Characterization of sugar metabolism in the stem of Tachisuzuka, a whole-crop silage rice cultivar with high sugar content in the stem

Yoichi Hashida, a, b*, Sakurako Kadoya, a, Masaki Okamura, a, Yu Sugimura, b, Tatsuya Hirano, b, Tatsuro Hirose, a, Satoshi Kondo, b, Chikara Ohto, b, Ryu Ohsugi, a and Naohiro Aoki, a

aGraduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan; bGraduate School of Agricultural Science, Meijo University, Nagoya, Japan; cCentral Region Agricultural Research Center, NARO, Niigata, Japan; Future Project Division, Toyota Motor Corporation, Toyota, Japan; Research Institute for Food and Agriculture, Ryukoku University, Otsu, Japan; 1Institute of Crop Science, NARO, Tsukuba, Japan; 2Mie Prefecture Matsuoka Agricultural Extension Center, Matsuoka, Japan

ABSTRACT
Tachisuzuka, a rice cultivar for whole-crop silage, is characterized by a small panicle and high sugar content in the stem. Our previous study suggests that the high sugar content in the stem of Tachisuzuka is due to a factor other than the small panicle. To characterize sugar metabolism in the stem of Tachisuzuka, here we compared carbohydrate content, enzyme activity, and the expression of genes involved in sugar metabolism in the stem between Tachisuzuka and its parental variety, Kusanohoshi. Thinning the panicles of Kusanohoshi increased the starch content in the leaf sheath and internode but did not increase the sucrose content in the leaf sheath to the same level as that of Tachisuzuka. This suggests that Tachisuzuka has high potential to accumulate sucrose in its leaf sheath. Comparison of enzyme activity showed that the hexokinase activities in the leaf sheath of Tachisuzuka tended to be higher in Tachisuzuka than Kusanohoshi or panicle-thinned Kusanohoshi, suggesting that glucokinase or fructokinase affects sugar accumulation in the stem of Tachisuzuka. Comparative transcriptome analysis revealed the differences in expression levels of carbohydrate-related genes between Tachisuzuka and Kusanohoshi. In particular, the expression levels of ISA2, which encodes starch-debranching enzyme, and TMT2, which encodes tonoplast monosaccharide transporter – both of which maybe involved in sugar accumulation in grass stems – were higher in Tachisuzuka than Kusanohoshi. Thus, these enzymes and transporters may contribute to the high sugar content in the stem of Tachisuzuka.

Introduction
In Japan, the production of forage rice is being promoted to overcome the low self-sufficiency ratio in livestock feed and increased abandonment of paddy fields (MAFF, 2015). Major way to utilize forage rice is whole-crop silage, wherein the whole forage rice plant including the stem is harvested and ensiled. For whole-crop silage production, high soluble sugar content in the stem parts namely the leaf sheaths and culms, is favorable because soluble sugars are substrates for fermentation. However, because starch mainly accumulates in the stem in rice (Park et al., 2011), low soluble sugar content can be problematic; in such cases, soluble sugars such as glucose are added to improve fermentation quality (Hattori et al., 1993; Li et al., 2010). Thus, forage rice must have high sugar content in the stem.

Previous studies have aimed to determine key factors underlying sugar accumulation in grass stems, specifically by examining sugar metabolism in the grass stems of sugarcane (Saccharum spp.) and sweet sorghum (Sorghum bicolor), which mainly accumulate soluble sugars in the stem (for reviews, see Bihmidine et al., 2013; Slewinski, 2012; Wang et al., 2013). Those studies identified key enzymes and transporters for sugar accumulation, such as sucrose-phosphate synthase (Botha & Black, 2000; Grof et al., 2007; Liu et al., 2013; Zhu et al., 1997), vacuolar acid invertase (Liu et al., 2013; McKinley et al., 2016; Zhu et al., 1997), and tonoplast sugar transporter (Bihmidine et al., 2016). However, sugar metabolism in rice stems has received less attention. Although the enzymes and transporters involved in soluble sugar and starch metabolism in rice stems are well documented...
Figure 1. Schematic model of the sucrose/starch metabolic pathway in rice stem.

Notes: Metabolites are shown in bold; ADPG, ADP-glucose; F6P, fructose 6-phosphate; Fru, fructose; G6P, glucose 6-phosphate; Glc, glucose; Suc, sucrose; UDPG, UDP-glucose. Enzymes (solid lines) and transporters (dashed lines) are shown in italics; AGP, ADPG pyrophosphorylase; AMY, amylases; BE, branching enzyme; CIN, cell-wall acid invertase; DBE, debranching enzyme; DST, disaccharide transporters (e.g. SUT, MT/MEX), and SWEET); FK, fructokinase; GK, glucokinase; GPT, G6P/ inorganic phosphate translocator; MST, monosaccharide transporters (e.g. MST, TMT, and SWEET); NIN, neutral/alkaline invertase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; SPS, sucrose-phosphate synthase; SPP, sucrose-phosphate phosphatase; SS, starch synthase; SUS, sucrose synthase; UGP, UDPG pyrophosphorylase; VIN, vacuolar acid invertase. The paths for sucrose-to-starch conversion and starch-to-sucrose conversion are shown in blue and red, respectively. The enzymatic reactions shown in purple are involved in both conversion paths.

(Chen & Wang, 2008; Hirano et al., 2005, 2016; Hirose et al., 2006; Scofield et al., 2007; Sugimura et al., 2015; Toyota et al., 2006; Watanabe et al., 1997) and the metabolic pathway involved in the interconversion between sucrose and starch is understood (Figure 1), the key factor underlying sugar accumulation in rice stems remains unknown.

Tachisuzuka (TS), a rice cultivar used for whole-crop silage, has a small panicle and high sugar content in the stem (Matsushita et al., 2011). We previously showed that its small panicle is due to the mutation of the sp1 gene (Hirose et al., 2017), which belongs to the NRT1/PTR family (Léran et al., 2014; Li et al., 2009). A small panicle is favorable for whole-crop silage because it reduces the number of grains, which cannot be digested by cattle. In addition, small panicle size is expected to increase the allocation of photoassimilates to the stem. Therefore, the high sugar content in the stem of TS maybe due to its small panicle. To test this hypothesis, we previously analyzed a sp1-knockout mutant from Nipponbare, a typical japonica rice cultivar (Hirose et al., 2017). Compared to the wild-type plant, the sp1 mutant did not have a higher soluble sugar concentration in the stem, although it had a higher starch concentration in the stem. This suggests that high soluble sugar concentration in the stem of TS is due to a factor other than the small panicle caused by the sp1 mutation.

Therefore, in the present study, we characterized sugar metabolism in the stem of TS to obtain clues to the cause of its high sugar concentration. To this end, we compared carbohydrate contents and enzyme activities involved in sugar metabolism in the stem between TS and parental variety, Kusanohoshi (KH) with or without panicle-thinning treatments. We also conducted transcriptome analysis to investigate the difference in the expressions of sugar metabolism-related genes in the stem between these two varieties.

Materials and methods

Plant materials, growth conditions, and sampling

Seeds of TS and KH were provided by the Western Region Agricultural Research Center, National Agricultural and Food Research Organization (WARC/NARO), Japan. The two forage rice cultivars were grown in an experimental paddy field at the Institute for Sustainable Agro-ecosystem Services (ISAS), The University of Tokyo (35°44’N, 139°32’E, altitude: 58 m), Tokyo, Japan, during the summer months in 2012 and 2013. One-month-old seedlings grown in a greenhouse were transplanted into the paddy field in late May. The plant density was 22.2 hills m$^{-2}$ (hill spacing: 0.30 × 0.15 m) with one seedling per hill. The plot size was 2.4 × 2.4 m (two replicates) in 2012 and 1.8 × 3.0 m (three replicates) in 2013. Compound fertilizer for paddy fields was applied at 6 g N m$^{-2}$, 8 g P$_2$O$_5$ m$^{-2}$, and 9 g K$_2$O m$^{-2}$ as a basal dressing.

To mimic the small panicle trait of TS, the panicles of some hills of KH were thinned at the full-heading stage in 2012 and 2013. Two types of thinning were conducted in 2012: partial removal (PR) treatment and whole removal (WR) treatment. In PR treatment (KH-PR hereafter), primary rachis-branches except the first to fourth from the top of the panicle were cut on each panicle of a hill. Meanwhile, in WR treatment (KH-WR hereafter), all panicles of a hill were cut from the panicle neck. Only PR treatment was conducted in 2013.
In 2012, the leaf sheaths and internodes of the main stems of TS, KH, KH-PR, and KH-WR were sampled at harvest (40–41 days after heading (DAH)) as follows: the leaf sheath of the leaf just below the flag-leaf (LS2), the leaf sheath of the second leaf below the flag-leaf (LS3), the second-top internode (Int2), and the third-top internode (Int3). In 2013, the same parts of leaf sheaths and internodes of TS and KH were sampled at the heading, (11 DAH), mid-ripening (21–22 DAH), and harvest stages (40–41 DAH), while KH-PR was sampled at the mid-ripening (22 DAH) and harvest stages (40 DAH). The stem parts were frozen immediately in liquid nitrogen, and stored at −80 °C until use. The samples were ground under cryogenic conditions using Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and weighed for each assay. In 2013, the panicles attached to the main stems were also sampled and weighed after drying at 80 °C for one week.

**Determination of carbohydrate content**

Carbohydrate content was determined as described previously by Okamura et al. (2013).

**Enzyme assays**

Ground samples (~100 mg) were suspended in extraction buffer (50 mM HEPES-KOH (pH = 7.5), 10 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 5 μL mL$^{-1}$ protease inhibitor cocktail (P9599, Sigma, St. Louis, MO, U.S.A.), 5 mg mL$^{-1}$ BSA, 1 μL mL$^{-1}$ Triton X-100, and 20 mg mL$^{-1}$ polyvinylpyrrolidone). After centrifugation at 15,000 × g for 5 min, the supernatant was collected and desalted by passage through a Sephadex G-25 column (GE Healthcare Japan, Tokyo, Japan). Desalted supernatant was used for the assays of ADP-glucose pyrophosphorylase (AGP), fructokinase (FK), glucokinase (GK), neutral/alkaline invertase (NIN), vacuolar invertase (VIN, i.e. soluble acid invertase), sucrose-phosphate synthase (SPS), and sucrose synthase (SUS) activities. The pellet was washed twice with 1 mL extraction buffer, resuspended in 0.5 mL the extraction buffer, and used for the assay of cell wall invertase (CWIN, i.e. insoluble acid invertase) activities.

AGP, SPS, and SUS activities were evaluated as described previously by Okamura et al. (2013), Hashida et al. (2016), and Hirose et al. (2008), respectively. CWIN, NIN, and VIN were evaluated as described previously by Ishimaru et al. (2005) with some modifications. Briefly, a 50-μL aliquot was added to 150 μL reaction mixture containing 100 mM HEPES-NaOH (pH = 7.5) and 20 mM sucrose for NIN or 100 mM sodium acetate buffer (pH = 4.5) and 20 mM sucrose for CWIN and VIN. After incubation at 37 °C for 30 min, the reaction was stopped by boiling for 2 min. For CWIN and VIN assays, 20 μL HEPES-KOH (pH = 7.5) was added before boiling. The resultant glucose was measured enzymatically using F-kit (#716,251, J. K. International, Tokyo, Japan). FK and GK activities were measured as described previously by Jiang et al. (2003) with some modifications to the reaction mixture. Briefly, the reaction mixture for FK contained 50 mM HEPES-KOH (pH = 7.5), 2.5 mM MgCl$_2$, 5 mM fructose, 2.5 mM ATP, 0.5 mM NADP, 2.0 U/mL G6PDH, and 2.0 U/mL FGI. The reaction solution for GK contained 50 mM HEPES-KOH (pH = 7.5), 2.5 mM MgCl$_2$, 5 mM glucose, 2.5 mM ATP, 0.5 mM NADP, and 2.0 U/mL G6PDH.

Alpha-amylase (AAM) and beta-amylase (BAM) activities were measured as described previously by Sugimura et al. (2015) and Hirano et al. (2016), respectively.

**Transcriptome analysis**

Total RNA was extracted from LS3 of TS and KH as described previously by Okamura et al. (2013). The RNA samples from at least four different plants were collected for each variety and analyzed as one sample. Next-generation sequencer-based RNA sequencing analysis and estimation of transcript expression levels were conducted by Takara Bio Inc. (Shiga, Japan). RNA libraries for sequencing were produced with the TrueSeq RNA Sample Preparation Kit v2 (Illumina Inc., San Diego, CA, U.S.A.). The libraries were sequenced using Illumina Hiseq 2000 as 100-bp single-end reads, which resulted in 93,984,630 reads for TS and 92,231,350 reads for KH. The read sequence data were mapped to the reference sequence of the rice genome (Os-Nipponbare-Reference-IRGSP-1.0; http://rapdb.dna.affrc.go.jp/download/archive/irgsp1) using Bowtie 1.0.0 (http://bowtie-bio.sourceforge.net/index.shtml; Langmead et al., 2009). The expression level of each transcript was calculated as reads per kilobase of exon model per million mapped reads (RPKM) (Mortazavi et al., 2008) using ERANGE 3.2 (http://woldlab.caltech.edu/html/software).

**Results**

**Effect of panicle removal on carbohydrate accumulation in KH stems**

In the field trial in 2012, TS had approximately 30% of the number of spikelets per panicle as KH. Meanwhile, in the KH-PR plants, the number of spikelets per panicle was reduced to approximately 20% of the control plants. Starch content at harvest was significantly higher in both the leaf sheaths and internodes in TS compared to KH (Figure 2(A)). The panicle-thinning treatments in KH, both KH-PR and KH-WR increased the starch contents in the leaf sheaths and internodes to the same levels as those in TS. Moreover, stem sucrose content was significantly
higher in TS than KH; thinning treatments in KH also increased stem sucrose content (Figure 2B). However, in the leaf sheaths, the sucrose levels in KH-PR and KH-WR were still significantly lower than those in TS. Meanwhile, in the internodes, there were no significant differences in sucrose levels among TS, KH-PR, and KH-WR. There were no significant differences in hexose contents in leaf sheaths or internodes between the two cultivars, and no significant effects of panicle-thinning treatments on KH were observed (Figure 2C). These results suggest that in TS, the higher sucrose content in the leaf sheath cannot be explained by its small panicle size. However, the small panicle can explain the higher starch content in the leaf sheath as well as the high starch and sucrose contents in the internodes.

**Carbohydrate accumulation pattern in leaf sheaths and internodes from heading to harvest**

To clarify the pattern of carbohydrate accumulation in the stem of TS, we compared the changes in carbohydrate contents in the leaf sheaths and internodes of TS, KH, and KH-PR from heading to harvest in 2013 (Figure 3). From heading to harvest, panicle weight in KH-PR was significantly lower than that in KH but higher than that in TS, although the difference between KH-PR and TS was not significant at harvest (Figure 3A). For each cultivar, changes in starch or soluble sugar content from heading to harvest were similar between LS2 and LS3, and between Int2 and Int3 (Figure 3B–M).

In TS, starch content in leaf sheaths decreased from 10 to 20 DAH, and then increased to harvest. In contrast, in KH, starch content in leaf sheaths decreased continuously from heading to harvest. In KH-PR, starch content in leaf sheaths was similar to that in TS and tended to be higher than that in KH at 20 DAH; it decreased slightly from 20 DAH to harvest and was significantly lower than that in TS but tended to be higher than that in KH at harvest (Figure 3B and E). In both TS and KH, starch content in internodes increased from heading to 10 DAH. In TS, starch content in internodes decreased slightly from 10 to 20 DAH and then increased to harvest. Meanwhile, in KH, starch content in internodes decreased continuously from 10 DAH to harvest. In KH-PR, starch content in internodes was significantly higher than that in KH and tended to be lower than that in TS at 20 DAH; it decreased slightly from 20 DAH to harvest and was significantly lower than that in TS at harvest but tended to be higher than that in KH (Figure 3H and K).

Sucrose contents in leaf sheaths and internodes showed opposite patterns in TS and KH from heading to harvest: it increased gradually in TS while it decreased gradually in KH. In KH-PR, stem sucrose content appeared to be almost constant from heading to harvest; at harvest, it was significantly lower than that in TS but tended to be higher than that in KH (Figure 3C, F, I, and L).

Hexose contents in leaf sheaths and internodes at the heading stage were significantly higher in TS than KH, but the difference disappeared at harvest. Panicle-thinning treatment did not affect stem hexose contents at harvest (Figure 3D, G, J, and M).

**Enzyme activity in leaf sheaths and internodes**

We compared the activities of 10 enzymes involved in sugar and starch metabolism in the leaf sheaths and internodes of TS, KH, and KH-PR at 20 DAH (Figure 4). SUS, CWIN, VIN, and NIN are involved in sucrose degradation. SUS catalyzes the degradation of sucrose to UDP-glucose and fructose, while CWIN, VIN, and NIN catalyze the degradation of sucrose to glucose...
and fructose (Strum & Tang, 1999). SUS activity in leaf sheaths was significantly higher in TS than KH, and there was no significant difference between TS and KH-PR (Figure 4(A)). SUS activity in internodes did not differ significantly between TS and KH but was significantly lower in KH-PR (Figure 4(B)). There were no significant differences in CWIN activity in leaf sheaths or internodes among TS, KH, and KH-PR (Figure 4(C) and (D)). VIN activity in leaf sheaths was significantly higher in TS than KH, and there was no significant difference between TS and KH-PR (Figure 4(E)). On the other hand, VIN activity in internodes was significantly lower in TS than KH, and there was no significant difference between TS and KH-PR (Figure 4(F)). The NIN activity in leaf sheaths tended to be higher in TS than KH, whereas there was no significant difference between that in TS and KH-PR (Figure 4(G)). The NIN activity in internodes did not differ among TS, KH, and KH-PR (Figure 4(H)).

SPS catalyzes the conversion of UDP-glucose and fructose 6-phosphate to sucrose 6-phosphate and is a key enzyme in sucrose synthesis (Huber & Huber, 1996). The SPS activity in leaf sheaths did not differ significantly between TS and KH, whereas it was significantly higher in TS than KH-PR (Figure 4(I)). The SPS activity in internodes in TS was significantly higher than that in KH but did not differ significantly from that in KH-PR (Figure 4(J)).

AGP catalyzes the conversion of glucose 1-phosphate to ADP-glucose and is a key enzyme in starch synthesis in rice stem (Okamura et al., 2013, 2015). AGP activities in the leaf sheaths and internodes of TS were significantly higher
Gene expression in leaf sheaths

Transcriptome analysis based on RNA sequencing with a next-generation sequencer was conducted to compare the expression levels of genes involved in sugar metabolism in the leaf sheaths of TS and KH at 20 DAH (Supplemental Table 1). Gene expression levels were normalized to RPKM values. To exclude genes with low expression levels, genes with RPKM > 2 in at least one variety were selected. These genes were classified by their biochemical role (Figure 5).

The expression levels of genes encoding SPS and sucrose-phosphate phosphatase (SPP), both of which are involved in sucrose synthesis, were not significantly different between TS and KH (log ratio < 1). Genes encoding CWIN, VIN, NIN, and SUS, which are involved in sucrose degradation, had different expression patterns among isogenes. The expression level of INV2/VIN1 was 2.4 times higher in TS than KH, whereas that of INV3/VIN2 was 2.0 times lower in TS than KH. The expression level of NIN4 was 2.4 times higher in TS than KH, whereas that of NIN6 was 2.3 times lower in TS than KH. Among SUS isogenes, only SUS1 was differentially expressed.

Figure 4. Enzyme activities related to carbohydrate metabolism in the stems of TS, KH, and KH-PR. Enzyme activities of SUS (A, B), CWIN (C, D), VIN (E, F), NIN (G, H), SPS (I, J), AGP (K, L), AAM (M, N), BAM (O, P), FK (Q, R), and GK (S, T) were measured in leaf sheaths (A, C, E, G, I, K, M, O, Q, S) and internodes (B, D, F, H, J, L, N, P, R, T), harvested at 20 DAH in 2013. Notes: LS2 and Int2 were used for the measurement of AAM and BAM activities, while LS3 and Int3 were used for the measurement of the other enzyme activities. Values (nmol min⁻¹ gFW⁻¹) are mean ± standard error (n ≥ 3). The same letters above bars indicate no significant difference (p ≥ 0.05) between lines determined by Tukey’s multiple comparison test.
between the cultivars: SUS1 expression was 2.6 times higher in TS than KH.

The expression levels of genes involved in starch synthesis and degradation tended to be higher in TS than KH. Specifically, the expression levels of genes involved in starch synthesis, i.e. AGPL1 and AGPS1 (which encode AGP); SS1, SS3b, GBSS1, and GBSS2 (which encode starch synthase); BE1 and BE2α (which encode branching enzyme); and ISA1, ISA2, and PUL (which encode debranching enzyme) were more than two times higher in TS than KH. The expression levels of genes involved in starch degradation, i.e. ISA3 (which encodes debranching enzyme); PHO1 and PHO2 (which encode starch phosphorylase); DPE1 and DPE2 (which encode debranching enzyme); Ramy4A (which encodes AAM); and BAM2 and BAM8 (which encode BAM) were more than two times higher in TS than KH. In contrast, Ramy2A expression (which encodes AAM) was 4.3 times lower in TS than KH.

The expression levels of GPT1, GPT2, and GPT2–3, which encode glucose 6-phosphate/Pi translocator, were more than two times higher in TS than KH. The expression levels of genes encoding SUT and triose phosphate/Pi translocator, glucose translocator, and ADP-glucose translocator did not differ between TS and KH. The expression level of MTI/MEX1, which encodes maltose translocator, was two times higher in TS than KH. Two genes encode tonoplast-localized monosaccharide transporter in rice: TMT1 and TMT2. The expression level of TMT2 was 3.2 times higher in TS than KH, whereas that of TMT1 did not differ between TS and KH. Among SWEET sugar transporters, the expression levels of SWEET2b, SWEET3a, SWEET11, and SWEET16 were more than two times higher in TS than KH, whereas that of SWEET1a was 3.6 times lower in TS than KH. Among genes encoding monosaccharide transporters, the expression level of MST1 is 2.8 times lower in TS than KH, whereas those of the other genes did not differ between TS and KH.

Among genes encoding UDP-glucose pyrophosphorylase (UGP), the expression level of UGP1 was two times higher in TS than KH, whereas that of UGP3 was 2.3 times lower in TS than KH. Finally, the expression levels of genes encoding hexokinase (FK/HXK), phosphoglucomutase (PGM), and phosphoglucoisomerase (PGI) did not differ between TS and KH.

Discussion

Potential accumulation of high levels of sucrose in the stem of Tachisuzuka

Our previous study suggests that the high sugar concentration in the leaf sheath of TS is due to a factor other than the small panicle (Hirose et al., 2017). Therefore, in the present study, we characterized sugar metabolism in the stem of TS to find candidates for the factor underlying the high sugar content in the stem. For comparison, we performed panicle-thinning treatment to investigate the effect of panicle size on carbohydrate accumulation in the stem of KH. The sucrose concentration in leaf sheaths of KH with thinning treatment was significantly higher than KH without thinning treatment but significantly lower than that in TS (Figures 2 and 3). This is consistent with Hirose et al. (2017), who hypothesized that TS has high potential to accumulate high sucrose content in the stem. On the other hand, in internodes, thinning treatment had inconsistent effects on sucrose accumulation between 2012 and 2013. Sucrose concentration in the internodes of TS was significantly higher than that in KH in 2012 and 2013 as well as that in KH-PR in 2013 but was not significantly different from those in KH-PR or KH-WR in 2012 (Figures 2 and 3). This result might suggest that the high sugar concentration in the internodes of TS is mainly due to its small panicle size. Nevertheless, further studies are required to determine if TS has the potential to accumulate high sugar content in its internodes.

Starch contents in the leaf sheaths and internodes were significantly higher in TS than KH in 2012 and 2013 as well as KH-PR in 2013, but there was no significant difference between TS and KH-PR in 2012 (Figures 2 and 3). Similarly, the enzyme activities of AGP, a key enzyme in starch synthesis (Okamura et al., 2013, 2015), in the leaf sheath and internodes of TS were significantly higher than those in KH but not significantly different from those in KH-PR (Figure 4(K) and (L)). These results suggest that the differences in starch content in the stem are mainly due to the amount of carbon translocated to the stems, which is affected by panicle size. Moreover, the higher expression levels of genes involved in starch synthesis and starch degradation in TS (Figure 5) might also be due to the difference in panicle size.

Candidates for the factor responsible for the high sugar accumulation in the stem of Tachisuzuka

Some studies suggest that high-sugar varieties of sugarcane and sweet sorghum are characterized by high SPS activity and low VIN activity (Liu et al., 2013; McKinley et al., 2016; Zhu et al., 1997). However, this is not applicable to TS, because SPS activity and gene expression levels in leaf sheaths did not differ significantly between TS and KH (Figures 4 and 5). In addition, SPS activity in internodes was significantly higher in TS than KH, but there was no significant difference between TS and KH-PR (Figure 4(L)). Thus, these results suggest that the difference in the SPS activity in the stem between TS and KH is due to the difference in panicle size. Similarly, the differences in VIN activities
in leaf sheaths and internodes between TS and KH could be explained by the difference in panicle size (Figure 4(E) and (F)).

GK activity in leaf sheaths was higher in TS than KH or KH-PR (Figure 4(S)); FK exhibited a similar trend (Figure 4(Q)). These results suggest that the higher hexokinase activity in the leaf sheaths of TS is the factor that determines sugar accumulation in the leaf sheaths of TS. However, there was no clear difference between TS and KH with respect to the expression levels of genes encoding hexokinase (HXK2−9), which have both glucokinase and fructokinase activities (Cho et al., 2006) (Figure 5). The hexokinase activity in rice stem may not be regulated at the transcriptional level, but at the translational and/or post-translational level.

A recent genome-wide association study on carbohydrate content in rice stems using diverse germplasm panels of tropical japonica rice showed that ISA2 genotype is associated with sucrose content in the stem at harvest (Wang et al., 2017). In the present study, the expression level of ISA2 was 6.8 times higher in TS than KH (Figure 5). However, it is unclear whether the difference in the expression level of ISA2 between TS and KH is due to the difference in ISA2 genotype and/or its regulatory region on the genome or panicle size.

Tonoplast sugar transporter (TST) was originally isolated as tonoplast-localized monosaccharide trans-porter (TMT) (Wormit et al., 2006). A TST in sugar beet, BvTST2.1, was recently demonstrated to function as an H+/sucrose antiporter that transports sucrose into the vacuoles and is responsible for sugar accumulation in taproot, which is the storage organ in sugar beet (Jung et al., 2015). In addition, TST is suggested to be important for sugar accumulation in sorghum stem (Bihmidine et al., 2016). Therefore, it is reasonable to assume that TSTs in other plants including rice can transport sucrose into the vacuole and are important for sugar accumulation, although this has not been demonstrated in any other plants including sorghum. There are two TSTs in rice: TMT1 and TMT2. TMT1 transports glucose, although

Figure 5. Expression levels of genes related to sugar metabolism in leaf sheaths of TS and KH.
Notes: LS3 harvested at 20 DAH was used for measurements. Genes related to carbohydrate metabolism with reads per kilobase of exon model per million mapped reads (RPKM) > 2 in at least one variety were selected. The scale shows log2 at −2.0 to 2.0. Details about each gene are shown in Supplemental Table 1.
it is unknown whether TMT1 can transport sucrose (Cho et al., 2010). Furthermore, the transport ability of TMT2 has not been investigated. In the present study, we found that the expression level of TMT2 was significantly higher in TS than KH (Figure 5), suggesting that TMT2 maybe a key factor underlying the high sugar accumulation in TS.

Although we found many genes that were expressed differently between TS and KH (Figure 5), these genes are not necessarily responsible for high sugar accumulation in TS because the observed differences in expression levels might result from the differences in sugar concentration between TS and KH. For example, CRCT (CO₂-responsive CCT protein; Os05g0595300) is a transcription factor that regulates the expression of genes related to starch metabolism such as AGPL1, BE1, and Pho1 in rice stem, and its expression is known to be up-regulated by sugars, especially sucrose (Morita et al., 2015). Consistent with the findings, the expression level of CRCT was 1.8 times higher in TS than KH (Supplemental Table 2). Since such sugar-responsive genes are included in the differentially expressed genes, it might be difficult to determine the candidate genes only by comparative transcriptome analysis. Different approaches such as genome-wide association analyses using backcross-derived populations of TS are needed to obtain some more clues for identification of the gene responsible for high sugar accumulation in the leaf sheath of TS.

**Conclusion**

This study revealed the differences in sugar metabolism between TS and KH, and between the leaf sheath and internode of TS. We also found possible candidates for the key factor that determines the capacity of sugar accumulation in the leaf sheath of TS, including hexokinases, ISA2, and TMT2. To further characterize the sugar metabolism in the stem of TS, concentrations of other metabolites involved in sugar metabolic pathway, e.g. G6P, F6P, UDPG, ADPG, should be compared between TS and KH. Also the other parental line of TS, Gokutsansui (a mutant line with extra-short panicles; Matsushita et al., 2011), would be worth for comparison. To clarify the key factor underlying sugar accumulation in rice stems, further studies such as functional analyses of candidate genes, and analyses using backcross-derived populations of TS, are required.

**Acknowledgements**

We would like to express our gratitude to the technical support staff of the ISAS at The University of Tokyo for their help in the cultivation and management of rice. Y.H. and M.O. received a fellowship from the Japan Society for the Promotion of Science (JSPS).

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by the JSPS KAKENHI [grant number 26292009] to N.A., T.H., and T.H.

**References**

Bihmidine, S., Hunter, C. T., III, Johns, C. E., Koch, K. E., & Braun, D. M. (2013). Regulation of assimilate import into sink organs: Update on molecular drivers of sink strength. *Frontiers in Plant Science*, 4, 177. doi:10.3389/fpscs.2013.00177

Bihmidine, S., Julius, B. T., Dweikat, I., & Braun, D. M. (2016). Tonoplast sugar transporters (SbTSTs) putatively control sucrose accumulation in sweet sorghum stems. *Plant Signaling & Behavior*, 11, e1117721. doi:10.1080/15592324.2015.1117721

Botha, F. C., & Black, K. G. (2000). Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. *Australian Journal of Plant Physiology*, 27, 81–85. doi:10.1071/PP990098

Chen, H. J., & Wang, S. J. (2008). Molecular regulation of sink-source transition in rice leaf sheaths during the heading period. *Acta Physiologiae Plantarum*, 30, 639–649. doi:10.1007/s11738-008-0160-8

Cho, J. I., Burla, B., Lee, D. W., Ryoo, N., Hong, S. K., Kim, H. B., … Jeon, J. S. (2010). Expression analysis and functional characterization of the monosaccharide transporters, OsTMTs, involving vacuolar sugar transport in rice (*Oryza sativa*). *New Phytologist*, 186, 657–668. doi:10.1111/j.1469-8137.2010.03194.x

Cho, J. I., Ryoo, N., Ko, S., Lee, S. K., Lee, J., Jung, K. H., … Jeon, J. S. (2006). Structure, expression, and functional analysis of the hexokinase gene family in rice (*Oryza sativa* L.). *Planta*, 224, 598–611. doi:10.1007/s00425-006-0251-y

Grolf, C. P. L., Albertson, P. L., Bursle, J., Perroux, J. M., Bonnett, G. D., & Manners, J. M. (2007). Sucrose-phosphate synthase, a biochemical marker of high sucrose accumulation in sugarcane. *Crop Science*, 47, 1530–1539. doi:10.2135/cropsic2006.12.0825

Hashida, Y., Hirose, T., Okamura, M., Hibara, K., Ohsugi, R., & Aoki, N. (2016). A reduction of sucrose phosphate synthase (SPS) activity affects sucrose/starch ratio in leaves but does not inhibit normal plant growth in rice. *Plant Science*, 253, 40–49. doi:10.1016/j.plantsci.2016.08.017

Hattori, I., Kumai, S., & Fukumi, R. (1993). The effect of saccharide additives on the fermentative quality of silage. *Journal of Japanese Grassland Science*, 39, 326–333. doi:10.14941/grass.39.326. In Japanese with English abstract.

Hirano, T., Higuchi, T., Hirano, M., Sugimura, Y., & Michiyama, H. (2016). Two β-amylase genes, OsBAM2 and OsBAM3, are involved in starch remobilization in rice leaf sheaths. *Plant Production Science*, 19, 291–299. doi:10.1007/s13439-016-11400-8

Hirano, T., Saito, Y., Ushimaru, H., & Michiyama, H. (2005). The effect of the amount of nitrogen fertilizer on starch metabolism in leaf sheath of japonica and indica rice varieties during the heading period. *Plant Production Science*, 8, 122–130. doi:10.1626/pps.8.122
Hirose, T., Kadoya, S., Hashida, Y., Okamura, M., Ohsugi, R., & Aoki, N. (2017). Mutation of the SP1 gene is responsible for the small-panicle trait in the rice cultivar Tachisuzuka, but not necessarily for high sugar content in the stem. *Plant Production Science, 20*, 90–94. doi:10.1080/134393X.2016.1260484

Hirose, T., Ohdan, T., Nakamura, Y., & Terao, T. (2006). Expression profiling of genes related to starch synthesis in rice leaf sheaths during the heading period. *Physiologia Plantarum, 128*, 425–435. doi:10.1111/j.1399-3054.2006.00758.x

Hirose, T., Scofield, G., & Terao, T. (2008). An expression analysis profile for the entire sucrose synthase gene family in rice. *Plant Science, 174*, 534–543. doi:10.1016/j.plantsci.2008.02.009

Huber, S. C., & Huber, J. L. (1996). Role and regulation of sucrose-phosphate synthase in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology, 47*, 431–444. doi:10.1146/annurev.arplant.47.1.431

Ishimaru, T., Hirose, T., Matsuda, T., Goto, A., Takahashi, K., Sasaki, H., ... Yamagishi, T. (2005). Expression patterns of genes encoding carbohydrate-metabolizing enzymes and their relationship to grain filling in rice (*Oryza sativa* L.): Comparison of caryopes located at different positions in a panicle. *Plant and Cell Physiology, 46*, 620–628. doi:10.1093/pcp/pcp066

Jiang, H., Dian, W., Liu, F., & Wu, P. (2003). Isolation and characterization of two fructokinase cDNA clones from rice. *Phytochemistry, 62*, 47–52. doi:10.1016/S0031-9422(02)00428-4

Jung, B., Ludewig, F., Schulz, A., Meißen, G., Wüstefeld, N., Flügge, U. I., ... Neuhaus, H. E. (2015). Identification of the transporter responsible for sucrose accumulation in sugar beet taproots. *Nature Plants, 1*, 14001. doi:10.1038/nplants.2014.1

Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology, 10*, R25. doi:10.1186/gb-2009-10-3-r25

Léran, S., Varala, K., Boyer, J. C., Chiurazzi, M., Crawford, N., Daniel-Vedele, F., ... Lacombe, B. (2014). A unified nomenclature of nitrate transporter 1/peptide transporter family members in plants. *Trends in Plant Science, 19*, 5–9. doi:10.1016/j.tplants.2013.08.008

Li, J., Shen, Y., & Cai, Y. (2010). Improvement of fermentation quality of rice straw silage by application of a bacterial inoculant and glucose. *Asian-Australasian Journal of Animal Sciences, 23*, 901–906. doi:10.5713/ajas.2010.9403

Li, S., Qian, Q., Fu, Z., Zeng, D., Meng, X., Kyozuka, J., ... Wang, Y. (2009). Short panicle1 encodes a putative PTR family transporter and determines rice panicle size. *The Plant Journal, 58*, 592–605. doi:10.1111/j.1365-313X.2009.03799.x

Liu, Y., Dun, B., Zhao, X., Yue, M., Lu, M., & Li, G. (2013). Correlation analysis between the key enzymes activities and sugar content in sweet sorghum (*Sorghum bicolor* L. Moench) stems at physiologically mature stage. *Australian Journal of Crop Science, 7*, 84–92.

MAFF. (2015). FY2015 Annual Report on Food, Agriculture and Rural Areas in Japan (Summary).

Matsushita, K., lida, S., Ideta, O., & Sunohara, Y. (2011). ‘Tachisuzuka’, a new rice cultivar with high straw yield and high sugar content for whole-crop silage use. *Breeding Science, 61*, 86–92. doi:10.1270/jsbbs.61.86

McKinley, B., Rooney, W., Wilkerson, C., & Mullet, J. (2016). Dynamics of biomass partitioning, stem gene expression, cell wall biosynthesis, and sucrose accumulation during development of Sorghum bicolor. *The Plant Journal, 88*, 662–680. doi:10.1111/tpj.13269

Morita, R., Sugino, M., Hatanaka, T., Misoo, S., & Fukayama, H. (2015). CO₃²⁻-responsive CONSTANS, CONSTANS-like, and Time of chlorophyll a/b binding protein expression1 protein is a positive regulator of starch synthesis in vegetative organs of rice. *Plant Physiology, 167*, 1321–1331. doi:10.1104/pp.15.00021

Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L., & Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods, 5*, 621–628. doi:10.1038/nmeth.1226

Okamura, M., Hirose, T., Hashida, Y., Ohsugi, R., & Aoki, N. (2015). Suppression of starch synthesis in rice stems splay tiller angle due to gravitropic insensitivity but does not affect yield. *Functional Plant Biology, 42*, 31–41. doi:10.1071/FP14159

Okamura, M., Hirose, T., Hashida, Y., Yamagishi, T., Ohsugi, R., & Aoki, N. (2013). Starch reduction in rice stems due to a lack of OsAGP11 or OsAPL3 decreases grain yield under low irradiance during ripening and modifies plant architecture. *Functional Plant Biology, 40*, 1137–1146. doi:10.1071/FP131105

Park, J., Kanda, E., Fukushima, A., Motobayashi, K., Nagata, K., Kondo, M., ... Tokuyasu, K. (2011). Contents of various sources of glucose and fructose in rice straw, a potential feedstock for ethanol production in Japan. *Biomass and Bioenergy, 35*, 3733–3735. doi:10.1016/j.biombioe.2011.05.032

Scofield, G. N., Hirose, T., Aoki, N., & Furbank, R. T. (2007). Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. *Journal of Experimental Botany, 58*, 3155–3169. doi:10.1093/jxb/erm153

Slewinski, T. L. (2012). Non-structural carbohydrate partitioning in grass stems: A target to increase yield stability, stress tolerance, and biofuel production. *Journal of Experimental Botany, 63*, 4647–4670. doi:10.1093/jxb/ers124

Strum, A., & Tang, G. Q. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science, 4*, 401–407. doi:10.1016/S1360-1385(99)01470-3

Sugimura, Y., Michiyama, H., & Hirano, T. (2015). Involvement of α-amylase genes in starch degradation in rice leaf sheaths at the post-heading stage. *Plant Production Science, 18*, 277–283. doi:10.1626/pps.18.277

Toyota, K., Tamura, M., Ohdan, T., & Nakamura, Y. (2006). Expression profiling of starch metabolism-related plastidic translocator genes in rice. *Planta, 223*, 248–257. doi:10.1007/s00425-005-0128-5

Wang, D. R., Han, R., Wolfrum, E. J., & McCouch, S. R. (2017). The buffering capacity of stems: Genetic architecture of nonstructural carbohydrates in cultivated Asian rice, *Oryza sativa*. *New Phytologist, 215*, 658–671. doi:10.1111/nph.14614

Wang, J., Nayak, S., Koch, K., & Ming, R. (2013). Carbon partitioning in sugarcane (*Saccharum* species). *Frontiers in Plant Science, 4*, 201. doi:10.3389/fpls.2013.00201

Watanabe, Y., Nakamura, Y., & Ishii, R. (1997). Relationship between starch accumulation and activities of the related enzymes in leaf sheath as a temporary sink organ in rice (*Oryza sativa*). *Australian Journal of Plant Physiology, 24*, 563–569. doi:10.1071/PP96107
Zhu, Y. J., Komor, E., & Moore, P. H. (1997). Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. *Plant Physiology, 115*, 609–616. doi:10.1104/pp.115.2.609

Wormit, A., Trentmann, O., Feifer, I., Lohr, C., Tjaden, J., Meyer, S., … Neuhaus, H. E. (2006). Molecular identification and physiological characterization of a novel monosaccharide transporter from *Arabidopsis* involved in vacuolar sugar transport. *The Plant Cell, 18*, 3476–3490. doi:10.1105/tpc.106.047290