The Effect of the Amount of Nitrogen Fertilizer on Starch Metabolism in Leaf Sheath of Japonica and Indica Rice Varieties during the Heading Period

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Abstract: The effects of the amount of nitrogen fertilizer on the starch metabolism of rice leaf sheath during the heading period in the japonica rice variety, cv. Nipponbare were compared with those in the indica varieties, cv. Tetep and Johna. The rice plants were grown under a low- (similar to the standard nitrogen level in paddy field) or high-nitrogen condition, and the starch content of the second leaf sheaths below the flag leaf was analyzed from the second leaf stage (growth stage 1) until 21 days after the heading (growth stage 7). The starch content of the plants grown under the high-nitrogen condition at the heading stage (growth stage 4) was lower than that under a low-nitrogen condition in all the varieties. The decrease in the activity of starch branching enzyme (SBE) was considered to be important for the repression of starch accumulation under a high-nitrogen condition. Under the high-nitrogen condition, Nipponbare accumulated more starch in the second leaf sheath than indica varieties at the heading stage. However, the phenomenon could not be accounted for by the activities of AGPase and SBE. Semi-quantitative RT-PCR analysis suggested that the lower activities of SBE in the second leaf sheath under the high-nitrogen condition may be due to, at least in part, the decrease in the expression level of RBE4.

Key words: ADP-glucose pyrophosphorylase, Debranching enzyme, Indica rice, Japonica rice, Leaf sheath, Nitrogen fertilizer, Starch, Starch branching enzyme.

Leaf sheath of rice serves as the temporary storage organ for photoassimilates. Starch accumulation in leaf sheaths reaches a maximum at the panicle development stage immediately before anthesis and starch content of the leaf sheath decreases rapidly during grain filling stage (Perez et al., 1971). Cock and Yoshida (1972) estimated that approximately 21% of the carbohydrate in the rice grain is derived from the starch accumulated temporarily in the culm including the leaf sheath before heading. Thus, in rice plants, it is important for improvement of the grain yield to elucidate the mechanisms of starch metabolism in leaf sheath.

Watanabe et al. (1997) suggested that the starch branching enzyme (SBE) is involved in the regulation of starch accumulation in the leaf sheath, possibly in collaboration with the other enzymes such as soluble starch synthase (SSS), granule-bound starch synthase (GBSS) and plastidial fructose-1,6-bisphosphatase. The mRNA levels of ADPglucose pyrophosphorylase (AGPase), SSS and SBE were highest around two weeks before heading when rapid starch accumulation was observed in the leaf sheath (Hirose et al., 1999). These reports indicate that some enzymes involved in the starch synthesis may regulate the starch accumulation in the rice leaf sheath during the heading period.

Starch debranching enzyme (DBE) catalyzes the cleavage of α-1,6-glucosidic linkage in amylopectin. However, analyses of mutants with the reduced starch content in endosperm in rice (Nakamura et al., 1996; 1997; Kubo et al., 1999), maize (James et al., 1995; Dinges et al., 2003), and barley (Burton et al., 2002) indicate that DBE plays a crucial role in the starch synthesis. Though a function of DBE in the rice leaf sheath is not clear, DBE may also be involved in the starch synthesis in leaf sheath.

Adaptability for heavy manuring is one of the important characteristics for the rice varieties. Generally, it is accepted that the yield of indica rice varieties with the low adaptability for heavy manuring is diminished with increasing the amount of nitrogen fertilizer application. Nagato et al. (1971) showed that in Tetep, an indica variety, the carbohydrate contents of culm and leaf sheath at the heading period were lowered by top-dressing at 15 days before heading, resulting in the lack of carbohydrate transported into...
the panicles after the anthesis. On the other hand, in
Kinmaze, a japonica variety, the contents were hardly
decreased by the top-dressing (Nagato et al., 1971).
These results indicate that the effects of nitrogen
fertilizer on the starch accumulation in the culm and
leaf sheath during the heading period may differ
between japonica and indica varieties.

Our objectives are to elucidate the mechanism
underlying the difference in the starch accumulation
in the leaf sheaths between japonica and indica
rice varieties, especially under the high-nitrogen
conditions. Here, we investigated the effect of the
heavy supply of nitrogen fertilizer on the starch
contents and the activities of starch-metabolizing
enzymes, AGPase, SBE and pullulanase-type DBE
in the japonica and indica rice leaf sheaths from
the second leaf stage to the grain filling stage. The
transcripts levels of AGPase and SBE genes were
also analyzed in the leaf sheath. We will discuss the
relationships between the starch content of the leaf
sheath and the starch-metabolizing enzymes under
excess nitrogen supply, and their differences between
the japonica and indica varieties.

Materials and Methods

1. Plant materials

Three varieties of rice (Oryza sativa L.) plants,
Nipponbare (japonica variety), Tetep (indica variety)
and Johna (indica variety), were used in this study.
Tetep that was used by Nagato et al. (1971) is a
typical indica variety. Johna is short in plant length
and early maturing variety as compared with Tetep.
Seeds sterilized with 1% sodium hypochlorite
solution containing 0.1% Tween-20 for 30 minutes
were germinated in distilled water for 48 hours at
30°C. The germinated seeds were sown on a nursery
box on 19 May, 2001, and grown until 3.5 leaf age
in a greenhouse. The two seedlings per pot were
transplanted separately into 1/5000a Wagner pots
containing 3.0 kg soil on 4 June, 2001, and grown
outdoors. The amount of fertilizer applied per pot was
0.3 g of P, 0.3 g of K and 0.3 g of N for a low-nitrogen
condition and 0.5 g of P, 0.5 g of K and 1.2 g of N for
a high-nitrogen condition. Top-dressing of nitrogen
fertilizer was conducted at the 10th leaf age and the
flag leaf stage with 0.15 g of N for a low-nitrogen
condition and 0.3 g of P, 0.3 g of K and 0.3 g of N for a high-nitrogen condition.
The low-nitrogen condition was similar to the standard
nitrogen level for cultivation in paddy field. When the
second leaf below the flag leaf expanded completely
(the second leaf stage; growth stage 1), the second
leaf sheath was harvested from the six plants in same
variety and nitrogen condition. Then the samplings
were carried out at the first leaf stage, the flag leaf
stage, the heading stage, 7 days after the heading, 14
days after the heading and 21 days after the heading,
which are referred to as growth stages 2, 3, 4, 5, 6 and
7, respectively, hereafter. Every sampling started at
8:00 a.m., and finished at 9:00 a.m. The harvested
leaf sheaths were longitudinally divided into two equal
parts, and then frozen in liquid nitrogen, and stored at
−80°C. The leaf sheaths of three plants were used for
the determination of starch content and the enzyme
assay. The remainder was used for extraction of total
RNA.

2. Determination of starch content

The frozen sample was ground with a mortar and
pestle in 5 mL of 80% (v/v) ethanol. The homogenate
was centrifuged at 1,500 × g for 15 minutes. The
supernatant was discarded, and the pellet was further
extracted with 2 mL of 80% (v/v) ethanol for 15
minutes at 70°C. The extract was centrifuged at 1,500
× g for 15 minutes, and then the pellet was used to
determine starch content according to the method
of Rufty and Huber (1983).

3. Enzyme assay

The frozen sample was homogenized with an ice-
cold mortar and pestle in 5 mL of extraction buffer
that contained 100 mM Tricine-HCl (pH 8.0), 8
mM MgCl₂, 2 mM EDTA, 50 mM 2-mercaptoethanol
and 12.5% (v/v) glycerol. The homogenate was
centrifuged at 10,000 × g for 5 min (< 4°C). The
supernatant was used as the crude enzyme solution
for the determination of AGPase and SBE activities
according to the method of Nakamura et al. (1989).
One unit of SBE activity was defined as the amount of
enzyme producing an increase of 0.1 in the absorbance
at 540 nm. For the determination of DBE (pullulanase)
activity, the supernatant was desalted by centrifugal
filtration on a Sephadex G-25 (Amersham Pharmacia)
column equilibrated with the grinding buffer minus
EDTA and glycerol (Hermerhorst and Stokes, 1980).
The activity of DBE was assayed by the method of
Nakamura et al. (1996).

4. Semi-quantitative RT-PCR and Southern blot
analysis

Total RNA was extracted from the frozen second
leaf sheath at the first leaf stage using Concert
Plant RNA Reagent (Invitrogen) according to the
manufacturers’ instructions. Twenty microgram of
total RNA was treated with 10 U of DNase and 40 U
of RNase inhibitor (TaKaRa) at 37°C for 30 min. The
reaction mixture was purified by phenol/chloroform
extraction, precipitated with 100% ethanol, and
dissolved in RNase free water. Two microgram of
DNase-treated total RNA was used as a template for
the first strand cDNA synthesis, which was performed
using Superscript II RNase H Reverse Transcriptase
(Invitrogen) in a reaction volume of 20 µl containing
1 × First-Strand buffer, 200 U of enzyme, 500 ng of
Oligo (dT)₁₂₋₁₈ primer, 0.5 mM dNTPs and 10 mM
DTT. The cDNA solution was treated with 2 U of E. coli RNase H (Invitrogen) at 37°C for 20 min, and then sterile, distilled water was added to the solution up to 50 µl.

Two cDNA fragments of rice AGPase small subunit genes have been reported on Genbank/EMBL/DDBJ Database. One (accession number: J04960) encodes putative cytosolic type small subunit (Anderson et al., 1989), and another (accession number: AY028315) encodes putative plastidial type small subunit (Sikka et al., 2001). Johnson et al. (2003) showed that AGPase small subunit genes of grass family were classified into two distinct subgroups, type 1 and type 2, which consist of cDNAs encoding the cytosolic or plastidial type small subunit, respectively. In this paper, we represented tentatively the cytosolic and plastidial type small subunit genes as AGPS1 and AGPS2, respectively.

The cDNA fragments of rice AGPase small subunit genes, AGPS1 (accession number: J04960) and AGPS2 (accession number: AY028315), rice starch branching enzyme genes, RBE1 (accession number: D11082) and RBE4 (accession number: AB023498) were amplified using AmpliTaq Gold polymerase (Applied Biosystems) and 1 µl of the first strand cDNA as a template. Rice actin 1 gene, Racl (accession number: X16280) was used as internal standard. Gene-specific primers were designed on the basis of the cDNA sequences of each gene. The pairs of primers of each gene were as follows: AGPS1 (5' - ACC TCG ACA CTT GCC TCC TT – 3', 5' - CTT ATA ATG CCC GCC TGG TG – 3'), AGPS2 (5' - ACC TCG ATA CTT GCC TCC AT – 3', 5' - GTG GTG ATT GTG TGC CGG CGG AT – 3'), RBE1 (5' - ACG TGG AGA TTT GGT TTT TG – 3', 5' - CAT TAG TAC AAG GGC ACC AAC AG – 3'), RBE4 (5' - TGA GTG TGG GCA TCC TGG AT – 3', 5' - GCA TAC AGC AGC GCC GTT CT – 3') and Racl (5' - TCG TCT GGG ATA ATG GAA CT – 3', 5' - ATC TTC GTT GCT CAT CCT GT – 3'). The cDNA fragments obtained after the amplification of 16, 18 and 20 cycles were electrophoresed on 1.5% (w/v) agarose gel and after the amplification of 16, 18 and 20 cycles were transferred onto a Hybond N+ nylon membrane. For the above-mentioned primers and the cDNA derived from the shoot of Nipponbare as template, and purified using QIAquick PCR Purification Kit (QIAGEN). Hybridization, probe labelling and signal detection were performed using the AlkPhos Direct Nucleic Acid Labelling and Detection System (Amersham Pharmacia) according to the manufacturer's recommendation. The hybridized signals were quantified with Lumivision Imager (AISIN SEIKI CO., LTD), and the mRNA level of each gene was standardized to the expression level of Racl.

**Table 1. Effect of the amount of nitrogen fertilizer on plant height at the heading stage, maximum tiller numbers and total dry weight at the heading stage.**

| Varieties     | Treatment | Plant height | Maximum tiller | Total dry weight |
|---------------|-----------|--------------|----------------|-----------------|
|               |           | (cm)         | numbers        | (g)             |
| Nipponbare    | Low nitrogen | 21.0a        | 17.9a          | 18.0a           |
|               | High nitrogen | 21.0b        | 39.6b          | 21.0b           |
| Tetep         | Low nitrogen | 14.7a        | 42.5a          | 14.7a           |
|               | High nitrogen | 17.0a        | 58.4b          | 17.0a           |
| Johna         | Low nitrogen | 16.7a        | 26.7a          | 16.7a           |
|               | High nitrogen | 22.3b        | 50.6b          | 22.3b           |

The values are the mean of results from three plants. Means followed by the different letter within a column of the same variety are significantly different at the 5% level by Student's T-test.

**Results**

1. **Effects of nitrogen fertilizer conditions on plant growth.**

In the present study, the flag leaf appeared as the 15th and 16th leaf, under low- and high-nitrogen conditions, respectively, in all the varieties. To analyze the second leaf sheath below the flag leaf, we used the 13th and 14th leaf sheaths of the plants grown under a low- and high-nitrogen conditions, respectively, in this experiment. Because the rate of leaf appearance differed among the three varieties, the effect of the amount of nitrogen fertilizer on the starch contents was compared among the varieties at each identical leaf age until the heading stage. The interval between each successive stage before the heading was six to twelve days.

The starch content of the second leaf sheath of Nipponbare grown under the low-nitrogen condition increased rapidly after growth stage 1 (Fig. 1). Subsequently, the content reached to the peak at growth stage 3, and then decreased gradually thereafter. In Tetep and Johna, the starch contents peaked at the heading period under the low-nitrogen
The heavy application of nitrogen fertilizer diminished the starch level in all the varieties. However, the amount of starch accumulation in Nipponbare grown under the high-nitrogen condition was as much as those in Tetep and Johna grown under the low-nitrogen condition. Under both nitrogen conditions, the second leaf sheath of Nipponbare always accumulated more starch than those of Tetep and Johna.

3. Changes in the activities of starch-synthesizing enzymes in the second leaf sheath

AGPase catalyzes the first unique step in the starch biosynthesis pathway, which produces ADP-glucose as the glucosyl donor from glucose-1-P and ATP, and plays a key role in the regulation of starch synthesis (Preiss et al., 1991). In Nipponbare, the AGPase activities increased rapidly from growth stage 1 to 2 under both nitrogen conditions (Fig. 2). Thereafter, the activity in the plants grown under low-nitrogen
condition decreased largely, while the activity under the high-nitrogen condition remained at a high level until growth stage 4. The activity from growth stage 2 to 4 was higher under the high-nitrogen condition than under the low-nitrogen condition. In Tetep, the AGPase activity under the high-nitrogen condition was higher than that observed under the low-nitrogen condition throughout the experimental period. In Johna, the maximum activities of AGPase were not different significantly between the plants under the two nitrogen conditions.

SBE is involved in the formation of α-1,6 glucosidic bond in the amylopectin biosynthesis. In Nipponbare, the SBE activity in the plants grown under the high-nitrogen condition was lower than that in those grown under the low-nitrogen condition throughout the experimental period (Fig. 3). In Tetep and Johna, a similar result was also observed, except for at growth stage 7. Under the low-nitrogen condition, the SBE activity in all the varieties peaked at growth stage 1, and then the activity decreased gradually. Under the low-nitrogen condition, the activity in Nipponbare
was higher than that in Tetep and Johna at all growth stages. Under the high-nitrogen condition, the activity in Johna was higher than that in Nipponbare and Tetep from growth stage 1 to 2.

There are two types of DBE, pullulanase and isoamylase, that hydrolyze the $\alpha$-1,6-glucosidic bond in amyllopectin. In addition, it is accepted that DBE also plays an important role in starch synthesis (James et al., 1995; Nakamura et al., 1996; 1997; Kubo et al., 1999; Burton et al., 2002; Dinges et al., 2003). We examined the activity of pullulanase-type DBE in rice leaf sheath during the heading period (Fig. 4). The DBE activity of the second leaf sheath in Nipponbare was much lower than that in Tetep and Johna under the low-nitrogen condition. The activity in the indica varieties was at a high level from growth stage 2 to 4 under the low-nitrogen condition, but was low under the high-nitrogen condition.

4. Expression analysis of AGPS and RBE genes in the second leaf sheath

In all varieties, the activities of AGPase and SBE remained at a high level before the heading stage (growth stage 4). So we analyzed their transcripts levels in the second leaf sheath at growth stage 2, when starch accumulated rapidly, and the activities of AGPase and SBE were high. The mRNA level of each gene was represented as the values standardized to the expression level of $\text{RAC}_1$, a constitutively expressed gene. The density of hybridized signal originated from the RT-PCR products for $\text{RAC}_1$ was nearly equal among each sample (data not shown).

Plant AGPase is a heterotetrameric enzyme composed of small and large subunits. The existence of two forms of AGPase small subunits, putative cytosolic and plastidial types, has been reported in rice (Anderson et al., 1989; 1991; Sikka et al., 2001). In the present study, we investigated the transcript level of rice type 1 AGPS ($\text{AGPS1}$) and type 2 AGPS ($\text{AGPS2}$), which encode the putative cytosolic and plastidial type small subunit, respectively (Johnson et al., 2003). The heavy application of nitrogen fertilizer enhanced slightly the mRNA level of $\text{AGPS1}$ in all the varieties, while it decreased the expression of $\text{AGPS2}$ markedly in Nipponbare and Johna (Fig. 5).

The cDNA clones encoding three SBE isoforms, $\text{RBE1}$, $\text{RBE3}$ and $\text{RBE4}$, have already been characterized in rice (Mizuno et al., 1992; 1993; 2001). The gene expression level of $\text{RBE1}$ in the leaves was found to be very low (Mizuno et al., 1992; 1993). The $\text{RBE4}$ was expressed in the leaves as well as in the developing seeds (Mizuno et al., 2001). Then, in this study, the transcripts level of $\text{RBE1}$ and $\text{RBE4}$ were analyzed in the second leaf sheath at growth stage 2 (first leaf stage) by semi-quantitative RT-PCR. The mRNA level of $\text{RBE1}$ was higher in Nipponbare than in Tetep and Johna under both nitrogen conditions (Fig. 5). In all the varieties, the heavy application of nitrogen fertilizer lowered the expression level of $\text{RBE4}$, compared with the low-nitrogen condition, while the amount of the application of nitrogen fertilizer did not affect the transcriptional level of $\text{RBE1}$.

Discussion

Nagato and Chaudhry (1969) showed that indica varieties contained less carbohydrate in leaf sheath and culm at heading stage, compared with japonica varieties. This report is consistent with our data on the starch contents of the second leaf sheath of rice.
plants grown under the low-nitrogen condition. In addition, as shown in Fig. 1, starch levels in the second leaf sheath during the heading period decreased under the high-nitrogen condition not only in indica varieties but also in the japonica variety. Nagato et al. (1971) demonstrated that the starch contents of the leaf sheath and culm were lowered by top-dressing at 15 days before heading in the indica rice varieties, cv. Tetep, but not in the japonica, cv. Kinnmaze. These inconsistent results in the starch contents of japonica varieties may be due to a difference in the method of heavy nitrogen application, basal dressing or top dressing and/or in the varieties, Nipponbare and Kinnmaze. In addition, whether or not these differences in the starch level in the leaf sheath between Nipponbare and two indica varieties are generally observed in other japonica and indica varieties should be examined with other varieties.

The plant length and maximum tiller numbers were higher under the high-nitrogen condition than under the low-nitrogen condition in all the varieties. This stimulated growth under the high-nitrogen condition may lead to the decrease in the starch accumulation in the leaf sheath at the heading stage. However, Scheible et al. (1997) proposed that nitrate acts as a signal molecule to control the starch synthesis. It is of interest to investigate whether or not nitrogen compounds, such as nitrate and ammonium, would regulate directly the starch accumulation in the rice leaf sheath.

Watanabe et al. (1997) described that the contribution of AGPase activity to developmental change of starch content is relatively less significant in rice leaf sheaths. In the present study, the activity of AGPase in the plants grown under the high-nitrogen condition with less starch content was higher than that observed under the low-nitrogen condition in all the varieties (Fig. 2). These results indicate that the AGPase activity is not responsible for the decrease of starch accumulation under the heavy nitrogen application in the leaf sheath at the heading stage. The effect of the heavy nitrogen application on the expression of AGPase gene differed between two genes encoding the AGPase small subunit, AGPS1 and AGPS2. The mRNA level of AGPS2 in Nipponbare and Johna was largely lowered under the high-nitrogen condition. However, the changes in the transcripts level of AGPS2 in response to the nitrogen condition was inconsistent with that of the AGPase activity. The expression level of AGPS1 rather than AGPS2 may be related to the changes in the AGPase activity in the rice leaf sheath. AGPase is known to be sensitive to allosteric regulation, being activated by glyceraldehyde-3-phosphate (3PGA) and inhibited by Pi (Sowokinos, 1981; Sowokinos and Preiss, 1982; Preiss, 1988). Tiessen et al. (2002) recently showed that potato tuber AGPase was under redox-dependent posttranslational regulation. These regulatory mechanisms as well as the transcriptional regulation are probably involved in the changes in the AGPase activity in the rice leaf sheath.

Before the heading, SBE activity under a high-nitrogen condition was lower than that under the low-nitrogen condition in all the varieties. SBE has been reported to be involved in the regulation of starch metabolism in leaf sheaths (Watanabe et al., 1997). Thus, the reduction in the SBE activity before the heading stage under the high-nitrogen condition may be one of important factors that lead to less starch accumulation in leaf sheaths at the heading period. The expression of RBE4, but not RBE1, in the second leaf sheath was repressed by the heavy application of nitrogen fertilizer in all the varieties. Thus, the lower activity of SBE under the high-nitrogen condition may be due to, at least in part, the decrease in the expression level of RBE4. In rice plants, although the expression level of RBE4 was similar to that of RBE1 in the leaves, RBE1 was expressed more intensively in seeds than in leaves and stems, while RBE4 was expressed in the leaves as well as in the developing seeds (Kawasaki et al., 1993; Mizuno et al., 2001). In Mutator insertional maize mutant of BEIIa type starch branching enzyme, to which RBE4 belongs, short chains of amylopectin were much diminished in leaf starch, but there was no difference in the chain-profile for amylopectin of kernel starch between the mutant and wild-type (Blauth et al., 2001). These results suggest that RBE4 may play a crucial role in the changes in the amount of starch accumulation and the SBE activity to the nitrogen conditions in the rice leaf sheath.

In endosperm during grain filling of cereal plants, DBE probably play a crucial role in the starch synthesis (James et al., 1995, Nakamura et al., 1996; 1997; Kubo et al., 1999; Burton et al., 2002; Dinges et al., 2003). As shown in Fig. 4, in indica varieties, the DBE activity of the second leaf sheath increased rapidly before the heading stage, when the starch accumulation was in progress, under the low-nitrogen condition. On the other hand, the DBE activity under the high-nitrogen condition remained at a low level through the sampling period. Therefore, in the leaf sheath of indica varieties, the pullulanase-type DBE seems to be involved in the starch synthesis as well as the amylopectin degradation, especially before the heading. The DBE activity of the second leaf sheath in Nipponbare was much lower than those observed in indica varieties. Recently, Umemo et al. (2002) proposed that the different alleles of starch synthase IIa gene may be responsible for the difference in the structure of amyllopectin between a japonica and an indica varieties. It is possible that the difference in the DBE activity among these rice varieties affects the structure of amylopectin in the leaf sheath.

Under the high-nitrogen condition, the starch content at the heading stage of the second leaf
sheath of Nipponbare was much higher than that in the indica varieties. However, in the present study, the phenomenon could not be accounted for by the activity of AGPase or SBE. The starch synthesis and degradation simultaneously occur in the temporary sink organ such as the rice leaf sheaths. In addition, the activities of SSS and GBSS are positively correlated with starch content, although the correlation coefficient was lower than that estimated between the SBE activity and starch content (Watanabe et al., 1997). The possibility that the starch degradation enzymes, SSS and/or GBSS may be related to the differences in the starch content of rice leaf sheaths between japonica and indica varieties under the high-nitrogen condition should be examined hereafter.

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