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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 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
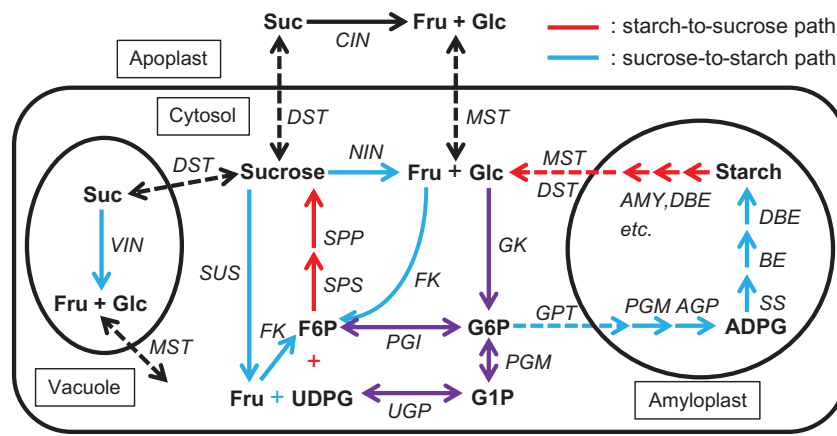

Figure 1. Schematic model of the sucrose/starch metabolic pathway in rice stem.

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/MEX1, 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} polyvinylpolypyrrolidone). 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 PGI. 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 TruSeq 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
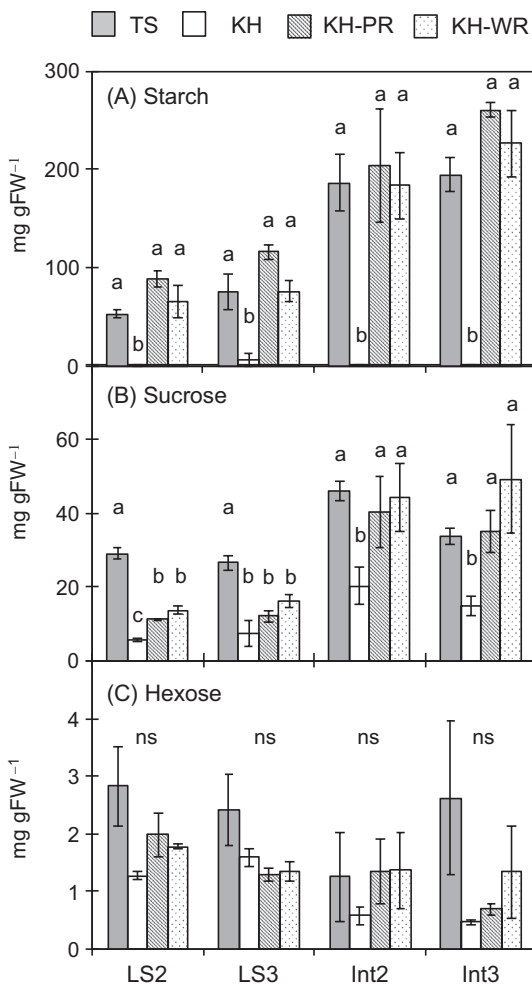
Figure 2. Carbohydrate content in leaf sheaths and internodes of Tachisuzuka and Kusanohoshi at harvest in 2012. Starch (A), sucrose (B), and hexose (C, glucose + fructose) contents in LS2, LS3, Int2, and Int3 of TS, KH, KH-PR, and KH-WR.

Notes: TS, Tachisuzuka; KH, Kusanohoshi; KH-PR, Kusanohoshi with partial removal of spikelets from panicles; KH-WR, Kusanohoshi with whole removal of panicles. Values are mean ± standard error (*n* = 3–4). The same letters above bars indicate no significant difference (*p* ≥ 0.05) between TS, KH, KH-PR, and KH-WR determined by the Tukey–Kramer multiple comparisons test. ns, no significant difference (*p* ≥ 0.05) between TS, KH, KH-PR, and KH-WR by ANOVA.

higher in TS than KH; thinning treatments in KH also increased stem sucrose content (Figure 2(B)). 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 2(C)). 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 3(A)). 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 3(B)–(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 3(B) 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 3(H) 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 and higher than that in KH (Figure 3(C), (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 3(D), (G), (J), (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
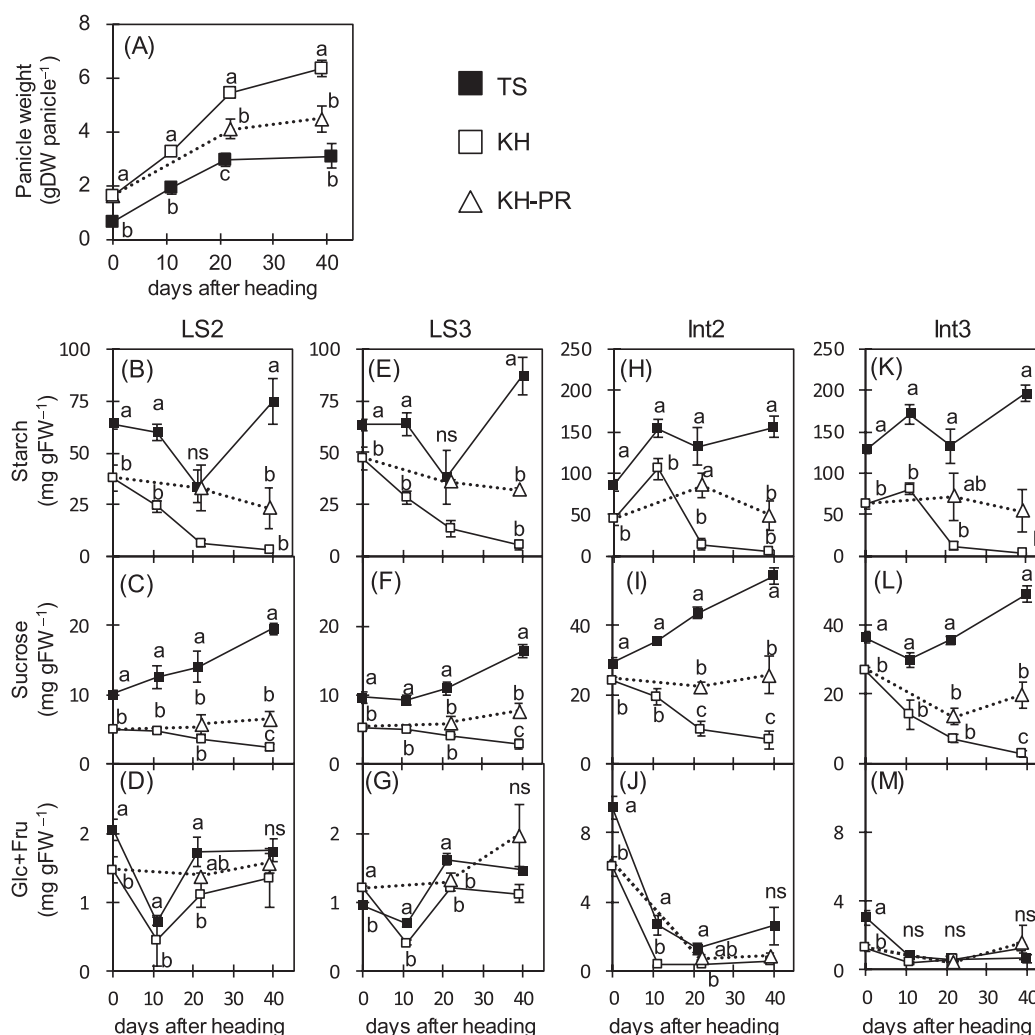
Figure 3. Panicle weight and carbohydrate contents in leaf sheaths and internode of TS, KH, and KH-PR from heading to harvest in 2013. (A) Panicle weight from heading to harvest. Panicles sampled from the main stem of each plant were used for measurement. The panicle weight of KH-PR at the heading stage was the same as that of KH. Values are mean ± standard error (n ≥ 4). Starch (B, E, H, K), sucrose (C, F, I, L), and hexose (D, G, J, M) contents in LS2 (B–D), LS3 (E–G), Int2 (H–J), and Int3 (K–M) of TS, KH, and KH-PR.

Notes: Values are mean ± standard error (n = 3–4). The values of KH-PR at the heading stage were the same as those of KH. The same letters indicate no significant difference (p ≥ 0.05) between lines determined by t-test or the Tukey–Kramer multiple comparisons test. ns, no significant difference (p ≥ 0.05) between lines by t-test or ANOVA.

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
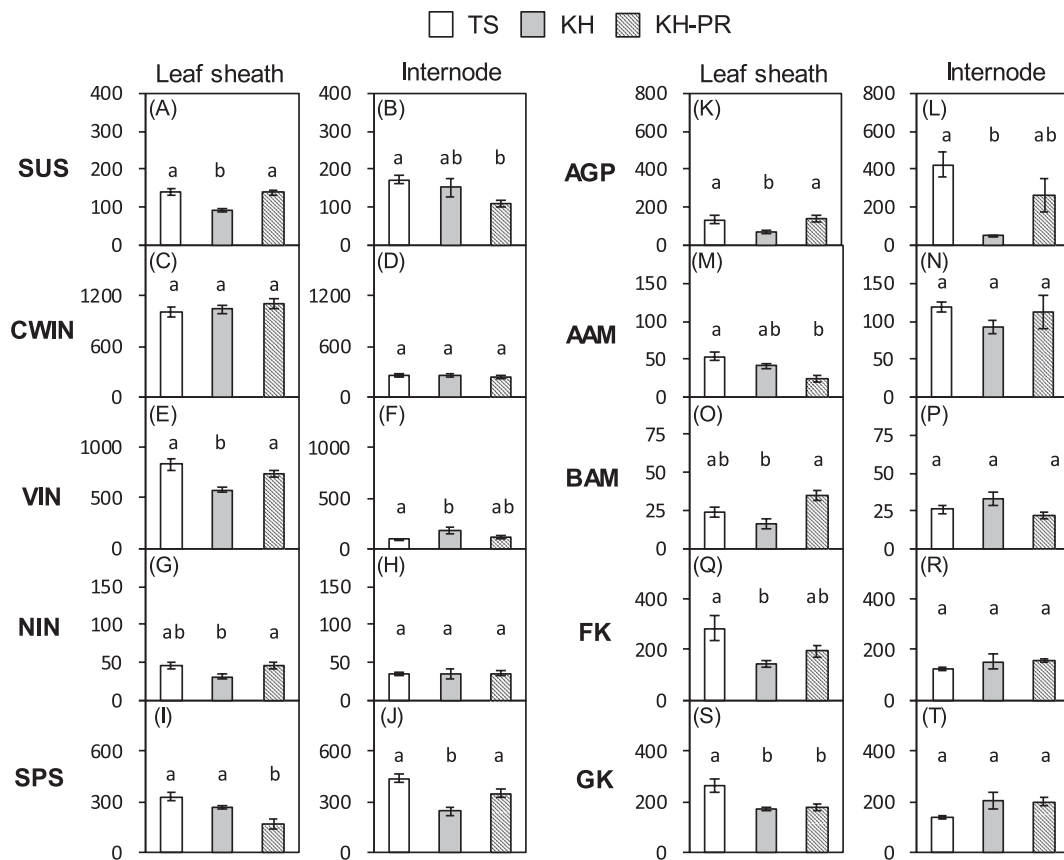
Figure 4. Enzyme activities related to carbohydrate metabolism in the stems of TS, KH, and KH-PR. Enzyme activities of SUS (A, B), CWIN (C, D), VIN (E, F), NIN (G, H), SPS (I, J), AGP (K, L), AAM (M, N), BAM (O, P), FK (Q, R), and GK (S, T) were measured in leaf sheaths (A, C, E, G, I, K, M, O, Q, S) 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 ($n \geq 3$). The same letters above bars indicate no significant difference ($p \geq 0.05$) between lines determined by Tukey's multiple comparison test.

than those in KH but not significantly different from those in KH-PR (Figure 4(K) and (L)).

AAM and BAM catalyze the hydrolysis of starch into sugars and are important for starch degradation in rice stems (Hirano et al., 2016; Sugimura et al., 2015). The AAM activity in leaf sheaths in TS did not differ from that in KH but was significantly higher than that in KH-PR (Figure 4(M)). AAM activity in internodes did not differ significantly among TS, KH, and KH-PR (Figure 4(N)). BAM activity in leaf sheaths in TS tended to be higher than that in KH but was not significantly different from that in KH-PR (Figure 4(O)). BAM activity in internodes did not differ significantly among TS, KH, and KH-PR (Figure 4(P)).

Both GK and FK are hexose kinases with different specificities to their substrate sugar (Jiang et al., 2003). GK activity in leaf sheaths in TS was significantly higher than those in KH and KH-PR (Figure 4(S)). FK activity in leaf sheaths showed a similar pattern, although the difference between TS and KH-PR was not significant after adjusting for multiple comparisons (Figure 4(Q)). In internodes, there were no differences in GK or FK activity among TS, KH, and KH-PR (Figure 4(R) and (T)).

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

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 *BE2a* (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 *MT/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), phosphoglucokinase (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(J)). 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
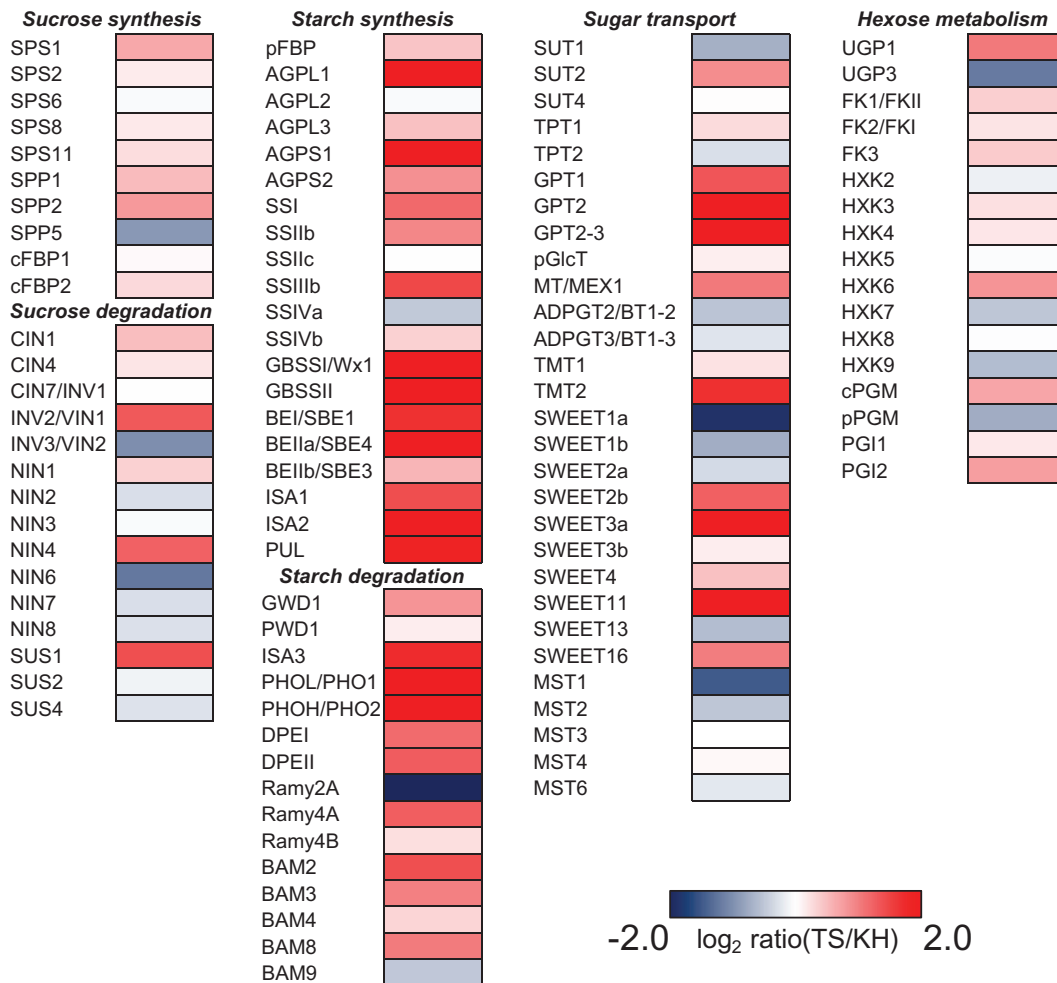
Sucrose synthesis
SPS1
SPS2
SPS6
SPS8
SPS11
SPP1
SPP2
SPP5
cFBP1
cFBP2

Sucrose degradation
CIN1
CIN4
CIN7/INV1
INV2/VIN1
INV3/VIN2
NIN1
NIN2
NIN3
NIN4
NIN6
NIN7
NIN8
SUS1
SUS2
SUS4

Starch synthesis
pFBP
AGPL1
AGPL2
AGPL3
AGPS1
AGPS2
SSI
SSIIb
SSIIc
SSIIIb
SSIVa
SSIVb
GBSSI/Wx1
GBSSII
BEI/SBE1
BEIIa/SBE4
BEIIb/SBE3
ISA1
ISA2
PUL

Starch degradation
GWD1
PWD1
ISA3
PHOL/PHO1
PHOH/PHO2
DPEI
DPEII
Ramy2A
Ramy4A
Ramy4B
BAM2
BAM3
BAM4
BAM8
BAM9

Sugar transport
SUT1
SUT2
SUT4
TPT1
TPT2
GPT1
GPT2
GPT2-3
pGlcT
MT/MEX1
ADPGT2/BT1-2
ADPGT3/BT1-3
TMT1
TMT2
SWEET1a
SWEET1b
SWEET2a
SWEET2b
SWEET3a
SWEET3b
SWEET4
SWEET11
SWEET13
SWEET16
MST1
MST2
MST3
MST4
MST6

Hexose metabolism
UGP1
UGP3
FK1/FKII
FK2/FKI
FK3
HXK2
HXK3
HXK4
HXK5
HXK6
HXK7
HXK8
HXK9
cPGM
pPGM
PGI1
PGI2

-2.0　$\log_2$ ratio(TS/KH)　2.0

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 $\log_2$ at −2.0 to 2.0. Details about each gene are shown in Supplemental Table 1.

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 transporter (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

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* (CO_2-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, Gokutansui (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.

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