Coumaric acid (0.03 mM) increased the rate of dopa oxidation to *ca* 45 μM μg^{-1} HRP min^{-1} and decreased the concentration of dopa required for the half-maximal rate to *ca* 0.1 mM.

The cinnamic acid-dependent stimulation of dopa oxidation (Fig. 1) suggests that cinnamic acid radicals can oxidize dopa, because the initial oxidation products of these cinnamic acids are the phenoxyl radicals when the oxidation was catalysed by POXs. The oxidation of dopa by the radicals results in the generation of dopa radicals which can transform to dopachrome via dopaquinone [23, 24] as shown below;

$$2(\text{cinnamic acid}) + H_2O_2 \rightarrow 2(\text{cinnamic acid radical}) + H_2O \quad (1)$$

$$\text{cinnamic acid radical} + \text{dopa} \rightarrow \text{cinnamic acid} + \text{dopa radical} \quad (2)$$

$$2(\text{dopa radical}) \rightarrow \text{dopa} + \text{dopaquinone} \quad (3)$$

$$\text{dopaquinone} \rightarrow \rightarrow \rightarrow \text{dopachrome} \quad (4)$$

Reaction (1) is catalysed by POX. The occurrence of the radical reaction (2) is supported by the results that degrees of the stimulation of dopa oxidation by cinnamic acids were related to the oxidation rates of these cinnamic acids (compare Figs 2 and 3). The decrease in the concentration of dopa required for the half-maximal rate of dopa oxidation by 4-coumaric acid suggests that dopa can react rapidly with the radicals of 4-coumaric acid producing dopa radicals and that the rapid transformation of the dopa radicals to dopachrome. IWF-dependent decrease in the concentration of dopa required for the half-maximal rate of dopa oxidation can also be explained by rapid reactions between dopa and radicals of apoplastic components which were small heat-stable molecules.

The presence of cinnamic acid derivatives in the apoplast has been reported with spinach leaves [9]. In epicotyls of *V. angularis* and tobacco leaves, an ester of 4-coumaric acid [25] and chlorogenic acid itself or a related compound (unpublished results), respectively, are present in the apoplast. Apoplastic POX from the respective plant tissues rapidly oxidizes the apoplastic components [9, 25].

It has been proposed that radicals of a phenolic compound that are generated by POX-dependent reactions can oxidize other phenolic compounds of which oxidations by POX are slow [15, 16]. For example, in cinnamic acids and cinnamyl alcohols, radicals of 4-hydroxyphenyl compounds can oxidize guaiacyl and syringyl compounds, and radicals of guaiacyl compounds can oxidize syringyl compounds. However, the reverse reactions are very slow even if they occur [16]. An ester of 4-coumaric acid that is an apoplastic component of epicotyls of *V. angularis*, enhanced the oxidation of sinapyl alcohol catalysed by apoplastic POX isoenzymes isolated from epicotyls of that plant [25]. These radical dependent oxidations of sinapyl alcohol are discussed in relation to the biosynthesis of lignins [15, 16, 25].

The radical-dependent oxidation of dopa in *V. faba* observed in this study may be important in the rapid formation of melanin-like compounds in the apoplast, because melanins are formed when this plant is injured by wounding or on infection. Further studies are required to identify the components that can enhance the POX-dependent oxidation of dopa in the apoplast and to determine the substrate specificities of apoplastic POX isoenzymes of *V. faba*. The elucidation of oxidation mechanisms of dopa in vacuoles is also necessary because this compound was also present in vacuoles (Table 1).

EXPERIMENTAL

Plant materials. Vicia faba L. cv. con Amore was grown from seeds in a greenhouse. Leaves were taken from the plants of which heights were *ca* 1 m. Leaves with 5 leaflets and those with 2 leaflets were used as young and aged leaves.

Preparation of intercellular washing fluid (IWF). IWF that contained components in the apoplastic aq. phase, was prepd by vacuum-infiltration (1 min) in 20 mM citric acid-Na citrate (pH 4) and centrifugation (200 *g*, 5 min). About 1 ml of IWF was obtained from

1 g fr. wt of leaves. This IWF was kept on ice and used for experiments as non-dialysed IWF.

In some experiments, IWF (5 ml) was dialysed against 1 l of 10 mM Na-Pi (pH 6) for 20 hr at 4° and used as dialysed IWF. To obtain molecules of low M_r in IWF, IWF (5 ml) was dialysed against 5 ml of 100 mM Na-Pi (pH 6). The fr. outside the dialysis tube was used as a fr. of components of low M_r.

Preparation of cell-free extracts. Leaves (1 g fr. wt) were homogenized in 4 ml of 5% metaphosphoric acid with a pestle and mortar. The homogenates were centrifuged at 2500 *g* for 5 min. The supernatants were used to determine cellular levels of AA, DHA and dopa.

Quantification of dopa. Dopa is oxidized to an orange product, dopachrome, via dopaquinone and cyclodopa [23, 24]. The level of dopa was estimated measuring the amount of dopachrome formed. The reaction mixt. (1 ml) contained 1 mM H_2O_2, 5 μg HRP, 2 μM chlorogenic acid and 100 μl of IWF or 10 μl of cell-free extracts in 100 mM Na-Pi (pH 6). The reactions were started by adding H_2O_2 and the amounts of dopachrome formed were calculated from the maximal *A* increases at 480 nm (cf. Fig. 3). The *E* coefficient used was 3.6 mM^{-1} cm^{-1}. This value was determined by postulating that transformation of dopa to dopachrome was much faster than the further transformation of dopachrome that led to the formation of melanin. Chlorogenic acid was added since that acid greatly enhanced HRP-dependent oxidation of dopa (see Results). The maximal *A* increase of an oxidation product of 2 μM chlorogenic acid was 0.0005 at 480 nm during the reactions. Since the *A* increases due to the formation of dopachrome were adjusted around 0.05 when dopa was quantified, oxidation of chlorogenic acid even if it occurred did not affect the quantification of the level of dopa.

Measurement of AA and DHA. Leaves of AA and DHA were measured in principle by the method described in ref. [9]. The reaction mixt. (1 ml) for the measurement of AA contained 0.06 unit of AA oxidase, 0.05 ml of IWF or 0.01 ml of cell-free extracts and 0.95 ml of 100 mM Na-Pi (pH 6.8). When levels of DHA in IWF were measured, DTT (final concn 2 mM) was added to mixts of 0.05 ml of IWF and 0.95 ml of 100 mM Na-Pi (pH 6.8) to reduce DHA to AA. *E* coefficient (265 m) of AA used was 15 mM^{-1} cm^{-1}.

POX-dependent oxidation. Dopa oxidation was measured in a reaction mixt. (1 ml) that contained 0.4 mM dopa, 1 mM H_2O_2 and POX in 100 mM Na-Pi (pH 6). Details of the reaction mixts are given in the legends to figs. POX-dependent oxidation of a cinnamic acid was measured in the reaction mixt. (1 ml) that contained 30 μM cinnamic acid, 1 mM H_2O_2 and 0.05 ml of dialysed IWF or 0.5 μg HRP in 100 mM Na-Pi (pH 6). The oxidation was measured by *A* decreases at 284, 310, 285 and 320 nm for 4-coumaric, ferulic, caffeic and chlorogenic acids, respectively.

Glucose-6-phosphate dehydrogenase. This enzyme was assayed in a reaction mixt. (1 ml) that contained 10 mM glucose-6-phosphate, 1 mM NADP, 5 mM $MgCl_2$ and 0.05 ml of cell-free extracts or IWF in 100 mM Tricine-NaOH (pH 7.6). Cell-free extracts were prepd by grinding leaves (0.3 g fr. wt) in a soln (2 ml) that contained 20 mg Na ascorbate and 0.1 g polyvinylpyrnolidone in 100 mM Na-Pi (pH 7.4). To examine the contamination of this enzyme in IWF, IWF was prepd with Na citrate (20 mM) because there is a possibility that if 20 mM Na citrate–citric acid (pH 4.0) was used for the infiltration soln, the enzyme might be inactivated.

Reagents. Caffeic, chlorogenic, 4-coumaric and ferulic acids and AA oxidase were from Sigma. HRP (170 unit mg^{-1} protein) and dopa were from Merck and Aldrich, respectively.

REFERENCES

1. Freudenberg, K., *Science*, 1965, **148**, 595.
2. Lewis, N. G. and Yamamoto, E., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1990, **41**, 455.
3. Iiyama, K., Lam, T. B.-T. and Stone, B. A., *Plant Physiology*, 1994, **104**, 315.
4. Albrecht, C. and Kohlenbach, H. W., *Protoplasma*, 1990, **154**, 144.
5. Takahama, U. and Oniki, T., *Plant Cell Physiology*, 1991, **32**, 745.
6. Takahama, U., *Phytochemistry*, 1992, **31**, 1127.
7. Jan, C.-Y., Takahama, U. and Kimura, M., *Biochimica et Biophysica Acta*, 1992, **1086**, 7.
8. Takahama, U., *Biochimca et Biophysica Acta*, 1986, **882**, 445.
9. Takahama, U. and Oniki, T., *Plant Cell Physiology*, 1992, **33**, 379.
10. Takahama, U., *Physiologia Plantarum*, 1993, **89**, 791.
11. Córdoba-Pedregosa, M. C., Gonzalez-Reyes, J. A., Canadillaas, M. S., Navas, P. and Córdoba, F., *Plant Physiology*, 1996, **112**, 1119.
12. Castillo, F. J. and Greppin, H., *Environmental and Experimental Botany*, 1988, **28**, 231.
13. Polle, A., Chakrabarti, K., Schücrmann, W. and Rennenberg, H., *Plant Physiology*, 1990, **94**, 312.
14. Luwe, M. W. F., Takahama, U. and Heber, U., *Plant Physiology*, 1993, **101**, 969.
15. Takahama, U., Oniki, T. and Shimokawa, H., *Plant Cell Physiology*, 1996, **37**, 499.
16. Takahama, U., *Physiologia Plantarum*, 1995, **93**, 61.
17. Takahama, U. and Egashira, T., *Phytochemistry*, 1991, **30**, 73.
18. Takahama, U. and Egashira, T., *Plant Cell Physiology*, 1990, **31**, 539.
19. Mehdy, M. C., Sharma, Y. K., Sathasivan, K. and Bays, N. W., *Physiologia Plantarum*, 1996, **98**, 365.
20. Luwe, M. and Heber, U., *Planta*, 1995, **197**, 448.

21. Moldau, H., Bichele, I., Kollist, H. and Padu, E., *Biologia Plantarum*, 1996, **38**, 229.
22. Pedreño, M. A., Ferrer, M. A., Gasper, T., Munoz, R. and Ros Barcelo, A., *Plant Peroxidase Newsletter*, 1995, **5**, 3.
23. Gracia-Carmona, F., Gracia-Canovas, F., Iborra, J. L. and Lozano, J. A., *Biochimica et Biophysica Acta*, 1982, **717**, 124.
24. Young, T. E., Griswold, J. R. and Hulbert, M. H., *Journal of Organic Chemistry*, 1974, **39**, 1980.
25. Takahama, U. and Oniki, T., *Plant Cell Physiology*, 1997, **38**, 456.