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SIGNIFICANCE OF CAFFEIC ACID-*O*-METHYLTRANSFERASE IN LIGNIFICATION OF CULTURED TOBACCO CELLS

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; callus and cell culture; *O*-methyltransferase; lignification; kinetin.

Abstract—Caffeic acid-*O*-methyltransferase activity was assayed in the callus and suspension cultured cells of tobacco. Lignification of cells was observed only in a kinetin (10^{-5} M) culture and not in an auxin (10^{-6} M 2,4-D or 10^{-5} M IBA) culture. Enzyme activity in the kinetin cultured cells was much higher than in the stock culture and the rise in enzyme activity coincided with the onset of lignification.

INTRODUCTION

Morphological differentiation of xylem elements from cultured plant cells have been studied, but the biochemical regulation of lignification is little understood. Using cultured cells for such studies has several advantages; the control of environmental conditions, homogeneous tissue and the mass production of lignified cells. Ferulic acid and sinapic acid have been shown to be potent lignin precursors in tracer experiments with radioisotopes [1] and since the discovery of *S*-adenosylmethionine:catechol-*O*-methyltransferase (OMT, E.C. 2.1.1.6) in plant cambial tissue [2], this enzyme has been considered an important factor in lignin biosynthesis. We have, therefore, examined the metabolic activity of xylem differentiation in cultured tobacco cells and the role of OMT as a controlling factor in lignification.

RESULTS AND DISCUSSION

The levels of auxins and cytokinins in the medium greatly affect the growth of plant cells. Generally, a high concentration of kinetin inhibits growth [3] and promotes differentiation and organization of cultured cells or tissues [4]. We have observed this same effect in the TB5 callus of tobacco. In a kinetin (10^{-5} M) culture, the growth of callus was suppressed (Fig. 1). Among a number of treatments combining various IBA and kinetin concentrations, the calluses on media containing 10^{-5} M of kinetin showed repressed growth, but after 30–50 days of culture, shoots were regenerated adventitiously and after 15–20 days of culture, organized xylem elements appeared. In contrast, very few unorganized lignified cells were scattered through IBA cultured tissue. Changes in the OMT activity and the percentage of lignified cells to the whole tissue are shown in Fig. 1. In both types of culture, OMT showed two peaks of activity during 30 days. The first occurred on the 2nd day after transfer in both and the second maximum was reached on the 20th day in the kinetin culture, but in the IBA culture a lower activity peak appeared after 10 days. In the kinetin culture, lignified cells increased markedly with the second rise in OMT activity, while the IBA cultured callus had a relatively low and constant number of lignified cells (Fig. 1).

Similar results were obtained with T5S cultured cells of tobacco in suspension. As with TB5 callus, similar patterns of growth and OMT activity were obtained during the culture of the T5S cells. Lignified cells appeared only in the kinetin culture. The percentage of these cells increased from 0·5% in 2,4-D cultured cells to 2·8% in 15 day-old kinetin cultured cells. In a kinetin culture

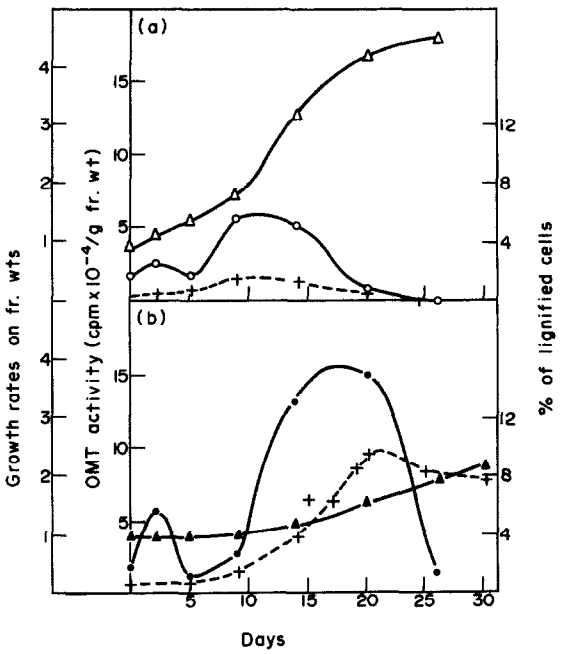

Fig. 1. Growth rates of calluses (triangles), the percentage of lignified cells (crosses) and the OMT activity of cells (circles) in an IBA culture (a), and in a kinetin culture (b) of TB5 callus.

of T5S cells, most lignified cells did not differentiate into tracheids but had thickened secondary walls with lignin, while most lignified cells in kinetin cultured TB5 callus differentiated into xylem elements. Many more lignified cells were observed by phloroglucinol–HCl staining than by chromic acid treatment in the kinetin culture of T5S cells. During successive kinetin cultures of T5S cells, changes in OMT activity maintained the pattern observed for the TB5 callus, and rigid aggregates of cells gradually increased. Tracheids were differentiated continuously in these aggregates with successive passages. Shoot formation appeared at about the 4th passage in the kinetin culture.

Microscopic observations of kinetin cultured TB5 callus suggest that cell division occurs before xylem differentiation. Fosket [5] has reported the inhibition of wound xylem formation by FUdR, mitomycin C and colchicine. In pea root segments, cell division in the cortical cells is induced by auxin-cytokinin treatment and this division is followed immediately, or after one more division cycle, by tracheary element formation [4]. These data suggest that DNA synthesis or cell division is a prerequisite for xylem differentiation. The period of lignification appears to be limited, however, to a particular stage of growth in kinetin cultures. The percentage of lignified cells reached a maximum at the 20th day of a kinetin culture (Fig. 1), though the total number of lignified cells increased after the 20th day. This indicates that the period from the 15th–20th day in the kinetin culture is the time at which lignification selectively occurs. The second rise in OMT activity is apparently related to lignification. The first rise in OMT activity may be the effect of transplantation because enzymes of phenolic acid biosynthesis (phenylalanine ammonia lyase (PAL), cinnamic acid-4-hydroxylase, etc.) are reported to be induced after the transfer of cells to new media [6,7].

Enzymes of phenolic acid biosynthesis, including OMT, are also known to be induced by light [8,9]. Under our dark conditions, however, OMT activity rose in the kinetin culture. Several reports have suggested that these enzymes, including PAL [10] and OMT [11,12], might be involved in the control of lignification, though negative data have also been reported on the contribution of OMT to lignification in wheat plants [13].

There appear to be unknown steps between lignification and tracheid differentiation, and between the appearance of tracheids and vessel elements in the biosynthesis of lignin, but this is the first report, using cultured cells, which shows the contribution of OMT to lignin biosynthesis.

EXPERIMENTAL

Tissue culture. Tobacco (*Nicotiana tabacum* var. Bright Yellow) callus TB5 was induced at our laboratory in 1972 from pith tissue of matured tobacco plants by 10^{-5} M indolebutylic acid (IBA) on the basal medium of Linsmaier and Skoog [14] with 1% (w/v) agar, 3% (w/v) sucrose, thiamine and inositol. It was subcultured on the same medium every 20 days in the dark at 26° (IBA culture). T5S strain [15] of the same variety of tobacco was subcultured every 13 days in a liquid medium containing 10^{-6} M 2,4-D with reciprocal shaking of 120–130 rpm (2,4-D culture). Other factors in the medium were the same as those used in the culture of TB5 strain. These 20-day old IBA cultured calluses and 13-day old 2,4-D cultured cells were transferred, respectively, to agar and liquid media containing 10^{-5} M kinetin (no auxins). TB5 cultures were started with an inoculum of 1·5 g on 20 ml of new agar medium in a 100 ml flask. Liquid cultures of T5S cells were started at a density of 5×10^4 cells/ml in 50 ml of new medium in a 300 ml Erlenmeyer flask.

Number of cells and lignified cells. The number of cells in an aliquot of callus or suspended cells was determined using chromic acid digestion [5]. Lignified cells, including tracheids or vessel members, were darker coloured under the UV microscope.

Extraction and assay of OMT. Deep frozen tobacco callus or cells was homogenized and extracted using the method of Shimada *et al.* [15]. The centrifuged supernatant of the extract was used as the crude enzyme. The reaction mixture consisted of 0·12 μmol of [Me-^{14}C]-*S*-adenosylmethionine (0·20 mCi/mmol), 0·25 μmol of caffeic acid, 5 μmol of $MgCl_2$, 5 μmol of Na-ascorbate, 50 μmol of K-Pi buffer (pH 7·5) and enzyme solution in a total volume of 500 μl. After the reaction, the radioactive product was extracted using the method of Shimada *et al.* [16].

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REFERENCES

1. Higuchi, T. and Brown, S. A. (1963) *Can. J. Biochem. Physiol.* **41,** 613.
2. Finkle, B. J. and Nelson, R. F. (1963) *Biochim. Biophys. Acta* **78,** 747.
3. Simpkins, I. and Street, H. E. (1970) *J. Exp. Botany* **21,** 170.
4. Torrey, J. G. and Fosket, D. E. (1970) *Am. J. Botany* **57,** 1072.
5. Fosket, D. E. (1968) *Proc. N.A.S.* **59,** 1089.
6. Hahlbrock, K. and Wellmann, E. (1973) *Biochim. Biophys. Acta.* **304,** 702.
7. Yamada, Y. and Sekiya, J. (1975) *Phytomorph.* (in press).
8. Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellmann, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* **244,** 7.
9. Ahmed, S. I. and Swain, T. (1970) *Phytochemistry* **9,** 2287.
10. Higuchi, T. (1966) *Agr. Biol. Chem.* **30,** 667.
11. Higuchi, T., Shimada, M. and Ohashi, H. (1967) *Agr. Biol. Chem.* **31,** 1459.
12. Shimada, M., Fushiki, H. and Higuchi, T. (1973) *Mokuzai Gakkaishi* **19,** 13.
13. Glass, A. D. M. and Bohm, B. A. (1972) *Phytochemistry* **11,** 2195.
14. Linsmaier, E. M. and Skoog, F. (1965) *Physiol. Plantarum* **18,** 100.
15. Sekiya, J. and Yamada, Y. (1974) *Agr. Biol. Chem.* **38,** 1101.
16. Shimada, M., Fushiki, H. and Higuchi, T. (1972) *Phytochemistry* **11,** 2657.