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CAFFEIC ACID: *O*-METHYLTRANSFERASES AND THE BIOSYNTHESIS OF FERULIC ACID IN PRIMARY CELL WALLS OF WHEAT SEEDLINGS

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Key Word Index—*Triticum aestivum*; Gramineae; lignin; *O*-methyltransferase; coniferyl alcohol dehydrogenase; ferulic acid; *p*-coumaric acid; ferulic acid ester-ether bridges; caffeic acid; 5-hydroxyferulic acid.

Abstract—The activity of *O*-methyltransferases (OMT) in tissues of wheat and the changes in their specificity for hydroxycinnamic acid substrates was followed during seedling development. In the early stages of development, prior to lignin deposition, the OMT showed a preference for caffeic acid as a substrate. This acitivity peaked at day 3. Later, after lignin deposition had begun, the activities of OMT and cinnamyl aldehyde dehydrogenase increased continuously and the OMT had a preference for 5-hydroxyferulic acid. It is suggested that the "early" OMT may be involved in the synthesis of ferulic acid destined for esterification of cell wall arabinoxylans whereas, the "late" OMT may be involved with the synthesis of ferulic and sinapic acids which are converted to the monolignol building blocks of lignin.

INTRODUCTION

Ferulic acid (FA) ester-ether bridges [1, 2] have been described between lignin and polysaccharides in cell walls of wheat (*Triticum aestivum*) and phalaris (*Phalaris aquatica*) internodes [3, 4]. These bridges probably involve FA esters to arabinoxylan components of the wall that are, in turn, joined to lignin monomers through ether linkages. The esterification of the wall polysaccharides by FA is believed to occur intra-cellularly prior to their deposition into the wall [5, 6]. The etherification of lignin to complete bridge formation is believed to occur extra-cellularly via peroxidase-catalysed reactions [7] during deposition of lignin in the walls. Although FA is well-established as the bridging molecule, there is no evidence for a similar role for *p*-coumaric acid (PCA) in either wheat or phalaris internodes [3]. There is now increasing evidence that FA ester–ether bridges contribute to the resistance of lignified walls of grasses to rumen digestion [8]. It is thus of interest to understand the route of biosynthesis of the FA ester–ether bridges. The specific involvement of FA in these bridges suggests that there may be a unique pathway for the synthesis of the FA involved in the bridges.

It is well-known that plant *O*-methyltransferases (OMT, EC 2.1.1.68) catalyse the *O*-methylation of caffeic acid (CA) and 5-hydroxyferulic acid (5-OHFA) to FA and sinapic acid, respectively. These reactions are key steps in the biosynthesis of guaiacyl and syringyl nuclei of lignins [9]. Some OMTs [10, 11] appear to *O*-methylate both CA and 5-OHFA, but others appear to be specific for CA [12]. We have determined the specificity of OMTs in tissues of developing wheat seedlings for CA and 5-OHFA and investigated their relationship to the formation of lignin and ester- and ether-linked PCA and FA in the cell walls. An improved procedure for the synthesis 5-OHFA is reported.

RESULTS AND DISCUSSION

Lignin content. Lignin was not detected in any tissue until after day 4 from sowing (Fig. 1). Between days 4 and 9 lignin concentration increased sharply and thereafter more slowly. The appearance of lignin indicates that the differentiation of lignified walls in the tracheary elements and fibres has commenced. In *Zinnia elegans* in suspension culture, lignin deposition occurs 72 hr [13] or 96 hr [14] after cell division.

Content of ester- and ether-linked hydroxycinnamic acids. For each tissue examined the total content of covalently-bound FA was always significantly higher than total PCA between days 3 and 10 (Fig. 2). Thereafter the covalently bound-PCA content increased steeply in roots and internodes, but less so in leaves. The increase in the concentrations of ester- and ether-linked PCA (Fig. 2) parallels the appearance of lignin. This is consistent with PCA being the major hydroxycinnamic acid associated with lignin in wheat [15]. In contrast, in the early stages

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of tissue development, when no lignin has been deposited, substantial ester-linked FA is present. FA is the major hydroxycinnamic acid associated with arabinoxylans in walls of wheat cells. The ester-linked FA probably arises from the FA-arabinoxylan complex that is synthesized and deposited prior to secondary wall formation and lignin deposition, and probably represents the precursor of the ether linked FA involved in polysaccharide-lignin ester-ether bridges [3] which can only be formed after lignin deposition. The appearance of ether-linked FA follows a course similar to PCA.

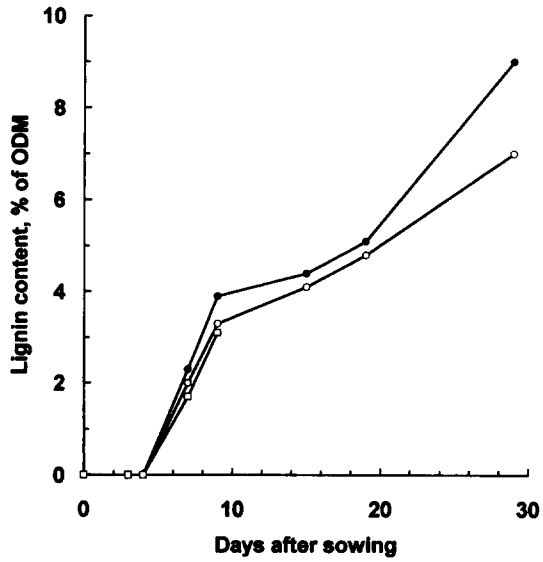

Fig. 1. Lignin content of roots, shoots and coleoptiles from developing wheat seedlings determined by an acetyl bromide procedure. —●—, roots; —○—, shoots; —□—, coleoptiles.

Changes in OMT and CAD activities. OMT was assayed using two substrates, CA and 5-OHFA (Fig. 3). The specific activity of OMT (CA substrate) reached a maximum at day 3 in all tissues tested. Roots showed the highest activity. A second, smaller peak, of activity was recorded at day 15 for roots and day 9 for shoots. The pattern of changes in the specific activity of OMT (5-OHFA substrate) was quite different, its activity was always much higher than for OMT (CA substrate) in the older roots and shoots than in their younger counterparts.

It is significant in relation to the timing of appearance and level of OMT (5-OHFA substrate) that its product sinapic acid does not occur covalently linked to wall polymers in wheat tissues [15, 16]. However sinapyl alcohol, derived from sinapic acid, is an important monolignol found in wheat lignin. The OMT with a preference for CA substrate ("early" OMT) is probably involved in the conversion CA to FA for esterification to arabinoxylan, whereas the OMT with the preference for 5-OHFA ("late" OMT) is probably involved in the genesis of both the FA and sinapic acid precursors of monolignols. It

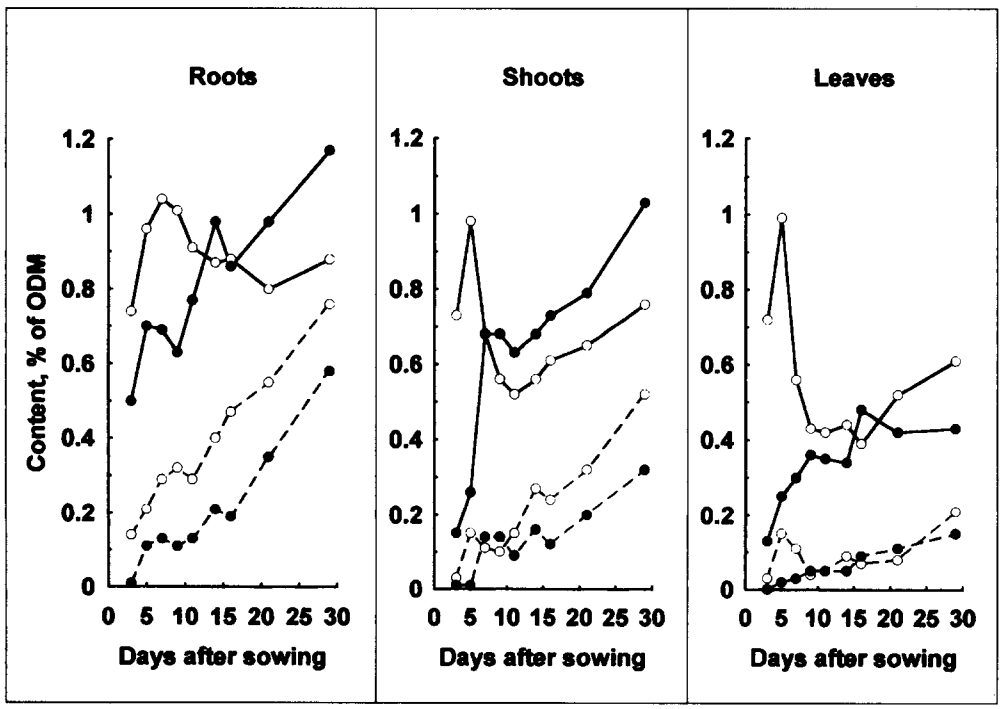

Fig. 2. Content of ester- and ether-linked hydroxycinnamic acids of roots, shoots and leaves from developing wheat seedlings. ——○——, ester-linked ferulic acid; ——●——, ether-linked ferulic acid; - - - -○- - - -, ester-linked *p*-coumaric acid; - - - -●- - - -: ether-linked *p*-coumaric acid.

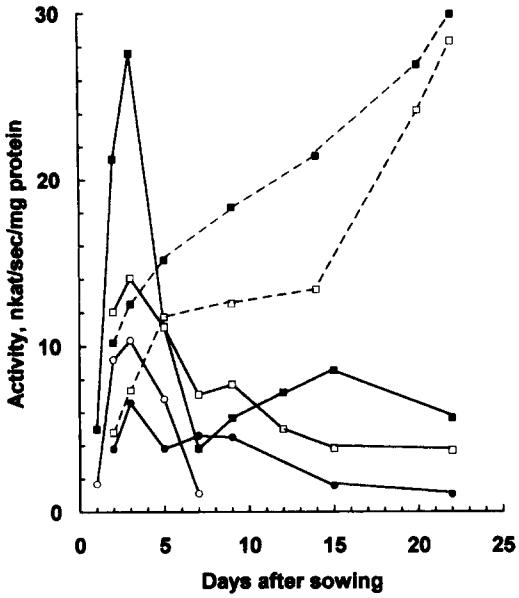

Fig. 3. Activity of *O*-methyltransferase (caffeic acid and 5-hydroxyferulic acid substrates) of roots, shoots, leaves and coleoptiles from developing wheat seedlings. ——■——, OMT-CA (roots); ——□——, OMT-CA (shoots); ——●——, OMT-CA (leaves); ——○——, OMT-CA (coleoptiles); ----■----, OMT-5-OHFA (roots); ----□----, OMT-5-OHFA (shoots).

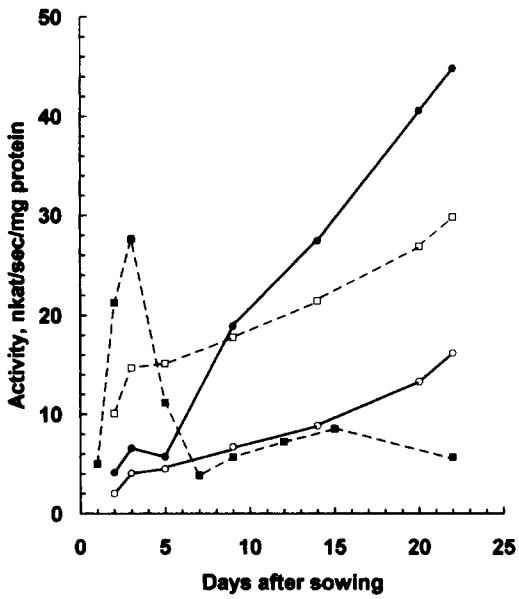

Fig. 4. Activity of cinnamyl alcohol dehydrogenase (reduction) of roots and shoots from developing wheat seedlings. ——●——, CAD (roots); ——○——CAD (shoots); ---■---, OMT-CA (roots); ----□----, OMT-5-OHFA (roots).

might be speculated that the "early" and "late" OMTs are located in different compartments of the cell, allowing further control of their activities.

The changes in the specific activity of CAD (Fig. 4), an enzyme specifically concerned with lignin precursor formation were similar to that of OMT (5-OHFA substrate). The specific activities of both enzymes were still increasing after 20 days in both roots and shoots. Taken together these results suggest that in developing wheat seedlings two forms of OMT are present. An "early" OMT, which in roots, appears to have a preference for CA as substrate and a "later" OMT which appears to prefer 5-OHFA. These *in vitro* results are interpreted in relation to the temporal development of lignin in the tissues (Fig. 2) and lead to proposal that the "early" OMT is implicated, in the formation of FA which is later esterified to arabinoxylans.

EXPERIMENTAL

Synthesis of 5-OHFA. 5-Bromovanillin was prepd by bromination of vanillin using a modification of the procedure of Puri *et al.* [17]. Vanillin (20 g, 0.13 M) was dissolved in acetic acid (80 ml), and bromine (15.8 g, 0.10 M) added dropwise. The mix. was stirred for 2 h at room temp. Within 15 min of the addition of the bromine an orange precipitate appeared. The mixt. was poured into ice water (200 ml), the ppt. collected by filtration and washed three times with cold water (50 ml each). The 5-bromovanillin was crystallized from ethanol. Yield 26.7 g (88%), mp 158°. ^{1}H NMR (MeOH-d6, 90 MHz): OCH_3 (3.6, 3H), aromatic H-2 (7.0, 1H), aromatic H-6 (7.3, 1H), aldehyde (9.4, 1H).

4,5-*Dihydroxy*-3-*methoxybenzaldehyde.* This was prepared by a modification of the procedure of Bradley *et al.* [18]. 5-Bromovanillin (9.0 g, 39 mmol), pptd copper (3.6 g, 57 mM) and 10% KOH (100 ml) were placed in a stainless steel, high pressure reaction vessel (Parr Instrument Co. Ltd. Chicago), and heated for 1 hr at 210° with stirring. After cooling, the dark-brown mixture was acidified to pH 2 with 1 M H_2SO_4, and the ppt. (mainly Cu) removed by filtration. The filtrate was extracted with diethyl ether (100 ml × 3), and the organic layer treated with activated carbon. After removal of the activated carbon by filtration, the solution was dried over Na_2SO_4, and evapd to dryness. The 4,5-dihydroxy-3-methoxybenzaldehyde was extracted from the syrup with benzene. After evapn of benzene, the compound was recrystallized from benzene. Yield 3.15 g (48.1%), mp 122°. ^{1}H NMR (MeOH-d6, 90 MHz): OCH_3 (3.6, 3H), arom H-2 (6.9, 2H), aldehyde (9.3, 1H). 5-Hydroxyferulic acid was prepd by condensing 4,5-dihydroxy-3-methoxy benzaldehyde with malonic acid as follows: 4,5-dihydroxy-3-methoxybenzaldehyde (2.6 g, 15.5 mmol) was dried in a vacuum oven, mixed with malonic acid (4.7 g, 45 mmol), dry pyridine (15 ml), and piperidine (0.5 ml). The mixt. was stirred, in the dark, for 3 weeks at room temp with exclusion of moisture. The mixture was poured into ice water (50 ml), evaporated *in vacuo* to remove pyridine. Water (50 ml) was added, and solution re-evapd to remove pyridine completely. Finally the aq. solution was adjusted to 50 ml, acidified with 6 M HCl to pH 1.0. The 5-OHFA was extracted with diethyl ether, the organic layer dried over Na_2SO_4, and evapd to dryness. The product was recrystallized from water. Yield 2.0 g (65%), mp 176°.

Plant materials. Seeds of wheat (*T. aestivum* cv. Millewa) were steeped in water for 3 hr, then germinated on filter paper (Whatman No. 50), in the dark at 25° for up to 5 days or on vermiculite: sand (50:50) for 7 days to 1 month in a greenhouse at 20–25°. Plants were harvested at intervals, separated into roots, shoots, coleoptiles and leaves, immediately frozen in liquid N_2 and stored at −20°.

Extract prepn and $(NH_4)_2SO_4$ fractionation. Each tissue (20 g fr. wt) was ground in liquid N_2 with a mortar and pestle and was homogenised for 3 × 1 min in 20 ml of 0.2 M Tris-HCl pH 7.5 buffer containing 5 mM EDTA and 28 mM 2-mercaptoethanol. The homogenate was filtered through two layers of cheesecloth and centrifuged (12,000 g, 30 min). The extract was fractionated by $(NH_4)_2SO_4$ ppn. The protein ppt. at 30–70% satn was collected by centrifugation, dissolved in buffer and dialysed against the same buffer overnight. All experiments were performed at 4°.

Determination of enzyme activities. The activity of *O*-methyltransferase with CA or 5-OHFA as substrate was assayed as described by Bugos *et al.* [11]. The standard reaction mixt. (200 μl) contained 1 mM substrate, 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$ and 20–100 μl of enzyme. After pre-incubation for 5 min at 30°, 65 nmol of [^{14}C-CH_3]*S*-adenosylmethionine (SAM) (1.85 GBq mmol^{-1}, Amersham) in 1 μl (final SAM concentration 0.325 mM) was added and the reaction allowed to proceed for 30 min at 30°. The reaction was terminated by addition of 20 μl of 2 M HCl and the products extracted with diethyl ether (1 ml). The ether extract was added to 5 ml scintillation liquid and the radioactivity measured using a Pharmacia Wallac 1410 Liquid Scintillation Counter.

Cinnamyl alcohol dehydrogenase activity was assayed spectrophotometrically using the procedure of Wyrambik and Grisebach [19] with coniferaldehyde (Aldrich) and NADPH (Aldrich) as co-substrates. The incubation mixt. contained 60–70 nmol coniferaldehyde dissolved in 10 μl 50% (v/v) ethanol, 200 mmol NADPH, protein 0.5 μg, and 200 mM potassium phosphate buffer (pH 6.5), in a total volume of 1 ml. The reaction mixture was incubated at 30° for 5 min in a 1 cm cuvette and the change in absorbance at 340 nm measured. The rate of coniferyl alcohol formation was calculated from the decrease in absorbance due to oxidation of NADPH. The changes in absorbance were linear for about 10 min.

Determination of hydroxycinnamic acids. The residue remaining after homogenization of wheat tissue was washed with acetone (100 ml, twice), and dried in a vacuum oven overnight at 40°. The esterified and total (esterified plus etherified) hydroxycinnamic acids in the dried residue were determined using the procedures of Lam *et al.* (1 M NaOH, room temp overnight) [15] and Iiyama *et al.* (4 M NaOH, 170° for 2 hr) [2], respectively.

Lignin determination. Lignin content was determined by the acetyl bromide procedure [20].

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