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Ontogeny and Hormonal Control of Polyphenoloxidase Isozymes in Tobacco Pith[1]

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ABSTRACT

Isozymes of tobacco pith polyphenoloxidases (*o*-diphenol oxidase, EC 1.10.3.1) were separated electrophoretically from fresh pith of intact plants and from cultured pith sections. Extracts of fresh pith contained a poorly resolved complex of two to three anodic bands after starch gel electrophoresis at alkaline pH. This anodic complex was more active with chlorogenic acid than with 3,4-dihydroxyphenylalanine and was found in greater activity per gram fresh weight of tissue in younger internodes than in older ones. The longitudinal gradient of activity was thus the opposite of that found for the constitutive isozymes of peroxidase.

A well defined cathodic band of polyphenoloxidase activity appeared after culture of pith in modified White's medium with shaking. This band, which was more active with 3,4-dihydroxyphenylalanine than with chlorogenic acid, could be detected after 1 to 2 days of incubation. Its appearance was enhanced by the addition of 10 μM indoleacetic acid; kinetin (1 μM tended to prevent this indoleacetic acid effect). Such hormonal control is opposite to that previously reported for the rapidly appearing new isozymes of peroxidase.

The pattern of the major isozymes associated with polyphenoloxidase activities differs from that of peroxidase.

Activities of both peroxidase and ascorbic acid oxidase have been reported to increase during culture of tobacco pith tissues (4, 10, 11). Both activities may involve isozymes and both are controlled, although in opposite ways, by IAA (1, 4, 10, 11). Recently, the pattern of isozymes of PPO[3] in tobacco tissues has been reported to be identical with or similar to that of peroxidase (14, 15). In the present work, the possibility of a complex of enzymes catalyzing the oxidation of phenolic compounds and the hormonal control of the newly appearing isozymes has been explored in cultures of tobacco pith. Since two separate PPO enzymes, one acting on CA and the other on classical substrates such as DOPA and catechol have been postulated in tobacco (3), both DOPA and CA have been used as substrates.

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[3] Abbreviations: PPO: polyphenoloxidase; CA: chlorogenic acid; DOPA: 3,4-dihydroxyphenylalanine; DO: DOPA oxidase; CAO: chlorogenic acid oxidase; PER: peroxidase.

MATERIALS AND METHODS

Plants and Culture of Pith Tissues. Greenhouse-grown plants of tobacco (*Nicotiana tabacum*, cv. Wisconsin-38) used in previous studies (4, 10) were the source of pith for analysis and aseptic culture. Both vegetative (about 100 cm high) and flowering plants were used. Internodes were excised at nodal regions, and the pith was removed aseptically with a No. 2 cork borer to give cylinders 0.5 cm in diameter. The pith was extracted directly for the constitutive enzymes or was cut into approximately 5-mm sections for culture either (*a*) on moist filter paper in air or (*b*) in flasks on a shaker, in liquid solutions of 2I-MW medium (4) with and without added kinetin and IAA. Sections were incubated at about 25 C under constant illumination (about 100 ft-c) for 2 to 7 days. After rinsing with distilled H_2O, the tissues were extracted directly or after freezing, without apparent differences in activities.

Enzyme Extracts. Tissues were ground with sand in a mortar in 50 mM phosphate buffer at pH 6; the ratio of fresh weight to buffer volume was 1:1. After straining through cheesecloth and centrifugation for 15 min at 30,000*g* the final supernatant was dialyzed against distilled water with continuous stirring overnight at about 5 C. Enzyme activities were generally assayed on unfrozen extracts. Gel electrophoresis was done either before or after freezing of extracts, without detectable differences. Other preparative methods such as grinding with Polyclar AT, ascorbic acid or cysteine, desalting with Sephadex G-25, or dialysis against phosphate buffer at pH 6 instead of distilled water caused no significant change in the enzymatic activities.

ASSAY METHODS

Spectrophotometric Assay of Total Activities in Crude Extracts.
Chlorogenic Acid Oxidase. Activity was measured by following the disappearance of CA at 326 nm in a 1.1-ml volume containing 100 mM phosphate buffer at pH 6, 100 μM CA, and 1 mM EDTA. The blank contained all components but CA. The reaction was started with the addition of enzyme. One-centimeter cuvettes in a Gilford spectrophotometer were used in this and the following assay.

DOPA Oxidase. This activity was measured by following the appearance of a yellow-brown product at 475 nm in a 1.1-ml volume containing 14 mM DL-DOPA in 100 mM phosphate buffer at pH 6. The blank lacked enzyme.

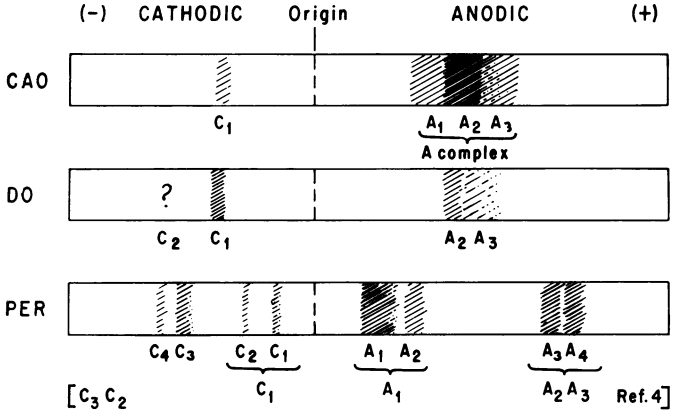
Peroxidase. This reaction was followed by measuring the formation of product absorbing at 470 nm in a 1.1-cm diameter test tube in a Bausch and Lomb Spectronic 20 colorimeter in a mixture of 15 mM guaiacol and H_2O_2 in 100 mM phosphate buffer at pH 5.8.

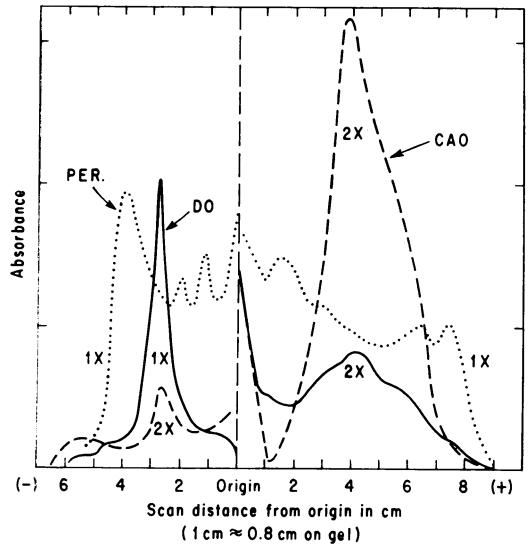
The above enzyme activities were calculated as the initial linear change in absorbancy per g fresh weight, per min in the case of PPO activities and per 15 sec for peroxidase. Linearity in the case

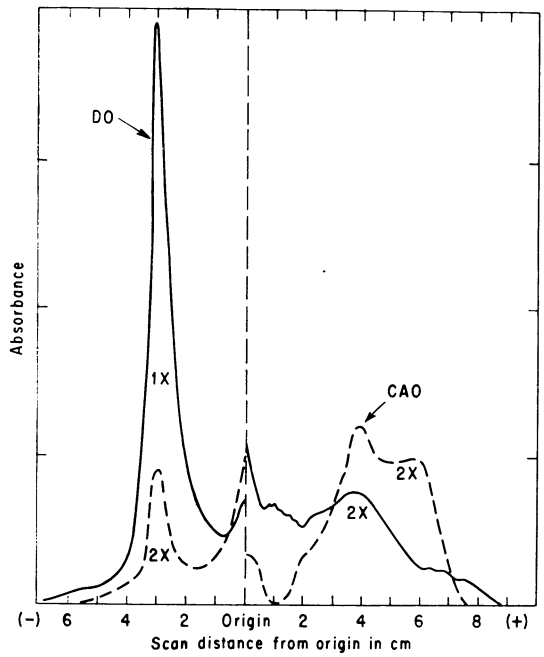
of the PPO substrates was generally restricted to the first 2 to 3 minutes of the reaction.

Isozyme Identification and Assay by Vertical Starch Gel Electrophoresis. Samples (40 μl) of the dialyzed extracts, used directly or after concentration under vacuum at 25 C, were placed in slots in a 26- × 16-cm gel of 10% starch made up in 24 mM borate buffer at pH 9. The gels were connected with electrode buffers of 300 mM borate at pH 8.3 and were developed for about 4 hr at 5 C at about 11 v/cm (300 v and about 40 amp).

Enzymatically active bands were identified by flooding the gels with one of the following solutions: 15 mM guaiacol and H_2O_2 in 100 mM phosphate buffer for peroxidase; 10 mM CA or 20 mM DL-DOPA in 100 mM phosphate buffer at pH 6 for the PPO activities. The more active peroxidase bands were developed only 2 to 5 min before fixing in a solution of 2 parts each of glycerin and glacial acetic acid and 5 parts each of methanol and H_2O. The PPO bands were developed for 1 to 3 hr before densitometer analysis without fixing. The products of the PPO activities were measured by scanning in a Beckman-Gilford 2000 spectrophotometer equipped with a linear transport attachment, at 470 nm for the brown and brown to black products of peroxidase and DO, respectively, and at 420 nm for the yellow products of CAO. In the latter case, the blank values were too high at the more appropriate lower wave lengths. Gel slabs cut from the original blocks were mounted directly on the sample carrier propped against a plastic window and were scanned with an 0.2 × 2.36 aperture slit plate. A white area of the gel was used as a blank, the background being neutralized by the Absorbance Control system. Estimates of the activities per band were made by approximating the area under each peak by calculating the area of a triangle coinciding with the peaks, but overlapping areas and diffuse colors between major bands were eliminated. Similar estimates could be made by weighing recorder paper cut from under the peaks. Such areas were used to compute the percentage of the total activity per gel attributed to each isozyme for one substrate, and the ratio of activities of each isozyme with the two PPO substrates.







RESULTS

PPO Isozymes in Intact and Cultured Pith Tissues. Two major PPO isozymes in extracts of pith tissues have been identified and compared with those for peroxidase (Figs. 1 and 2). The well defined cathodic band migrated to an area between peroxidase bands C_2 and C_3 and was more active with DOPA than with CA as substrate in the enzymatic staining techniques used for gels. The anodic band was diffuse and may represent a complex of at least three poorly resolved isozymes. The area was more active with CA than with DOPA in the gel assay method. The central and probably major band migrated slightly faster than the poorly resolved A_2 band for peroxidase and was clearly distinct from either the major A_1 and A_3 peroxidase isozymes. The PPO-active A_1 and A_3 were sometimes seen as shoulders or separate peaks in densitometer tracings of the gels (Fig. 3). These were not clearly enough resolved to be sure that they do not coincide with A_2 and A_3 of peroxidase. No major differences in relative activities with CA or DOPA have been observed within this complex. Ammonium sulfate precipitation and resuspension of the crude extracts did not improve the resolution; other purification or

FIG. 1 (top). Diagram of the relative positions of isozymes of tobacco pith tissues. Note that PER isozymes of Reference 4 have been renumbered.

FIG. 2 (middle). Densitometer tracings of gels stained for enzyme activities with DOPA (——) and CA (-----) as PPO substrates and guaiacol-H_2O_2 (· · · ·) for PER. See data for gels 3 and 4 in Table III. The same amount of extract was used for peroxidase as for both PPO assays, but a much shorter incubation period was involved; better resolution for peroxidase bands would be obtained with 1/10 to 1/100 dilution. Absorbancy readings were made on two scales, designated 1x and 2x. The scan distance in cm is equal to 0.8 cm on the gel.

FIG. 3 (bottom). Densitometer tracings of gels stained for enzyme activities in Figure 2. See gels 5 and 6 in Table III for data.

electrophoretic methods will be necessary to resolve this complex. Possible weak bands were occasionally seen on each side of C_1 at approximately 4.2 and 1.7 cm from the origin, plus ill defined areas near both origins. Catechol was occasionally used as a substrate for PPO activity on gels; its pattern appeared to reflect that of DOPA, but the colored product was very unstable.

Isozyme Pattern and Total Activities of the Intact Pith (Constitutive Forms). Only the anodic complex of PPO isozymes was detected after starch gel electrophoresis of extracts of pith from intact plants. The total PPO activities varied from plant to plant, but the gradient of activity was always the same. Illustrative data are shown in Table I. The gradient of total activity per g fresh weight with both substrates was from a high value in the youngest tissues (upper internodes) to a low activity in the oldest tissue (lower ones), a gradient that is just the opposite of that for total peroxidase activity (Table I and Ref. 10). Since the number of cells per unit area of pith was also greater in the upper internodes than in the lower (10), the PPO activity per cell might actually be approximately constant along the longitudinal axis. Mixing experiments, where pith tissues from upper and lower internodes were ground and extracted together, indicated that this gradient was not due to the presence of an inhibitor in the lower pith tissues.

Even though the two assay methods for PPO activity are not directly comparable, the ratio of the total activities due to CA and to DOPA in the crude extract is a useful parameter that is relatively independent of variations of activities from plant to plant. This ratio is approximately 15:1 in the upper two-thirds of the plant, and 8:1 in the bottom third. This lower ratio could be due to the presence of small amounts of the DOPA-rich cathodic isozyme or to variations within the anodic complex in the relative specificities with the two substrates.

Isozyme Pattern after Culture in Nutrient Solution with and without Added Hormones. A well defined cathodic band, characterized by a high DOPA activity, was detectable in pith tissue incubated for 4 to 6 days in 2I-MW nutrient medium. This appearance was reflected in the spectrophotometric assay of the crude extracts by an increase in total DOPA oxidase activity. In some experiments, occasional high values of the new DOPA activity were obtained from pith isolated from the upper internodes incubated in nutrient media without the addition of IAA; this effect was always associated with a relatively high value for the constitutive anodic forms. Consistently high values with middle or lower internodes were obtained only in the presence of 10 μM IAA, as shown in Table II. The presence of 1 μM kinetin tended to counteract this increase due to IAA, but kinetin had no detectable effect without added IAA. The total peroxidase activity (the increase due mainly to the new cathodic isozymes) tended to be greater in the presence of kinetin and was inhibited by IAA, as previously reported (4).

The ratio of CAO to DO activities in crude extracts is a better indicator of the changes that have occurred because of the variations in the amounts of the constitutive types from plant to plant and the gradient along the axis. But such ratios can be misleading if the anodic complex is in great excess of the cathodic form. A more useful calculation involves correcting the total DO activity for that part due to the constitutive or anodic isozymes. This can be approximated either by subtracting a zero time control from a pooled sample of sections or by making use of the ratio of CAO to DO activities. Since the ratio of the constitutive activities is close to 10 to 1, the value for the total DO activity can be corrected by subtracting $\frac{1}{10}$ of the total CAO activity. The remainder is an estimate of the "new" DO activity attributable to the new cathodic enzyme. Both methods give similar results, and such calculated values are given in Table II. Gel electrophoresis always indicated the presence of the new major cathodic band.

An approximation of the relative contribution of the major isozymes to the total activity for each substrate can be made by estimating the areas under the peaks after scanning the developed gel preparations with a densitometer. Typical data obtained from such scanning records are shown in Figures 2 and 3 and Table III. The values, expressed as percentages of the total absorbancy under the major peaks, tend to be too high for the weak bands relative to the stronger ones, because the latter are probably being measured after they have passed their linear phase of enzyme activity. Minor peaks such as the possible C_2 band account for less than 2% of the total activity per gel. The ratios determined for each isozyme by the gel assay method are about 0.2 for the cathodic and 3 for the anodic ones, a clear indication of the reciprocal specificities of the cathodic and anodic forms. These ratios based on the gel assay will differ from those based on the total activity in a crude extract, because the CAO activity was followed by the appearance of the product in the former case and the disappearance of the substrate in the latter, more sensitive method.

If a tissue can be induced to form a new isozyme that is not normally present, the question arises as to whether that isozyme is a constitutive component of another part of the plant. Extracts

Table I. *Chlorogenic Acid Oxidase, DOPA Oxidase, and Peroxidase Activities of Pith Extracts from Intact Plants*

Two nonflowering plants about 100 cm high were cut into three parts, and internodes of each part were combined. See text for details of procedures.

Position of Internodes in Plant	CAO	DO	PER	CAO/DO
	total activity/g fresh wt			
Upper ⅓	6.2	0.50	0.13	13
Middle ⅓	2.2	0.15	1.1	15
	2.5	0.17	1.3	15
	3.2	0.20	1.6	16
Lower ⅓	0.61	0.09	6.5	7
	0.93	0.10	7.5	9

Table II. *Effect of Added IAA and Kinetin on PPO and Peroxidase Activity*

Plants were about 100 cm high and were just starting to flower. Middle internodes were cut into sections and 17 to 20 sections (about 2 g fresh weight) were removed at random and incubated with shaking in 10 ml of a modified White's nutrient solution at 25 C in continuous light, with (+) and without (−) added hormone. Nutrients were changed at 2 and 4 days. The data are the average of two to three samples each.

Addition		CAO	DO	PER	CAO/DO	New DO	PER/New DO
IAA, 2 mg/liter	Kinetin, 0.2 mg/liter						
		total activity/g fresh wt					
Freshly excised pith		0.51	0.04	0.2	12	...	...
4-day incubation							
−	−	0.46	0.34	50	1.2	0.30	158
−	+	0.37	0.30	73	1.3	0.26	250
+	−	0.49	1.75	39	0.3	1.70	23
+	+	0.20	0.58	42	0.6	0.54	75
7-day incubation							
−	−	1.04	1.43	129	1.8	1.4	100
−	+	0.59	1.6	131	0.4	1.5	94
+	−	0.44	6.8	72	0.08	6.7	11
+	+	0.36	3.9	105	0.1	3.8	76

Table III. *Approximation of Relative Activities of Various Isozymes in Selected Gels Obtained by Measuring the Area under Each Major Peak of Absorbancy*

Figures represent percentages of total activity per gel which is due to the activity of each isozyme, and the ratio of total activity per isozyme due to CA with that due to DOPA. Gels scanned after 1 to 2 hr of incubation. See Figures 2 and 3 for actual scanning records for gels 3 through 6.

	Cathodic C_1		Anodic Complex		CAO	DOPA
		CAO/DO		CAO/DO[1]		
	% total act/sub		*%*		*total activity/g fresh wt in crude extract*	
Freshly excised pith						
Gel 1, CA	0		100		6.2	...
Gel 2, DOPA	0	...	100	3	...	0.5
After 4 days incubation						
Gel 3, CA[2]	5		93		3.0	...
Gel 4, DOPA	73	0.2	27	4	...	2.2
After 6 days incubation						
Gel 5, CA	19		81		3.6	...
Gel 6, DOPA	82	0.2	18	2	...	5.3
After 6 days incubation						
Gel 7, CA	26		76		2.2	...
Gel 8, DOPA	88	0.2	12	3	...	8.4

[1] Note that ratios based on total activity of crude extracts will be expected to differ from those based on the gel assay (see text).

[2] Plus 2% in position of C_2.

Table IV. *A Time Study of the Early Development of PPO and Peroxidase Activities in Tobacco Pith Cultures*

Internodes were from the middle of plants about 120 cm high with nonelongated flower buds. Twenty sections per flask containing 10 ml of nutrient solution with kinetin or IAA were incubated for 1 to 7 days with shaking. Values are averages from two to three samples of pooled sections.

Treatment	CAO	DO	PER	New DO
	total activity/g fresh wt			
Freshly excised pith	2.2	0.2	2.3	...
+Kinetin (0.2 mg/liter)				
1 day	1.1	0.1	8	0
2 days	1.2	0.1	15	0
3 days	1.2	0.2	26	0.13
4 days	1.2	0.2	38	0.11
+IAA (2 mg/liter)				
1 day	1.5	0.1	2	0.05
2 days	0.8	0.3	6	0.19
3 days	1.5	1.0	13	0.86
4 days	1.2	2.6	17	2.5

of secondary roots did contain a well defined DOPA-rich cathodic band in a position comparable to that found in incubated pith sections, along with several anodic bands. In preparations of young leaves, however, only the anodic forms were found.

Early Development of PPO and Peroxidase Activities. The increase in DO and the total peroxidase activities (due mainly to the new peroxidase isozymes) were compared at different time intervals in the presence of kinetin, favoring peroxidase formation, or IAA, favoring PPO formation (Table IV). An increase in peroxidase activity appeared within the first 24 hr in the presence of kinetin, while the increase in DO activity in the presence of IAA was not significant until about 48 hr. The increase in activities of both enzymes was delayed about 24 hr when suboptimal hormonal conditions were used.

A related phenomenon, observed during culture of excised pith sections, was an increase in the browning reaction of the extracts. While extracts of pith from intact plants remained quite pale, a yellow-brown color appeared in extracts of incubated tissues, part of which remained with the protein after dialysis. An estimate of this browning of protein was made by a direct spectrophotometric assay of dialyzed extracts at 475 nm. The increase in absorbancy at 475 nm was linear to about 4 days of incubation, followed by a leveling off. By 6 days, the tissues themselves appeared slightly brown, especially if they were frozen before extraction. But freezing had no significant effect on the enzymatic activities studied. Only traces of lignin, detectable with phloroglucinol-HCl, were observed in sections of 6-day-incubated sections. The browning of the protein extracts, possibly an iron-protein-chlorogenic acid or a scopoletin complex as identified in leaves (20), was lower in the presence of added IAA. It appeared to be more closely related in time with the production of new peroxidase activity than with PPO, although the latter is considered the agent of oxidation. The addition of ascorbic acid during preparation of the extracts also reduced the browning, but browning occurred readily when the ascorbic acid was removed, as if the reduced phenolics were already associated with the protein.

Effect of Cutting of Tissues on PPO Activity. When sections of pith were incubated without any nutrient additions on moist filter paper in Petri dishes, only small amounts of the new DO activity were formed (highest value assayed was 0.4 per g fresh weight). But this new DO activity appeared only when the internodes were incubated as 0.5-cm sections rather than as the whole internodes (about 4 cm in length). Such an effect of the degree of cutting was not observed when the pith was cultured in nutrient solutions with shaking.

DISCUSSION

There is no evidence in the present work of any basic similarity between peroxidase and PPO zymograms, and the amount of extract necessary for detection of PPO activity on gels or in the assay for total activity was far greater than that required for peroxidase. This contrasts with the data of Sheen (14, 15), who reported that the zymograms were identical with either CA or DOPA as substrate and that the PPO patterns in a variety of tobacco tissues, including pith, were similar to those of peroxidase. His pith tissues also differed in containing as constitutive enzymes major activities of cathodic as well as anodic forms. Sheen's variety of tobacco, electrophoretic buffer and medium (acrylamide gels), and isozyme detection methods (substrates in alcoholic solutions) differed from those used in the present study, which might account for some of the differences.

PPO exists in some plants as polymeric forms separable into monomers (7, 9). The subunits may differ in their monophenol *versus* diphenol activities. No monophenol activity has been observed in tobacco preparations (3), but the possibility of polymeric forms needs investigation. The poor resolution of the anodic complex might be an indication of slightly unstable polymeric forms, and the cause of the apparent loss in activity of the anodic forms is unknown (Table IV). The possibility of artifacts associated with the presence of phenolic complexes also needs to be explored.

The separation of PPO activities with different substrate specificities has been demonstrated by others in tobacco tissues with classical techniques. Both Clayton (3) and Sisler and Evans (17) reported that purification of leaf extracts led to fractions with altered ratios of CA to catechol activities. Substrate differ-

ences among isozymes have also been observed in potato tissues (12).

The physiological function of a PPO is not clear, and the above difference in substrate specificities of the isozymes adds to the complexity. There appears to be no simple correlation in tobacco between phenolic content and the PPO activity (2, 15). Chlorogenic acid is found in pith tissue (14, 19) and could be considered a physiological substrate, but neither DOPA nor catechol has been identified in tobacco tissues, and tyrosine is apparently not hydroxylated to DOPA (3). A clue to the physiological function may lie in the relationship to peroxidase and the difference in hormonal control in tissue culture.

Peroxidase and PPO activities are similar in that the constitutive isozymes of intact pith do not increase upon excision. Instead, for reasons that are unclear, new and more positively charged isozymes are activated or synthesized. Although the degree of cutting of the pith cylinders may be important in the development of the new DO activity when the pith is incubated in moist air, no such effect was observed after incubation in solution culture with shaking. Under the latter conditions, excision alone may be sufficient. Cutting effects on PPO activity have been observed in tissues such as sweet potato (5, 8), and PPO activities have been found in latent or bound forms (7). So-called wounding effects due to the release of phenolic compounds from damaged vacuoles or as a result of fungal infection have also been reported to increase PPO activities (13). The new DOPA-rich activity described here, however, cannot be attributed to microbial contamination, since analysis of tissues from contaminated cultures always showed a much reduced increase in the new activity.

PPO activities differ from those of peroxidase in the direction of the longitudinal gradient of activity of the constituent forms and the timing and mode of hormone control of the newly developed isozymes. The IAA-dependent increase in PPO activities appears to be similar to that previously reported for a wall-localized ascorbic acid oxidase (11). All of these enzymatic changes, along with an IAA-related increase in nucleic acids (16) and changes in phenolic compounds (19), precede the major growth phase that can occur after about 4 days of culture in the presence of IAA (4).

Intermediate ratios of IAA to kinetin (2 mg/liter IAA to 0.2 mg/liter kinetin) are optimal for maintaining tobacco callus cultures in an undifferentiated but growing state, and a shift in these ratios leads to organogenesis (6, 18). In tissue culture, these intermediate ratios are suboptimal for the development of the new activities of either peroxidase or PPO. Both constitutive and new isozymes of PPO appear to be more characteristic of younger pith tissues, or where there is a relatively high IAA to kinetin ratio. Peroxidase isozymes, on the other hand, appear more characteristic of older and differentiated pith tissues, or where there is a lower ratio of IAA to kinetin. A balance between these hormonally regulated enzymes and their phenolic substrates could be a critical aspect of early stages of growth and development in tobacco tissue and callus cultures.

PPO could be regulatory by virtue of its oxidase activity in controlling the level of *o*-diphenols, or in conjunction with a quinone reductase, the ratio of NADP to NADPH. Since the latter would alter the rates of both pentose and shikimic acid pathways leading to IAA and phenolic acids, an interaction would be expected with IAA oxidase (peroxidase), an activity in which monophenols are cofactors and diphenols are inhibitors. The various isozymes of PPO and peroxidase might vary in their ability to catalyze these reactions and be confined to different cell compartments. But the direct, causal relationships between these hormones, enzymes, and phenolic compounds in growth and differentiation remain an intriguing and unsolved puzzle.

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