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Research Information

Effects of semi-dwarf and glaucousness genes on sugar contents in liquid hydrolysates and saccharificated acid-insoluble residues from wheat straw

Ryoko Ohno[1], Hiroshi Teramura[1], Chiaki Ogino[2], Akihiko Kondo[1,2,3], Shigeo Takumi[4]*

[1]Graduate School of Science, Technology and Innovation, Kobe University, Nada-ku, Kobe, Hyogo 657-8501, Japan
[2]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Nada-ku, Kobe, Hyogo 657-8501, Japan
[3]RIKEN Biomass Engineering Program, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[4]Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe, Hyogo 657-8501, Japan

*Corresponding author: Shigeo Takumi (E-mail: takumi@kobe-u.ac.jp)

Wheat straw is one of the major attractive resources for low-cost raw materials for renewable energy, biofuels and biochemicals. However, there has been little emphasis on developing the non-food components for biorefining purposes in wheat (Ho et al. 2014). For effective biomass utilization, pretreatment is required to break down the structure of the biomass, and dilute acid pretreatment has been applied to wheat straw (Saha et al. 2005). Recent advances in rice showed that variations in the biomass properties after dilute acid-hydrolysis pretreatment of rice straw were observed among cultivars (Matsuda et al. 2011; Goda et al. 2016). Previously, we applied the same approach to obtain glucose and xylose from wheat straw, and found significant variations among wheat cultivars in the biomass properties (Ohno et al. 2017). By-products of organic acids may significantly disturb xylose fermentation (Palmqvist and Hahn-Hägerdal 2000), and the contents of these by-products in rice liquid hydrolysates varied widely among rice cultivars (Matsuda et al. 2011). In liquid hydrolysates from wheat straw, three by-products, acetic acid, formic acid and furfural, in the liquid hydrolysate showed wide variation among cultivars (Ohno et al. 2017).

Plant height has been a main target for wheat breeding, and many semi-dwarfing alleles including *Rht-B1* and *sphaerococcum* (*s*) have been identified and introduced to modern cultivars (Hedden 2003). Cuticular wax production on aerial surfaces of plants called as glaucousness is one of the wheat domestication traits, and the allelic combinations at the *W* and *Iw* loci affect wax morphology on the plant surface and the composition of chemicals in waxes including β-diketones, aldehydes and alcohols (Adamski et al. 2013; Zhang et al. 2013). A recent study showed that wax removal from wheat straw enhanced enzymatic hydrolysis yield (Kádár et al. 2015). These allelic combinations might be related to cultivar differences of structural and chemical components affecting the biomass properties of wheat straw. Therefore, effects of the four genes, *Rht*, *s*, *W* and *Iw*, on the liquid hydrolysate xylose content and the glucose content after enzymatic saccharification of the acid-insoluble residues from wheat straw were examined using near-isogenic lines (NILs).

NILs of hexaploid wheat cultivar Novosibirskaya 67 (ANBW lines) and tetraploid wheat cultivar LD222 (ANDW lines) were used for studies involving plant height and glaucousness (Watanabe et al. 2003). *Rht-B1* and *s* alleles are known semi-dwarfing genes, with *Rht-B1* and *s* respectively assigned to chromosomes 4B and 3D

(Börner et al. 1996; Salina et al. 2000). Cuticular wax production on the leaf and stem surfaces is observed in most wheat cultivars, and glaucousness is mainly controlled by two loci, *W1* and *Iw1* on chromosome 2B, in tetraploid wheat (Tsunewaki 1966; Yoshiya et al. 2011). The dominant allele *W1* controls wax production, and the dominant allele *Iw1* contributes non-glaucousness. Four *Rht-B1* NILs of Novosibirskaya 67, three *s* NILs of LD222, and *w1* and *Iw1* NILs of LD222 were used. The wheat lines were grown in a greenhouse of Kobe University. 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.

For analysis of the liquid hydrolysates, dilute acid pretreatment was performed according to the method reported previously in rice straw (Goda et al. 2016). After pretreatment, the liquid hydrolysate was separated from acid-insoluble residue by centrifugation at 12,000 g for about 5 s, and then the sugar concentrations in the liquid hydrolysate (neutralized to pH 5.0) were determined by gas chromatography-mass spectrometry (GC-MS). Enzymatic saccharification of the acid-insoluble residue was performed according to the method reported previously (Ohno et al. 2017). After addition of 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, the reaction mixture was incubated at 50°C for 72 h with agitation at 120 rpm under thetetracycline- and cycloheximide-containing

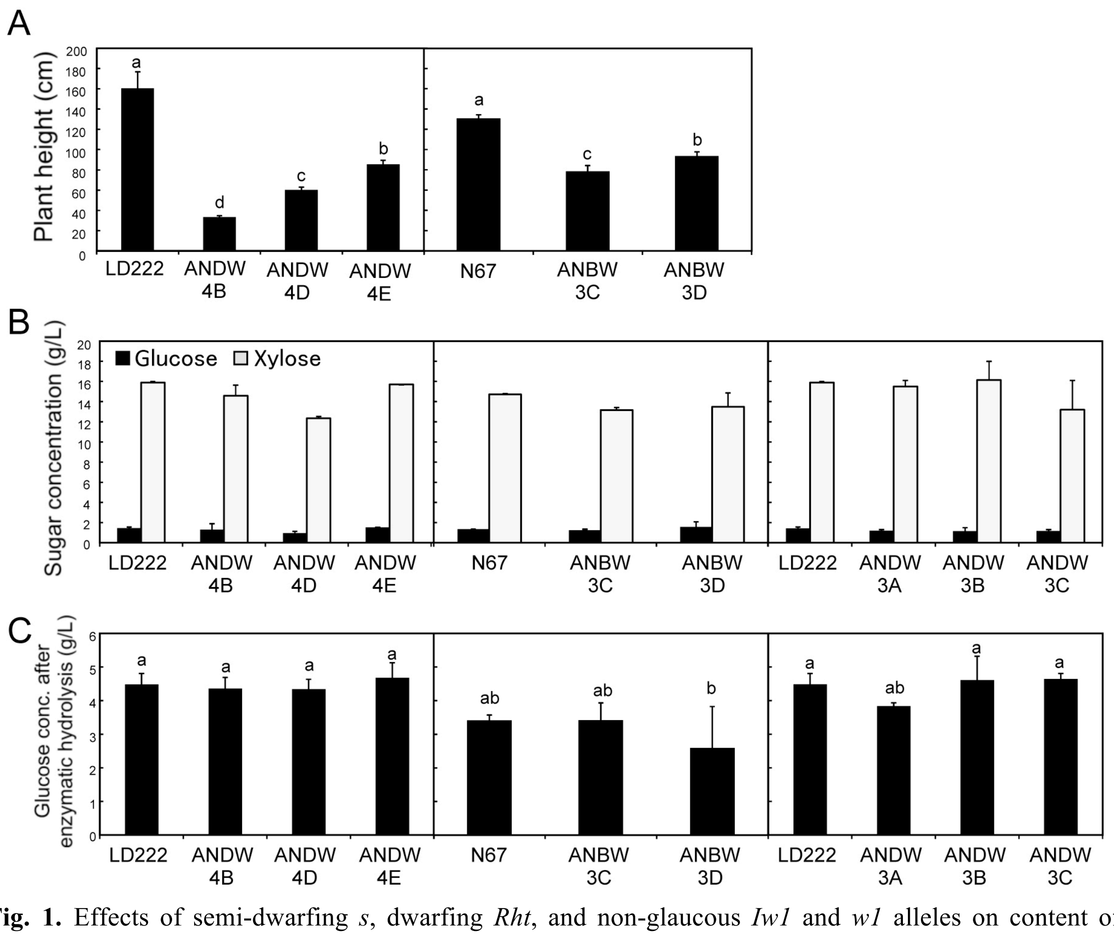

Fig. 1. Effects of semi-dwarfing *s*, dwarfing *Rht*, and non-glaucous *Iw1* and *w1* alleles on content of soluble sugars in liquid hydrolysate and glucose after enzymatic hydrolysis. (A) Comparison of plant height (cm) for the *s* and *Rht* NILs. (B) Concentrations of xylose and glucose in liquid hydrolysate of straw from lines carrying the *s*, *Rht*, *Iw1* and *w1* alleles. (C) Glucose concentration after enzymatic hydrolysis of straw from lines carrying the *s*, *Rht*, *Iw1* and *w1* alleles. Error bars indicate standard deviations over triplicate samples. Means with the same letter were not significantly different ($P > 0.05$, Tukey-Kramer HSD test).

condition.

The sugars in the supernatant were analyzed as previously described (Ohno et al. 2017). Each liquid hydrolysate sample and enzymatically saccharificated sample (1.5 μL) was mixed with 1.5 μL 0.1% (w/w) ribitol, and the mixture was dried. 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-trimethylsilyltrifluoro-acetamide 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 GC-MS-2010 plus system (Shimadzu, Kyoto, Japan) under the condition described previously (Matsuda et al. 2011).

Three NILs of the tetraploid wheat cultivar LD222, ANDW4B, ANDW4D and ANDW4E, contain the *Rht-B1c*, *Rht-B1e* and *Rht-B1f* alleles, respectively. ANDW4B, ANDW4D and ANDW4E showed semi-dwarfism compared with their parental LD222 (Fig. 1A). To examine the effect of structural changes to the stem on content of soluble sugars in liquid hydrolysates and glucose after enzymatic hydrolysis of acid-insoluble residues, NILs for the gibberellin (GA)-insensitive alleles of *Rht-B1* were used. No significant differences in the glucose or xylose level were observed in the liquid hydrolysates of LD222, ANDW4B, ANDW4D or ANDW4E (Fig. 1B), and the glucose content after enzymatic hydrolysis in ANDW4B, ANDW4D and ANDW4E was similar to that in LD222 (Fig. 1C).

Similarly, two NILs of the common wheat cultivar Novosibirskaya 67 for another semi-dwarf allele *s*, ANBW3C and ANBW3D, were used to examine the effects of stem structure. ANBW3C and ANBW3D showed a significant reduction in plant height compared with Novosibirskaya 67 (Fig. 1A). These two NILs showed no significant differences from Novosibirskaya 67 in glucose or xylose level of liquid hydrolysates (Fig. 1B). The two NILs and Novosibirskaya 67 exhibited similar levels of glucose content after enzymatic hydrolysis (Fig. 1C). These results indicated that neither the soluble sugar content in liquid hydrolysates nor the saccharification of acid-insoluble residues is affected by semi-dwarfism in wheat.

In tetraploid wheat, a *W1* dominant allele positively controls wax production, and a *Iw1* dominant allele is sufficient to inhibit the glaucous phenotype even in a *W1*-containing genotype (Tsunewaki and Ebana 1999). To examine effects of the chemical components on plant surfaces on the content of soluble sugars in liquid hydrolysates and glucose from acid-insoluble residues, we used three LD222 NILs for glaucousness, ANDW3A, ANDW3B and ANDW3C. While the genotype of the glaucous accession LD222 is *W1W1iw1iw1*, ANDW3A and ANDW3C have the *W1W1Iw1Iw1* non-glaucous genotype, and ANDW3B shows the *w1w1iw1iw1* non-glaucous genotype. No significant differences in glucose or xylose level in the liquid hydrolysates were observed between LD222 and the three NILs (Fig. 1B). After enzymatic hydrolysis, the three NILs showed similar glucose levels to LD222 (Fig. 1C). Thus, cuticular wax production had no influence on the soluble sugar content in the liquid hydrolysate or on the saccharification of acid-insoluble residue in wheat straw.

Cultivar differences in the liquid hydrolysate xylose content and the glucose content after enzymatic hydrolysis of the acid-insoluble residue imply breeding potential for development of wheat cultivars with straw that offers high bioethanol productivity (Ohno et al. 2017). Here, the effects of plant height and cuticular wax on the sugar content were evaluated in liquid hydrolysates and acid-insoluble residues using NILs. Two traits, plant height and glaucousness, have been targets for selection of new wheat varieties during wheat breeding. The *Rht* and *s* semi-dwarf genes dramatically repress cell elongation in the wheat column (Tsunewaki and Koba 1979; Youssefian et al. 1992), and glaucousness is related to the wax composition on the cuticle of plant aerial organs (Adamski et al. 2013; Zhang et al. 2013). Our results indicate that semi-dwarfism and non-glaucousness have no significant influence on the sugar content of wheat straw. Therefore, cell structure and the chemical components affected by *Rht*, *s*, *Iw* and *w* genes do not appear to be directly related to the biomass properties of wheat straw for bioethanol production, and the effects of these genes are at least limited through the protocol used in the present study. These results provide a basis for future wheat breeding to improve biomass-related traits in wheat straw.

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