Lignocellulosic Characterizations of Indonesian Rice and The Expression Analyses of *OsCAD2* and *OsMYB55/61* Related To Lignin Biosynthesis

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Abstract

Plant cell walls as sources of lignocellulosic biomass are abundant and used in diverse applications. Lignocellulosic contents manipulation can be applied to develop biomass plants. This study aimed to determine the lignocellulosic components, expressions patterns of *OsCAD2* and *OsMYB55/61* related to lignin biosynthesis, and deposition patterns of lignin in previously determined three low and three high total lignin content rice accessions based on TGA method. The total Klason and acid-soluble lignin showed variations between 18.03-24.53%, consistent with the TGA method. The ash content of the low lignin content (12.50-21.68%) were significantly differed from the high lignin content (24.84-27.91%) accessions. The extractive, α-cellulose, and hemicellulose contents in each accession showed almost similar proportion. Py-GC-MS proved that the S/G ratio and the syringyl contents were directly proportional to the total lignin contents. *OsCAD2* expression levels at the heading stages were consistent with their total lignin contents and their lignin histochemical assay results in the vascular bundle and cortical fiber, suggesting its important roles in rice lignin biosynthesis. However, *OsMYB55/61* showed inconsistent expression patterns to total lignin contents, which may also upregulate not only lignin biosynthesis but also cellulose biosynthesis.

Keywords: lignin, lignocellulosic, *OsCAD2*, *OsMYB55/61*, rice

Introduction

Plant cell wall is the most abundant source of lignocellulosic biomass and has been utilized in various applications such as food sources, clothing, lumber or timber, and energy. Cell walls are a complex and dynamic biological tissue consists of interacting polysaccharides, proteins, polyphenols, minerals, and water, which determine morphological traits and characteristics during plant development and protection against biotic and abiotic stresses. Cell walls can be classified based on their composition and function into three types; primary cell walls, secondary cell walls and gelatinous layer walls (Anderson & Kieber, 2020). The secondary cell wall is constructed between the primary cell wall and plasma membrane and is particullarly thickened in tissues such as xylem and sclerenchyma (Loix *et al*., 2017; Meents *et al*., 2018). The secondary cell wall dominantly composes lignocellulosic biomass and consists mainly of cellulose, hemicelluloses, and lignin.

Lignin is a phenolic biopolymer. The cinnamate/monolignol biosynthetic pathway provides three monolignols, lignin precursors, *p*-coumaroyl, coniferyl, and sinapyl alcohols, which are incorporated into *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignins, respectively, via oxidative radical coupling (Weng *et al*., 2008; Chanoca *et al*., 2019). Gymnosperm lignin consists dominantly of G units with a small number of H units, whereas

dicotyledonous plants of angiosperms additionally have S units with a small amounts of H units. (Baucher *et al*., 1998; Boerjan *et al*., 2003; Vanholme *et al*., 2019). Grass lignins share S, G, and H units with dicotyledonous lignins, although they also incorporate unique structures such as *p*-hydroxycinnamates (Ralph, 2010) and flavon tricin (del Rio *et al*., 2012; Lan *et al*., 2015; Lam *et al*., 2021)

Cellulose and hemicelluloses are cell-wall polysaccharide components in lignocellulose. Cellulose is a glucose polymer with a β-1,4 bond and the largest component in the secondary cell wall. Unlike cellulose, hemicelluloses consist of various monosaccaharide units and are relatively easy to be hydrolyze (Demirbas, 2009; Li *et al*., 2010). Cellulose and hemicelluloses can be degraded into monosaccharides which will be able to be fermented into different valuable products such as bioethanol, lactic acid, and detergent (Vanholme *et al*., 2013). On the other hand, although lignin has been regarded as an obstacle inhibiting the use of cell-wall polysaccharides (Zhao *et al*., 2020), it is also a potent source of beneficial aromatic chemicals (Umezawa, 2018)

Bioengineering approaches for structural modification and/or reduction of lignin to improve utilization characteristics of lignocellulosic biomass have been reported have been reported (Umezawa, 2018, Yoo *et al*., 2018; Park *et al*., 2022). Among them, of lignin content and structures in grasses, which generally show the high biomass productivity, such as rice (*Oryza sativa*), sorghum (*Sorghum* spp.), and sugarcane (*Saccharum* spp.) can be applied as a promising feedstock for lignin-oriented biorefinery (Umezawa, 2018; Umezawa *et al*., 2020; Kumar *et al*., 2021; Scopel & Rezende, 2021). Promoted utilization of grass biomass may be also useful for the save of timber, enhanced utilization of marginal lands, and beneficial use of crop residues (Antar *et al*., 2021; He *et al*., 2022).

Our deeper understanding of grass lignin biosynthesis may be important for bioengineering toward promoted applications of grass lignocellulosic biomass. Attempt to manipulate lignin contents and structures of grass biomass can be performed by engineering genes involved in the lignin biosynthetic pathway (Umezawa, 2018; Umezawa *et al*., 2020). Several genes and transcription factors have been identified to play roles in grasses lignin biosynthesis (Cesarino *et al*,. 2016; Umezawa, 2018; Zhao *et al*., 2019; Miyamoto *et al*., 2020; Umezawa *et al*., 2020)

OsCAD2 (Zhang *et al*., 2006; Hirano *et al*., 2012; Koshiba *et al*., 2013; Martin *et al*., 2019) and *OsMYB55/61* (Koshiba *et al*., 2017; Zhao *et al*., 2019; Miyamoto *et al*., 2020) have been indicated to play roles in lignin biosynthesis in rice. Twelve CAD genes (*OsCAD1-7*, *OsCAD8A-D* and *OsCAD9*) have been predicted in rice, *O. sativa ssp. Japonica* cv. Nipponbare. Among them, *OsCAD2* showed the highest expression levels in the internode in Nipponbare, indicating its important role in cell wall deposition, thus lignin biosynthesis in lignin biosynthesis in rice (Hirano *et al*., 2012; Koshiba *et al*., 2013; Martin *et al*., 2019).

Overexpressing *OsMYB55/61* enriched lignin in vascular bundles and activated expression of a gene encoding CAD (Hirano *et al*., 2013). In plants, most of the MYB protein family members of R2R3-MYB are transcription factors involved in the regulation of different plant responses, such as biotic and abiotic stress, hormonal signals, phenylpropanoid biosynthesis, meristematic cell development, differentiation regulations and cellular morphogenesis (Zhang *et al*., 2018). The orthologue of *OsMYB55/61* in *Arabidopsis thaliana* (*AtMYB61*) was identified to play roles in lignin and cellulose biosynthesis in the secondary cell wall formation mediated by gibberellin (Koshiba *et al*., 2017; Rao and Dixon, 2018).

The aims of this study is to elucidate the compositions of structural and non-structural lignocellulose components as well as lignin tissue localization and their relationship with expression levels of *OsCAD2* and *OsMYB55/61*. Six Indonesian rice accessions, which represent rice biomass with high and low levels of the thioglycolid acid (TGA) lignin (Unpublished), were used. Lignin unit composition was determined by using pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS). The contents of ash and extractives, which are important to determine the biomass heating value, were also analyzed. The information obtained will be useful for genetic manipulation of lignin biosynthesis in grasses for future applications of their biomass.

Materials and Methods

Rice Genetic Materials. Indonesian rice accessions used were low lignin content rice cv Tukad Petanu (8.38%), Ciherang (10.67%) and Cigenit (11.04%), and high lignin content rice cv Inpago7 (16.98%), Lampung Kuning (18.76%) and Gebang (20.75%), based on TGA method (Unpublished). Those rice accessions were grown in the glass house belonging to the Research Center for Genetic Engineering, BRIN. The plants were grown in pots containing 10 kg of growing media consisted of soil and manure (3:1). Plants maintenance including watering, fertilizer application and pest controls, were done routinely until harvesting followed standard procedure.

Biomass Preparation. The biomass of the six rice accessions were harvested at seed maturation stages when approximately 85% of seeds were ripe and then dried at 55°C in a drying oven for 5–7 days until constant weight was achieved. Leaves and leaf sheaths were removed, and the culms were chopped into fine pieces (5–10 mm) using scissors and placed in grinding jars. The tissues were pulverized in TissueLyser (Qiagen, Hilden, Germany) at 26 Hz for 2.5 minutes at room temperature. The powder was transferred into a 10 mL dark glass bottles and kept in a desiccator until further use.

Chemical Analysis of Biomass. Chemical components of the biomass were analyzed for their ash, extractive in alcohol-benzene (1:2), Klason lignin (acid-insoluble lignin), acid-soluble lignin, and holocellulose (α-cellulose and hemicellulose) contents. Ash contents were determined by heating the biomass in a furnace at 525±25°C for 6 hours following the TAPPI T211 om-02 method (TAPPI, 2002). Extractive contents were obtained by the Soxhlet extraction method using ethanol:benzene (1:2) for ± 6 hours. The free extractive sample was then analyzed for lignin and holocellulose contents. The extractive contents were then determined gravimetrically after evaporating the solvent according to the TAPPI T204 om-97 method (TAPPI, 1997). The Klason lignin contents as the residual sulfuric acid hydrolysis were determined using the NREL LAP 003 method (Templeton and Ehrman, 1995). Acid-soluble lignin was measured by spectrophotometry using Multiskan GO (Thermo Fisher Scientific, USA) at 205 nm based on the NREL LAP 003 method (Ehrman, 1996). Holocellulose (hemicellulose and α-cellulose) was isolated from the extracted samples by delignification using the acid-chlorite method (Wise *et al*., 1946). Analysis of α-cellulose content was determined by removing hemicellulose from holocellulose by alkaline extraction (Rowell., 2005). Three replicates were analyzed for each sample.

Relative Quantification of Lignin Monomers by Pyrolysis - Gas Chromatography - Mass Spectrometry (Py-GC-MS). Lignin monomer was determined using pyrolysis gas chromatography mass spectrometry (Py-GC-MS) GC/MS system QP-2020 NX (Shimadzu, Kyoto, Japan) equipped with a multi-shot pyrolyzer EGA/PY-3030D. Approximately 5 mg of rice culm powder was placed into the SF PY1-EC50F eco-cup tube and covered by glass wool. The eco-cup tube was pyrolyzed at 500°C for 0.6 minutes using helium as the carrier gas and SH-Rxi-5Sil MS column (30 m × 0.25 mm id, 0.25 µm film thickness). The Py-GC-MS temperature program used was 50°C for 1 minute, 5°C/minute to 280°C, and 13 minutes at 280°C. The mass spectrum was taken at 70 eV with a pressure of 20.0 kPa, the total flow of 15.9 mL/min and column flow of 0.61 mL/minute. The pyrolysis products were identified by mass spectra and retention times using the library data in NIST LIBRARY 2017. Three biological replicates per accession were bulk, and two technical replicates were analyzed per accession.

Total RNA Isolation and cDNA Synthesis. Total RNA was isolated from 100 mg of the uppermost internode at the heading stage (Hirano *et al*., 2012) following the Trizol procedure (Invitrogen, Carlsbad, CA, USA). The total RNA concentration was measured using µDrop™ Plate, Microplate Spectrophotometer (Thermo Fisher Scientific, USA) and the quality was evaluated using agarose gel electrophoresis. Total RNA was treated with *DNaseI* (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer recommendation. cDNA was synthesized using 1 µg of total RNA as template, 4 µL 5×TransAmp Buffer, 1 µL

reverse transcriptase and nuclease-free water in 20 µL total volume with the SensiFast cDNA Synthesis Kit (Bioline, London, UK). The qPCR program consists of primer annealing (25°C, 10 minutes), reverse transcription (42°C, 15 minutes), incubation (48°C, 15 minutes), inactivation (85°C, 5 min) and cooling at 4°C.

***OsCAD2* and *OsMYB55/61* Expression Pattern Analysis.** Expression analysis in each accession was performed with three biological replicates and three technical replicates using SensiFAST™ SYBR No-Rox Kit (Bioline, London, UK) qPCR kit in the Eco Real-Time PCR System (Illumina, San Diego, CA, USA). The primers used to amplify the *OsCAD2* gene (LOC_Os02g09490; AK105011) were F 5'-TGT GTG AGA CTC TGA CGA CTT GTC-3' and R 5'-CAT ATA TTG CGA GGC CGA ATT T-3' (Koshiba *et al*., 2013). Amplification of transcription factor *OsMYB55/61* (LOC_Os01g18240; AK111803) was performed using primers F 5'-TGT TCT GGG AAA CAG GTG GTC TCA-3' and R 5'-TGG GTG TTT GGG TCA ATG CCT TTC-3'. The rice *actin-1* gene (LOC_Os03g50885) was used as an internal control using primers F 5'-AAG GCC AAT CGT GAG AAG ATG ACC-3' and R 5'-CAG AGT CCA ACA CAA TAC CTG TGG- 3' (Nurdiani *et al*., 2018). Each reaction consisted of 5 µL 2×SensiFAST SYBR No-Rox Mix, 0.4 µL 10 µM forward primer and 0.4 µL reverse primer (10 µM), 2 µL cDNA and nuclease-free water in a total volume of 10 µL. The qPCR was carried out in cycle steps of 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds (denaturation), 60–65°C for 10 seconds (annealing) and 72°C for 15 seconds (extension). The expression level was calculated based on the Ct value obtained from each gene and normalized to the Ct value of the reference gene. The two values were then formulated using the $2^{-\Delta\Delta Ct}$ calculation (Livak and Schmittgen, 2001) to obtain the relative expression values .

Lignin Histochemical Staining. The second uppermost internode at the heading stage was cut and then stored in FAA solution (Formalin : Acetic acid : Ethyl Alcohol). The culm tissue was cut to approximately 0.5 mm using blade knife. Staining was performed using the Wiesner test (phloroglucinol-HCl). The culm sections were incubated for 10 minutes in phloroglucinol solution (1% in 70% ethanol, v/v), and 18% HCl for 5 minutes (Li *et al*., 2009) at room temperature. Sections of the tissue were photographed and documented using Dino-Lite Eye Piece AM7025B light microscope (Dino-Lite, Hsinchu, Taiwan).

Results

Chemical Analysis of Biomass

The total lignin content (Klason lignin and acid-soluble lignin) of the six accessions used in this study ranged from 18.03-24.53%. The total lignin contents of Inpago7, Lampung Kuning, and Gebang (20.72-24.53%) were higher than those of Tukad Petanu, Ciherang, and Cigenit (19.38-20.55%). The tendency of difference in lignin content is consistent with the results of TGA lignin.

Table 1. Percentage of lignocellulose component in six rice accessions

Accessions	Klason lignin (%)	Acid-soluble lignin (%)	α-cellulose (%)	Hemicellulose (%)
Tukad Petanu	17.63 ± 0.29^{a}	0.45 ± 0.05^{a}	38.22 ± 0.24^{b}	33.52 ± 0.59^{a}
Ciherang	17.60 ± 0.37^{a}	0.43 ± 0.03^{a}	33.60 ± 0.24^{a}	31.55 ± 0.99^{a}
Cigenit	18.93 ± 1.62^{ab}	0.45 ± 0.04^{a}	33.43 ± 1.05^{a}	31.74 ± 2.01^{a}
Inpago 7	20.20 ± 1.16^{b}	0.52 ± 0.13^{a}	37.41 ± 1.13^{b}	25.83 ± 2.13^{b}
Lampung Kuning	21.01 ± 1.88^{b}	0.55 ± 0.14^{a}	33.36 ± 1.61^{a}	32.70 ± 1.07^{a}
Gebang	23.94 ± 1.41^{c}	0.59 ± 0.14^{a}	35.16 ± 0.54^{a}	33.83 ± 0.85^{a}

The letters a,b,c,d in the numbers indicate a significant difference in the DMRT test (α = 0.05). Data is the mean value of n =3

On the other hand, among the six accessions, the acid-soluble lignin content has no significant difference (Table 1). The α-cellulose and hemicellulose contents generally showed no correlation with the total lignin content. The highest α-cellulose content was found in the low lignin accession Tukad Petanu (38.22%) followed by the high lignin

content accession Inpago 7 (37.41%) with no significant difference. The hemicellulose content of all the accessions tested ranged 25.83–33.83%. The hemicellulose content of Inpago7 (25.83%) was significantly lower than those of the other five accessions (Table 1).

Table 2. Percentage of ash and extraction content.

Accessions	Ash content (%)	Extractive content (%)
Tukad Petanu	14.31 ± 0.20^{a}	3.74 ± 1.22^{a}
Ciherang	12.50 ± 1.99^{a}	4.16 ± 1.02^{ab}
Cigenit	21.68 ± 0.05^{b}	4.28 ± 0.09^{ab}
Inpago 7	24.93 ± 1.48^{c}	3.47 ± 1.43^{a}
Lampung Kuning	27.91 ± 2.71^{d}	6.19 ± 1.49^{b}
Gebang	24.84 ± 0.59^{c}	4.07 ± 0.66^{a}

The letters a,b,c,d in the numbers indicate a significant difference in the DMRT test ($\alpha = 0.05$). Data is the mean value of n =3

The ash contents showed a significant difference between low lignin and high lignin rice accessions (Table 2). Low lignin content accessions had relatively lower ash contents than high lignin contents accessions. In contrast to the ash content, the extractive components of the six accessions tended to vary in the range of 3.47–6.19%. The exctractive contents generally showed no correlation with the total lignin and ash content.

Lignin Monomer Analysis Using Py-GC-MS

In general, the lignin monomer composition S, G, and H of the six rice accessions were consistent with the composition of lignin monomers of Gramineae. The largest percentage of lignin monomer composition was G (50.00–67.43%), followed by S (16.30–40.18%), and H (6.42–16.60%) (Table 3). The rice accessions with low and high lignin contents showed different S/G monomer composition. The value of S/G ratio of the six accessions is in the range of 0.31–0.80 and potitively correlated with the total lignin content.

Relative Expressions of *OsCAD2* and *OsMYB55/61*

The relative expressions of *OsCAD2* in the uppermost internode at the heading stage showed consistency with the total lignin content (Figure 1A). The *OsCAD2* relative expression in high lignin content accessions were significantly higher than those of the low lignin content accessions. The relative expressions of *OsCAD2* in Gebang, which has the highest lignin was 14 times that of Tukad Petanu (the lowest lignin accession), followed by Lampung Kuning (12 times) and Inpago7 (9 times). The relative expressions of *OsCAD2* of the low lignin content rice accessions showed that Cigenit has almost similar relative expressions as Tukad Petanu. Ciherang has the highest *OsCAD2* relative expression, approximately four times that of Tukad Petanu. In contrast to *OsCAD2*, the relative expression of *OsMYB55/61* did not positively correlate with the lignin contents (Figure 1B). For example, Ciherang, one of the low lignin content accessions, showed relative expression higher than those of two high lignin content accessions, Lampung Kuning and Gebang (Figure1).

Table 3. Percentage of lignin monomer and S/G ratio using Py-GC-MS

Accessions	%H	%G	%S	S/G ratio
Tukad Petanu	16.60 ± 1.78	61.32 ± 4.13	19.14 ± 6.50	0.31 ± 0.13
Ciherang	15.73 ± 2.36	67.43 ± 1.87	16.30 ± 1.25	0.24 ± 0.01
Cigenit	16.14 ± 1.26	56.98 ± 0.17	26.86 ± 1.10	0.47 ± 0.01
Inpago 7	8.67 ± 0.64	55.81 ± 0.89	33.32 ± 0.23	0.60 ± 0.01
Lampung Kuning	6.42 ± 2.30	52.24 ± 5.47	38.74 ± 2.59	0.74 ± 0.13
Gebang	8.05 ± 0.87	50.00 ± 0.79	40.18 ± 2.59	0.80 ± 0.03

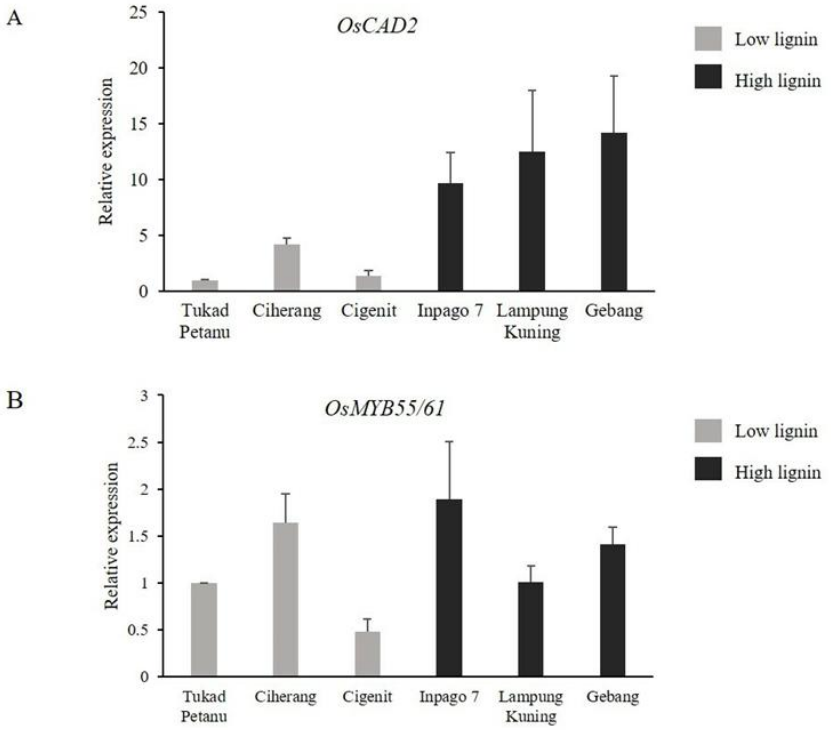

Figure 1. The relative expression of (A) *OsCAD2* and (B) *OsMYB55/61* in the uppermost internode at the heading stage normalized by *Actin-1*. Numbers indicate multiples of the mean value of the expression relative to Tukad Petanu ± standard deviation (n = 3).

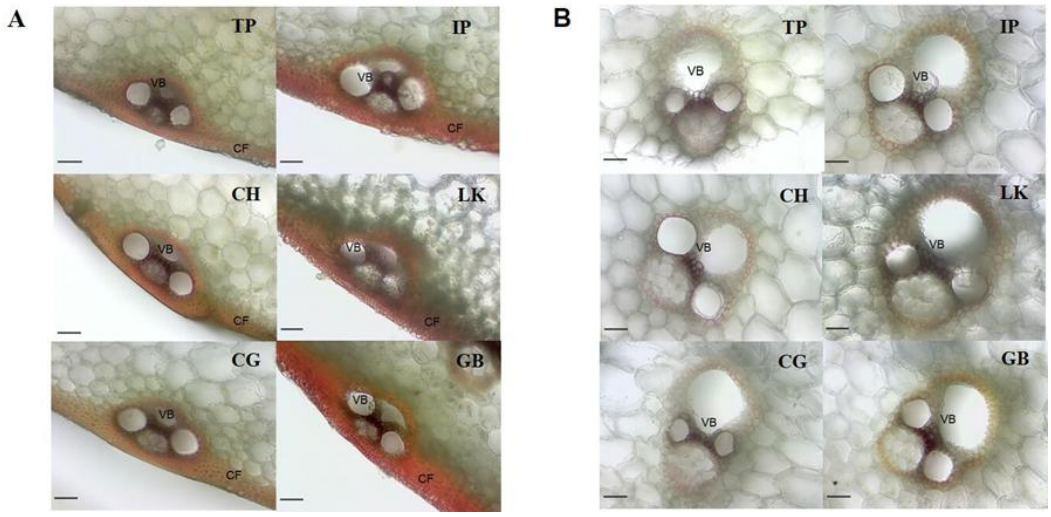

Figure 2. Lignin depositions in the second uppermost internode using Phloroglucinol-HCl staining (A) Outer culm (B) Inner culm (VB: Vascular Bundle, CF: Cortical Fiber, TP: Tukad Petanu, CH: Ciherang, CG: Cigenit, IP: Inpago 7, LK: Lampung Kuning, GB: Gebang). Scale bar = 100 µm.

Lignin Deposition

The histochemical analysis in a vertical cross-section of the culm showed lignin dominant accumulations in the vascular bundle (VB) and cortical fiber (CF) (Figure 2). The intensity of the reddish coloration was higher and lower in therice accessions with high and low lignin contents, respectively, which agrees with the result of Klason lignin method (Table 1).

Discussion

Variations in total lignin content (Klason lignin and acid-soluble lignin) in the six rice accessions were consistent with total lignin in the previous study using TGA, starting from the lowest to highest lignin rice accessions. However, the percentages of total lignin contents using the Klason and acid-soluble lignin method (18.03–24.53%) in this study are consistently higher than the total lignin content using the TGA method in the previously reported (8.38–20.75%), for the same rice accessions. The TGA method has been the most suitable method for isolating and determining lignin in Gramineae (Suzuki *et al*., 2009). However, another study reported that some lignin components were lost using the TGA method due to high specificity reactions with the ether bonds in lignin (Moreira-Vilar *et al*., 2014). In addition, there are other linkages besides the β-O-4 , the main linkage of the monolignol chain that does not react with thioglycolic acid (Moreira-Vilar *et al*., 2014). The results of these studies indicated that although there are consistencies using the two methods, there are differences in terms of total lignin content due to different characters of the two methods, and therefore can be used as a consideration when choosing the right method for lignin isolation and characterization. Based on the required samples and consumable times, we suggest that the TGA is more efficient than the Klason method. The TGA method requires only 10–20 mg of a sample when compared to the Klason method (>200 mg). On the other hand, the Klason method presented higher lignin recovery than the TGA method (Moreira-Vilar *et al.*, 2014). Based on the component analysis, the six accessions contained similar percentages of cellulose and hemicellulose but with varying percentages of total lignin. Genetics and environmental factors influence the variation of components that construct the secondary cell wall in plants (Wood *et al*., 2018; Vaahtera *et al*., 2019). Environmental factors such as soil nutrient content (Rivai *et al*., 2021; Rivai *et al*., 2022), biotic and abiotic stresses (Hamann, 2012; Gall *et al*., 2015; Tenhaken, 2015; Houston *et al.*, 2016) in plants also affect variations in secondary cell walls components.

Ash is an inorganic material or mineral dominated by Ca, Mg, and K in organic samples that are left behind during the ashing process. The ash contents in high lignin content rice accessions, in general, were higher than in low lignin content rice accessions. Ash content can be influenced by soil conditions that allow plants to absorb inorganic materials under different conditions (Yunanta *et al*., 2014). The extractive contents in six rice accessions showed inconsistency with the total lignin and ash content (Table 2). This may be due to the differences in the extractive substances of each accession. Extractive substances such as waxes, fats, terpenes, flavonoids, and dyes in plants also affect the color, smell, taste, and resistance of plant cell walls in the process of decay (Bajpai, 2018). These two non-structural lignocelluloses are essential parameters that directly affect the heating value of the biomass (Demirbas, 2002), which is important when carrying out energy analysis of any bioenergy systems. High ash content reduces the heating value due to the loss of carbon content in the form of ash (Rahmadani *et al*., 2017). In addition, high ash content could result in clogs in the boiler during combustion process, which will increases handling costs (Hytönen and Nurmi, 2015). On the other hand, extractive contents raises the heating value of the biomass.

The composition of S lignin monomers in high lignin content rice accessions were relatively higher than in low lignin content rice accessions (Figure 3).

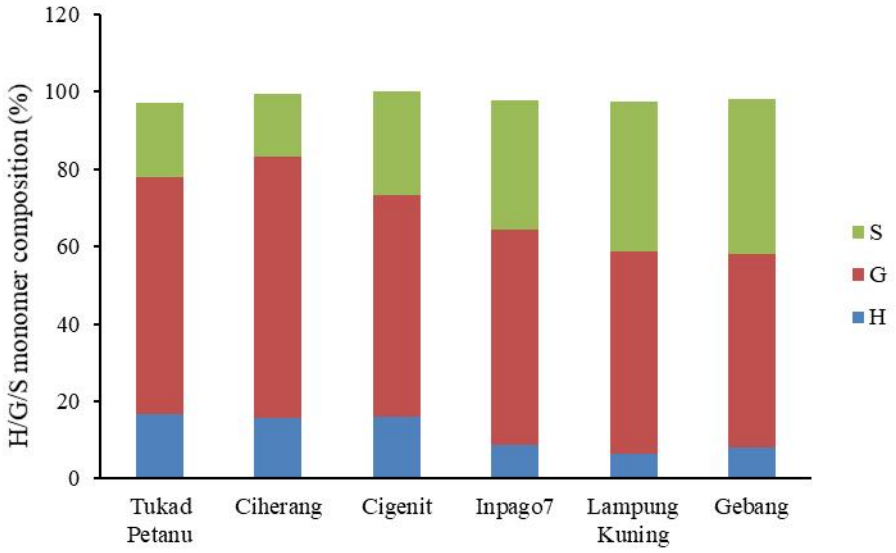

Figure 3. Composition of H, G, and S monomers in six rice accessions

In this study, the S percentage was correlated with acid-soluble lignin and total lignin content. S proportion and S/G ratio positively correlate with acid-soluble lignin and total lignin content (Nawawi *et al*., 2019; Rosado *et al*., 2021). S monomer has a higher methoxyl content than G monomer. The composition of the lignin monomer is a critical parameter in the delignification process. In the delignification reaction, especially in the pulp industry, the S/G ratio affects the ease of lignin being degraded. The S monomer is easier to be hydrolyzed than G in delignification (Zhao *et al*., 2020). In this study, S/G ratio of the high lignin content accessions (0.60–0.80) were higher than the low lignin content accessions (0.24–0.47). We suggest that the high lignin content rice accessions may be easier to be delignified than the low lignin content rice accessions. This information can be used in the application of rice biomass for different purposes. In the future, more in-depth characterization of lignin structures using 2D-NMR (del Rio *et al*., 2009; Kim & Ralph, 2010; Mansfield *et al*., 2012) and thioacidolysis techniques (Lapierre *et al*., 1986; Yamamura *et al*., 2012) should be performed.

The relative expressions of *OsCAD2* are consistent with the total lignin content in the six rice accessions tested (Figure 1A). The data supports the notion that *OsCAD2* is one of the genes that play important roles in rice lignin biosynthesis (Zhang *et al*., 2006; Hirano *et al*., 2012; Koshiba *et al*., 2013; Martin *et al*., 2019). In other studies, the co-expression network analysis also showed that *OsCAD2* had the highest relative expression among other CAD genes in rice and was expressed together with several other lignin biosynthetic genes (Hirano *et al*., 2012). The total RNA was extracted from the uppermost internode tissues at the heading stage, when lignin content increases (Hirano *et al*., 2012). Although previous studies have reported that *OsCAD2* is expressed in culms, it is also expressed in other lignified tissues such as roots and panicles (Park *et al*., 2018).

Our histochemical analysis observed the greater reddish color intensity in the high lignin content than in the low lignin content rice accessions, which is formed in a reaction between phloroglucinol HCl (Wiesner reagent) and the aldehyde group of lignin polymers (Pomar *et al*., 2002). The coloration additionally indicated particular lignification of the vascular bundle and cortical fiber tissues of all the tested samples.

The relative expressions of *OsMYB55/61* in the six accessions did not correlate with their total lignin contents of the tested samples, although the transcript levels in the two high lignin content accessions (Inpago 7 and Gebang) were relatively higher than those of the low lignin content accessions (Figure 1B). The transcription factor *OsMYB55/61* may also upregulate not only lignin biosynthesis but also cellulose biosynthesis through activation of gene(s) encoding CESA (cellulose synthase) (Huang *et al*., 2015; Rao and Dixon, 2018), suggesting that a higher expression of *OsMYB55/61* may not always enrich lignin in cell walls. In addition, lignin biosynthetic genes are regulated by multiple genes (Zhao *et al*.,

2019). Thus, the association of transcription factors with lignin content would be complicated.

An earlier study showed that a heterogenous expression of the *OsMYB55/61* homolog *AtMYB61* augmented the percentages of syringyl, *p*-coumarate, and tricin lignin units (Koshiba *et al*., 2017). In conclusion, our results revealed a variation of lignocellulose composition of rice accessions and a correlation between lignin content and *OsCAD2* expression in the tested samples. In addition, the lignin content positively correlated with the S/G lignin unit ratio in the accessions. These may provide insight into marker-assisted breeding and gene manipulation strategy to alter lignin content and/or lignin composition in grass biomass plants.

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