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G-lignin and hemicellulosic monosaccharides distinctively affect biomass digestibility in rapeseed

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HIGHLIGHTS

- Four rapeseed species showed distinct cell wall composition in 19 samples.
- H_2SO_4 and lime pretreatments led to a diverse biomass digestibility in rapeseeds.
- G-lignin had strongly negative effect on biomass saccharification.
- Hemicellulosic monosaccharides positively affected biomass digestibility.
- *Brassica napus* showed more efficient biomass digestion and ethanol production.

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ABSTRACT

In this study, total 19 straw samples from four *Brassica* species were determined with a diverse cell wall composition and varied biomass enzymatic digestibility under sulfuric acid or lime pretreatment. Correlation analysis was then performed to detect effects of cell wall compositions and wall polymer features (cellulose crystallinity, hemicellulosic monosaccharides and lignin monomers) on rapeseeds biomass digestibility. As a result, coniferyl alcohol (G-lignin) showed a strongly negative effect on biomass saccharification, whereas hemicellulosic monosaccharides (fucose, galactose, arabinose and rhamnose) were positive factors on lignocellulose digestions. Notably, chemical analyses of four typical pairs of samples indicated that hemicellulosic monosaccharides and G-lignin may coordinately influence biomass digestibility in rapeseeds. In addition, *Brassica napus* with lower lignin content exhibited more efficiency on both biomass enzymatic saccharification and ethanol production, compared with *Brassica junjea*. Hence, this study has at first time provided a genetic strategy on cell wall modification towards bioenergy rapeseed breeding.

1. Introduction

The global energy crisis and greenhouse gas emissions call for the development of renewable energy resource. Biomass and biofuel show promising solution for the replacement of fossil fuel for future transportation. Agricultural wastes, such as wheat straw (Sun et al., 2000; Ruiz et al., 2013), rice straw (Sun et al., 2000), barley straw (Sun and Sun, 2002), maize stems (Xiao et al., 2001) and sugarcane bagasse (Sun et al., 2004) have been investigated during the last decade as bioresource for biofuel and biochemical production. The use of agricultural residues for energy generation reduces the proportion of crop residues burnt in the field, improves the overall value of each crop species, especially for China with limited cultivation area of food crop and huge demand for sustainable energy supply.

Rapeseed has long been cultivated for oil production with various desired properties (Svärd et al., 2015). Due to the fact that rapeseed straw is not suitable for cattle feed, it is left in the field after harvest. Utilization of rapeseed straw depends on the under-

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standing of its composition in order to develop suitable processing technology for the production of biofuel or biochemicals. Mature straw is mainly consisted of secondary cell wall with three type of polymers-cellulose, hemicellulose and lignin. Extensive work has been done with cereal crops, different cell wall polymer features have been shown to influence biomass digestibility. For example, cellulose features including crystallinity index (CrI) and degree of polymerization (DP) have been characterized as major features affecting biomass enzymatic hydrolysis (Zhang et al., 2013). For hemicellulose, the arabinose substitution on xylan backbone has been shown to be a positive factor for biomass digestibility, both in rice and Miscanthus (Xu et al., 2012; Zhang et al., 2013), i.e. more arabinose substitution on xylan backbone would lead to a higher saccharification rate. Lignin is associated with cellulose or hemicellulose to form a cell wall network that is extremely recalcitrant for enzyme penetration and degradation (Achyuthan et al., 2010). Dual effects of lignin on biomass enzymatic hydrolysis has been suggested (Fu et al., 2011; Studer et al., 2011; Wu et al., 2013), but much remains unknown in rapeseed.

Rapeseed (including *Brassica napus*, *Brassica rapa*, *Brassica carinata* and *Brassica junjea*) has different cell wall structure compared to cereal/monocot plants. For example, the major type of side chain substitution on xylan backbone is glucoronoarabinoxylan in rice and Miscanthus, but glucuronoxylan instead in rapeseed. The relative abundance of *p*-coumaryl alcohol (H-lignin), coniferyl alcohol (G-lignin), and sinapyl alcohol (S-lignin) is also significantly different. The fundamental cell wall composition difference between monocot and dicot plants is reflected by the impact of pectin, hemicellulose and lignin on enzymatic hydrolysis after various pretreatment methods. One of the most frequently used pretreatment involves mild acidic or alkaline pretreatment, which partially removes hemicellulose or lignin and give access of cellulose to cellulase during enzymatic hydrolysis/saccharification (Garlock et al., 2011; Hendriks and Zeeman, 2009; MacDonald et al., 1983). Biomass digestibility can be measured by saccharification rate, which measures hexose released from enzymatic hydrolysis in relation to cellulose content or dry mass of the starting material.

Considerable genotypic and phenotypic variation exists within *Brassica* species; some of these genetic differences would also influence lignocellulosic cell wall composition (Luo et al., 2011). Although process-dependent differences have been explored using *B. napus* straw (Wood et al., 2014; Ryden et al., 2014), little is known about how variations in straw cell wall polymer composition would influence enzymatic saccharification and biofuel production. Studies using steam explosion-pretreated rapeseed straw has revealed that retention of uronic acid from pectin fractionation and removal of xylose from hemicellulose were important for initial hydrolysis rate and overall reducing sugar yield (Wood et al., 2014). It has been shown that variation exists between different *B. napus* cultivars on fermentation inhibitor released upon steam-explosion pretreatment (Wood et al., 2015).

The purpose of this work is to determine differences in rapeseed straw cell wall composition between different cultivars, but also to correlate the differences of cell wall polymer features with enzymatic saccharification to reveal key structural determinant for biomass digestibility. To do this, straw samples from 19 rapeseed cultivars from four *Brassica* species were subjected to sulfuric acid or lime pretreatment followed by enzymatic saccharification. Correlation analysis was performed between the cell wall compositions of different rapeseed straw samples with saccharification efficiency and between cell wall polymer features. In detail, level of cellulose, hemicellulose and lignin content, hemicellulose monosaccharide composition, and G, H, S-monolignin contents were correlated with saccharification efficiency following H_2SO_4 or CaO pretreatment, to determine the most important cell wall feature for rapeseed straw biomass digestibility. Yeast fermentation and bioethanol production was also determined in a subset of samples, to illustrate the impact of saccharification efficiency on bioethanol production. Our data suggested that galactose of hemicellulose and G-lignin monomer content are the two most predominant factors influencing biomass digestibility in rapeseed straw. The mechanism of how these cell wall features influence rapeseed straw digestibility is discussed.

2. Methods

2.1. Collection of rapeseed straw samples

A total of 19 rapeseed cultivars were grown in Hubei experimental field in 2013, and the mature straw was harvested from 200 to 220 days after sowing. The straw tissues were dried at 50 °C for at least 1 week until the dry weight is constant. The dry straw was ground into powders, passed through a 40 mesh screen, and stored in a sealed falcon tubes until further use. For each rapeseed straw, at least three biological replicates were prepared for composition analysis.

2.2. Plant cell wall fractionation

The procedure for cell wall fractionation was described by Peng et al. (2000) and modified by Wu et al. (2013). The soluble sugar, lipid, starch and pectin were removed by potassium phosphate buffer (pH 7.0) followed by extraction with chloroform–methanol (1:1, v/v), DMSO–water (9:1, v/v) and 0.5% (w/v) ammonium oxalate. The remaining pellet was extracted with 4 M KOH with 1.0 mg/mL sodium borohydride, followed by extraction with H_2SO_4 (67%, v/v) to completely dissolve crystalline cellulose. Hexoses and pentoses released after each extraction step were quantified later by colorimetric assay. Hemicellulose content was calculated based on hexose and pentose released during 4 M KOH extraction, and pentose released from 67% H_2SO_4 extraction. Cellulose content was calculated according to hexose released from 67% H_2SO_4 extraction. The experiments were conducted in triplicate.

2.3. Colorimetric assay of hexose and pentose

Hexoses were quantified by the anthrone/H_2SO_4 method (Fry, 1988), and pentoses by orcinol/HCl method (Dische, 1962). UV–VIS spectro-photometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China) was used for absorbance measurement. D-glucose and D-xylose (Sinopharm Chemical Reagent Co., Ltd.) were used as standard for hexose and pentose. Considering the pentose can affect hexose readings at 620 nm, deduction of pentose was carried out at 660 nm and a calibration curve was conducted to correct for hexose values with pentose values. All experiments were performed in triplicate.

2.4. Determination of hemicellulose monosaccharide composition by GC–MS

Monosaccharide composition of hemicellulose was determined by GC–MS as described previously (Xu et al., 2012). The hemicellulose fraction was dissolved in 0.5 mL 2 M TFA and heated in a sealed tube at 121 °C for 1 h. 2.00 mg/mL *myo*-inositol was used as internal standard. 800 μL distilled water and 400 μL freshly made $NaBH_4$ solution (100 mg/mL in 6.5 M aqueous NH_3) were added to cooled sample. Sample was capped, mixed well and incubated at 40 °C for 30 min. Excess $NaBH_4$ was decomposed by addition of 800 μL absolute acetic acid. 400 μL sample was transferred to a 25 mL glass tube. 4 mL acetic anhydride was added, followed

by 600 μL 1-methylimidazole. The sample was allowed to stand for 10 min, and excess acetic anhydride was decomposed by adding 10 mL distilled water. Then dichloromethane (3 mL) was added, mixed gently, and centrifuged. The upper phase was discarded, the lower phase was washed three times with 20 mL distilled water each time. Lower phase was collected and dehydrated by adding anhydrous sodium sulfate and stored at −20 °C.

SHIMADZU GCMS-QP2010 Plus was used for GC–MS analysis. Analytical Conditions: Restek Rxi-5ms, 30 m × 0.25 μm df column; carrier gas: helium; injection method: split; injection port: 250 °C; interface: 250 °C; injection volume: 1.0 μL; temperature program: from 170 °C (held for 12 min) to 220 °C (held for 8 min) at 3 °C/min; ion source temperature: 200 °C; ACQ mode: SIM. The mass spectrometer was operated in EI mode with ionization energy of 70 eV. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 m/z in 0.45 s. Calibration curves of all analytes routinely yielded correlation coefficients of at least 0.999.

2.5. Quantification of total lignin and lignin monomers

Total lignin level of biomass samples was detected by two-step acid hydrolysis method according to NREL analytical LAP protocol (Sluiter et al., 2008). The acid-insoluble lignin was calculated gravimetrically after correction for ash, and the acid-soluble lignin was measured using UV spectroscopy. Monolignin determination was essentially according to Li et al. (2014b). H-lignin, G-lignin and S-lignin were purchased from Sinopharm Chemical Reagent Co., Ltd. as standards during HPLC analysis. Kromat Universal C18 column (4.6 mm × 250 mm, 5 μm) was used for HPLC analysis with SHIMADZU LC-20A machine with a UV-detector at 280 nm. CH_3OH:H_2O:HAc (16:63:1, v/v/v) was used as mobile phase (flow rate: 1.1 mL/min), the injection volume was 20 μL. All experiments were carried out in triplicate.

2.6. Analysis of cellulose CrI and DP

Crystalline cellulose was prepared according to Zhang et al. (2013). CrI was measured by X-ray diffraction (XRD) method using Rigaku-D/MAX instrument (Ultima III, Japan). The CrI was estimated using the equation: $CrI = 100 \times (I_{200} - I_{am})/I_{200}$. I_{200} is intensity of the 200 peak (I_{200}, $\theta = 22.5°$), which represents crystalline cellulose. I_{am} (I_{am}, $\theta = 18.5°$) is the intensity at the minimum between the 200 and 110 peaks, which corresponds to amorphous cellulose. The routine STDEV of CrI measurement was at the level of 0.05–0.15 (%). DP was determined using the viscosity method subjective to the equation: $DP^{0.905} = 0.75\ [\eta]$ as described previously (Li et al., 2014a). All experiments were performed in triplicate at 25 ± 0.5 °C using an Ubbelohde viscosity meter.

2.7. Rapeseed straw pretreatment and enzymatic hydrolysis

Acidic or alkaline pretreatment were described by Huang et al. (2012) with minor modifications. H_2SO_4 pretreatment: the well-mixed powder of the rapeseed straw sample (0.3000 g) was added with 6.0 mL H_2SO_4 at three concentrations (1%, 2%, 4%, v/v), respectively. A tube with 6.0 mL distilled water was used as control. The tube was sealed and heated at 121 °C for 20 min, shaken at 150 rpm for 2 h at 50 °C and centrifuged. The supernatant was collected and 1.0 mL was used to measure hexose released during pretreatment. The pellet was washed at least three times with distilled water until pH 7.0, and kept at −20 °C for enzymatic hydrolysis. All experiments were carried out in triplicate. CaO pretreatment: the well-mixed powder of rapeseed straw sample (0.3000 g) was pretreated with four concentrations of CaO solutions (2.5%, 5.0%, 7.5%, 10.0% w/w). The sample tube with CaO solution was sealed and shaken at 50 °C, 150 rpm for 48 h. The supernatant was neutralized to pH 7.0 with H_2SO_4, and shaken at 50 °C for 2 h then centrifuged. Supernatant was used for hexose measurement, and pellet was washed by distilled water until neutral and stored in −20 °C.

The pellets from the above pretreatments were washed 5 times with distilled water, once with 10 mL cellulase reaction buffer (0.2 M acetic acid–sodium acetate, pH 4.8), and added with 1.6 g/L crude cellulase (Imperial Jade Bio-technology Co., Ltd, containing β-glucanase $\geqslant 2.98 \times 10^4$ U, cellulase $\geqslant 298$ U, and xylanase $\geqslant 4.8 \times 10^4$ U) in a final volume of 6 mL. A tube with 6 mL reaction buffer was used as control. Samples were shaken at 50 °C, 150 rpm for 48 h. After centrifugation, supernatant was collected for hexose measurement. All experiments were carried out in triplicate.

2.8. Yeast fermentation and bioethanol yield determination

Yeast fermentation was performed with *Saccharomyces cerevisiae* (purchased from Angel yeast Co., Ltd., Yichang, China) according to Li et al. (2014b). The sample pretreatment method with 1% H_2SO_4 or 5% CaO was the same as described above. The pretreated-samples were adjusted to pH 4.8, crude-cellulases was added to a final concentration of 1.6 g/L. The fermentation medium consists of supernatants and the pellets after pretreatment and sequential enzymatic hydrolysis. *S. cerevisiae* was added to a final concentration of 0.5 g/L, fermentation was performed at 37 °C for 48 h. The fermentation liquid was distilled at 100 °C to collect ethanol liquor. Ethanol was measured by $K_2Cr_2O_7$ method (Fletcher and van Staden, 2003). Absolute ethanol was used for standard curve.

The sugar-bioethanol conversion rate was calculated according to the formula: $S\text{–}E = E/A/H \times 100\%$ [S–E: sugar to ethanol conversion rate; E: total ethanol weigh (g) at the end of fermentation; $A = 51.11\%$ according to theoretical conversion rate from glucose to ethanol in *S. cerevisiae*; H: total hexose weight (g) at the beginning of fermentation]. All experiments were carried out in triplicate.

2.9. Calculation of correlation coefficient

Correlation coefficients were calculated by SPSS software (SPSS 17.0) for all pairs of parameters. Correlation coefficient values were calculated by performing Spearman rank correlation analysis for all pairs of the measured aspects (or traits, factors) across the whole populations. The measured aspects were derived from the average values of duplications. The box plot, histogram and line graph presented in the study were generated by using software (Origin 8.0).

3. Results and discussion

3.1. Diversity of cell wall polymers in rapeseed straw samples

According to the classification of *Brassica* genus, rapeseed cultivars present today belongs to four different species: *B. napus* (*Bn*), *B. rapa* (*Br*), *B. carinata* (*Bc*) and *B. junjea* (*Bj*). Among those, *B. napus* and *B. rapa* are the most widely grown species for rapeseed cultivation worldwide. In this study, we collected 19 rapeseed straws, which included 7 *Bn* cultivars, 5 *Br* cultivars, 5 *Bj* cultivars and two *Bc* cultivars (Table A.1). Mature straw was collected and cell wall polymer composition of the raw material was analyzed (Fig. 1A). A diverse cell wall composition (cellulose, hemicelluloses, and lignin) was observed between different straw samples. The coefficient of variation (CV) values for cellulose, hemicellulose, and lignin were 51.3%, 17.3% and 44.0%, respectively (Table A.1).

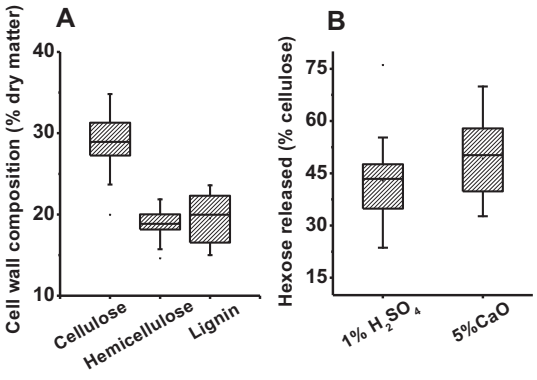

Fig. 1. Variation of cell wall composition and enzymatic saccharification in 19 rapeseed straw samples. (A) Diversity of three major cell wall polymers-cellulose, hemicellulose and lignin. (B) Hexoses released from enzymatic saccharification after 1% H_2SO_4 or 5% CaO pretreatment.

Content of cellulose and hemicellulose were not significantly different between the four rapeseed species, however total lignin content in *B. junjea* and *B. carinata* rapeseed straws was significantly higher ($P < 0.01$) than that from *B. napus* and *B. rapa* rapeseed straw (Fig. 2). Lignin content is a very important cell wall feature for rapeseed, higher lignin level could affect both physical parameter of the mature stem and lodging index. The large variation on cell wall polymer composition offers a possibility of analyzing correlation of cell wall composition with biomass digestibility.

3.2. Effects of wall polymers on biomass enzymatic digestibility

We have shown in previous studies that biomass enzymatic digestibility (enzymatic saccharification) can be estimated by calculating the hexose yield (% cellulose) released during enzymatic hydrolysis of pretreated lignocellulosic material (Xu et al., 2012). In the present study, saccharification rates of rapeseed straw after 1% H_2SO_4 pretreatment or 5% lime pretreatment were determined (Fig. 1B). The rapeseed straw samples displayed large variation of hexoses yield from 23.65% to 76.10% in H_2SO_4-pretreated samples, while the hexose yield in lime pretreated samples varied from 32.64% to 69.91% (Table A.2). Compared with previous studies of enzymatic saccharification with 1% H_2SO_4 pretreatment, rapeseed straw had a relatively higher biomass digestibility compared to wheat and Miscanthus as lignocellulosic materials (Xu et al., 2012).

Hemicellulose content, especially the extent of side chain substitution by arabinose or other sugar modifications, has been demonstrated to be a dominant positive factor for biomass digestibility in wheat, rice and Miscanthus (Li et al., 2013; Xu et al., 2012). The hemicellulose side chain substitution would lead to lower CrI, which would offer favorable access of cellulase enzymes to the substrate and therefore higher biomass digestibility. Another correlation of cell wall polymers was shown between lignin level and biomass digestibility, although the situation for lignin seems to be more complicated and different in monocot and dicot plants (Fu et al., 2011). Correlation analysis was performed between cellulose, hemicellulose and lignin content from 19 rapeseed straw raw material and enzymatic saccharification rate (hexose released as% cellulose of raw material) after H_2SO_4 or lime pretreatment and sequential enzymatic hydrolysis (Fig. 3). As a result, the cellulose level was not correlated with the hexose yield from either pretreatments (Fig. 3A and D), but hemicellulose showed negative correlation in both cases (Fig. 3B and E). A negative correlation between lignin level and hexose yield was revealed both in H_2SO_4- and lime-pretreated rapeseed straw samples (Fig. 3C and F). The negative correlations between hemicellulose and lignin content with hexose yield were quite strong (at $P < 0.01$), suggesting that higher lignin content or hemicellulose would be unfavorable for a better biomass digestibility in rapeseed straw.

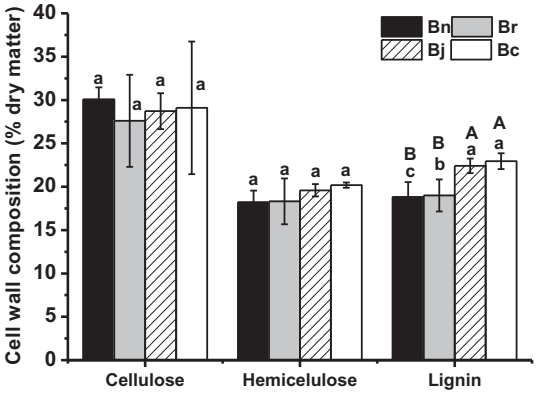

Fig. 2. Comparison of cell wall polymers between different rapeseed species. Small letters indicate significance at $P < 0.05$, capital letters indicate significance at $P < 0.01$.

3.3. Correlation of mono lignin and hemicellulose sugar composition with biomass digestibility

Given their structural diversity and chemical heterogeneity of lignin, evaluation of lignin effect on biomass digestibility could be difficult (Fu et al., 2011; Xie and Peng, 2011). Mono lignin content of the 19 rapeseed straw samples was determined (Table 1). In contrast to cereal plant, G-lignin and S-lignin are the major mono lignin in rapeseed cell wall, these two constitute more than 95% of total lignin (Table 1). When the mono lignin content was correlated with rapeseed straw digestibility, a strong negative correlation was found between G-lignin and enzymatic saccharification rate (Fig. 4A). S-lignin also showed a significant negative correlation in H_2SO_4 pretreated rapeseed straw ($P < 0.05$, $n = 19$), but not in lime pretreated samples. The reason why there was a difference for S-lignin effect on rapeseed straw sample digestibility after H_2SO_4 or CaO pretreatment is most likely due to the different mechanism of acidic versus alkaline pretreatment for cell wall destruction. The S-lignin effect on biomass digestibility, as measured by hexose released during pretreatment and crude cellulose hydrolysis, is probably through hemicellulose–cellulose network rigidity, therefore acidic pretreated samples was more affected than alkaline pretreated samples.

Hemicellulose sugar composition is also significantly different between monocot and dicot (Pauly and Keegstra, 2010). Monosaccharide composition in rapeseed straw samples was determined by GC–MS (Table 2). Hemicellulose side chain substitution rate (xylose/arabinose, Xyl/Ara) was also included in Table 2. When correlation analysis was performed with hemicellulose monosaccharides with enzymatic saccharification, rhamnose (Rha), arabinose (Ara), fucose (Fuc) and galactose (Gal) showed positive correlation both in H_2SO_4- and lime-pretreated samples (Fig. 4B). Under both pretreatments, the highest correlation coefficient was observed for Fuc, followed by Gal, Ara and Rha, respectively (Fig. 4B). Interestingly, although arabinose is not the major side chain substitution for xylan in dicot plant, a negative correlation was observed between Xyl/Ara and enzymatic saccharification (Fig. 4B), as previously shown in Rice and Miscanthus (Li et al., 2015; Wu et al., 2013). It is possible that arabinose monosaccharide from hemicellulose extraction represents a more branched conformation of cellulose–hemicellulose–lignin cell wall polymer network, which

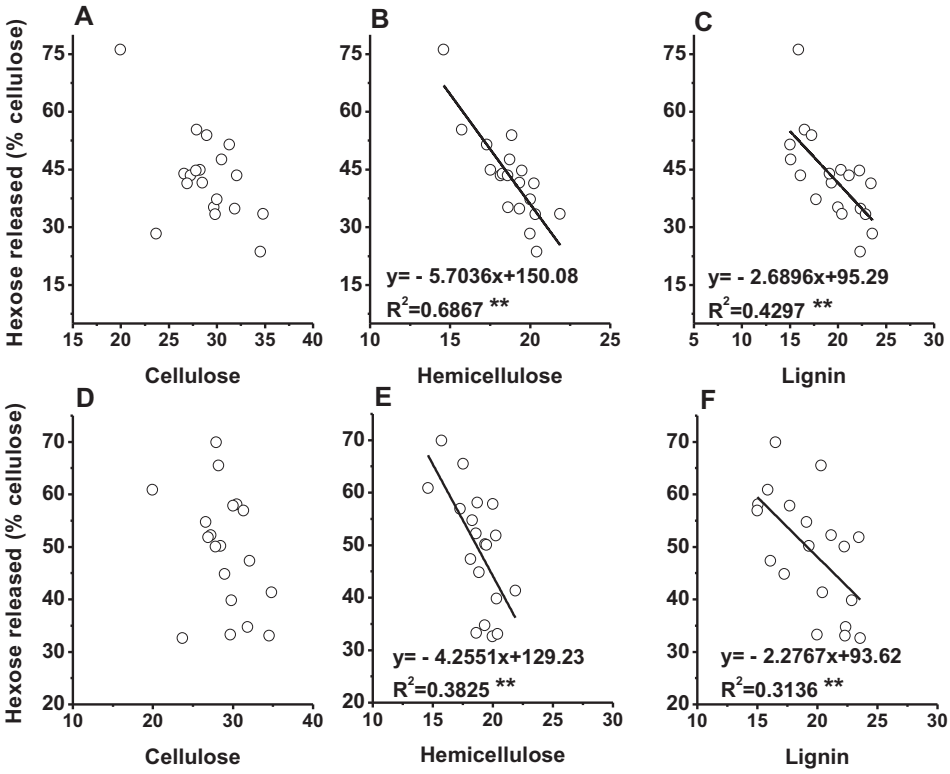

Fig. 3. Correlation coefficient between cell wall polymer content and enzymatic saccharification after 1% H_2SO_4 (A–C) or 5% CaO (D–F) pretreatment ($n = 19$). * and ** indicated significant correlation at $P < 0.05$ and 0.01, respectively.

Table 1
Mono-lignin content (μmol/g dry mass) of 19 rapeseed straw samples.

	H	G	S	H/G	S/G	S/H	H (%)	G (%)	S (%)
Bn01	22.84	527.94	640.78	0.04	1.21	28.05	1.92	44.31	53.78
Bn02	20.48	349.23	521.43	0.06	1.49	25.46	2.30	39.19	58.51
Bn04	15.79	405.34	622.44	0.04	1.54	39.42	1.51	38.84	59.65
Bn06	11.72	391.53	548.93	0.03	1.40	46.83	1.23	41.12	57.65
Bn09	14.21	363.13	510.80	0.04	1.41	35.93	1.60	40.89	57.51
Bn18	23.29	417.05	543.35	0.06	1.30	23.33	2.37	42.40	55.24
Bn10	12.94	494.50	525.24	0.03	1.06	40.59	1.25	47.89	50.86
Br01	18.08	484.21	643.46	0.04	1.33	35.59	1.58	42.26	56.16
Br02	18.83	371.55	548.20	0.05	1.48	29.11	2.01	39.59	58.41
Br03	41.93	465.78	631.89	0.09	1.36	15.07	3.68	40.87	55.45
Br04	23.00	539.84	649.18	0.04	1.20	28.22	1.90	44.54	53.56
Br05	22.70	510.25	678.85	0.04	1.33	29.90	1.87	42.11	56.02
Bj01	30.34	476.94	808.2	0.06	1.69	26.64	2.24	44.57	53.20
Bj02	11.09	393.00	825.33	0.03	2.10	74.40	0.90	31.97	67.13
Bj03	11.53	482.50	876.42	0.02	1.82	75.99	0.84	35.21	63.95
Bj04	12.33	570.91	770.00	0.02	1.35	62.43	0.91	42.19	56.90
Bj05	8.25	532.81	770.22	0.02	1.45	93.38	0.63	40.63	58.74
Bc01	31.47	627.59	749.08	0.05	1.19	23.80	2.24	44.57	53.20
Bc02	36.09	721.65	597.13	0.05	0.83	16.55	2.66	53.26	44.07

would allow more accessibility of enzyme penetration and therefore higher digestibility.

CrI was found to be negatively correlated with enzymatic saccharification, both in H_2SO_4- or CaO-pretreated samples (Fig. 4C and Table A.3). However, DP showed no significant correlation (Fig. 4C).

3.4. Detail analysis of four pairs of rapeseed straw samples

To test the effects of lignin and hemicellulose, in particular effects of G-lignin and hemicellulosic monosaccharide (Fuc, Gal, Ara and Rha) on biomass digestibility, four pairs of rapeseed straw samples were selected based on their cell wall content and biomass digestibility (Table 3). Each pair consisted of two samples with high (H) or low (L) biomass digestibility, difference on cell wall features between samples were highlighted in bold (Table 3). Difference in relevant parameters between samples within each pair was calculated as percentage of the lower value. For simplicity, Gal was chosen as a representative for hemicellulosic monosaccharide. Pair I differed in G-lignin content, pair II in Gal monosaccharide, pair III in hemicellulose content and Gal monosaccharide, pair IV differed in both lignin content, G-lignin and Gal content. The comparison of pair I would indicate G-lignin effect on biomass digestibility, since hemicellulose and lignin content, as well as

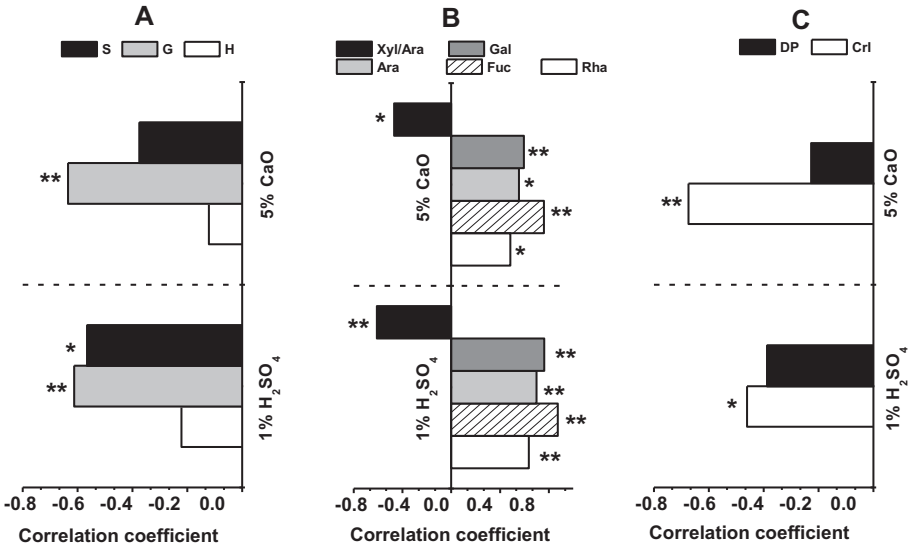

Fig. 4. The correlation between cell wall features with enzymatic saccharification. (A) Lignin monomer, (B) hemicellulose monosaccharides, (C) cellulose CrI, DP.

Table 2
Hemicellulose monosaccharide composition (μmol/g dry mass) of 19 rapeseed straw samples.

	Rha	Fuc	Ara	Xyl	Man	Glu	Gal	Xyl/Ara
Bn01	11.73	n.a.*	21.17	598.41	13.05	33.22	19.94	28.27
Bn02	21.27	3.70	25.14	581.33	10.14	32.88	29.31	23.12
Bn04	17.59	3.00	27.62	942.65	13.09	39.26	27.91	34.13
Bn06	14.14	3.17	21.54	879.00	13.78	38.14	26.90	40.81
Bn09	19.93	3.98	26.32	854.11	15.90	55.63	37.36	32.45
Bn18	25.81	4.80	49.54	957.13	17.66	46.62	42.97	19.32
Bn10	20.55	4.39	29.65	989.37	18.00	59.61	41.78	33.37
Br01	11.91	2.28	16.16	815.19	13.27	41.81	22.70	50.43
Br02	22.81	4.28	35.22	590.90	16.35	48.36	41.02	16.78
Br03	15.49	2.39	20.30	1132.07	16.21	53.86	26.44	55.77
Br04	9.32	2.50	11.26	877.91	14.70	62.45	25.64	77.95
Br05	10.01	2.17	18.45	1235.15	16.67	79.40	26.36	66.94
Bj01	19.69	1.87	12.47	1271.37	22.46	87.37	27.26	101.97
Bj02	19.53	2.49	20.42	996.18	18.55	63.25	23.64	48.79
Bj03	12.42	1.50	20.32	1050.38	13.59	46.98	21.61	51.70
Bj04	10.40	1.58	9.91	973.19	23.74	68.38	24.58	98.23
Bj05	13.56	2.20	16.39	993.72	20.11	63.35	27.18	60.63
Bc01	7.58	0.71	10.36	879.35	9.16	30.44	10.64	84.90
Bc02	16.97	1.23	15.57	738.75	26.02	80.43	20.34	47.45

* n.a.: not available.

hemicellulosic monosaccharides were not significantly different otherwise. Similarly, pair II focused on galactose effect, pair III on both hemicellulose content and Gal, difference on both G-lignin and Gal in pair IV would hopefully reveal whether there was an additive effect on enzymatic saccharification.

Series H_2SO_4 or lime pretreatment was performed on the 4 pairs of rapeseed straw samples. Within the range of H_2SO_4 or CaO pretreatment, hexose release after enzymatic hydrolysis in the sample with higher biomass digestibility was always high, the difference was maintained throughout the series for all pairs of samples (Fig. 5). For H_2SO_4 pretreatment series, the maximum difference occurred at 2% concentration except for pair III (Fig. 5A). The hexose yield between pairs varied from 1.41 to 1.76-fold (Fig. 5A and Table A.4), with pair IV showing the maximum difference (1.76×). Pair IV samples differed in both galactose, G-lignin and total lignin content (Table 3), the hexose yield at different H_2SO_4 concentration between Bn02 and Bn01 was 1.35–1.76-fold (Table A.4). The second affected pair was pair II, where the main difference was Gal-level (Br04 28.6% higher than Bn01). Accordingly, hexose yield from Br04 was 1.28–1.68-fold of that from Bn01 (Table A.4). In contrast, pair I sample which differed only in G-lignin (Bj04 42.8% higher than Bj02), showed a maximum 1.41-fold difference on hexose yield (Table A.4).

For lime pretreatment, the maximum difference occurred mostly at 5% CaO concentration, except for pair III (Fig. 5B). The most affected was pair II (1.36–1.97-fold, Table A.5), followed by pair IV (1.38–1.75-fold, Table A.5), similar to that observed from H_2SO_4 series (Fig. 5A and Table A.4). From both H_2SO_4 and CaO pretreatment series, Gal and G-lignin (and total lignin) content exhibited significant effect on enzymatic saccharification. Based from the fold of change between pairs, Gal was a more prominent factor than G-lignin (compare pair I with pair II), and there was an additive effect between Gal and G-lignin (Fig. 5, Tables A.4 and A.5).

3.5. Impact of enzymatic hydrolysis on bioethanol production in rapeseed

The ultimate goal for improvement on hexose yield after pretreatment and enzymatic hydrolysis is for biofuel production via yeast fermentation (Lopez-Linares et al., 2015). To compare the influence of enzymatic hydrolysis on bioethanol production, we chose two samples (Bn18 and Bj04) with a vast difference on enzymatic saccharification, to compare their ethanol yield upon yeast fermentation. Bn18 had the highest hexose yield (55.29%) and Bj04 had the lowest (33.38%) at 1% H_2SO_4 pretreatment (Table A.4). If there is no difference on inhibitor production during the pretreatment and enzymatic hydrolysis, the difference on saccharification would be reflected or even amplified on bioethanol production. If there is an increase or decrease of ethanol yield between these two samples, that is most likely due to the production of inhibitors that was different between the two samples. 1% H_2SO_4 and 5% CaO pretreatment was used followed by enzymatic hydrolysis with crude cellulose mixture at 1.6 g/L final concentration. Hexose released during pretreatment and enzymatic hydrolysis was collected together, and the mixture was inoculated with *S. cerevisiae* at a final concentration of 0.5 g/L for fermentation. The ethanol yield varied from 7.76 to 10.63 g/100 g biomass, the ethanol yield from CaO pretreatment was slightly higher than that from H_2SO_4 pretreatment. The enzymatic saccharification after 1% H_2SO_4 pretreatment was 55.29% in Bn18 and 33.38% in Bj04, in contrast the ethanol yield from Bn18 was only 22% higher than Bj04

Table 3
Cell wall features of 4 pairs of selected rapeseed straw samples.

Group	Accession	Cellulose (g/100 g biomass)	Hemicellulose (g/100 g biomass)	Diff.[b] (%)	Lignin (g/100 g biomass)	Diff. (%)	G (μmol/g dry mass)	Diff. (%)	Gal (μmol/g dry mass)	Diff. (%)
I	Bj04(L)[a]	29.81 ± 0.87	20.32 ± 2.08		22.86 ± 0.72		**570.91**	**42.8**	24.58	
	Bj02(H)	27.25 ± 1.38	18.59 ± 0.67		21.15 ± 0.71		**399.69**		23.64	
II	Bn01(L)	29.66 ± 1.34	18.62 ± 1.86		19.98 ± 0.29		527.94		**19.94**	**−28.6**
	Br04(H)	28.22 ± 0.79	17.53 ± 0.63		20.32 ± 0.35		539.84		**25.64**	
III	Bn04(L)	29.13 ± 0.37	**20.01 ± 0.35****	**27.3**[b]	17.70 ± 0.51		405.34		**27.91**	**−54.0**
	Bn18(H)	27.89 ± 1.03	**15.72 ± 0.66**		16.53 ± 0.13		417.05		**42.97**	
IV	Bn01(L)	29.66 ± 1.34	18.62 ± 1.86		**19.98 ± 0.29****	**32.7**	**527.94**	**51.1**	**19.94**	**−47.0**
	Bn02(H)	30.49 ± 1.55	18.72 ± 0.95		**15.06 ± 0.20**		**349.23**		**29.31**	

± stands for standard deviation of three technical replicates.
[a] (H) or (L) indicated the sample in the pair with relatively high (H) or low (L) biomass digestibility.
[b] Percentage difference (Diff.) is calculated by pair-difference within each pair divided by low values.
** A significant difference by *t*-test at $P < 0.01$ ($n = 3$).

(Table 4). For 5% CaO pretreatment, an almost two fold-difference in hexose yield resulted in 33% difference only on ethanol yield (Table 4). In this three-step setup (pretreatment, enzymatic hydrolysis and fermentation), all sugars from pretreatment and enzymatic saccharification steps were combined together, as substrate for yeast fermentation. Enzymatic saccharification was calculated based from hexose released from enzymatic hydrolysis after pretreatment, the sugar-ethanol conversion and enzymatic saccharification were two different parameters to consider during the whole process of biomass–biofuel conversion. In bottom line,

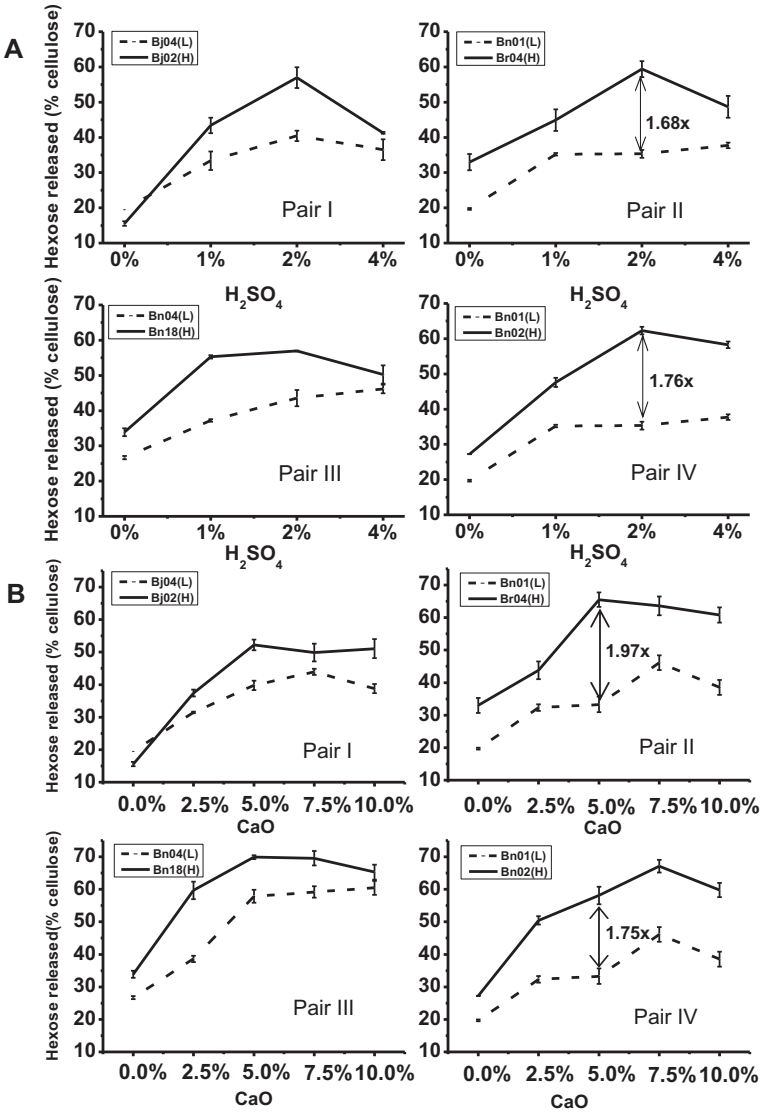

Fig. 5. Hexose yield from enzymatic saccharification of 4 pairs of rapeseed straw samples after series of H_2SO_4 (A) or CaO (B) pretreatments.

Table 4
Yeast fermentation and ethanol yield from two selected rapeseed samples.

Pretreatment	Accession	Enzymatic saccharification (% cellulose)	Hexose released[a] (% dry matter)	Ethanol yield (% dry matter)	Diff.[b] (%)
1% H_2SO_4	Bn18(H)	55.29 ± 0.47	20.85 ± 1.43	9.45 ± 0.21*	22
	Bj04(L)	33.38 ± 2.62	15.29 ± 1.70	7.76 ± 0.53	
5% CaO	Bn18(H)	69.91 ± 0.57	19.62 ± 1.42	10.63 ± 0.75*	33
	Bj04(L)	39.79 ± 1.44	14.49 ± 0.30	8.01 ± 0.12	

± stands for standard deviation of three technical replicates.
[a] Hexose released from pretreatment and enzymatic saccharification.
[b] Diff.: percentage of difference between the two samples within each pair, calculated by subtraction of two samples divided by low value.
* Significant difference between the two samples by *t*-test ($P < 0.05$).

Bn18 material showed a higher enzymatic saccharification rate and higher ethanol yield than the Bj04 material.

In general, hexose released from H_2SO_4 or CaO pretreatment from *B. napus* material was always higher than from *B. junjea* material (Table A.2), suggesting that *B. napus* rapeseed straw might represent a cell wall structure composite that is easier to be degraded. From cell wall composition, a higher lignin level was obvious in *Bj* group compared to *Bn* group (Fig. 2 and Table A.1). Taken consideration of a negative correlation between total lignin and G mono-lignin with enzymatic saccharification, the data is consistent with the idea that a higher lignin and G mono-lignin in rapeseed straw would result in lower biomass digestibility and also bioethanol yield.

3.6. Mechanism of cell wall feature and biomass digestibility in rapeseed

Rapeseeds represent typical dicot plant cell wall, the major hemicellulosic polysaccharide is glucuronoxylan (GX) in secondary cell wall and xyloglucan (XG) in primary cell (Scheller and Ulvskov, 2010), instead in grass walls glucoronoarabinoxylan (GAX) is the major hemicellulosic polysaccharide. Therefore, monosaccharide composition from dicot and monocot walls is different: arabinose content in cell wall material from rapeseed straw is considerably less than from rice and Miscanthus. Xyl/Ara ratio has been used for a measure for hemicellulose side chain substitution, however this might be inappropriate since Ara is not a major hemicellulosic side chain sugar in dicot walls. Studies in steam-explosion treated rapeseed suggested that the initial hydrolysis rate was determined by remaining amount of pectic uronic acid, total sugar yield was mostly dependent on xylan removal from the substrate, and proportion of rapidly hydrolysable carbohydrate was positively correlated with lignin abundance (Wood et al., 2014). However, percentage of H-, G- and S-lignin in rapeseed is different compared to grasses. H-lignin only represents less than 2% of total lignin in cell wall material from mature rapeseed straw, G- and S-lignin together stand for more than 95% of total lignin monomers (Table 1). The percentage of H-lignin in rice, corn and Miscanthus is significantly higher (Jia et al., 2014; Li et al., 2014b; Xu et al., 2012).

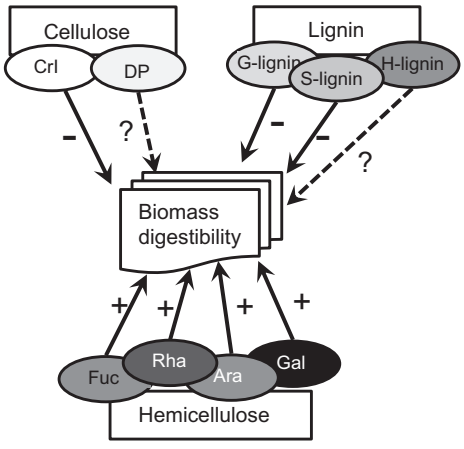

Fig. 6. Influence of cell wall polymer features on rapeseed biomass digestibility. "+" indicated positive effects for biomass digestibility, "–" represented negative effects. Unknown effect was shown by dashed lines with "?".

From correlation analysis between rapeseed cell wall polymer features and enzymatic saccharification, hemicellulose and lignin were found to be negative factors on biomass digestibility in rapeseed cultivars. However, content of hemicellulosic monosaccharides (Fuc, Gal, Ara and Rha) were positively correlated with enzymatic saccharification efficiency. This result might be interpreted by the difference of abundance of hemicellulosic monosaccharides on crystalline cellulose region versus amorphous region. The abundance of hemicellulose is negatively correlated with biomass digestibility, i.e. more hemicellulose coating around cellulose fibers would prevent cellulase access to the substrate. However, certain hemicellulosic monosaccharides on amorphous cellulose region would probably allow more cellulase and other enzyme penetration for cell wall destruction. Therefore, several hemicellulosic monosaccharides, including Fuc, Gal, Ara and Rha, are positive factors on rapeseed biomass digestibility.

It is generally accepted that for cell wall destruction, especially for cellulose microfiber to be accessed by cellulase and other degrading agents, substantial amount of lignin and hemicellulose surrounding cellulose microfibers must be removed. The exact crossing network between cellulose, hemicellulose and lignin is far from clear (Handford, 2006; Scheller and Ulvskov, 2010). With the knowledge now we could only tentatively speculate that certain hemicellulose monosaccharides might be more important for cellulose–hemicellulose–lignin network and therefore secondary cell wall recalcitrance (Fig. 6). We don't have enough information to deduce the network between certain lignin monomers with certain hemicellulosic monosaccharide. Given the extreme complex sugar chain composition of hemicellulose and the potential of G-lignin and S-lignin to crosslink with different sugars, the structure detail underlying the significance of hemicellulosic monosaccharides and G-lignin for rapeseed biomass digestibility is still to be explored.

4. Conclusions

Straw samples from four rapeseed species were analyzed for cell wall composition, enzymatic saccharification after H_2SO_4 or lime pretreatment. Correlation analysis was performed between enzymatic saccharification and cell wall features. Hemicellulose and lignin content (especially G-lignin) showed negative correlation with enzymatic saccharification, whereas several hemicellulosic monosaccharides (Gal, Ara, Fuc and Rha) showed positive correlation. Detail analysis of four pair of samples suggested that

both G-lignin and hemicellulosic monosaccharides were important determinants for rapeseed straw digestibility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.12.072.

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