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VARIATION IN CAFFEIC ACID *O*-METHYLATION IN WHEAT PLANTS DURING GROWTH

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Abstract—Caffeic acid *O*-methylase activity was assayed in wheat plants from age 0–75 days. The enzyme activity reached a maximum at 10 days, dropped, rose to a second peak at about 25–30 days, then fell off and remained essentially level. Phenylalanine, cinnamic acid and ferulic acid had no influence upon the activity of the enzyme. The results are discussed in terms of the problem of control of lignification.

INTRODUCTION

THE RESULTS of extensive studies[1] employing ^{14}C-labelled precursors have clearly demonstrated that aromatic compounds such as phenylalanine, cinnamic acid, ferrulic acid, and coniferyl alcohol are all good precursors of lignin in higher plants. Such studies have also indicated the sequence phenylalanine → cinnamic acid → *p*-coumaric acid → caffeic acid → ferulic acid; these cinnamic acids are believed to be reduced to the corresponding alcohols prior to dehydrogenation and polymerization.[2,3] Further support for the existence of this pathway has come from the isolation of certain key enzymes in the sequence.[4–8]

One important aspect of the lignification process which is little understood as yet is the mechanism(s) for control. The present study was undertaken for the examination of the possible role of *S*-adenosylmethionine catechol *O*-methyltransferase (E.C.2.1.1.6) as a controlling factor in lignification in wheat.

A variety of factors, both environmental and endogenous, have been found to exert an effect upon the process of lignification. Thus, Siegel[9] observed that the peroxidase-mediated polymerization of various lignin precursors was inhibited by IAA. This observation was interpreted as a possible mechanism for the control of lignification since in young, non-

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[4] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2962 (1961).
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[6] D. W. RUSSELL and E. E. CONN, *Arch. Biochem. Biophys.* **122**, 256 (1967).
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[9] S. M. SIEGEL, *Physiol. Plantarum.* **6**, 134 (1953).

lignifying tissue auxin levels tend to be high. Phillips[10] showed that when ash shoots were shaded for several weeks, the wood formed during the period gave only a weak lignin reaction. Higuchi *et al.*[11–16] have investigated several of the enzymes of the lignin pathway and attempted to correlate enzyme activity with lignin content of various portions of the bamboo plant. The results of these experiments have been interpreted to suggest that enzymes such as phenylalanine ammonia lyase (PAL) and catechol *O*-methyltransferase (COMT) may be important in controlling lignification. Since a considerable amount of information is available on phenolic acid metabolism and lignification in wheat, we decided to employ this plant in an examination of the possible regulator role of COMT in the process. Stone and Blundell[17] showed that there is a spectacular rise in the rate of lignification between 60 and 80 days after planting. Our study of COMT deals with the activity of this enzyme prior to and during this period of rapid lignification as well as the influence of various cinnamic acids which might be predicted to act as activators or inhibitors of this enzyme. Since data were available dealing with PAL activity in wheat[18] and changes in phenolic acid concentrations with time for wheat,[19] we have prepared a composite figure (Fig. 3) with a view to clarifying or possibly establishing interrelationships.

RESULTS AND DISCUSSION

The results of the time study are shown in Fig. 1. In preliminary experiments buffered extracts of the plant material were prepared according to the procedure described by Finkle.[8] The use of acetone powders, however, resulted in a greater yield of enzyme activity and was adopted for the study.

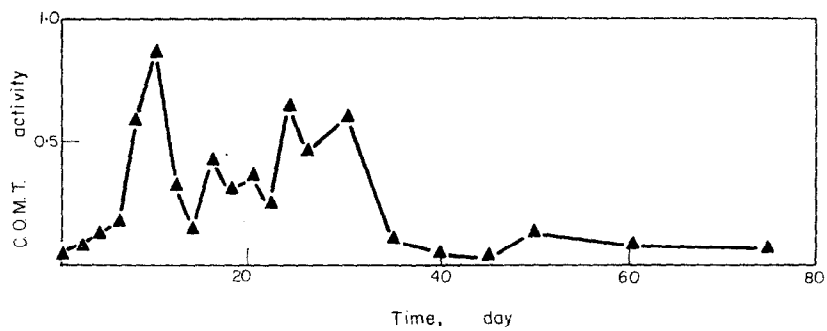

FIG. 1. ACTIVITY OF CAFFEIC ACID-*O*-METHYLTRANSFERASE SYSTEM IN WHEAT AS A FUNCTION OF TIME. Measured as activity of ^{3}H-methyl ferulic acid/g dry wt of plant material.

A most striking observation is the similarity between COMT activity and the concentration of ferulic acid as a function of age. El-Basyouni's samples for ferulic acid determina-

[10] E. W. J. PHILLIPS, *Nature, Lond.* **174,** 85 (1954).
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[19] S. Z. EL-BASYOUNI, Ph.D. Thesis, McGill University (1964).

tion were taken at 10 day intervals after the first 15 days. This possibly results in an oversimplification of the pattern of accumulation and the similarity between this accumulation pattern and COMT activity are not immediately apparent. When our COMT data were plotted at 10 day intervals so as to correspond with El-Basyouni's sampling times there was a striking correlation between ferulic acid concentration and COMT activity. It is possible that this enzyme might determine the levels of ferulic acid present in wheat. Alternatively, ferulic acid might act as an inhibitor or repressor of the enzyme. In order to examine the possibility of activation or inhibition of COMT by ferulic acid, we determined COMT activity in the presence of various concentrations of ferulic acid, and found that concentrations ranging from 5×10^{-6} to $1{\cdot}5 \times 10^{-4}$ M it had no influence upon enzyme activity.

The maximum COMT activity was observed at 10 days. PAL activity in wheat, as determined by Young[18] reached a maximum at 6 days. Similarly, studies by Grisebach *et al.*[20] have shown that maximum levels of PAL and other enzymes involved in flavone glycoside biosynthesis in parsley are reached quite early in the growing season. Could it be that a product of the PAL reaction serves as an activator or inducer of the COMT system? Both phenylalanine and cinnamic acid were found to be without influence upon COMT activity at concentrations ranging from 5×10^{-6} to $1{\cdot}5 \times 10^{-4}$ M. Furthermore, the COMT preparation from wheat (Fig. 2) failed to exhibit the sigmoidal response to increasing concentrations of substrate which might be diagnostic of allosteric interaction. However, these observations clearly do not exclude the possibility of enzyme induction or repression by intermediates of the lignin pathway.

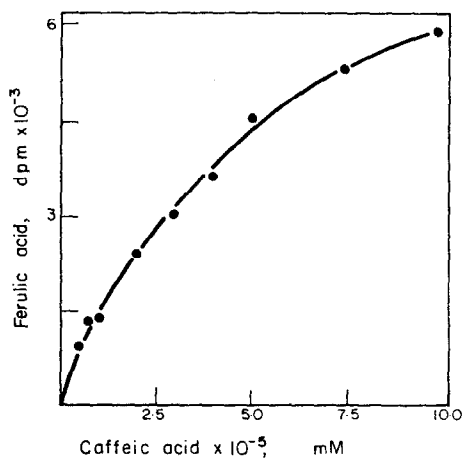

FIG. 2. THE AFFECT OF INCREASING AMOUNTS OF SUBSTRATE (CAFFEIC ACID) ON THE ACTIVITY OF CAFFEIC ACID-*O*-METHYLTRANSFERASE.

Measured as activity of ^{3}H-methyl ferulic acid/g dry wt of plant material.

Considerable evidence supports the view that PAL may be an important regulator of phenolic biosynthesis. This enzyme has been shown to be sensitive to feedback repression and feedback inhibition through its product cinnamic acid.[21] The studies of Maier and

[20] K. HAHLBROOK, A. SUTTER, E. WELLMANN, R. ORTMANN and H. GRISEBACH, *Phytochem.* **10**, 109 (1971).
[21] K. R. HANSON, M. ZUCKER and E. SONDHEIMER, in *Phenolic Compounds and Metabolic Regulation* (edited by B. J. FINKLE and V. C. RUNECKLES), Appleton–Century–Crofts, New York (1967).

Hasegawa[22] indicate an extremely high degree of correlation between PAL activity and the rate of naringenin accumulation in grapefruit. Furthermore, physiological studies indicate that the phytochrome-mediated enhancement of flavonoid biosynthesis is accompanied by corresponding increases in PAL activity and incorporation of ^{14}C-labelled phenylalanine into flavonoids in *Pisum*.[22] However, the notion of a single pathway proceeding from phenylalanine and diverging variously to give rise to the flavonoids, lignins, etc. is almost certainly an over-simplification. Smith *et al.*[23] have obtained evidence to suggest that pathways to the various phenylalanine-derived end products may be biochemically or perhaps spatially distinct. Thus observations relating to flavonoid biosynthesis should not *a priori* be taken to apply to lignification.

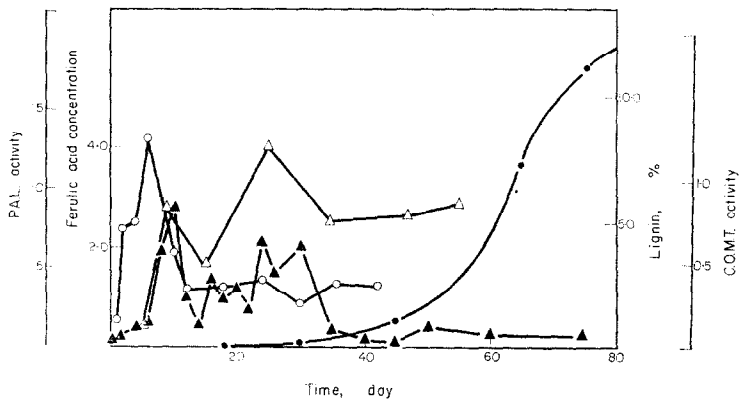

FIG. 3. COMPOSITE PICTURE OF PHENYLALANINE AMMONIA LYASE ACTIVITY, FERRULIC ACID CONCENTRATION, CAFFEIC ACID-*O*-METHYLTRANSFERASE, AND LIGNIFICATION IN WHEAT AS A FUNCTION OF TIME.
○—PAL expressed as % turnover/0·2 g acetone powder; ▲—caffeic acid-*O*-methyltransferase expressed as activity of ^{3}H-methyl ferulic acid/g dry wt; △—ferulic acid concentrations expressed as mg/g dry wt; ●—lignin content expressed as % of dry wt.

Higuchi *et al.*[11,12] have observed a tendency towards higher PAL and COMT activities in older more highly lignified tissues of bamboo, and it has been suggested that these enzymes might therefore be involved in the control of lignification. In wheat, however, the highest levels of PAL and COMT activity occur within 10 days of germination at a time when lignification is at a minimum and it is therefore difficult to imagine that either of these enzymes could function in a regulatory capacity. The studies by El-Basyouni[19] have shown that cinnamic acid derivatives such as ferulic acid are available in high concentrations early in the growing season. Thus it would appear unlikely that the availability of precursors represents a limiting factor in lignification in this plant. Once again, however, the possibility of distinct compartments destined specifically for flavonoid, or lignin biosynthesis cannot be evaluated in studies that involve gross determinations of enzyme activity or concentrations of substrates. It is unfortunate that all the time studies on wheat referred to in this paper, with the exception of the COMT study, were terminated prior to the period when lignification is at its height. It would have been interesting to observe, for example, any changes in the levels of cinnamic acids or PAL during this later period.

[22] V. P. MAIER and S. HASEGAWA, *Phytochem.* **9**, 139 (1970).
[23] D. B. HARPER, D. J. AUSTIN and H. SMITH, *Phytochem.* **9**, 497 (1970).

EXPERIMENTAL

Enzyme preparation. Wheat plants grown in soil (12 hr day, ambient temp. 20° ± 3) were harvested at the times indicated in Fig. 1. Plant material (10 g) was blended in acetone (−20°) and an acetone powder prepared. The acetone powder was extracted with 10 ml of 0·1 M $NaHCO_3$ for 30 min. The powder and buffer were then filtered through 2 layers of cheese cloth and the filtrate centrifuged at 10 000 rpm in a refrigerated centrifuge. The supernatant was brought to 50% $(NH_4)_2SO_4$ saturation (previous studies had shown that the major COMT activity resided in 30–50% fraction). Following centrifugation the protein precipitate was resuspended in 2·5 ml of 0·05 M phosphate buffer.

Assay procedure. Incubation media contained 0·5 ml of the buffered enzyme preparation, 2·5 μmol of caffeic acid, 1 μmol $MgCl_2$, 1μmol *S*-adenosylmethione (^{14}C methyl labelled—0·05 μCi), 4·0 μmol ascorbic acid in a total volume of 1·0 ml of 0·05 M phosphate buffer. The enzyme was incubated at 37° for 30 min. The reaction was terminated by the addition of two drops of 6 N HCl, 10 ml of distilled water were then added and the aqueous solution extracted with Et_2O (2 × 25 ml). The ethereal phase was dried and evaporated to dryness in a scintillation vial. Radioactivity of the ether fraction was determined in a Nuclear Chicago Scintillation counter.

Proof of identity of product. Using ^{14}C labelled ferulic acid (gift from Dr. Warren Steck) it was demonstrated that 80–91% of ferulic acid was extracted from aqueous solution by a single treatment with Et_2O. Using a scaled-up enzyme preparation, chromatography of the ether extract indicated that 95% of the activity corresponded to the ferulic acid fraction. At most 4% of the activity located in the ether fraction was due to *S*-adenosylmethionine. The radioactive band corresponding to ferulic acid was eluted from the chromatogram with 80% ethanol. To this ethanolic solution was added 1 g of authentic ferulic acid. The ferulic acid was recrystallized 3 times, to constant specific activity.