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# Genotypic effects on sugar and by-products of liquid hydrolysates and on saccharification of acid-insoluble residues from wheat straw

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Wheat straw is one of the major attractive resources for low-cost raw materials for renewable energy, biofuels and biochemicals. However, like other sources of lignocellulosic biomass, straw is a heterogeneous material due to its mixed origin from different tissue and cell types. Here, to examine the genotypic effects on biorefinery usage of wheat straw, straw obtained from different wheat cultivars and experimental lines was pretreated with dilute acid. Significant differences between cultivars were observed in the concentrations of glucose and toxic by-products of the liquid hydrolysates. A higher content of xylose than glucose was found in liquid hydrolysates from wheat straw, and the xylose content appeared to be affected by both environmental and genetic factors. Analysis using chromosome substitution lines of the common wheat cultivar Chinese Spring showed that chromosomes 2A and 3A from other wheat cultivars, Hope and Timstein, significantly increased the xylose content. However, no significant relationship was observed between the liquid hydrolysate xylose content and the glucose content obtained from enzymatic saccharification of the acid-insoluble residue. These results highlight the potential of wheat breeding to improve biomass-related traits in wheat straw.

**Key words:** biomass, biorefinery, chromosome substitution lines, wheat straw

## INTRODUCTION

Agricultural lignocellulosic residues are attractive major resources for the production of renewable energy, biofuels and biochemicals (Ho et al., 2014; Li et al., 2014). Sugar composition and concentration in the lignocellulosic hydrolysates vary widely due to distinct compositions of different sources of lignocellulosic biomass and varying methods of extraction. Wheat straw is an abundant source of lignocellulosic hydrolysates from large-scale wheat cultivation, and contains 30–45% cellulose, 20–30% hemicellulose and 8–15% lignin (Peiji et al., 1997; Saha and Cotta, 2006; Chen and Liu, 2014). Lignocellulosic biomass, even from a single species such as wheat, is heterogeneous (Lindedam et al., 2010, 2012; Larsen et al., 2012; Collins et al., 2014), and its chemical composition may vary due to agronomic conditions, geographical location and local climate in addition to heritable variation (Liu et al., 2010).

For effective biomass utilization, pretreatment is required to break down the structure of the biomass. Much research has studied biorefining technologies for pretreatment, enzymatic saccharification and fermentation of the constituent sugars from wheat straw (Talebnia et al., 2010; Li et al., 2014). Among the various pretreatment methods, dilute acid pretreatment has been widely conducted because of its general inexpensiveness, convenience and effectiveness for a broad range of lignocellu-

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losic biomass (Zheng et al., 2009), and the effect of dilute sulfuric acid pretreatment has also been evaluated in wheat straw (Saha et al., 2005). A slurry obtained from dilute acid pretreatment of wheat straw can be separated into an acid-insoluble residue and liquid hydrolysate; the acid-insoluble residue is mainly composed of cellulose and acid-insoluble lignin (Wei et al., 2012), while the liquid hydrolysate contains glucose, xylose and various by-products (Redding et al., 2011). Recently, the properties of rice straw from many cultivars were evaluated in samples after dilute acid hydrolysis pretreatment (Matsuda et al., 2011; Goda et al., 2016).

Analysis of straw-derived lignocellulosic hydrolysates among a wide range of genetic resources could also provide an important basis for further cereal breeding not only for biofuel production but also for other potentially renewable products. There has been little emphasis on developing the non-food components for biorefining purposes in wheat (Ho et al., 2014), and little information on wheat cultivar differences is available on the use of wheat straw for biorefineries. Among rice cultivars, genetic variation in the liquid hydrolysate glucose content was recently observed (Matsuda et al., 2011; Tanger et al., 2015; Goda et al., 2016). In maize lines, hydrolysis by cellulolytic enzymes varies, and is related to cell wall properties (Li et al., 2015). Thus, genetic variation in the glucose content of dilute acid-pretreated straw liquid hydrolysates should also be observed among wheat cultivars. Here, we applied the method of evaluation of the liquid hydrolysate sugar content of rice straw to wheat straw. Research objectives of the present study were (1) to clarify line differences within hexaploid wheat cultivars and between tetraploid and hexaploid wheat in sugar and by-product composition after dilute acid pretreatment of wheat straw, and (2) to identify the chromosomes controlling the sugar content using chromosome substitution lines.

## MATERIALS AND METHODS

**Plant materials** In this study, one tetraploid wheat accession, *Triticum turgidum* L. ssp. *carthlicum* KU-138, and 11 hexaploid wheat accessions of *T. aestivum* L. were used. Two sets of wheat chromosome substitution lines, CS/Hope and CS/Tim, in which each chromosome pair was substituted with the corresponding homologous pairs from the Hope and Timstein (Tim) donor cultivars in a Chinese Spring (CS) recipient background, were also used. Seeds of the 21 CS/Hope substitution lines (Hope1A to Hope7D, accession numbers KT729 to KT749), and the 21 CS/Tim substitution lines (Tim1A to Tim7D, accession numbers KT708 to KT728), were supplied by the National BioResource Project-Wheat, Japan (http://shigen.nig.ac.jp/wheat/komugi/).

The wheat cultivars and lines were grown in the 2013–2014 and 2014–2015 seasons in a greenhouse of Kobe University. Selfed seeds were sown on the same day in November, and harvested on the same day in May. Spikes were removed, and wheat straw from at least three individuals was powdered using a WB-1 blender (TGK, Tokyo, Japan) fitted with a 2-mm screen. Three biological replicates were conducted for each experiment mentioned below.

**Dilute acid pretreatment** For analysis of the liquid hydrolysates, dilute acid pretreatment was performed according to the method reported previously in rice straw (Goda et al., 2016). The reactor had a total volume of 100 ml and was equipped with an electric heater and was capable of magnetic agitation. Wheat straw powder (150 mg), dried at 80 °C overnight, was suspended in 2 ml 1% (v/v) sulfuric acid, and then incubated at 180 °C for 60 min with agitation at 100 rpm. After pretreatment, the liquid hydrolysate and acid-insoluble residue were separated by centrifugation at 12,000 × *g* for about 5 s. The liquid hydrolysate was neutralized to pH 5.0 by addition of calcium hydroxide powder. The concentrations of sugar and fermentation inhibitors in the liquid hydrolysate were determined by gas chromatography-mass spectrometry (GC-MS).

For both evaluation of the liquid hydrolysates and enzymatic saccharification of the acid-insoluble residues, 6 g of wheat straw powder was first suspended in 80 ml 1% (v/v) sulfuric acid and incubated at 180 °C for 45 min with agitation at 200 rpm, and then incubated at 180 °C for 45 min with agitation at 200 rpm according to Matsuda et al. (2011). The acid-insoluble residue was washed with deionized water, neutralized to pH 7.0, and then dried.

**Measurement of sugar content in liquid hydrolysates** For sugar analysis, each sample (1.5 µl) was mixed with 1.5 µl 0.1% (w/w) ribitol, and the mixture was then dried in a 7810010 vacuum concentrator (Labconco, Kansas City, MO, USA). The dried residue was dissolved in 20 mg/ml methoxyamine hydrochloride in 100 µl pyridine and incubated at 30 °C for 90 min, after which 50 µl N-methyl-N-trimethylsilyltrifluoroacetamide was added and the sample was incubated at 37 °C for 30 min. A 10-µl aliquot of the sample solution was run on a CG-MS-2010 plus system (Shimadzu, Kyoto, Japan) under the following conditions: column, Agilent CP-Sil 8 CB-MS (30 m × 0.25 mm); carrier gas, helium; injection temperature, 230 °C; oven temperature, 80 °C at $t$ = 0 to 2 min, then to 330 °C at 15 °C/min according to Matsuda et al. (2011).

**Estimation of by-products in liquid hydrolysates** Acetone (900 µl) was added to 100 µl liquid hydrolysate and mixed thoroughly, and then the sample was centri-

fuged at 21,880 × *g* at room temperature for 10 min. An aliquot (10 µl) of the resulting supernatant was analyzed by GC-MS on a GC-MS-2010 plus instrument. The following conditions were used for analysis of 5-hydroxymethyl furfural (5-HMF) and furfural: column, Agilent CP-Sil 8 CB-MS; carrier gas, helium; injection temperature, 250 °C; oven temperature, 50 °C at $t$ = 0 to 5 min, then to 280 °C at 20 °C/min. Formate was analyzed under the following conditions: column, Agilent DB-FFAP; carrier gas, helium; injection temperature, 250 °C; oven temperature, 100 °C at $t$ = 0 to 5 min, then to 230 °C at 10 °C/min.

**Enzymatic saccharification** Enzymatic saccharification of the acid-insoluble residue (10% dry weight) was performed by adding 0.3 M citrate buffer (pH 4.8) and cellulase (Cellic CTec2, Novozymes, Bagsvaerd, Denmark) to the residue at a concentration of 66 filter paper units/g dry biomass. Tetracycline (40 µg/ml) and cycloheximide (30 µg/ml) were added to prevent the growth of microbial contaminants. The reaction mixture was incubated at 50 °C for 72 h in a PPS2000 ChemiStation (Tokyo Rikakikai, Tokyo, Japan) with agitation at 120 rpm. Enzymatic saccharification was stopped by rapid chilling on ice, followed by centrifugation at 21,880 × *g* for 10 min at 4 °C. The sugars in the supernatant were analyzed as described in the sugar analysis section.

## RESULTS

**Variation in soluble sugars and by-products in liquid hydrolysates** In dilute acid treatment, xylose and

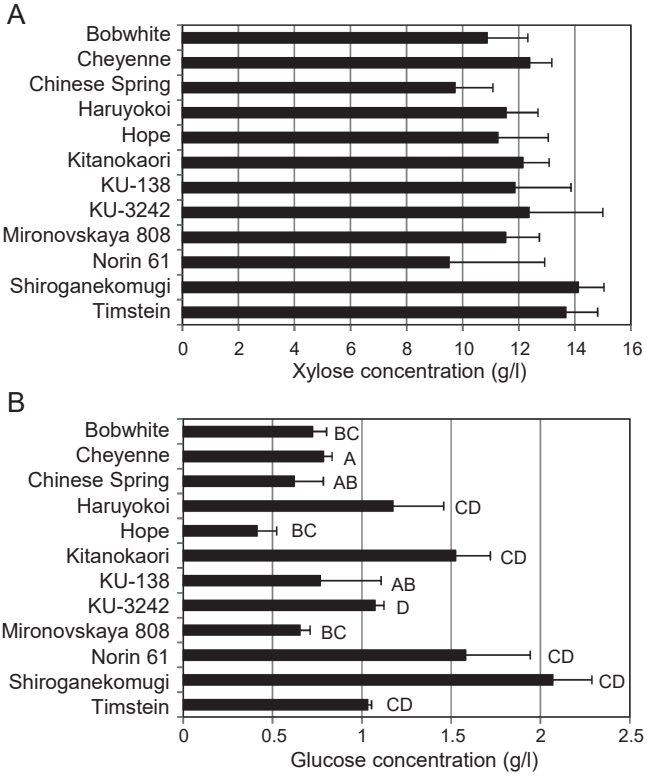


Fig. 1. Concentrations of xylose and glucose in liquid hydrolysates of wheat straw grown in 2014 and pretreated with dilute acid. The levels of xylose (A) and glucose (B) were determined by gas chromatography-mass spectrometry using the external standard method. Error bars are standard deviations over triplicate samples. Means with the same letter were not significantly different ($P$ > 0.05, Tukey-Kramer HSD test).

Table 1. Concentrations of by-products in liquid hydrolysate of wheat cultivars

| Cultivar | Acetic acid (mM) | Formic acid (mM) | Furfural (mM) | 5-HMF (mM) |
|---|---|---|---|---|
| Bobwhite | 6.3 ± 0.4$^{d}$ | 8.0 ± 2.6$^{a}$ | 0.36 ± 0.20$^{b}$ | 0.018 ± 0.013$^{b}$ |
| Cheyenne | 13.2 ± 4.3$^{ab}$ | 5.8 ± 1.9$^{abc}$ | 0.68 ± 0.19$^{b}$ | 0.021 ± 0.006$^{b}$ |
| Chinese Spring | 7.6 ± 0.6$^{cd}$ | 3.6 ± 1.7$^{bc}$ | 0.17 ± 0.21$^{b}$ | 0.007 ± 0.008$^{b}$ |
| Haruyokoi | 12.4 ± 0.0$^{abc}$ | 3.1 ± 0.8$^{c}$ | 0.55 ± 0.38$^{b}$ | 0.002 ± 0.003$^{b}$ |
| Hope | 9.2 ± 0.4$^{bcd}$ | 2.1 ± 0.4$^{c}$ | 0.27 ± 0.21$^{b}$ | 0.005 ± 0.003$^{b}$ |
| Kitanokaori | 9.8 ± 0.7$^{bcd}$ | 5.0 ± 1.8$^{abc}$ | 1.66 ± 1.14$^{ab}$ | 0.0022 ± 0.018$^{b}$ |
| KU-138 | 13.0 ± 2.2$^{ab}$ | 3.5 ± 2.0$^{bc}$ | 0.32 ± 0.38$^{b}$ | 0.007 ± 0.007$^{b}$ |
| KU-3242 | 12.8 ± 0.7$^{ab}$ | 3.7 ± 0.2$^{bc}$ | 0.70 ± 0.46$^{b}$ | 0.009 ± 0.006$^{b}$ |
| Mironovskaya 808 | 12.4 ± 1.1$^{abc}$ | 2.6 ± 0.2$^{c}$ | 0.38 ± 0.22$^{b}$ | 0.005 ± 0.003$^{b}$ |
| Norin 61 | 12.1 ± 0.0$^{abc}$ | 4.2 ± 0.9$^{abc}$ | 0.61 ± 0.53$^{b}$ | 0.005 ± 0.005$^{b}$ |
| Shiroganekomugi | 10.9 ± 0.5$^{abcd}$ | 7.4 ± 1.7$^{ab}$ | 2.97 ± 2.05$^{a}$ | 0.037 ± 0.026$^{ab}$ |
| Timstein | 15.4 ± 1.2$^{a}$ | 3.5 ± 0.4$^{bc}$ | 0.47 ± 0.15$^{b}$ | 0.0063 ± 0.021$^{a}$ |
| RSD (%) | 22.2 | 40.4 | 99.9 | 101.1 |

Data are represented as mean ± standard deviation.
Mean values with the same letters were not significantly different ($P$ > 0.05) (Tukey-Kramer's HSD test).

glucose are liberated from hemicellulose and cellulose, respectively (Binod et al., 2010). However, depending on the temperature, the pretreatment usually produces sugar degradation products, such as furfural and 5-HMF, which are inhibitory to fermentative microorganisms (Palmqvist and Hahn-Hägerdal, 2000). In this study, we treated wheat straw with dilute acid under the same conditions as rice straw (Matsuda et al., 2011; Goda et al., 2016). These conditions produced good yields of xylose, whereas they generated less glucose and by-products in the liquid hydrolysates from wheat straw (Fig. 1 and Table 1). The amount of glucose varied significantly among cultivars, although wheat straw liquid hydrolysates generally had a small amount of glucose. For example, the glucose level in liquid hydrolysates of Shiroganekomugi (2.1 g/l) was approximately 5 times more than Hope (0.4 g/l) (Fig. 1B), with a relative standard deviation (RSD) of 44.7%. The xylose content also varied in the liquid hydrolysates from wheat straw among cultivars. The xylose content ranged from 9.5 to 14.1 g/l and the RSD was 11.1%. Large variation among wheat cultivars was also observed for several by-products (furfural, 5-HMF, acetic acid and formic acid) released into the liquid hydrolysate, and significant differences in by-product composition were observed in the liquid hydrolysate among the wheat accessions (Table 1). On the other

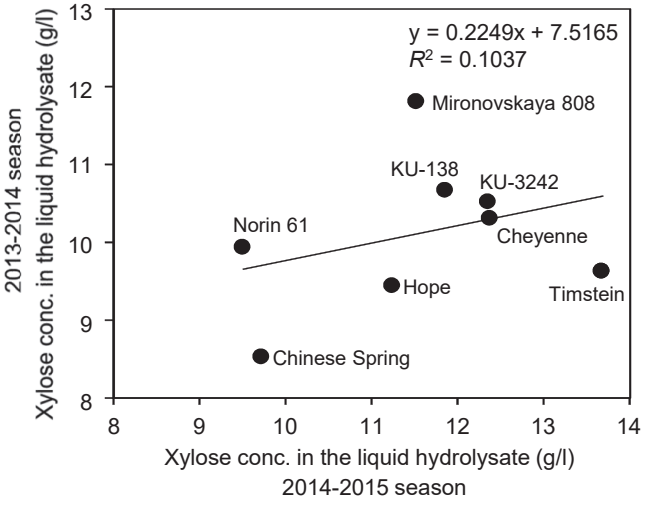


Fig. 2. Comparison of the liquid hydrolysate xylose content in wheat cultivars grown in 2013 and 2014.

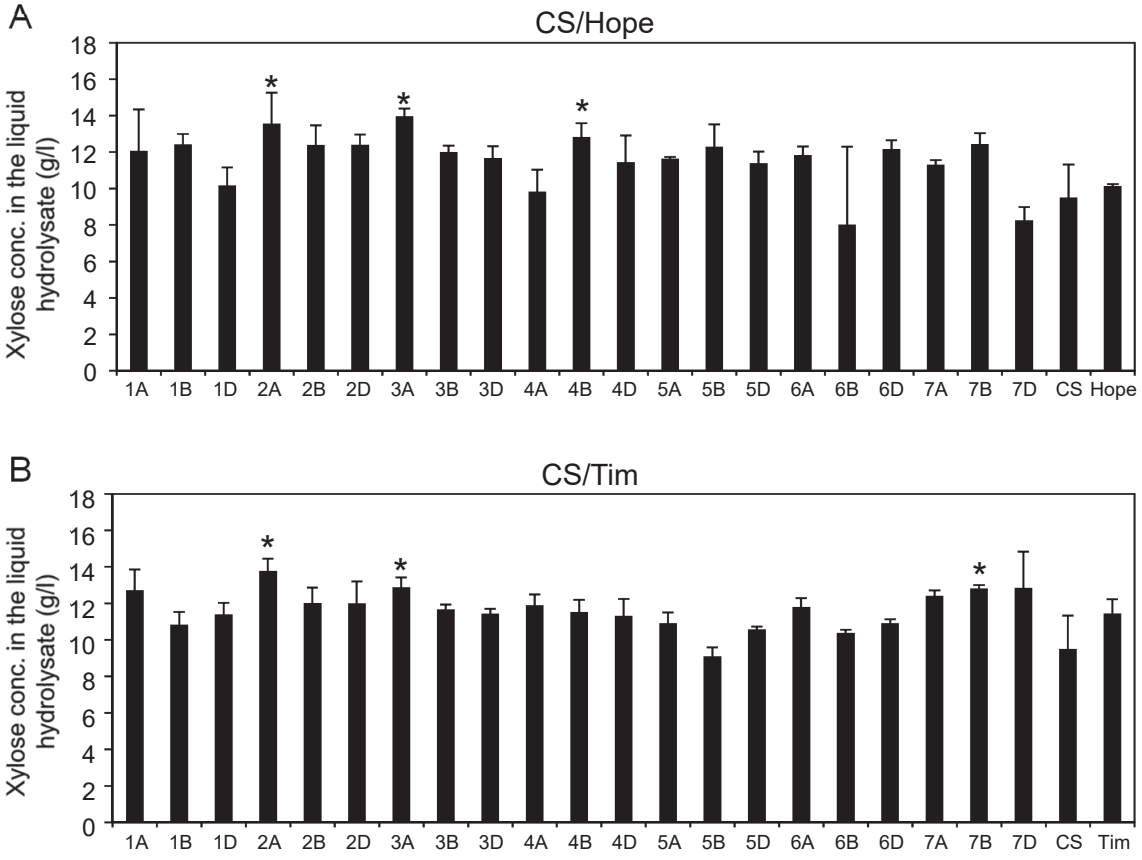


Fig. 3. Concentration of xylose in liquid hydrolysates of two sets of chromosome substitution lines grown in 2014. The mean ± SD of triplicate measurements of xylose content is shown for CS chromosome substitution lines with Hope (A) and Timstein (Tim) (B) chromosomes. Student's *t*-test was used to test for statistical significance (*$P < 0.05$) between CS and each chromosome substitution line.

hand, no clear differences in sugar and by-product composition were found between a tetraploid wheat accession (KU-138) and the 11 hexaploid wheat accessions (Fig. 1 and Table 1).

While narrower variation was observed, more xylose than glucose was obtained from wheat straw (Fig. 1). Therefore, in the following experiments, we analyzed mainly the xylose content in the liquid hydrolysate. The xylose content in the liquid hydrolysate from wheat straw in the 2014–2015 season was compared with that in the 2013–2014 season (Fig. 2) to reveal whether it depended more on genetic or environmental factors. Yearly variation in the xylose content was observed in two of the eight wheat cultivars examined, Cheyenne and Timstein, but the other cultivars showed no significant differences in xylose content between the two seasons. Xylose content of Cheyenne and Timstein grown in the 2014–2015 season was higher than that in the 2013–2014 season (Student's $t$-test, $P < 0.05$ for Cheyenne and $P < 0.01$ for Timstein). In addition, xylose content in the liquid hydrolysates of CS was lower than that in the other cultivars in both years (Fig. 2).

**Chromosomes affecting the xylose content in wheat straw** To identify the wheat chromosomes affecting the xylose content in liquid hydrolysates of straw, we used chromosome substitution lines of CS with a Hope (CS/Hope) and Timstein (CS/Tim) chromosome pair. The xylose content varied in the two sets of chromosome substitution lines, CS/Hope and CS/Tim (Fig. 3). The xylose levels in the liquid hydrolysates from wheat straw of chromosome substitution lines with chromosome 2A of Hope (Hope2A), Hope3A and Hope4B were significantly higher than that of CS (Fig. 3A). Similarly, higher xylose levels were observed in chromosome substitution lines with chromosome 2A of Timstein (Tim2A), Tim3A and Tim7B than in CS (Fig. 3B). Thus, chromosomes 2A and 3A from both Hope and Timstein affected the xylose content in the CS background.

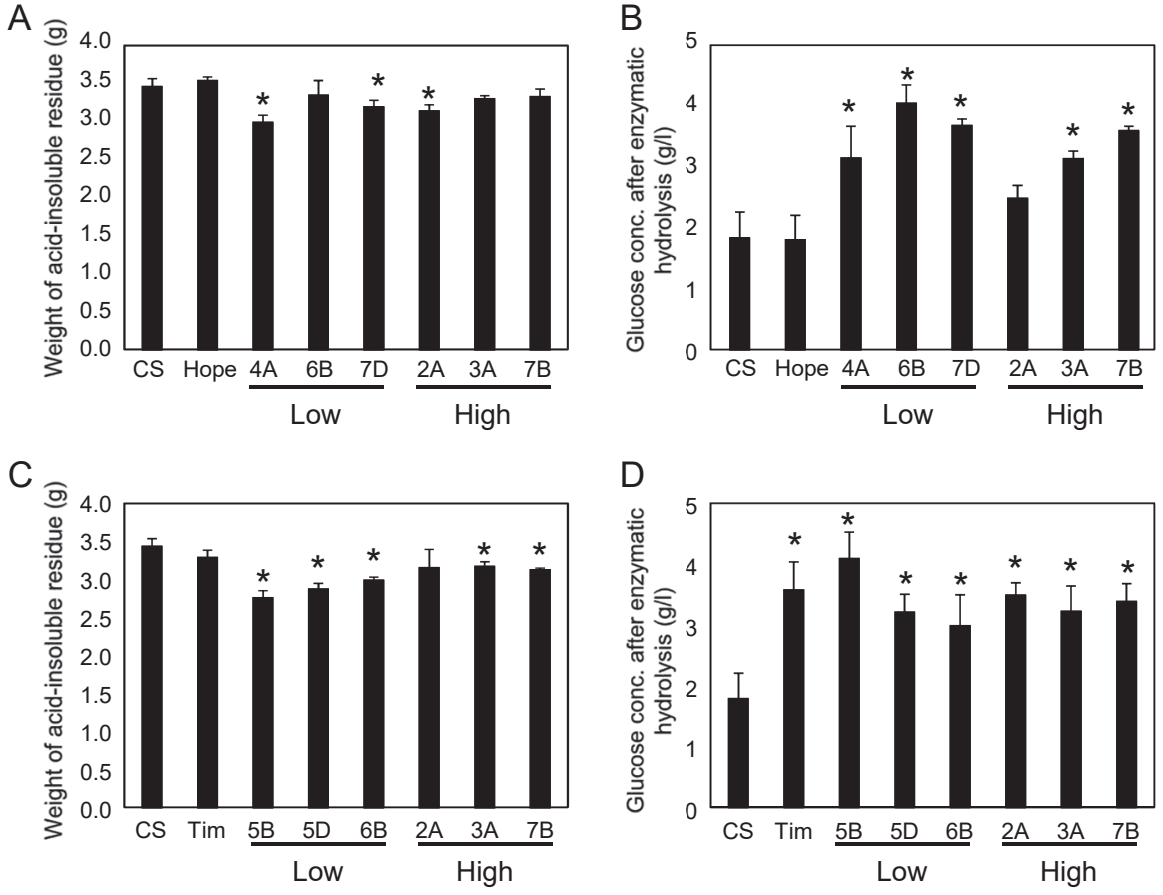


Fig. 4. Relationship between xylose content in the liquid hydrolysate and glucose content after enzymatic hydrolysis. The glucose concentration after enzymatic hydrolysis was analyzed in low (Low) and high (High) xylose content lines. (A, C) Weight of acid-insoluble residues of CS chromosome substitution lines with Hope (A) and Timstein (Tim) (C) chromosomes. (B, D) Glucose content after enzymatic hydrolysis of chromosome substitution lines with Hope (B) and Timstein (Tim) (D) chromosomes. The mean ± SD of triplicate measurements is shown. Student's $t$-test was used to test for statistical significance (*$P < 0.05$) between CS and each chromosome substitution line.

**Glucose content after enzymatic hydrolysis in the acid-insoluble residues** The acid-insoluble residue remaining after separation from the liquid hydrolysate is the target biomass for enzymatic saccharification to obtain glucose (Matsuda et al., 2011). Next, we analyzed the relationship between the glucose content after enzymatic hydrolysis and the xylose content in the liquid hydrolysates using the three lowest and highest xylose content lines from the CS/Hope and CS/Tim chromosome substitution lines. Similar levels of acid-insoluble residue were not necessarily obtained from wheat straw of CS, Hope, Timstein and the 12 chromosome substitution lines (Fig. 4A, 4C). In the CS/Hope substitution lines, the glucose content after enzymatic hydrolysis of the acid-insoluble residue was significantly higher in the high xylose lines, Hope2A, Hope3A and Hope7B, than in CS and Hope (Fig. 4B). A higher glucose content was also observed in the low xylose lines, Hope4A, Hope6B and Hope7D. Moreover, the acid-insoluble residue of Timstein generated higher glucose content after enzymatic hydrolysis than did that of CS (Fig. 4D), although no significant difference in glucose content was observed between CS and Hope (Fig. 4B). The three high xylose lines, Tim2A, Tim3A and Tim7B, and the three low xylose lines, Tim5B, Tim5D and Tim6B, all had significantly higher glucose content after hydrolysis than did CS (Fig. 4D).

## DISCUSSION

There was significant variation among wheat cultivars in term of biomass properties, although these traits are affected by both genetic and environmental factors. In rice, xylose levels were similar to glucose levels in liquid hydrolysates from various types of dilute acid-pretreated straw (Matsuda et al., 2011), whereas the liquid hydrolysate glucose content varied among cultivars (Goda et al., 2016). Here, we applied the same approach to obtain glucose and xylose from wheat straw. Xylose from liquid hydrolysates was more abundant than glucose in wheat straw (Fig. 1A). Similarly to rice, cultivar differences were observed in the glucose content of the liquid hydrolysates (Fig. 1B). Due to the limited glucose content compared with that in rice (Matsuda et al., 2011), however, only the xylose content was examined further in genetic analyses. Organic acid by-products may significantly disturb xylose fermentation (Palmqvist and Hahn-Hägerdal, 2000). In liquid hydrolysates from wheat straw, three by-products, acetic acid, formic acid and furfural, in the liquid hydrolysate showed wide variation among cultivars, whereas little 5-HMF was generated (Table 1). The content of these by-products in rice liquid hydrolysates, obtained by the same method, varied widely among rice cultivars (Matsuda et al., 2011), although the content was much lower in wheat straw hydrolysates. Similarly to the glucose-to-xylose ratio in liquid hydrolysate, therefore, the wheat straw by-products are distinct from those of rice straw. These observations suggest that wheat straw liquid hydrolysate should be a useful resource for obtaining the xylose, and that the method developed in rice straw to extract xylose from liquid hydrolysate works well for this purpose. For glucose extraction from the liquid hydrolysate, further optimization of the dilute sulfuric acid-pretreatment process may be required in wheat straw.

The xylose content in liquid hydrolysates varied to a limited extent in wheat cultivars, and significant correlation was observed between the two growth seasons (Fig. 2). The cultivar differences in xylose content are at least partly affected by genetic factors, with the yearly differences apparently due mainly to environmental factors. Thus, the liquid hydrolysate xylose content is affected by both genetic and environmental factors. Norin 61 and CS, with low levels of xylose in liquid hydrolysates (Fig. 2), are well known as comparatively early-flowering cultivars, implying that the length of the vegetative phase partially contributes to the xylose content in liquid hydrolysates from wheat straw. Xylose content in the liquid hydrolysates varied in both sets of chromosome substitution lines (Fig. 3), suggesting that xylose content is a quantitative trait regulated by multiple loci. Substitution of chromosomes 2A and 3A from Hope and Timstein significantly increased the liquid hydrolysate xylose content in the CS background. Chromosomes 2A and 3A should thus harbor genes controlling the xylose content, with the Hope and Timstein alleles contributing positively. Quantitative trait locus analysis of the xylose content in liquid hydrolysates may identify genetic loci on these chromosomes that are responsible for the cultivar differences.

The acid-insoluble residue remaining after separation from the liquid hydrolysate is the target biomass for enzymatic saccharification to obtain glucose (Matsuda et al., 2011). No significant differences in weight of the acid-insoluble residue or glucose content after enzymatic saccharification were observed between CS and Hope, whereas the glucose content was much higher in Timstein than in CS (Fig. 4). These results indicated the presence of genetic variation among wheat cultivars in the glucose content from the acid-insoluble residue. The glucose content after enzymatic saccharification was compared using selected chromosome substitution lines with high and low xylose content in liquid hydrolysates. However, most of the substituted chromosomes examined contributed to significantly higher glucose content in both the CS/Hope and CS/Tim chromosome substitution lines, indicating no significant relationship of the liquid hydrolysate xylose content to the glucose content after enzymatic hydrolysis of the acid-insoluble residue. The glucose content after enzymatic saccharification is apparently controlled in a

different way from the liquid hydrolysate xylose content in wheat.

Cultivar differences in the liquid hydrolysate xylose content and the glucose content after enzymatic hydrolysis of the acid-insoluble residue imply breeding potential for the development of wheat cultivars with straw that offers high bioethanol productivity. These results provide a basis for future wheat breeding to improve biomass-related traits in wheat straw.

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