Involvement of $\alpha$-Amylase Genes in Starch Degradation in Rice Leaf Sheaths at the Post-Heading Stage

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Abstract: Identifying the mechanisms regulating starch remobilization after heading in rice leaf sheaths is essential to understand the capability of the source for grain filling. In the present study, the changes in starch content and expression levels of $\alpha$-amylase genes in the third leaf sheaths of Takanari, a high-yielding indica cultivar, were compared with those of Nipponbare, a standard japonica cultivar, during the post-heading stage to examine the starch remobilization characteristics in the leaf sheath of a high-yielding cultivar. Starch content in Takanari tended to decrease at a faster rate than in Nipponbare starting 3 days after heading. The decrease in starch content during 12 days after heading was greater in Takanari than in Nipponbare. Of eight genes predicted to encode $\alpha$-amylase in the rice genome, $\text{RAMy2A}$ and $\text{RAMy3C}$ were primarily expressed in the leaf sheaths after heading. Moreover, $\text{RAMy2A}$ mRNA level peaked at 9 days after heading in both cultivars. Particularly in Takanari, the $\text{RAMy2A}$ mRNA levels rapidly increased from 3 to 9 days after heading. In addition, $\alpha$-amylase activity was significantly higher in Takanari than in Nipponbare at 9 days after heading. Our results suggest that the rapid degradation of starch in the leaf sheaths of Takanari at the post-heading stage may be attributed, at least in part, to the enhancement of $\alpha$-amylase activity caused by an increase in $\text{RAMy2A}$ transcription level.

Key words: $\alpha$-Amylase, Leaf sheath, Oryza sativa L., Starch degradation.

Heavy panicle type rice cultivars have been recently bred in Japan due to the increase in demand of rice as a forage as well as a food crop. Heavy panicle type cultivars generally have lower grain ripening percentages because of the increased demand for carbohydrates to fill the grains. Thus, the ability to supply carbohydrates for grain filling must be enhanced to improve the grain ripening percentage. In rice, carbon sources for grain filling comprise non-structural carbohydrates (NSCs) in culms and leaf sheaths before heading and carbohydrates supplied from photo-assimilates after heading. Approximately 30% of rice grain carbohydrates are derived from NSC (Cock and Yoshida, 1972). Weng et al. (1982) suggested that NSCs in the culms and leaf sheaths influence the increase in panicle weight by contributing to the production of active caryopses. Thus, elucidating the mechanisms regulating starch synthesis before heading and starch remobilization after heading in culms and leaf sheaths is essential to understand the capability of the source for grain filling.

Many studies have been made on the key enzymes related to starch synthesis in rice leaf sheaths (Watanabe et al., 1997; Hirose et al., 1999; He et al., 2005; Hirano et al., 2005; Hirose et al., 2006). Hirose et al. (2006) demonstrated that $\text{AGPL1}$ and $\text{AGPS1}$ encoding ADP-glucose pyrophosphorylase (AGPase), $\text{SSI}$, $\text{SSIb}$, $\text{SSIIIb}$ and $\text{GBSSII}$ encoding starch synthase (SS), and $\text{BEIIa}$ encoding starch-branching enzymes (BE) are involved in starch synthesis in leaf sheaths. In contrast, a few reports have addressed the relationship between starch degradation enzymes and changes in starch content after heading in leaf sheaths. Ishimaru et al. (2004) reported that $\alpha$-amylase activity is consistent with the degree of starch degradation in leaf sheaths at the heading stage. The transcription level of $\text{RAMy2A}$, which encodes $\alpha$-amylase, increases in leaf sheaths after heading (Chen and Wang, 2008). These results indicate that $\alpha$-amylases may play an important role in starch degradation after heading in leaf sheaths.

Takanari, a semi-dwarf indica cultivar developed from a cross between Milyang 42 and Milyang 25, has a high percentage of grain ripening despite the large number of spikelets per panicle (Xu et al., 1997a), suggesting that it possesses a high capability to fulfill the carbohydrate demand for its large sink. The quantity of NSCs...
translocated from the culms and leaf sheaths to the panicles after heading is higher in Takanari than that in the japonica cultivar, Nipponbare (Xu et al., 1997b). However, Nagata et al. (2001) reported that NSC plays a role compensating for the shortage of carbohydrate supplied from photo-assimilates after heading in Takanari. In contrast, although the net quantity of translocated NSC in Takanari is similar to that in japonica-dominant high-yielding cultivars, the indica-dominant high-yielding cultivars including Takanari showed an earlier decrease in NSC content during the grain filling stage than that in japonica-dominant high-yielding cultivars (Yoshinaga et al., 2013). Thus, Takanari may possess a high capability to remobilize reserved starch, a major NSC, in culms and leaf sheaths.

The present study was carried out to evaluate the involvement of α-amylase genes in starch remobilization in leaf sheaths. In particular, to examine the characteristics of starch remobilization in the high-yielding cultivar Takanari, we compared the changes in leaf sheath starch content in Takanari with that in the standard cultivar, Nipponbare, during the post-heading stage. Furthermore, the transcriptional levels of the α-amylase genes \( \text{RAmy2A} \) and \( \text{RAmy3C} \), which are preferentially expressed in leaf sheaths and culms, were analyzed in detail in Nipponbare and Takanari leaf sheaths.

## Materials and Methods

### 1. Plant materials

Two cultivars of rice (\textit{Oryza sativa} L.), Nipponbare (standard japonica cultivar) and Takanari (high-yielding indica cultivar), were used in this study. The seeds were sterilized in 1% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20 for 30 min and immersed in distilled water for 2 days at 30°C. The germinated seeds were sown in a nursery box on May 24, 2013. Three seedlings per pot were separately transplanted and grown outdoors in 1/5000 a Wagner pots containing 3.0 kg soil on June 7, 2013. As a basal fertilizer, chemical fertilizer consisting of N (15%), P (15%), and K (10%) was applied at 2.0 g per pot. Nitrogen fertilizer in the form of ammonium sulfate (0.3 g of N per pot) was also applied at the 10th leaf stage. The second leaf sheaths below the flag leaf (i.e., the third leaf sheaths) were harvested at the flag leaf-emerging stage, the heading stage (Nipponbare: August 17, Takanari: August 19), and 3, 6, 9, 12, 18, 24, and 40 days after the heading stage. The harvested samples were longitudinally divided into two equal parts, immediately frozen in liquid nitrogen, and stored at −80°C. After heading, the panicles on the main stems were harvested and dried at 80°C for 5 days to determine their dry weight.

### 2. Determination of starch and sugar contents

The frozen samples were ground with a mortar and pestle in 5 mL of 80% (v/v) ethanol. The homogenate was centrifuged at 1,500 × g for 15 min, and the supernatant was decanted into a glass tube. The pellet was further extracted with 3 mL of 80% (v/v) ethanol for 15 min at 75°C, and the extract was centrifuged at 1,500 × g for 15 min. The supernatant was mixed with the first supernatant and dried by evaporation. Starch content of the pellet was determined according to the method of Hirano et al. (2005). The dried supernatant was dissolved in 2 mL distilled water and extracted with 2 mL chloroform. After centrifugation, the upper aquatic phase was collected and filtered through a membrane filter. Sucrose, glucose, and fructose contents of the filtered solution were determined using high performance liquid chromatography (Prominence Reducing Sugar Analysis System, Shimadzu) equipped with Shim-Pack ISA-07/s2542 column (Shimadzu), using 0.1 M potassium borate buffer (pH 8.0) and 0.4 M potassium borate buffer (pH 9.0) as the mobile phase and 1% (w/v) arginine and 3% (w/v) boric acid solution as the reaction reagent.

### 3. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extraction and cDNA synthesis were
performed according to the method of Hirano et al. (2011). The transcription levels of eight α-amylase genes were analyzed by semi-quantitative RT-PCR using the gene specific primers listed in Table 1. An aliquot of cDNA solution corresponding to 40 ng total RNA extracted from various organs was used as the template. The reaction was performed using AmpliTaq Gold DNA polymerase (Life Technologies) under the following conditions: 26 – 35 PCR cycles of treatment for 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The amplified fragments were electrophoresed on a 1.5% (w/v) agarose gel.

4. Quantitative RT-PCR

RAmy2A and RAmy3C mRNA levels in the third leaf sheaths were analyzed by quantitative RT-PCR using the gene specific primers listed in Table 1. An aliquot of cDNA solution corresponding to 40 ng total RNA extracted from the third leaf sheaths was used as the template. Quantitative RT-PCR was carried out using a StepOnePlus Real-Time PCR System (Life Technologies) in a 20 μL reaction mixture containing Power SYBER Green PCR Master Mix (Life Technologies). A serial dilution series of plasmid DNA containing the partial cDNAs of RAmy2A or RAmy3C was prepared to create a standard curve for each gene. The mRNA level of each gene estimated from the obtained standard curve was standardized to the expression of OsEF1α as a housekeeping gene, which encodes the translation elongation factor 1A.

5. Determination of α-amylase activity

Leaf sheath samples at 9 days after heading were ground and extracted with 50 mM malic acid buffer (pH 5.4) containing 50 mM sodium chloride, 2 mM calcium chloride, and 0.005% (w/v) sodium azide on ice. α-Amylase activity in the samples was measured according to the Ceralpha™ assay kit instructions (Megazyme) using blocked p-nitrophenyl maltoheptaoside (BPNPG7) as the substrate.

Results

1. Changes in panicle dry weight

In Nipponbare, dry weight of the panicle on the main stem gradually increased from 6 to 24 days after heading (Fig. 1). In contrast, in Takanari, the panicle dry weight increased from 3 to 18 days after heading and at a faster rate than that in Nipponbare. Consequently, the panicle dry weight in Takanari was approximately twice that in Nipponbare at 18 days after heading.

2. Changes in starch and sugar contents of the third leaf sheaths

Starch content of the third leaf sheaths in Nipponbare increased from the flag leaf emerging stage to the heading stage but decreased from 3 to 9 days after heading (Fig. 2). Subsequently, the starch content increased again from 12 to 18 days after heading. Starch content tended to decrease at a faster rate in Takanari than in Nipponbare starting 3 days after heading. Moreover, the starch content continued to decrease until 12 days after heading, so that the starch content was significantly lower in Takanari than in Nipponbare at 12 days after heading. The starch content in Takanari remained at a substantially low level since 12 days after heading.

Sucrose content of the third leaf sheaths in Nipponbare decreased from 6 to 9 days after heading and then tended to gradually increase (Fig. 3). The sucrose content in Takanari was higher than that in Nipponbare at the flag leaf emerging stage, but it rapidly decreased until 3 days after heading. Subsequently, sucrose content gradually decreased.
increased from 3 to 9 days after heading but decreased again from 9 to 12 days after heading. Glucose and fructose contents in Nipponbare peaked at 6 and 12 days after heading. Glucose and fructose contents in Takanari were higher than in Nipponbare until 3 days after heading, but decreased to the same extent as those in Nipponbare at 6 days after heading. Their contents increased again from 6 to 9 days after heading and rapidly decreased thereafter.

3. **α-Amylase gene expression analysis in various organs**

α-Amylase isoforms have been shown to be encoded by a multiple gene family in rice (Mitsui and Itoh, 1997), and ten genes encoding α-amylase isoforms were identified in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp). Among these ten genes, the two genes (locus ID: Os02g0765400 and Os04g0403300) were not analyzed in the present study, because the sufficient information on their transcripts was not listed in the Rice Annotation Project Database. Thus, we analyzed the expression levels of eight genes (RAmy1A, RAmy1B, RAmy2A, RAmy3A, RAmy3B, RAmy3C, RAmy3D, and RAmy3E), which have been annotated in the Oryzabase (http://www.shigen.nig.ac.jp/rice/oryzabase/), in various organs. The locus ID of their genes is shown in Table 1.

RAmy1A, RAmy1B, RAmy3B, and RAmy3D were specifically expressed in germinating seeds (Fig. 4), whereas RAmy3A was specifically expressed in developing caryopses. The RAmy3E transcript was detected in both germinating seeds and developing caryopses. RAmy2A was clearly expressed not only in developing caryopses but also in leaf sheaths before heading, leaf sheaths after heading, and internodes after heading. The RAmy3C transcript was clearly detected in germinating seeds. In addition, its transcript was detected at low levels in all other organs.

4. **Changes in RAmy2A and RAmy3C mRNA levels in the third leaf sheaths**

The RAmy2A mRNA level was higher than the RAmy3C level in both cultivars throughout the experimental period (Fig. 5). The RAmy2A mRNA level in Nipponbare was constant from the heading stage until 3 days after heading. The levels significantly decreased until 6 days after heading.

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**Fig. 3.** Changes in sucrose (a), glucose (b) and fructose (c) contents of the third leaf sheaths. Values are the means ± standard errors of three replications.

**Fig. 4.** Expression analyses of the α-amylase genes family in various organs. OsEF1α1, encoding translation elongation factor 1A, was used as the internal standard.
but significantly increased from 6 to 9 days after heading. In contrast, the \( R\text{Amy}2\text{A} \) mRNA level in Takanari rapidly increased starting 3 days after heading and peaked at 9 days after heading. These mRNA levels were significantly higher in Takanari than in Nipponbare since 3 days after heading.

\( R\text{Amy}3\text{C} \) mRNA levels increased in both cultivars from the flag leaf emerging stage to the heading stage and remained constant until 6 days after heading. \( R\text{Amy}3\text{C} \) mRNA level in Nipponbare increased from 6 to 9 days after heading but decreased 9 to 12 days after heading. \( R\text{Amy}3\text{C} \) mRNA level in Takanari increased from 6 to 9 days after heading but decreased from 12 to 18 days after heading.

5. \( \alpha \)-Amylase activity in the third leaf sheaths

\( \alpha \)-Amylase activity was measured in the third leaf sheaths at 9 days after heading when \( R\text{Amy}2\text{A} \) mRNA level was at its maximum. \( \alpha \)-Amylase activity was significantly higher in Takanari than that in Nipponbare (Table 2).

Table 2. \( \alpha \)-Amylase activity in the third leaf sheath at 9 days after heading.

| Cultivar   | \( \alpha \)-amylase activity (\( \mu \text{mol hr}^{-1} \text{gFW}^{-1} \)) |
|------------|--------------------------------------------------------------------------------|
| Nipponbare | 1.71 ± 0.79                                                                     |
| Takanari   | 7.94 ± 0.92                                                                     |

*Values are means ± standard errors of three replications. *, significant at the 5% level by Student’s \( t \)-test.

Discussion

Starch content of the third leaf sheaths decreased starting 3 days after heading in both cultivars (Fig. 2). In addition, the starch content in Takanari tended to decrease at a faster rate than in Nipponbare starting 3 days after heading. Moreover, the starch content continued to decrease until 12 days after heading, so that the starch content was significantly lower in Takanari than in Nipponbare at 12 days after heading. These results indicated that the decrease in starch content is greater in Takanari than in Nipponbare. Yoshinaga et al. (2013) reported that the decrease of NSCs in the culms and leaf sheaths during the post-heading stage occurs earlier in Takanari than in Nipponbare but the net amount of the NSC decrease in Takanari remains similar to that in Nipponbare. Thus, the decrease in the amount of starch content in the present study did not agree with the findings reported by Yoshinaga et al. (2013). This inconsistency may be partly explained by the differences in the plant organs (third leaf sheaths vs culms and leaf sheaths) used for the analysis of starch content. Our results indicated that starch degradation in the third leaf sheaths was probably activated starting 3 days after heading, and the activity was higher in Takanari than in Nipponbare. Panicle dry weight increased at a faster rate in Takanari than in Nipponbare (Fig. 1). The indica-dominant high-yielding cultivars such as Takanari showed an earlier decrease in NSC content during grain-filling and a larger increase in panicle weight (Yoshinaga et al., 2013). Thus, our results suggest that high capability to degrade starch in the third leaf sheaths after heading may also, at least in part, lead to a larger increase in panicle dry weight in Takanari. Furthermore, sucrose content of the third leaf sheaths in Takanari increased from 3 to 9 days after heading (Fig. 3), indicating that sucrose temporarily accumulates in the third leaf sheaths, probably due to excess starch degradation relative to the amount of sucrose exported to the ripening grains.

The functions of \( \alpha \)-amylase genes in germinating seeds have been examined in detail (Huang et al., 1990; Karrer, et al., 1991, 1992; Ranjhan et al., 1992; Thomas and Rodriguez, 1994; Umemura et al., 1998). \( R\text{Amy}3\text{D} \) gene
expression is induced in the scutellum when the dry rice seeds begin to germinate (Karrer, et al., 1991; Ranjhan et al., 1992). Subsequently, the \textit{RAmy}1A transcript level sharply increases in the aleurone layer as the embryo grows and \textit{RAmy}3B, \textit{RAmy}3C, and \textit{RAmy}3E are also gradually expressed (Thomas and Rodriguez, 1994). In the present study, the \textit{RAmy}1A, \textit{RAmy}3B, \textit{RAmy}3C, \textit{RAmy}3D, and \textit{RAmy}3E transcripts were detected in germinating seeds (Fig. 4). Few studies have examined the expression levels of \(\alpha\)-amylase genes in the leaf sheaths and culms, although Ishimaru et al. (2004) reported that \(\alpha\)-amylase activity is consistent with the degree of starch degradation at the heading stage. Amy1-1, encoded by \textit{RAmy}1A, is involved in starch degradation in rice leaves (Asatsuma et al., 2005). However, we did not detect the \textit{RAmy}2A transcript in leaf sheaths or culms (Fig. 4). In contrast, \textit{RAmy}2A was clearly expressed in the leaf sheaths before heading, the leaf sheaths after heading, and the internodes after heading (Fig. 4). In addition, the \textit{RAmy}3C transcript was detected in all analyzed organs (Fig. 4). The \textit{RAmy}2A transcript has been detected in germinating seeds, roots, etiolated leaves, immature seeds, and callus (Huang et al., 1992). Chen and Wang (2008) showed that the \textit{RAmy}2A transcriptional level increases in leaf sheaths after heading. Furthermore, our results indicated that \textit{RAmy}2A and \textit{RAmy}3C are the \(\alpha\)-amylase genes primarily expressed in leaf sheaths and culms. \textit{RAmy}2A mRNA level peaked at 9 days after heading in both cultivars (Fig. 5). The result agreed with the previous study of Chen and Wang (2008). In contrast, the mRNA level of \textit{RAmy}3C was lower than that of \textit{RAmy}2A throughout the experimental period (Fig. 5). Therefore it may be mainly \textit{RAmy}2A, and not \textit{RAmy}3C, that functions in starch degradation in leaf sheaths after heading. The \textit{RAmy}2A mRNA level in Takanari rapidly increased from 3 to 9 days after heading (Fig. 5) when starch content rapidly decreased (Fig. 2). Moreover, \(\alpha\)-amylase activity was significantly higher in Takanari than in Nipponbare at 9 days after heading (Table 2). In conclusion, our results suggest that the rapid starch degradation in leaf sheaths of Takanari at the post-heading stage is attributed, at least in part, to enhanced \(\alpha\)-amylase activity caused by an increase in the \textit{RAmy}2A transcriptional level.

We propose that \textit{RAmy}2A, encoding one of the \(\alpha\)-amylase isoforms, may play an important role in leaf sheath starch degradation at the post-heading stage. In Takanari, the percentage of grain ripening is high although the number of spikelets per panicle is large (Xu et al., 1997a). Thus, the rapid starch degradation in leaf sheaths after heading induced by the increased \textit{RAmy}2A expression may be related to high grain-filling ability. Further studies are needed to elucidate the function of \textit{RAmy}2A in starch degradation in leaf sheaths after heading and to investigate varietal differences, such as those between japonica and indica.

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