Temporal and Spatial Variations of Carbohydrate Content in Rice Leaf Sheath and Their Varietal Differences

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Abstract: In rice plant, carbohydrates accumulated in leaf sheaths before heading are translocated to grain and affect yield formation greatly. To clarify the intrinsic mechanism of carbohydrate metabolism in the leaf sheath, we investigated the temporal and spatial variations of carbohydrate metabolism in the third leaf sheath counted from the top and their varietal differences. The results revealed that the amount of carbohydrate decreased from the base to the tip of the leaf sheath, irrespective of variety and developmental stage. However, the proportion of starch content in the basal one-fifth of the leaf sheath to that in the whole sheath varied from 35% to 60% with the variety. Comparing the activities of enzymes related to starch metabolism at the base, middle and tip of the leaf sheath in IR65598-112-2 (New plant type) with those in Nipponbare, the activities of ADP-glucose pyrophosphorylase, branching enzyme and granule-bound starch synthase (GBSS) showed varietal differences. Particularly, the activity of GBSS may play an important role in the varietal difference in spatial variation of starch content in the leaf sheath. In IR65598-112-2, the sucrose content in the leaf sheath was extremely high, suggesting that sucrose may be one of the carbohydrate reserves in this line.

Key words: Carbohydrate, Enzyme, Leaf sheath, Spatial variation, Temporal change, Variety.

In rice plant, carbohydrate temporarily stored in leaf sheaths and culms is translocated to grain after heading and contributes to rice yield greatly (Yoshida and Ahn, 1968; Song et al., 1990; Ishikawa et al., 1993). This non-structural carbohydrate was effective in improving the relative growth rate of grain in the initial 10 days of grain filling (Tsukaguchi et al., 1996) and in increasing the percentage of ripened grains (Weng et al., 1982). The leaf sheaths have a sink size similar to or larger than that of the culms (Fujita and Yoshida, 1984) and carbohydrate in the leaf sheaths is translocated to grains more completely than that in the culms (Baba and Kitsutaka, 1953; Togari and Sato, 1954). Thus, the leaf sheaths provide more carbohydrate for grain. Therefore, it is of great importance to elucidate the mechanism of carbohydrate accumulation in leaf sheaths in view of their contribution to the yield.

There are varietal differences in the amount of non-structural carbohydrate in leaf sheaths (Fujita and Yoshida, 1984). In the leaf sheath of japonica rice, the basal part started to accumulate starch earlier and accumulated more than the apical part (Baba and Kitsutaka, 1953; Togari and Sato, 1954; Sato and Ebara, 1974). However, there is no information about the spatial variation in the amount of starch in the leaf sheath in varieties other than japonica, and therefore it still remains unclear whether the varietal difference in the amount of starch accumulation in the leaf sheath correlates with that in the spatial variation.

In the process of starch synthesis, sucrose translocated from source organs is cleaved to UDP-glucose and fructose by sucrose synthase (SuSy), and metabolized as a carbon source in rice grain (Nakamura et al., 1989). ADP-glucose pyrophosphorylase (AGPase), starch synthase and branching enzyme (BE) catalyze substrate production, chain elongation and branching, respectively. In higher plants, there are two types of starch synthase: the granule-bound one (GBSS), which tightly binds to the starch granules and functions specifically to elongate amylose, and the soluble one (STS), which mainly exists in plastid stroma and contributes to amylpectin synthesis (see Preiss and Sivak 1996 for review). These enzymes generally play important roles in the regulation of starch synthesis.

In rice leaf sheaths, Perez et al. (1971) first investigated the activities of enzymes involved in carbohydrate metabolism in total leaf sheaths at different growth stages, and showed that the activity of GBSS closely paralleled the starch content of leaf sheaths. Watanabe et al. (1997) examined the starch content of the leaf sheaths at different nodal positions, and found that the second leaf sheath from the flag...
leaf sheath accumulated 4-fold more starch than the sixth leaf sheath, and that the starch content closely correlated with the activities of BE, STS, GBSS and AGPase. The mRNA levels of AGPase, STS and BE also correlated with a rapid starch accumulation in the third leaf sheath of rice (Hirose et al., 1999). These enzymes might also be responsible for the spatial variation of starch accumulation in the leaf sheath and its varietal differences.

In this study, we examined the temporal and the spatial variations of carbohydrate metabolism in the third leaf sheath, counting the flag leaf sheath as the first one, as well as their differences among 4 rice varieties different in grain filling percentage or ecotype.

**Materials and Methods**

1. **Plant materials**
   - In 2002, IR72 (indica), Takanari (indica), IR65598-112-2 (tropical japonica) and Nipponbare (temperate japonica) were sown on Apr. 23, and transplanted on May 21 with a hill spacing of 30×15 cm and one seeding per hill in the experimental paddy field of Field Production Science Center, Graduate School of Agricultural and Life Sciences, the University of Tokyo in West Tokyo City. Basal fertilizers, 60 kg ha⁻¹ N, 90 kg ha⁻¹ P₂O₅, and 80 kg ha⁻¹ K₂O were supplied on May 12. The third leaf sheath, counting the flag leaf sheath, was transplanted on May 21 with a hill spacing of 30×15 cm and one seeding per hill in the experimental paddy field of Field Production Science Center, Graduate School of Agricultural and Life Sciences, the University of Tokyo in West Tokyo City. Basal fertilizers, 60 kg ha⁻¹ N, 90 kg ha⁻¹ P₂O₅, and 80 kg ha⁻¹ K₂O were supplied on May 12. The third leaf sheath, which finished elongation in late July, was harvested at intervals of 3 or 4 days until early September and divided into five segments with the same length (called seg 1 to seg 5 from the top). Five samples for determination of carbohydrate content were frozen in liquid nitrogen, lyophilized, weighed and then stored in a desiccator at room temperature. Five samples for enzyme assay were frozen in liquid nitrogen and stored at −80 °C until use. Heading date was determined as the day when 50% panicles headed.

2. **Determination of carbohydrate content**
   - Lyophilized samples were powdered with a multi-bead shocker (Yasui Corp.). Approximately 10 mg powder was homogenized with 80% ethanol in a mortar. The homogenate was collected in an Eppendorf tube, and heated at 80°C for 3 min for sugar extraction. After centrifugation at 10,000 g for 5 min, the supernatant was transferred to another Eppendorf tube and sugar was further extracted from the pellet twice with 80% ethanol. Finally, the total supernatant was dried in vacuo with a rotary evaporator and used for determination of sucrose, glucose, and fructose by the enzymatic method (Nakamura and Miyachi, 1982). The ethanol-insoluble fraction was boiled in distilled water for 4 hr. The gelatinized starch was then digested with glucoamylase at 60°C for 1.5 hr and the resultant glucose was measured according to the method mentioned above. The carbohydrate concentration derived on the basis of dry weight was then multiplied by the dry weight of the respective segment to obtain the carbohydrate content. Carbohydrate content of the whole leaf sheath was the sum of those in the 5 segments.

3. **Determination of enzyme activities**
   - Approximately 100 mg fresh samples were ground in liquid nitrogen in a mortar with a pestle, using silica sand to obtain a fine powder. It was homogenized in 0.8 ml buffer solution containing 100 mM Tricine-NaOH (pH 8.0), 8 mM MgCl₂, 2 mM EDTA, 50 mM 2-mercaptoethanol, 125 mM L⁻¹ glycerol and 20 mg polyvinylpyrrolidone-40. The homogenate was centrifuged at 10,000 g for 5 min, and the supernatant was used as the enzyme preparation of SuSy, AGPase, STS and BE. The precipitate was washed twice with the buffer solution to remove the soluble enzymes, suspended in a 0.8 ml buffer solution, and used for assay of GBSS. All procedures for enzyme extraction were performed on ice. All assays were carried out at 30°C, in the reaction mixtures described below (Nakamura et al., 1989). Assays were conducted in the range of the concentrations of enzymes where the activities increased linearly with increase in the amount of enzyme preparations used and the reaction time. The background values were routinely taken as the activities detected at a reaction time of zero.

   **SuSy**: The assay was conducted in a 70 µl mixture of 50 mM HEPES (pH 7.4), 16 mM MgCl₂, 8 mM UDP-glucose, 8 mM fructose and enzyme preparation. After a 30-min incubation, the reaction was terminated by adding 70 µl of 1 N NaOH. The resulting solution was put into boiling water for 10 min to destroy the unreacted fructose. Sucrose (+sucrose phosphate) was estimated according to the method of Rorem et al. (1960) by adding 250 µl of 0.1% resorcinol (ethanolic) and 750 µl of 30% HCl, and incubating the solution at 80 °C for 8 min. The reaction was stopped on ice and absorbance at 520 nm was measured.

   **AGPase**: The assay was conducted in a 650 µl mixture of 100 mM HEPES-NaOH (pH 7.4), 1.2 mM ADPglucose, 3 mM Ppi, 3 mM PGA, 5 mM MgCl₂, 4 mM DTT, and enzyme preparation. After a 20-min incubation, the reaction was terminated by heating the mixture in boiling water for 30 s. The resulting solution was transferred to an Eppendorf tube and centrifuged at 15,000 rpm for 10 min. A portion (500 µl) of the supernatant was mixed with 15 µl 10 mM NADP. The activity was assayed by measuring the increase in absorbance at 340 nm after addition of 1 µl (0.35 unit) each of P-glucomutase and G6P dehydrogenase.

   **STS and GBSS**: The assay was conducted in a 280 µl mixture of 50 mM HEPES-NaOH (pH 7.4), 1.6 mM ADPglucose, 0.7 mg amylpectin, 15 mM DTT and enzyme preparation. After 20 min, the reaction was...
terminated by heating the mixture in boiling water for 30 s. Then 100 µl solution containing 50 mM HEPES-NaOH (pH 7.4), 4 mM PEP, 200 mM KCl, 10 mM MgCl₂ and pyruvate kinase (1.2 unit) was added and incubated for 30 min. The resulting solution was put into boiling water for 30 s and then centrifuged at 15,000 rpm for 10 min. The supernatant (300 µl) was mixed with a 200 µl solution of 50 mM HEPES-NaOH (pH 7.4), 10 mM glucose, 20 mM MgCl₂ and 2 mM NADP. The activity was assayed by measuring the increase in absorbance at 340 nm after addition of 1 µl each of hexokinase (1.4 unit) and G6P dehydrogenase (0.35 unit).

BE: The assay was conducted in a 200 µl mixture of 50 mM HEPES-NaOH (pH 7.4), 5 mM G1P, 1.25 mM AMP, phosphorylase (54 unit) and enzyme preparation. The reaction was terminated by addition of 50 µl of 1 N HCl. The solution was mixed with 500 µl of dimethylsulfoxide and then 700 µl of 0.1% I₂ in 1% KI was added. The activity was assayed by measuring the increase in absorbance at 540 nm. One unit was defined as the amount causing an increase in absorbance of one unit at 540 nm.

The activities were calculated on the basis of fresh weight, and multiplied by fresh weight of the respective segment to obtain the activity in the segment.

Results

1. Varietal difference in the carbohydrate content of the whole third leaf sheath

After full elongation, the starch content of the whole third leaf sheath increased, reached maximum around heading, and decreased gradually thereafter to near zero (Fig. 1A). The maximum starch contents of the whole third leaf sheath were 132, 125, 102, 88 mg in Nipponbare, IR65598-112-2, Takanari and IR72, respectively. Sucrose content in IR65598-112-2 was approximately 4-fold higher than that in the

Fig. 1. Temporal changes of carbohydrate contents of the whole third leaf sheath in 4 rice varieties. Arrows represent heading date, and vertical bars indicate SE of means (n=4-5).
other varieties till late Aug. (Fig. 1B), and showed a temporal change similar to that of starch content. Sucrose contents in the other varieties showed only a slight temporal change. Both glucose and fructose contents decreased before heading and then increased a little after heading followed by a fall again in all the varieties. IR65598-112-2 had about 3-fold higher glucose and fructose contents than the other varieties from late July to early Aug. (Fig. 1C, D). Since the dry weight of the leaf sheath in IR65598-112-2 was approximately twice that in the other varieties (892 mg in IR65598-112-2 in contrast to 499, 375 and 354 mg in Nipponbare, IR72, Takanari respectively on Aug. 13), the maximum starch concentration in the third leaf sheath in IR65598-112-2 was about half of that in the other varieties (data not shown).

2. Spatial variation of carbohydrate content in the third leaf sheath

To investigate the spatial variation of carbohydrate content, we divided the third leaf sheath into five segments, seg. 1 to 5, with the same length, and measured the carbohydrate contents in every segment. In all varieties, the starch content was lowest in the apical segment (seg. 1) and increased toward the basal segment irrespective of developmental stage (Fig. 2). The starch content in the basal segment varied among the varieties, showing the maximum value of 40, 51, 60 and 72 mg in IR65598-112-2, IR72, Takanari and Nipponbare, respectively. On the contrary, the starch content in the apical segment ranged between 1 and 8 mg. Since the proportions of starch content in each segment to that in the whole leaf sheath were stable before Aug. 16 in each variety (data not shown), the proportions from July 31 to Aug. 16 were averaged in each segment and shown in Fig 3. The basal segment occupied 35%, 50%, 60% and 60% of starch in the whole leaf sheath in IR65598-112-2, Nipponbare, IR72 and Takanari, respectively. On the contrary, the apical segment occupied 7% in IR65598-112-2, and around 1% in the other varieties. In contrast to starch content, the proportions of sucrose content in each segment
were stable among the varieties (Fig. 3).

3. Activities of enzymes related to carbohydrate metabolism

Activities of enzymes related to carbohydrate metabolism were compared between Nipponbare and IR65598-112-2, since the temporal and the spatial variations in the carbohydrate content in the third leaf sheath were similar in IR72, Takanari and Nipponbare. The activities of enzymes were measured when starch contents in the whole leaf sheaths increased rapidly, i.e. July 31 in Nipponbare and Aug. 9 in IR65598-112-2 (Fig. 4). The activities of all enzymes, except GBSS in IR65598-112-2, increased from the apical to the basal segment in both varieties. Differences in the activities of AGPase, GBSS, and BE between the apical and basal segments were larger in Nipponbare than in IR65598-112-2, but that of SuSy were larger in IR65598-112-2. Compared with Nipponbare, IR65598-112-2 showed lower activities of AGPase and GBSS in the basal segment, and higher activities of GBSS and BE in the apical segment. Impressively, activities of SuSy in the apical and the basal segments of the leaf sheath, were 6- and 15-fold higher, respectively, in IR65598-112-2 than in Nipponbare.

Discussion

In this study, we examined the third leaf sheath because it accumulated much more starch than the flag leaf sheath (Hirose et al., 1999), and the leaf sheaths at a higher nodal position translocate more starch to the panicle than those at a lower nodal position (Togari and Sato, 1954; Watanabe et al., 1997).

Predominant accumulation of starch in the basal segment in the leaf sheath was reported in the previous works using temperate japonica varieties (Baba and Kitutaka 1953; Togari and Sato, 1954; Sato and Ehara, 1974). Our results confirmed that it was also the case in other ecotypes such as tropical japonica and indica varieties throughout the developmental stage (Fig. 2). To find the regulator of the starch content in each segment of the leaf sheath, we measured the activities of SuSy, AGPase, STS, GBSS and BE. SuSy cleaves sucrose to provide carbon source for starch and the other four enzymes are closely related to starch metabolism in leaf sheath (Perez et al., 1971; Watanabe et al., 1997; Hirose et al., 1999). The activities of SuSy, AGPase, STS, and BE increased from the apical segment to the basal segment in the leaf sheath of both Nipponbare and IR65598-112-2 (Fig. 4), which may be one of the reasons for the base-concentrated starch accumulation pattern in the leaf sheath. In addition, Sato and Ehara (1974) reported that the parenchyma tissue which contained amyloplast became larger basipetally in the leaf sheath.

The spatial variation of the starch accumulation in the leaf sheath, however, differed greatly among varieties (Fig. 3). This is mainly due to the significant varietal difference of the starch content in the basal segments. Among the five enzymes examined, the activity of GBSS was significantly lower in the basal segment in IR65598-112-2 than in Nipponbare, corresponded well with the difference in the starch content of the basal segment between the two varieties, suggesting that it may be the most important enzyme regulating starch metabolism in the leaf sheath. This is consistent with the report of Perez et al (1971).

It is intriguing that sucrose content and its temporal change in IR65598-112-2 were different from those in the other varieties (Fig. 1B). IR65598-112-2 had a sucrose content approximately 4-fold higher than the other varieties. And the ratio of sucrose content to starch content during the examined period was 0.45 to 0.61 in IR65598-112-2, but 0.08 to 0.19 in the other varieties. The temporal change of sucrose content was
similar to that of starch content in IR65598-112-2, but not in the other varieties. These results suggested that sucrose also serves as a storage carbohydrate like starch in IR65598-112-2. The sucrose may remain uncleaved, and the low activities of AGPase and GBSS may directly lead to the low starch content of the basal segment in IR65598-112-2.

The activity of SuSy in IR65598-112-2 was dramatically higher than that in Nipponbare (Fig. 4). SuSy catalyzes a reversible reaction, but mainly works in the direction of sucrose breakdown in sink organ (Borisjuk et al., 2003; Yang et al., 2003; Tomlinson et al., 2004). The activity depended on removal of the cleaved products and was inhibited by free hexoses (Ross and Davies, 1992; Weber et al., 1996). The sucrose content in IR65598-112-2 was high and almost constant during late July to mid August when SuSy activities were also high, compared with that in Nipponbare (Fig. 1B, 4), which conflicts with the suggestion that SuSy also cleave sucrose in the leaf sheath. Therefore, the role of SuSy in this line remains to be further elucidated including the possibility that SuSy functions to form sucrose at least in IR65598-112-2.

IR65598-112-2 was bred from tropical japonica germplasms as a new plant type with large panicles to increase the yield potential (Peng et al., 1999). However, it turned out to have a low grain-filling percentage (Peng et al., 1999; Yang et al., 2000). Grain-filling percentage is affected in part by the amount of carbohydrate stored in the stem (leaf sheaths and culms) before heading (Weng et al., 1982) and may be determined by the amount of stored carbohydrate per grain available in the initial 10 days of grain filling (Tsukaguchi et al., 1996). The maximum starch content in the whole third leaf sheath before heading was nearly the same in Nipponbare and IR65598-112-2. However, the maximum storage carbohydrate content in the third leaf sheath per grain was much lower in IR65598-112-2 (0.3 mg and 0.44 mg when sucrose was added) than in Nipponbare (0.96 mg), which may be related to the low grain-filling percentage in IR65598-112-2. Thus, the grain-filling percentage in IR65598-112-2 can be improved by increasing the starch accumulation in the basal segment of the leaf sheath.

In conclusion, the basal part of the leaf sheath accumulates the most starch in the leaf sheath,
irrespective of variety and developmental stage. However, the proportion of starch in the base to that in the whole sheath differed considerably among varieties. Activities of GBSS may regulate both the spatial variation and the varietal difference of starch content in the leaf sheath. Sucrose may be one of the carbohydrates stored before heading in IR65598-112-2.

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