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5-HYDROXYGUAIACYL NUCLEI AS AROMATIC CONSTITUENTS OF NATIVE LIGNIN

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Key Word Index—*Sorghum bicolor*; *Pennisetum americanum*; *Zea mays*; tramineae; lignin; lignin biosynthesis; 5-hydroxyguaiacyl nuclei; pyrolysis-gas chromatography-mass spectrometry; brown midrib mutant.

Abstract—The pathway of lignin biosynthesis has been well documented. However, there are still questions because of the lack of knowledge about the exact chemical structure of lignin, caused by the restriction of analytical procedures used and/or the interpretation of analytical results. The presence of 5-hydroxyguaiacyl nuclei in lignin of a brown midrib mutant (*bm3*) of maize has been established using a thioacidolysis. In this paper, it was confirmed using pyrolysis-gas chromatography-mass spectrometry (PY-GC/MS) that in addition to *bmr* mutants of some tropical grasses, lignins of their normal counterparts and some temperate and tropical angiosperms woody plants are composed of 5-hydroxyguaiacyl nuclei, in addition to guaiacyl and syringyl nuclei. Based on the results, it is suggested that 3(3,4-dihydroxy-5-methoxyphenyl)-propen-1-ol, which is synthesised from 3(3,4-dihydroxy-5-methoxyphenyl)-propionic acid (5-hydroxyferulic acid) is also involved in dehydrogenative polymerisation by perioxidase during the biogenesis of lignin of some species of plants.
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INTRODUCTION

The pathway of lignin biosynthesis has been well documented and most of the enzymes involved in lignin biosynthesis have been purified and characterised [1]. In addition, genes encoding these enzymes have been isolated and focused as targets for gene manipulation [1]. However, some parts of the pathway of lignin biogenesis are still unclear. For example, it is not clear when and how genes for lignin biosynthesis are triggered, where the anchor for lignification is [2] and what is the final stage for monolignol biosynthesis—monolignol-glycoside or not [2].

Other questions about lignin biogenesis originate from a lack of knowledge of the exact chemical structure of lignin, which is due to the restrictions of the analytical procedures used and/or the interpretation of analytical results. Lignin in walls of grasses has been characterised by the presence of *p*-hydroxyphenyl nuclei; which mostly originated from *p*-coumaric acid covalently linked to wall polymers. *p*-Hydroxybenzaldehyde is produced by alkaline nitrobenzene oxidation of *p*-coumaric acid [3].

The presence of 5-hydroxyguaiacyl nuclei, which could not be detected by conventional analytical procedures, such as an alkaline nitrobenzene oxidation, in lignin of stem cell walls of a brown midrib mutant (*bm3*) of maize (*Zea mays*) together with normal guaiacyl and syringyl nuclei has been published by Lapierre and co-workers [4] using thioacidolysis. They also detected trace amounts of 5-hydroxyguaiacyl nuclei in lignin of stem cell walls of the corresponding normal strain of maize [4]. These nuclei have been quantified in lignins of *bm3* mutants of three cultivars of maize using thioacidolysis [5, 6], but not in lignins of the *bm2* mutant or its normal counterparts [6]. It has been suggested from these results that 5-hydroxyguaiacyl nuclei are incorporated into the lignin macromolecule because of significantly lower *O*-methyltransferase activity in the *bm3* mutant [7, 8] than that in the normal counterparts [4–6].

In the present paper, in addition to maturing walls from stems of *bmr* mutants and their normal counterparts of sorghum (*Sorghum bicolor*: *bmr6* and *bmr18*), pearl millet (*Pennisetum americanum*) and maize (*Z. mays*: *bm3*), walls of some temperate and tropical woody angiosperms were analysed using pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) to address the presence or absence of 5-hydroxyguaiacyl nuclei in their lignins.

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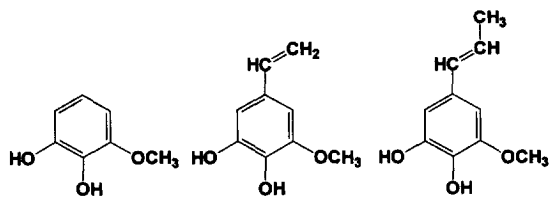

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RESULTS AND DISCUSSION

Compositional characteristic of lignin from brown midrib mutants

It has been reported that the lignin contents of *bmr* mutants of tropical C4 grasses are significantly lower than their normal counterparts [9–12] when determined by the acid–detergent lignin procedure [13]. However, lignin contents determined by the acetyl bromide procedure [14] and also by sulphuric acid digestion (sum of Klason and acid-soluble lignins) showed no differences between *bmr* mutants and their normal counterparts (Table 1) [15]. The lignins of *bmr* mutants are characterised by lower molar ratios of syringyl nuclei to guaiacyl nuclei (S/V ratio) [5, 6, 15, 16] and lower total yields [15, 17] of alkaline nitrobenzene oxidation products than those of normal strains (Table 1). These characteristics have been confirmed by biochemical investigations which showed lower *O*-methyltransferase (OMT) [7, 8] and coniferyl alcohol dehydrogenase (CAD) [8, 18] activities in *bmr* mutants than in their normal counterparts. Based on these chemical and biochemical investigations, genes encoding OMT and CAD have been set as target genes for manipulation to obtain plants having 'low lignin concentration'.

Lapierre and coworkers [4] applied thioacidolysis to characterise lignin of maize *bmr* mutant (*bmr*3). They found 5-hydroxyguaiacyl nuclei as a new aromatic structure in addition to normal guaiacyl and syringyl nuclei in lignin of maize *bmr*3 mutant and also trace amounts in the lignin of corresponding normal strain. It was suggested that 5-hydroxyguaiacyl nuclei are incorporated in the *bmr* mutant lignin due to significantly low OMT activity. The presence of 5-hydroxyguaiacyl nuclei was confirmed by Chabbert *et al.* [5, 6] in lignins of the *bm*3 mutant of three cultivars of maize, but not in the *bm*2 mutant or the corresponding normal counterpart.

PY-GC/MS analysis of lignin from brown midrib mutants

5-Hydroxyguaiacyl nuclei have not been detected by conventional analytical procedures, such as an alkaline nitrobenzene oxidation, because of the stability of the catechol structure to alkaline medium. Lignins from the *bmr* mutants of some tropical grasses and their normal counterparts were analysed using PY-GC/MS. Pyrograms of the walls of *bmr*6 mutant of sorghum are shown in Fig. 1. In addition to pyrolysed products with guaiacyl and syringyl nuclei, which were identified [19–24], 2-methoxycatechol [compound **1**, *m/z*, 140[M]$^+$, 125[M-15]$^+$, 107, 97 and 79], 2-methoxy-4-vinylcatechol [compound **2**, *m/z* 166[M]$^+$, 151[M-15]$^+$, 137, 123, 107 and 95] and 2-methoxy-4-propenylcatechol [compound **3**, *m/z* 180[M]$^+$, 165[M-15]$^+$, 147, 137 and 119], which are so-called 5-hydroxyguaiacyl compounds, were detected and identified by mass-fragmentation by comparison with those of corresponding authentic compounds. These products were detected not only in all *bmr* mutants examined, but also in their normal counterparts. The detection of 2-methoxy-4-vinylcatechol in walls may suggest the presence of 5-hydroxyferulic acids esterified and/or etherified to wall polymers, because it was confirmed by pyrolysis of the authentic compound that 5-hydroxyferulic acid selectively gives 2-methoxy-4-vinylcatechol as a pyrolysis product.

The quantities of these products cannot be determined by PY-GC/MS because an internal standard is not available. The relative amounts of 2-methoxy-

Table 1. Lignin contents and alkaline nitrobenzene oxidation products

Sample		AcBr lignin content, % of ODM	Nitrobenzene oxidation products: Total yield % of AcBr	Nitrobenzene oxidation products: S/V molar ratio
Sorghum	*bmr*6	10.5	16.4	0.74
	normal	10.9	31.2	0.92
Sorghum	*bmr*18	6.2	20.0	0.31
	normal	8.5	25.1	1.10
Pearl millet	*bmr*	10.4	17.7	1.17
	normal	10.8	31.3	1.09
Maize	*bm*3	10.0	31.2	0.49
	normal	11.0	41.6	1.83

ODM: original dry matter, S/V: molar ratio of syringyl nuclei to guaiacyl nuclei.

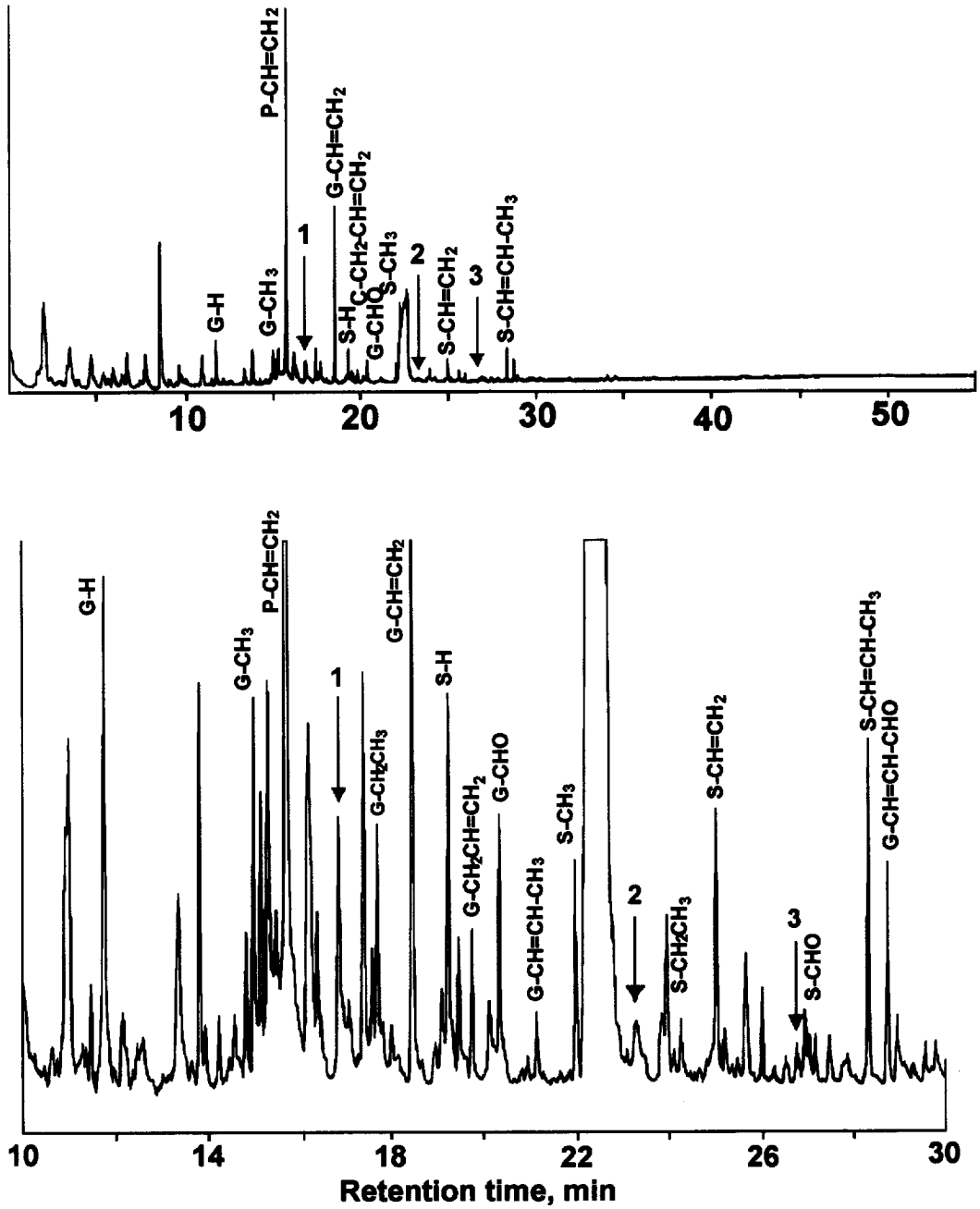

Fig. 1. Pyrograms of walls of *bmr* mutant of sorghum (*bm6*).

catechol (5OHG) to guaiacol (G_1) and syringol (S_1) in the pyrolysis products were calculated from the peak areas as $5OHG/G_1$ and $5OHG/S_1$, respectively, and summarised in Table 2, together with the relative values of 5OHG in the lignins of *bmr* mutants and those of normal strains [$(5OHG/G_1)_{bmr}/(5OHG/G_1)_{normal}$ and $(5OHG/G_1)_{bmr}/(5OHG/G_1)_{normal}$]. The ratios of S_1 to G_1, which would be relevant to the S/V ratios of alkaline nitrobenzene oxidation products, are also listed in Table 2.

It may be suggested that the above compounds could be produced by demethylation of the methoxyl groups of syringyl nuclei. We tested many syringyl lignin model compounds by pyrolysis under the same conditions, but no demethylation of methoxyl groups were detected, the same as in the reaction during thioacidolysis [4]. In addition, if demethylation is caused during pyrolysis, walls of samples with higher S/V ratio should give much more abundant of 5OHG derivatives as pyrolysis products. However, walls of *bmr* mutants in which S/V ratios are lower than those of normal strains, except pearl millet, gave higher quantities of the products by pyrolysis than those of normal counterparts. These observations strongly

Table 2. Relative amounts of 5-hydroxyguaiacyl nuclei in lignins of *bmr* mutants and their normal counterparts of tropical grasses

Sample	Area ratio			Relative are ratio based on normal strain		
	5OHG/G_1	5OHG/S_1	S_1/G_1	5OHG/G_1*	5OHG/S_1†	S_1/G_1‡
Sorghum						
*bmr*6	0.52	0.65	0.80	3.32	3.69	0.90
normal	0.16	0.18	0.88			
Sorghum						
*bmr*18	0.26	0.46	0.58	1.53	2.41	0.64
normal	0.17	0.19	0.91			
Pearl millet						
bmr	0.29	0.27	1.09	2.58	2.11	1.22
normal	0.11	0.13	0.89			
Maize						
*bm*3	0.45	0.88	0.52	1.11	3.88	0.29
normal	0.41	0.23	1.80			

5OHG: 5-hydroxyguaiacol, G_1: guaiacol, S_1: syringol.

* and †: Relative values of 5OHG in lignin of *bmr* mutants to those of normal strains, $[(5OHG/G_2)_{bmr}/(5OHG/G_1)_{normal}]$ and $[(5OHG/G_1)_{bmr}/(5OHG/S_1)_{normal}]$, respectively.

‡ Relative value of S_1/G_1 in lignin of *bmr* mutants to those of normal strains.

support the presence of 5-hyroxyguaiacyl nuclei in native lignins of *bmr* mutants and their normal counterparts of sorghum, pearl millet, and maize. It is suggested based on the results that 3(3,4-dihydroxy-5-methoxyphenyl)-propen-1-ol which is synthesised from 3(3,4-dihydroxy-5-methoxyphenyl)-propionic acid (5-hydroxyferulic acid), is also involved in dehydrogenative polymerisation by peroxidase during biogenesis of lignin of some species of plants.

5-Hydroxyguaiacyl nuclei were also detected in the lignins of ash (*Fraxinus mandshurica*), some tropical angiosperms woody plants, phdiek (local names in Papua New Guinea) (*Anisoptera laevis*), amberoi (*Pterocymbium beccarii*), taun (*Pometia pinnata*) and burst fibre of Manila hemp (*Abaca textilis*). However, these structures were not detected in lignins of some temperate angiosperms woody plants, toneriko (*Fraxinus japonica*), painted maple (*Acer mono*), yamaguruma (*Trochodendron araliodes*, which is characterised by pseudo vessels), mountain ash (*Eucalyptus regnans*), and some tropical woods, malas (*Homalium foetidum*), jelutong (*Dyera* sp.), kamerere (*Eucalyptus deglupta*), calophyllum (*Calophyllum inophyllum*) and kuila (*Intsia bijugai*) (Table 3).

EXPERIMENTAL

Plant cell walls. Ground stem-bases of *bmr* mutants and their normal counterparts of sorghum *bmr*6 and *bmr*18 (harvested at the watery- to milky-grain stage), pearl millet (harvested at the soft-dough stage) and maize *bmr*3 (harvested at the early-dent stage) were provided by Dr D. R. Buxton and co-workers (Agriculture Research Service, USDA, USA) [9] and analysed previously for lignin and hydroxycinnamic acids (Table 1) [15]. Samples were extracted with boiling 80% aq. EtOH (1 hr, × 3) and the residues dried in a vacuum oven at 40°. In addition, extract-free ground walls of burst fibre of Manila hemp (*Abaca textilis* L.), some species of temperate angiosperms woody plants, ash (*Fraxinus mandshurica* Rupr.), toneriko (*F. japonica* Blume), painted maple (*Acer mono* Maxim.), yamaguruma (*Trochoden-dron aralioides* Sieb. et Zucc.), mountain ash (*Eucalyptus regnans* F. Muell.), which were extracted with EtOH–benzene (1:2) using a Soxhlet extractor, tropical angiosperms woody plants, phdiek (common name used in Papua New Guinea) (*Anisoptera laevis* Ridl), amberoi (*Pterocymbium beccarii* K. Schum.), taun (*Pometia pinnata* Forster), amals (*Homalium foetidum* (Roxb.) Benth), jelutong (*Dyera* sp.), kamerere (*Eucalyptus deglupta* Blume), calophyllum (*Calophyllum inophyllum* Linn.) kuila (*Intsia bijugai* (Colebr.) O. Kuntze), which were gifted by Dr K. Shimada of the Forestry and Forest Products Research Institute, Ministry of Agriculture, Forestry and Fishery, Tsukuba, Japan, and extracted with boiling 80% aq. MeOH (1 hr, × 3), were examined.

Chemical analyses. Lignin contents of extract-free samples was determined by an acetyl bromide (AcBr) procedure [11]. Alkaline nitrobenzene oxidation of the samples was by the procedure of ref. [3]. Yields of *p*-hydroxybenzaldehyde, vanillin and *p*-hydroxybenzoic acid were corrected for the products from esterified and/or etherified *p*-coumaric acid, ferulic acid and *p*-hydroxybenzoic acid, respectively [3, 25]. All analyses were duplicated.

Chemicals. Commercially available 3-methoxycatechol (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) and previously synthesised 5-hydroxyferulic acid [26] were used as the authentic compounds having

Table 3. Characteristics of lignin in walls of angiosperms and the presence of 5-hydroxyguaiacyl nuclei

Plant	Lignin content* % of ODM	Alkaline nitrobenzene oxidation products S/V molar ratio	Alkaline nitrobenzene oxidation products Total yield % of lignin†	Py-GC/MS analysis 5OHG nuclei
Manila hemp (burst fibre) *Abaca textilis*	18.9	5.22	41.0	+
Temperate woody angiosperms				
Ash *Fraxinus mandshurica*	21.2	2.32	40.3	+
Mountain ash *Eucalyptus regnans*	20.5	3.25	42.1	–
Toneriko *Fraxinus. japonica*	22.9	2.09	39.7	–
Yamaguruma *Traochoden-dron aralioides*	28.4	1.88	32.8	–
Painted maple *Acer mono*	20.1	3.45	41.5	–
Tropical woody angiosperms				
Phdiek *Anisoptera laevis*	24.6	1.57	42.2	+
Amberoi *Pterocymbium beccarii*	24.5	0.96	37.8	+
Taun *Pometia pinnata*	31.3	1.62	38.0	+
Malas *Homalium foetidum*	33.6	0.73	29.0	–
Jelutong *Dyera* sp.	27.9	1.08	42.6	–
Kamerere *Eucalyptus deglupta*	28.5	1.73	41.3	–
Calophyllum *Calophyllum inophyllum*	30.1	0.58	35.0	–
Kuila *Intsia bijugai*	28.2	0.80	38.3	–

*: Determined by acetyl bromide procedure [14].
†: Based on lignin.

5-hydroxyguaiacyl nuclei for PY-GC/MS. In addition, commercially available monomeric syringyl compounds were examined, together with some dimeric compounds, such as syringylglycerol-β-syringol, provided by Dr Y. Matsumoto, University of Tokyo.

Pyrolysis-gas chromatography-mass spectrometry (PY-GC/MS). Extract-free walls (*ca* 100 μg) were pyrolysed at 500° for 4 s using a Curie-Point Pyrolyser JHP-3 (Japan Analytical Industry Co. Ltd.) and the products analysed by GC-MS and by FID-GC. The conditions of GC-MS and GC were as follows. Column: neutrabond-1 (0.25 mm I.D. × 30 m), column temp.: 1 min at 50°, then programmed at 5° min^{-1} to 270°, carrier gas: He, transfer temp.: 270°. An Ultra Alloy-(8H)-1 (0.8 mm I.d. × 30 m) column was also used for PY-GC analysis under the following conditions. Column temp.: 1 min at 50°, then prog. at 5° min^{-1} to 270°, carrier gas: He, detector: FID.

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