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Tyrosine and Phenylalanine Ammonia Lyase Activities during Shoot Initiation in Tobacco Callus Cultures[1]

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ABSTRACT

Both phenylalanine ammonia lyase and tyrosine ammonia lyase were detected in tobacco (*Nicotiana tabacum* L. Wisconsin 38) callus. The enzymes were separated from each other by Sephadex G-200 column chromatography. Increased activity of tyrosine ammonia lyase was observed during culture of tobacco callus under shoot-forming conditions, while activity of phenylalanine ammonia lyase increased during culture under non-organ-forming conditions. Confirmation of these findings was obtained by examining the incorporation of [^{14}C]tyrosine and [^{14}C]phenylalanine into *p*-coumarate and *trans*-cinnamate, respectively.

Aromatic amino acids and simple phenylpropanoids have been known to be involved in organogenesis for some time. L-Tyrosine was shown to enhance the formation of both shoots and roots in tobacco callus and to allow organogenesis to occur at more marginal levels of auxin or cytokinin (15). Several substituted phenols were found to be capable of partially replacing L-tyrosine in stimulating shoot formation in tobacco callus (11). The effectiveness of these compounds could be correlated with their capacity to increase IAA oxidation (12). It was proposed that L-tyrosine participated in this process indirectly by its conversion into simple phenylpropanoids. Furthermore, SF[2] tobacco callus grown in the dark required the addition of L-tyrosine as well as adenine sulfate and increased levels of Pi for optimum organogenesis (8, 21).

In SF tobacco callus, previous studies have shown that there was greater incorporation of label from [^{14}C]shikimate into tyrosine than into phenylalanine (1, 2). Also higher rates of net turnover were found in tyrosine in SF tissue than in growing callus (2). Although tyrosine can be used directly in a variety of ways, one possibility was its direct deamination. PAL (EC 4.3.1.5) catalyzes the elimination of NH_3 from L-phenylalanine to give *trans*-cinnamic acid, and TAL (EC 4.3.1) catalyzes the conversion of L-tyrosine into *p*-coumaric acid. While PAL has been found in all plants examined, TAL has been found mainly in grasses, and in some fungi (5, 7). It was not known whether TAL was present in tobacco. Furthermore, if TAL activity was detected, it would be necessary to determine if it was a separate enzyme and not merely part of the PAL complex (5, 7), which has been shown to have relatively broad specificity toward aromatic amino acids in plants (7).

In this paper, studies were carried out on the presence and separation of the arylalanine ammonia lyases, as well as their activities and direct deaminating capacities in tobacco callus. The callus was grown under SF, NSF, and SFG conditions. GA_3 has been shown to repress shoot formation in tobacco callus (13).

MATERIALS AND METHODS

Culture Conditions. Tobacco (*Nicotiana tabacum* L. Wisconsin 38) callus was isolated from stem pith segments and maintained on a modified Murashige and Skoog medium (14), following the method reported earlier (21). The SF medium contained Murashige and Skoog salts, thiamine-HCl (0.40 mg/L), myoinositol (100 mg/L), IAA (10 μM), kinetin (10 μM), adenine sulfate (90 mg/L), L-tyrosine (50 mg/L), and $NaH_2PO_4 \cdot H_2O$ (170 mg/L). The SFG medium was the same as the SF medium, except that it contained GA_3 at a level of 50 μM. The NSF medium was the same as that used to isolate the pith callus and contained 10 μM IAA and 2 μM kinetin. Media were solidified with Difco Bacto agar (0.9% w/v). Supplements were added to the medium before autoclaving. For study, three to five pieces of 4- to 6-week-old callus, each measuring about 2 × 4 × 4 mm, were placed in each 125-ml Erlenmeyer flask containing 50 ml of medium and maintained in the dark at 27 ± 1°C.

Extraction of Enzymes. All steps in the preparation of the enzyme extract were carried out at 0 to 4°C. Tissue of the same culture age from the same treatment was pooled (1.0 g) and was ground in a mortar with 3 ml 0.05 M Tris-HCl buffer (pH 8.4) containing 15 mM 2-mercaptoethanol. The homogenate was centrifuged for 20 min at 40,000*g*. Subsequently, the supernatant was passed through a Biogel P2 column equilibrated with 0.05 M Tris buffer (pH 8.4). In the 18-d periodic studies, both PAL and TAL assays for a particular culture day were performed separately on three different samples taken from the same enzyme extract. Each experiment was carried out three times. For the purification and separation of PAL and TAL the method described earlier was followed (9). Nine-d-old tissue was ground and the resulting homogenate was centrifuged at 30,000*g* for 15 min. The extract was then purified by solid $(NH_4)_2SO_4$ fractionation. The 35 to 65% fraction of a two-step process was retained. Its precipitate was dissolved in buffer and subjected to a chromatography on Biogel CM-100, Sephadex G-25, and Sephadex G-200 sequentially in order to remove phenolic compounds and to separate the high mol wt peaks. The protein content of the extracts was determined by the Coomassie blue method (4).

Enzyme Assays. In the periodic studies, PAL and TAL were assayed spectrophotometrically on a Hitachi Perkin-Elmer Coleman 124 double beam recording spectrophotometer by measuring the amount of *trans*-cinnamic acid formed at 290 nm for PAL and the amount of *p*-coumarate formed at 333 nm for TAL. The reaction mixture consisted of either 6 μmol of L-

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[2] Abbreviations: SF, shoot forming; PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; NSF, non-shoot-forming; SFG, shoot forming plus GA_3.

phenylalanine (for PAL) or 5.5 μmol of L-tyrosine (for TAL), 500 μmol of Tris-HCl buffer (pH 8.01), and 100 μl of enzyme extract in a final volume of 1 ml (Figs. 2 and 3). After 70 min at 37°C, the reaction was stopped by the addition of 0.05 ml 5 N HCl.

Sephadex G-200 chromatography was used to separate PAL and TAL, which were assayed by a radioactive chromatographic method (9). Simultaneous assays were done by adding both [U-^{14}C]phenylalanine and [U-^{14}C]tyrosine to each assay mixture (Fig. 1). The products, *trans*-cinnamic acid and *p*-coumaric acid, were separated by TLC.

Radioactive Feeding Studies. Callus pieces were incubated in 3.0 ml of the appropriate half-strength medium with 0.37 mBq of [U-^{14}C]tyrosine (19.37 GBq/mmol) or 0.37 mBq [U-^{14}C] phenylalanine (18.87 GBq/mmol, New England Nuclear) in a 50-ml Erlenmeyer flask. The flasks were shaken in the dark for 1 h. The tissues were then removed, rinsed three times with half-strength medium, blotted dry, and placed in 2 ml methanol/chloroform/water (12:5:3) at −78°C (ethanol/dry ice bath). The tissues were extracted following the method of Bieleski and Turner (3). The dried extract was taken up in 2 ml H_2O and applied to a Dowex 1 × 8 column (acetate form). *p*-Coumaric acid and cinnamic acid were eluted from the column with 0.5 N acetic acid in 2 ml fractions. They were then separated by TLC. The compounds were spotted on a silica gel plate (Silica Gel G with 0.25 mm thickness from Analtech) which was developed in benzene/acetic acid (4:1, saturated with water). UV detected spots were scraped off and suspended in water and sampled for radioactivity.

^{14}C determination was carried out on a Mark III Scintillation System model 6881. The scintillation fluid consisted of 1 L Triton X-100, 2 L toluene, and 4 g/L Omnifluor. The results are expressed in kBq per mg proteins.

RESULTS

Both PAL and TAL activities were detectable in tobacco callus grown under the different culture conditions. These enzymes could also be detected in pith-phloem tissue and leaves of greenhouse-grown tobacco plants (results not shown). Use of a radioactive assay allowed for the unequivocal determination of the separate enzymes. Chromatography on Sephadex G-200 resulted in the separation of two distinct high mol wt peaks from the callus, the first having predominantly TAL activity and the second peak having mainly PAL activity (Fig. 1, A and B). The level of these peaks varied depending on the medium on which the tissue was growing. Callus grown on NSF medium had a higher level of PAL activity than of TAL activity (Fig. 1A), while callus grown on SF medium exhibited the reverse trend, *i.e.* a higher level of TAL than of PAL activity (Fig. 1B). Rechromatography of the peaks did not result in any loss of homogeneity.

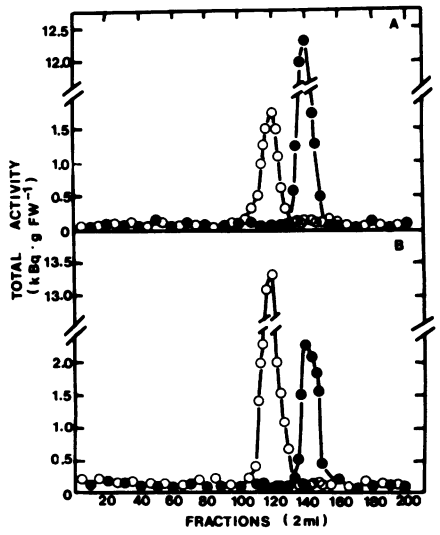

FIG. 1. Sephadex G-200 chromatography of ammonia lyases from 9-d-old tobacco callus grown on NSF (A) and SF (B) media. Radioactivity detected in *trans*-cinnamic acid (PAL activity, ●), and *p*-coumaric acid (TAL activity, O). Each point is the mean of three independent experiments.

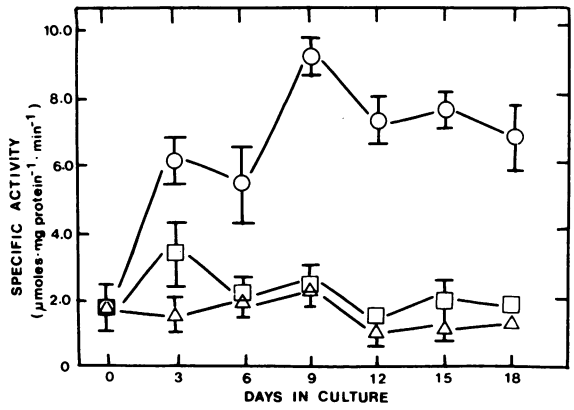

FIG. 2. The specific activity (μmol·mg^{-1} protein·min^{-1}) ± SE of tyrosine ammonia lyase in dark-grown SF (O), NSF (□), and SFG (△) tobacco callus at different times during culture. Each point is the mean of nine determinations from three independent experiments each with triplicate samples.

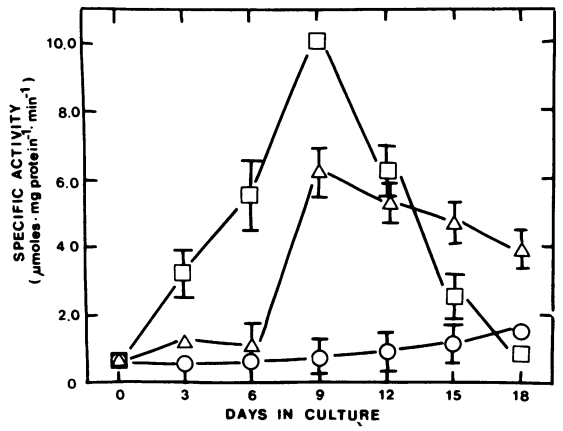

FIG. 3. The specific activity (μmol·mg^{-1} protein·min^{-1}) ± SE of PAL in dark-grown SF (O), NSF (□), and SFG (△) tobacco callus at different times during culture. Each point is the mean of nine determinations from three independent experiments each with triplicate samples.

A comparison of TAL and PAL activities in SF, NSF, and SFG tobacco callus is shown in Figures 2 and 3. Beginning at day 3, there were significant differences in activity in both PAL and TAL between the tissues. TAL had a higher level of activity in SF than in NSF and SFG tissues (Fig. 2). This activity peaked at day 9 and then decreased slightly to day 12 before leveling off. NSF and SFG tissues both exhibited similar trends and did not vary much over the 18-d culture period.

PAL showed a significant increase in activity in NSF tissue by day 3 (Fig. 3); and the level of activity increased linearly until day 9, where it peaked. This was followed by a drastic decline. At day 18, the level of PAL activity in NSF tissue had returned to values similar to that of day 0. SF tissue did not exhibit an increase in PAL activity until day 15 where an increase began to occur. Activity in SFG increased only slightly by day 6 and exhibited a trend similar to SF tissue up to that point. Activity

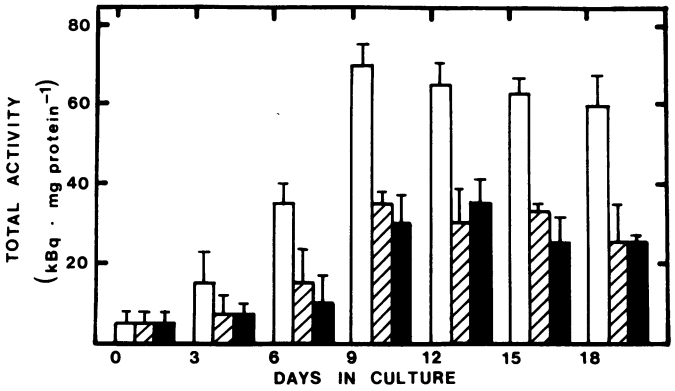

FIG. 4. Incorporation (kBq·mg^{-1} protein) ± SE from [^{14}C]tyrosine into *p*-coumarate after 1 h incubation in dark-grown SF (□), NSF (▨), and SFG (■) tobacco callus at different times during culture. Each point is the mean of nine determinations from three independent experiments each with triplicate samples.

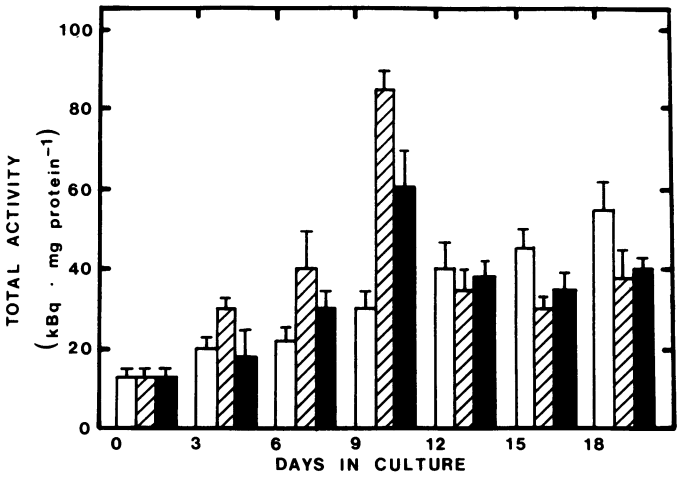

FIG. 5. Incorporation (kBq·mg^{-1} protein) ± SE from [^{14}C]phenylalanine into *trans*-cinnamate after 1 h incubation in dark-grown SF (□), NSF (▨), and SFG (■) tobacco callus at different times during culture. Each point is the mean of nine determinations from three independent experiments each with triplicate samples.

peaked on day 9 where the level was 5 times higher than that found in day 6 tissue. PAL activity decreased slightly over the rest of the culture.

The incorporation of [^{14}C]tyrosine into *p*-coumarate is shown in Figure 4. All tissues began to show increased incorporation from day 3, and a greater amount of label, up to 2 times more at day 9, was found in SF tissue than in the non-organ-forming controls. From day 9 onward, a nonsignificant reduction in incorporation was observed in all tissues.

Incorporation of [^{14}C]phenylalanine into *trans*-cinnamate was determined (Fig. 5). There was generally a greater incorporation of label into NSF and SFG tissues than into SF tissue up to day 9. This difference in incorporation occurred as early as day 3 and peaked at day 9 at a value of 3 times greater for NSF callus than for SF tissue. At day 12, the rate of incorporation of [^{14}C] phenylalanine in both NSF and SFG tobacco dropped significantly, but no further decline occurred later in culture. On the other hand, in SF tissue incorporation increased continuously over the 18-d period, so that at days 15 and 18 the level of labeling was greater than in the NSF and SFG tissues.

DISCUSSION

PAL and TAL are important enzymes in the biosynthesis of compounds possessing phenylpropanoid skeletons such as chlorogenic acid, caffeic acid, and lignin. They are involved in diverting the flow of carbon from proteins into phenolic compounds. Although it has been shown that protein levels are higher in SF than NSF tissue (20), this study indicates a probable role for phenolic compounds in the organogenetic process.

TAL activity increased in SF tobacco tissue during days 6 to 18. This period coincides with the key histogenic events found in the tissue. Histologically, shoot formation begins with the appearance of zones of preferential cell division at discrete distances from the medium in the lower half of the callus by 8 d in culture. Meristemoids or meristem-like masses of cells arise in these zones in 8- to 14-d-old tissue. By 10 d in culture, meristemoids begin to form shoot primordia which emerge from the basal portion of the callus by 14 d in culture. Eventually, the primordia form leafy vegetative shoots, which can be counted by day 28.

The shikimic acid pathway which provides the substrates required for PAL and TAL activity has been shown to increase in activity in SF tissue (1). Radioactive incorporation studies have shown that label from [^{14}C]shikimate preferentially went into tyrosine in SF tissue and into phenylalanine in non-organ-forming tissue. Turnover studies have also shown that these compounds rapidly disappeared (2). This paper supports these findings in that TAL activity increased in SF tissue and PAL activity increased in NSF and SFG tissues during the culture period. The enzyme activity data were supported by incorporation of ^{14}C from labeled tyrosine and phenylalanine into *p*-coumarate and *trans*-cinnamate. This indicates that synthesis of phenolic metabolites was occurring in the tissue. Since incorporation of label from [^{14}C]tyrosine was into *p*-coumarate and from [^{14}C]phenylalanine was into *trans*-cinnamic acid, the presence of both TAL and PAL, respectively, was confirmed. Thus, the aromatic amino acids can enter secondary metabolism directly. The importance of tyrosine metabolism in shoot formation in tobacco callus is therefore reinforced, although the actual role of tyrosine in the process remains unclear (8).

A basic regulatory mechanism underlying plant organ initiation involves a balance between auxin and cytokinin (15). A relatively low level of auxin and a high level of cytokinin result in shoot differentiation, and the reverse in root initiation. It has also been suggested that the endogenous auxin to cytokinin ratio must be reduced to allow shoot initiation. It was suggested that IAA oxidase (part of the isoperoxidase complex) was stimulated by phenolic compounds and thus reduced the auxin/cytokinin ratio (11). Since increases in activities of PAL and TAL, leading to the formation of phenolic compounds, were observed in this study, it is possible that the simple phenolics formed could stimulate IAA oxidase, as hypothesized previously (12). The increase in phenolics might be greater than the increase in IAA from tryptophan which would occur via anthranilate synthase; as the enzyme leading to the formation of phenylalanine and tyrosine, chorismate mutase, had a greater increase in activity than the enzyme leading to the formation of anthranilate, anthranilate synthase, in SF tobacco callus (1). As well the synthesis and turnover of tryptophan were less than those of tyrosine and phenylalanine in this tissue (1, 2). The end result would be a decrease in the availability of IAA, or the total auxin concentration, making the situation more compatible for shoot formation. As shoot primordia develop, lignification would also be important in the formation of vascular tissue in the shoots (19).

The detection of TAL activity in tobacco callus is a major finding of this study. Although it is sometimes believed to be a separate enzyme found mostly in monocots, TAL activity could not be demonstrated without also demonstrating activity with phenylalanine (22). It was also found that TAL activity could not be demonstrated in many PAL preparations and often the activity, if it exists, was lost during preparation (10). In *Ustilago*

hordei, TAL activity was not found in crude or purified PAL preparations and *p*-coumarate was not formed when tyrosine was administered *in vivo* (16). Although two separate enzymes have been found in castor bean endosperm (6), the question of the presence or absence of TAL in dicots has not been answered conclusively (5, 7). Chromatography on Sephadex G-200 resulted in the separation from tobacco callus of two distinct high mol wt fractions with independent TAL and PAL activities, thus indicating the presence of two separate enzymes in tobacco. Furthermore, the level of these enzymes varied as a function of the culture condition, *i.e.* higher TAL activity in SF tissue and higher PAL activity in non-organ-forming tissues. This differential modulation of enzyme capacity further confirms the presence of two independent enzymes.

To date, comparative studies on metabolism in SF and non-organ-forming tobacco have indicated generally an enhancement of enzyme capacity and metabolite production during shoot initiation (17, 18). However, with respect to aromatic amino acid metabolism, not only is the synthesis of tyrosine and phenylalanine different in SF and proliferating tissue, but also their deamination. Why this variation and coordination in aromatic amino acid metabolism should exist is intriguing, since deamination of either amino acid would allow for simple phenylpropanoid metabolism.

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