A simple method for squeezing juice from rice stems and its use in the high-throughput analysis of sugar content in rice stems

Masaki Okamura, Yoichi Hashida, Tatsuro Hirose, Ryu Ohsugi and Naohiro Aoki

ABSTRACT
Sugar content in rice (Oryza sativa L.) stem is an agronomically important trait for rice used in straw silage or whole-crop silage. However, the mechanisms underlying sugar accumulation in rice stems remain unclear, mainly due to the time-consuming method for measuring sugar content. Here, we established a simple method for squeezing stem juice from rice plants, similar to that used during breeding selection in sugarcane or sorghum. The Brix value of the stem juice, which can easily be measured using a portable refractometer, significantly correlated with the soluble sugar contents in the stem juice and tended to correlate with those in stem tissues. This indicates that the Brix value of the stem juice can be used for estimating the sugar content in rice stems. This simple estimation method will be a useful tool for high-throughput analysis of sugar content in rice stems during mutant screening, QTL analysis, and breeding selection.
(ca. 10 cm long) was immediately rolled up as shown in Figure 1(a), placed in a strong squeezer (KS-10, Fujiwara Scientific, Tokyo, Japan), and squeezed to obtain juice (Figure 1(b) and (c)). The squeezed juice was poured into the measuring hole of a portable refractometer (PAL-1, ATAGO, Tokyo, Japan) and, then, the Brix value was measured (Figure 1(d)).

**Juice squeezing from stems and Brix value determination**

Productive rice stems were harvested from the ground on 17 October 2014 (42, 46, 60, and 64 d after heading for Tachisuzuka, Kusanohoshi, Nipponbare, and agpl1, respectively). The basal portion of a harvested stem (ca. 10 cm long) was immediately rolled up as shown in Figure 1(a), placed in a strong squeezer (KS-10, Fujiwara Scientific, Tokyo, Japan), and squeezed to obtain juice (Figure 1(b) and (c)). The squeezed juice was poured into the measuring hole of a portable refractometer (PAL-1, ATAGO, Tokyo, Japan) and, then, the Brix value was measured (Figure 1(d)).

**Determination of carbohydrate content in stem juice and tissues**

The same juice used in the determination of the Brix value was collected in microtubes soon after being extracted and frozen in dry ice. Culms and leaf sheaths were sampled from the basal part of productive stems corresponding roughly to the part squeezed that same day for the stem juice and, then, immediately frozen in dry ice. The fourth internode from the neck internodes of the productive stems in Tachisuzuka and Kusanohoshi cultivars, and the third internode in Nipponbare cultivar and agpl1 mutant line were collected for culm analysis. The fourth leaf sheath from the flag leaves in Tachisuzuka and Kusanohoshi cultivars, and the third leaf sheath in Nipponbare cultivar and agpl1 mutant line were collected for leaf sheath analysis. All the juice and tissue samples were stored at −80 °C until use. Frozen juice samples were thawed on ice and then centrifuged at 11,000× g for 5 min, and the resultant supernatants were used in the soluble sugars assays. Frozen culms and leaf sheaths were ground under cryogenic conditions using multi-bead shocker (Yasui Kikai, Osaka, Japan). The ground samples were weighed (50–70 mg) and extracted twice.
with 1.0 mL of 80% (v/v) ethanol at 80 °C. After centrifugation at 11,000×g for 5 min, the supernatant was dried in vacuo, dissolved in distilled water, and used for the soluble sugars (i.e. sucrose, glucose, and fructose) assays. The pellet was resuspended in distilled water and boiled for more than 2 h. After that, the starch was degraded into glucose by adding 0.2 volume (v/v) 50 U mL⁻¹ glucoamylase (Toyobo, Osaka, Japan) in acetate buffer (pH = 4.5), and used for the starch assay. The soluble sugars in juice and tissues were measured using an enzymatic method with the F-kit #716,260 (J. K. International, Tokyo, Japan) and a microplate spectrometer (Viento XS, DS Pharma Biomedical, Osaka, Japan).

Chemical mutagenesis of rice seeds

Dry seeds of Nipponbare cultivar were immersed overnight in distilled water and air dried briefly, before they were soaked into 0.1 M ethyl methanesulfonate (eMS; Sigma-Aldrich, St. Louis, MO, USA) for 5 h at room temperature. After thoroughly being washed with distilled water overnight, the mutagenized seeds were sown in plastic trays (56 cm × 27 cm × 2 cm) filled with nursery soil and grown as M0 plants in a naturally lit glasshouse until the fifth leaf emerging stage. The M0 seedlings were transplanted to the paddy field of the Hokuriku Research Center, in the NARO Agricultural Research Center (37°06′N, 138°16′E, altitude: 11 m) in mid May 2012, grown under the customary management and harvested in late September 2012. The seeds from each individual M0 plant were harvested and used in this study. For each of the M1 populations, 20 seedlings were transplanted to the paddy field as described above.

Statistical analysis

Statistical analysis for correlation was performed using the SPSS statistical software (IBM, Chicago, IL, USA). Tukey’s test was conducted after one-way analysis of variance by using the SPSS statistical software. An analysis of correlation was conducted using a bivariate correlation procedure from the SPSS statistical software.

Results

Brix value and sugar contents in stem juice

More than 100 μL of stem juice was obtained from a stem by the methods described above (Figure 1). Brix value and sugar contents of the stem juice are presented in Figure 2. Kusanohoshi showed a significantly lower Brix value, sucrose content, and soluble sugar content than the other cultivars and mutant line. In all cultivars and mutant line, Brix values were higher by 2–3% (w/v) than soluble sugar contents, and glucose and fructose contents were much lower than sucrose contents.

The correlation between Brix values and sugar contents was analyzed using the data from each juice (Figure 3). Brix values correlated strongly and significantly with the
the agpl1 and lowest in Kusanohoshi, while those in Tachisuzuka and Nipponbare showed intermediate values. In leaf sheaths, the starch content of Tachisuzuka was significantly higher than those of Kusanohoshi and Nipponbare, whereas the starch content of agpl1 was the lowest (Figure 4(b)). Although we observed no significant differences among cultivars and mutant line with respect to sucrose and soluble sugar contents, these contents were the lowest in Kusanohoshi.

The correlation between the Brix value of stem juice and soluble sugar contents in culm or leaf sheath tissues was analyzed (Figure 5). We analyzed the correlation by using the mean value of each cultivar and mutant line because the Brix value and soluble sugar contents could not be measured using the same stem as a result of the destructiveness of both methods. The Brix value of the juice tended to correlate with the soluble sugar contents in both the culm and leaf sheaths.

Screening of sweet stem-juice mutants

To provide an example of the applications of Brix value measurement in rice stem juice, we attempted to screen the M1 populations of eMS-mutagenized Nipponbare to select individuals with a high stem Brix value, namely ‘sweet stem-juice mutants’. By employing the procedure shown in Figure 1, approximately 100 plants could be measured in a day, taking an hour of daytime labor under paddy field conditions. More than 1000 plants (at least 5 individual plants per population) were measured during the ripening period of these M1 populations. Brix values varied widely between plants from .9 to 12.3. Because Brix values tended to decrease after heading and then increase from 3 wk after heading until harvest, we selected plants showing relatively high Brix values in a single day and confirmed the test replicability by measuring Brix value in another stem of the same plant. Among the plants screened, we found a plant showing relatively a high Brix value (6.7, on 22 August when mean Brix value was approximately 3)

Carbohydrate contents in culm and leaf sheath, and their correlation with the juice Brix values

Carbohydrate contents in culm and leaf sheath tissues varied among the cultivars and mutant line (Figure 4). In culms, the starch content of Kusanohoshi was significantly lower than that of Tachisuzuka and Nipponbare, although the starch content of agpl1 was the lowest (Figure 4(a)). Sucrose and soluble sugar contents were highest in sucrose and soluble sugar contents, but not with those from glucose and fructose (data of glucose and fructose are not shown). The correlation coefficient with soluble sugars was higher than with sucrose.

Figure 4. Carbohydrate contents in culms (a) and leaf sheaths (b). SS means soluble sugars (sum of sucrose, glucose, and fructose). NSC means non-structural carbohydrate (sum of starch and soluble sugars). Values represent means ± S.D. (n = 6). Different letters above the bars indicate statistically significant differences between lines at p < .05 determined using Tukey’s test.

Figure 5. Correlations between Brix value in stem juice, and soluble sugar contents in culms or leaf sheaths. (a) Brix value in juice vs. SS in culm. (b) Brix value in juice vs. SS in leaf sheath. SS means soluble sugars (sum of sucrose, glucose, and fructose). r means Pearson product-moment correlation coefficient, and there was no significant correlation (p < .05).
The soluble sugar content in squeezed juice strongly correlated with its Brix value, which can easily be measured using a portable refractometer (Figure 3). Although the Brix value was higher than soluble sugar content by 2–3% probably due to soluble impurities in the juice, Brix value can be regarded as an accurate index of the sugar content in the stem juice.

Furthermore, the Brix value of the stem juice tended to correlate with the soluble sugar content in the culm and leaf sheath tissue (Figure 5). However, the correlations were not significant (Figure 5 (a)). The Brix value in the stem of agpl1 was the same as those of Tachisuzuka and Nipponbare, while the soluble sugar content in the culm was significantly higher in agpl1 (Figures 2 and 4). Therefore, the Brix value may affect factors other than soluble sugars in the stem tissue, such as moisture content.

Measurement of the Brix value in the stem juice is easy and quick and can be done in the field. It takes less than 1 min to harvest the stem and record the values, and it only needs two instruments: a squeezer and a portable refractometer. Compared with the conventional enzymatic method used to determine soluble sugars in the stem, which requires a couple of days from harvest to measurement and requires the grinding and extraction of plant tissue, biochemical reagents, and expensive instruments such as a high-speed centrifuge and spectrophotometer, Brix value measurement is extremely quick and has a low cost. Owing to these advantages, we were able to measure the Brix value of more than a 1000 plants in 2 mo and screen for sweet stem-juice mutants. From this screening, a candidate plant – 165-1 – that showed low starch and high sucrose content in the culm was obtained.

Although further investigation of 165-1 is needed to confirm the inheritability of the phenotype, its identification demonstrates the usability of this screening method. In other words, measurement of the Brix value of the stem juice can be used for estimating the sugar content in rice stems. Although further investigations are required for estimating the accuracy and applicability of the method, this simple estimation method will be a useful tool for a high-throughput analysis of the sugar content in rice stems during mutant screening, QTL analysis, and breeding selection, and it will contribute to the study of the mechanisms underlying sugar accumulation in rice stems and practical rice breeding, especially for WCS cultivars.

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No potential conflict of interest was reported by the authors.

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