Identification of Two Plastid-Targeted $\beta$-Amylases in Rice

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Abstract: In order to identify $\beta$-amylase isoforms acting inside plastids in rice (Oryza sativa L.), in the rice genome database we searched the genes predicted to encode $\beta$-amylase-like proteins, and designated them OsBAM1–9, OsBAM1, OsBAM7, and OsBAM9 genes were mainly expressed in germinating seeds and developing caryopses. In contrast, the transcripts of OsBAM2, OsBAM3, OsBAM4 and OsBAM5, which have a putative chloroplast transit peptide in their N-terminal regions, were mainly detected in leaf blades, leaf sheaths and internodes after heading. Soluble protein fractions prepared from Escherichia coli containing recombinant OsBAM2 and OsBAM3 proteins had significant $\beta$-amylase activity. Furthermore, OsBAM2-GFP and OsBAM3-GFP fusion proteins introduced into epidermis of Allium cepa scaly bulb were localized to the plastids. These results strongly suggest that OsBAM2 and OsBAM3 are plastid-targeted active $\beta$-amylase.

Key words: $\beta$-Amylase, Oryza sativa L., Starch metabolism.

Starch is the primary photoassimilate in many plants. It is transiently stored in the chloroplasts in leaves during the day and degraded during the following night as a carbon source to support metabolism (Zeeman et al., 2007). In rice, starch is stored at a high level in leaf sheaths prior to the heading stage and remobilized for grain filling during post-heading stage (Perez et al., 1971). Approximately 30% of the final grain yield is estimated to be derived from the carbohydrate accumulated transiently in the leaf sheaths and culms (Cock and Yoshida, 1972). Therefore, elucidation of the mechanism of starch remobilization at the post-heading stage in leaf sheaths is important for improving the rice grain yield. However, the molecular mechanism of enzymes involved in starch degradation in rice leaves has not fully been elucidated.

Exoamylolysis has been recently identified to play a crucial role in the degradation of starch accumulated in leaf chloroplasts. Scheidig et al. (2002) have demonstrated that the downregulation of a gene encoding a chloroplast-targeted $\beta$-amylase results in excess starch accumulation in potato leaves. In the Arabidopsis genome, nine genes (BAM1–BAM9) encode the $\beta$-amylase-like proteins (Smith et al., 2004). Of these isoforms, at least BAM1, BAM2, BAM3 and BAM4 are chloroplast-targeted $\beta$-amylase (Lao et al., 1999; Sparla et al., 2006; Fulton et al., 2008). A loss of BAM3 leads to elevated levels of starch in leaves (Kaplan and Gov, 2005). Fulton et al. (2008) reported that bam1 and bam3 double mutants have a more pronounced starch-excess phenotype than the bam3 single mutant, even though the starch level in leaves of the bam1 single mutant is normal. Furthermore, despite the fact that a mutant lacking BAM4 shows elevated starch accumulation in leaves, BAM4 has no detectable $\beta$-amylase activity (Fulton et al., 2008; Li et al., 2009). Hence, these facts reveal the significance and complexity of the $\beta$-amylase function in starch degradation in Arabidopsis leaves.

On the other hand, only a few studies on molecular basis of $\beta$-amylase function have been reported in rice (Wang et al., 1996; Yamaguchi et al., 1999; Saika et al., 2005). In Nipponbare, japonica rice cultivar, expression of the gene encoding the $\beta$-amylase-like protein (NCBI Protein Accession Number: BAC83773) has been reported to be deficient in the seedlings (Yamaguchi et al., 1999). This phenomenon results from insertion of a Mutator-like transposon in the promoter region of BAC83773 gene (Saika et al., 2005). However, since these studies were performed in germinating seeds and seedlings, little information is available concerning plastid-targeted $\beta$-amylase proteins in rice. In addition, although nine genes in the Arabidopsis genome have been shown to encode multiple $\beta$-amylase-like proteins (Smith et al., 2004), the molecular details of the $\beta$-amylase gene family...
in rice remain largely unknown.

To identify one or more β-amylase isoforms acting inside plastids in rice, we obtained information on nine genes predicted to encode β-amylase-like proteins in the rice genome, and focused on the two isoforms, OsBAM2 and OsBAM3. We demonstrate that OsBAM2 and OsBAM3 indeed function as plastid-targeted β-amylase.

Materials and Methods

1. DNA database search and phylogenetic analysis

We searched for genes encoding β-amylase-like proteins in the Rice TOGO Browser (http://agri-trait.dna.affrc.go.jp/), and found ten relevant genes. However, one of them (Os02g0129600) encoded a distinctly smaller protein than the other genes. Consequently, we analyzed nine genes predicted to encode β-amylase-like proteins. The phylogenetic tree was generated from an alignment of full-length β-amylase-like proteins in rice and Arabidopsis using the neighbor-joining method.

2. Plant materials

Germinated seeds of rice (Oryza sativa L. cv. Nipponbare) were sown in a nursery box and grown in a greenhouse until the 3.5-leaf stage. Three seedlings per pot were transplanted separately into 1/5000a Wagner pots containing 3.0 kg soil and grown outdoors. 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 was also applied at the 10th leaf stage at 0.15 g of N per pot. For analysis of the expression level of β-amylase genes, various organs were harvested, frozen immediately in liquid nitrogen, and stored at −80°C until use.

3. Semi-quantitative RT-PCR analysis

The frozen samples were ground to a fine powder in a mortar and pestle. Total RNA was extracted using PureLink Plant RNA Reagent (Invitrogen) according to the manufacturer’s instructions. A sample of 20 µg of total RNA was treated with 10 U of DNase (TaKaRa) at 37ºC for 30 min to eliminate genomic DNA contamination. The reaction mixture was purified by phenol/chloroform extraction, precipitated with 100% ethanol and dissolved in RNase-free water. The purified RNA was quantified by absorbance at 260 nm using a UV spectrophotometer.

A 2 µg sample of DNase-treated total RNA was used as a template for first-strand cDNA synthesis. The reaction was performed using SuperScript™ III Reverse Transcriptase (Invitrogen) with 50 pmol of Oligo (dT)20 Primer (Invitrogen). After the reaction, the cDNA solution was treated with 2 U of Escherichia coli RNase H (Invitrogen) at 37°C for 20 min.

Semi-quantitative RT-PCR was performed using the gene-specific primers listed in Table 1 and an aliquot of cDNA solution corresponding to 40 ng of total RNA was used as a template. The reaction was performed using PrimeSTAR™ HS DNA polymerase (TaKaRa) under the following conditions: 26–32 cycles of 10 s at 98°C, 5 s at 55°C, and 1 min at 72°C. The expression level of rice actin 1 gene (Rac1, GenBank accession number; X16280) was also examined as an internal control for quantification of the relative amount of cDNA used in PCR. The amplified fragments were

Table 1. Gene-specific primers used in this study.

| Gene name | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|-----------|--------------------------|--------------------------|
| For semi-quantitative RT-PCR |
| OsBAM1 | GTGCTGAGATGAGGGATTCTGAAC | CAGGAGGCTATCTTCTGTGATG |
| OsBAM2 | GGTCTGCGCTTGTCACGCAGA | TAAGGCTGCTGCTGCGAGG |
| OsBAM3 | CTTCCCTCTCTCTTCTGTGTA | TTCCTCTGTGCTGCTGAGG |
| OsBAM4 | GCTTCGCCGCGTCGACAGG | GCATGCTGCTGCTGCTGAGG |
| OsBAM5 | GCTTCGCCGCGTCGACAGG | GCATGCTGCTGCTGCTGAGG |
| OsBAM6 | GCTTCGCCGCGTCGACAGG | GCATGCTGCTGCTGCTGAGG |
| OsBAM7 | CTTCCCTCTCTCTTCTGTGTA | TTCCTCTGTGCTGCTGAGG |
| OsBAM8 | CTTCCCTCTCTCTTCTGTGTA | TTCCTCTGTGCTGCTGAGG |
| OsBAM9 | CTTCCCTCTCTCTTCTGTGTA | TTCCTCTGTGCTGCTGAGG |
| RAc1 | CCTACATGCGCTGCTGCTGTA | GGCTACACACACTGAGGAAG |

For construct of the GFP expression vector

| Gene name | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|-----------|--------------------------|--------------------------|
| OsBAM2 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM3 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM4 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM5 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM6 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM7 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM8 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM9 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| RAc1 | CCTACATGCGCTGCTGCTGTA | GGCTACACACACTGAGGAAG |

For construct of the expression vector of recombinant protein in E. coli

| Gene name | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|-----------|--------------------------|--------------------------|
| OsBAM2 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |
| OsBAM3 | ACGCTGCGCTGCTGCTGCTGTA | CAGGAGGCTATCTTCTGTGATG |

Other information:
The cDNA sequences encoding OsBAM2 and OsBAM3 were amplified by PCR using full-length cDNA clones obtained from the Rice Genome Resource Center (Tsukuba, Ibaraki, Japan) as a template with the gene specific primer pairs listed in Table 1. In each case, the amplified OsBAM2 and OsBAM3 cDNA sequences encoded 505 and 521 amino acid residues, respectively, from N-terminal methionine.

## 4. Subcellular localization of OsBAM2 and OsBAM3

The cDNA sequences of OsBAM2 and OsBAM3 were amplified by PCR using full-length cDNA clones obtained from the Rice Genome Resource Center (Tsukuba, Ibaraki, Japan) as a template with the gene specific primer pairs listed in Table 1. The amplified OsBAM2 and OsBAM3 cDNA sequences encoded 505 and 521 amino acid residues, respectively, from N-terminal methionine. Two PCR products were digested with SsaI and BamHI and ligated into the same restriction sites of a GFP expression vector (pUC18/CaMV35S/sGFP(S65T)/nos) (Chiu et al., 1996), resulting in two chimeric constructs, OsBAM2-GFP and OsBAM3-GFP, with GFP fused to their C termini. The constructs were introduced into epidermis of Allium cepa scaly bulb by microprojectile bombardment using 0.6 μm gold particles and the gene delivery system (PDS-1000, Bio-Rad). As a positive control for plastid targeting, a vector containing a chimeric construct, AtrecA-DsRed2 (Imaizumi-Anraku et al., 2005) was introduced together with OsBAM2-GFP or OsBAM3-GFP. After incubation in darkness at 25°C for 24 hr, the epidermis was peeled from the bombarded bulb segment, and the fluorescence of GFP and DsRed2 were observed with a fluorescence microscope (BX50, Olympus) through U-MNIBA and U-MWIG cubes (Olympus), respectively.

## 5. Expression of recombinant OsBAM2 and OsBAM3 in *E. coli*

The cDNA sequences encoding OsBAM2 and OsBAM3 were amplified by PCR using full-length cDNA clones obtained from the Rice Genome Resource Center (Tsukuba, Ibaraki, Japan) as a template with the gene specific primer pairs listed in Table 1. In each case, the sequence encoding the predicted chloroplast-targeted signal peptide was removed. The amplified PCR products were cloned into the expression vector pET100/D-TOPO® (Invitrogen) according to the manufacturer’s instructions. The resulting constructs pET100/OsBAM2 and pET100/OsBAM3, encoded the respective OsBAM with a 6× His-Tag fused to their N-terminus. The recombinant OsBAM2 and OsBAM3 proteins was expressed at 25 or 20°C, respectively, in *E. coli* BL21 Star™ (DB3) (Invitrogen) by induction with IPTG. The cells expressing the His-tag-OsBAM2 or -OsBAM3 fusion proteins were cultured for 24 or 34 hr, respectively, after the induction in 25 mL of Luria-Bertani medium containing 50 μg mL⁻¹ carbenicillin, and then collected by centrifugation. As a control, cells harboring pET100/D/lacZ (Invitrogen) that encode a lacZ with a 6× His-Tag fused to its N-terminus were used. The total soluble protein fraction was prepared from the collected cells resuspended in 6 mL maleate (pH 6.2) buffer containing 1 mM EDTA, 0.1% BSA, and 0.02% sodium azide using a sonicator on ice.

## 6. Determination of β-amylase activity in *E. coli* soluble protein fraction

The activity of β-amylase was determined using a Betamyl®® Kit (Megazyme) with PNP-β-G3 as a substrate. Betamyl®® substrate solution (0.1 mL) was dispensed into glass test tubes and pre-incubated for 5 min at 40°C. Assays were started by adding 0.1 mL of the soluble protein fraction prepared from *E. coli* cells at 40°C and stopped by adding 1.5 mL of 1% (w/v) Trizma-base. Absorbance of the reaction solutions was analyzed at 400 nm.

## 7. Western blot analysis

The soluble proteins (30 µg) prepared from *E. coli* cells expressing the recombinant OsBAM2 or OsBAM3 were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Antibodies against OsBAM2 and
OsBAM3 proteins raised in rabbit with Thermo Scientific were used in 1:10,000 dilutions to detect the recombinant OsBAM2 and OsBAM3, respectively. The antigen-antibody complex was visualized with a peroxidase-conjugated goat-anti-rabbit antiserum and the ECLplus system according to the manufacturer’s instructions (GE Healthcare).

Results

1. β-amylase gene family in rice

Saika et al. (2005) reported nine putative β-amylase genes in the rice genome. By searching the Rice TOGO Browser (http://agri-trait.dna.affrc.go.jp/), we also identified nine genes that are predicted to encode β-amylase-like proteins and designated those genes OsBAM1-9 (Table 2). The presence of possible chloroplast transit peptides in the N-terminal regions of the nine β-amylase-like proteins was analyzed using the ChloroP program (http://www.cbs.dtu.dk/services/ChloroP/). OsBAM1 and OsBAM7 do not have chloroplast transit peptides in their N-terminal regions (Table 2).

We performed a phylogenetic tree analysis for the full-length amino acid sequences of the predicted β-amylase-like proteins in Arabidopsis (Smith et al., 2004; Fulton et al., 2008) and rice. In Arabidopsis, β-amylase-like proteins have been classified into four major subfamilies (Fulton et al., 2008). The results of our phylogenetic tree analysis revealed that rice β-amylase-like proteins were also classified into four subfamilies identical to Arabidopsis (Fig. 1). OsBAM2, OsBAM3, OsBAM4 and OsBAM5 belong to subfamily II corresponding to AtBAM1 and AtBAM3 that encode chloroplast-targeted active enzymes in Arabidopsis (Lao et al., 1999; Sparla et al., 2006). OsBAM7 and OsBAM9 belong to subfamily III. OsBAM1 and OsBAM6 belong to subfamily I, and OsBAM8 belongs to subfamily IV.

2. Expression analysis of OsBAM genes in various organs of rice

Semi-quantitative RT-PCR was performed to investigate the expression levels of OsBAM genes in various organs. OsBAM1 and OsBAM7 were specifically expressed in germinated seeds and developing carpopyses, respectively (Fig. 2). OsBAM9 was expressed in germinated seeds, internodes after heading and developing carpopyses. OsBAM2 was expressed in roots, leaf sheaths, leaf blades, internodes after heading and developing carpopyses; however, it was found at particularly high levels in leaf blades and internodes after heading. Transcripts of
but primarily in leaf sheaths, leaf blades, internodes after heading and developing caryopses.

3. \( \beta \)-amylase activity of recombinant OsBAM2 and OsBAM3 proteins

The cDNA regions coding for the predicted mature protein of OsBAM2 and OsBAM3 (Fig. 3a) were expressed at high levels in \( E. \ coli \) as a His-tag fusion protein. After

\( OsBAM3 \) and \( OsBAM5 \) were detected in germinating seeds, roots, leaf sheaths, leaf blades, young panicles, internodes after heading and developing caryopses, but were found at particularly high levels in leaf blades. \( OsBAM4 \) was distinctly expressed in leaf blades, whereas it was slightly expressed in leaf sheaths, internodes after heading and developing caryopses. \( OsBAM6 \) was mainly transcribed in leaf blades. \( OsBAM8 \) was expressed in all organs examined, but primarily in leaf sheaths, leaf blades, internodes after heading and developing caryopses.

3. \( \beta \)-amylase activity of recombinant OsBAM2 and OsBAM3 proteins

The cDNA regions coding for the predicted mature protein of OsBAM2 and OsBAM3 (Fig. 3a) were expressed at high levels in \( E. \ coli \) as a His-tag fusion protein. After
induction with IPTG, soluble protein fractions were prepared from the E. coli cell culture containing either pET100/OsBAM2 or pET100/OsBAM3. In each soluble protein fraction, the His-tag-OsBAM2 and -OsBAM3 fusion proteins corresponding to the putative molecular weights were detected by immunoblot analysis with the OsBAM2 and OsBAM3-specific antibody, respectively (Fig. 3b, c). The expression level of the recombinant OsBAM2 protein was lower than that of OsBAM3. Beta-amylase activity was determined using PNPβ-G3, the specific substrate for β-amylase. Soluble protein fractions containing either the OsBAM2 or OsBAM3 fusion protein reacted with PNPβ-G3, whereas those prepared from the E. coli cell culture containing pET100/D/lacZ did not (Fig. 3d).

4. Subcellular localization of OsBAM2 and OsBAM3

Chimeric constructs of OsBAM2 and OsBAM3 with GFP fused to their C termini, the expression of which was driven by the CaMV35S promoter, were introduced into epidermis by particle bombardment. The construct of AtrecA-DsRed2 targeted to plastids (Imaizumi-Anraku et al., 2005) in the epidermis cells (Fig. 4). The expression level of the recombinant OsBAM2 protein was lower than that of OsBAM3. Beta-amylase activity was determined using PNPβ-G3, the specific substrate for β-amylase. Soluble protein fractions containing either the OsBAM2 or OsBAM3 fusion protein reacted with PNPβ-G3, whereas those prepared from the E. coli cell culture containing pET100/D/lacZ did not (Fig. 3d).

Discussion

Phylogenetic tree analysis indicated that rice β-amylase-like proteins are classified into four subfamilies identical to Arabidopsis β-amylase-like proteins (Fig. 1). In Arabidopsis, AtBAM1 and AtBAM5 belonging to subfamily II target chloroplasts (Lao et al., 1999; Sparla et al., 2006). In addition, total β-amylase activity is significantly reduced in leaves of Arabidopsis bam1 and bam3 mutants, and bam1 and bam3 double mutants have a severe starch-excess phenotype (Fulton et al., 2008). In rice, OsBAM2, OsBAM3, OsBAM4 and OsBAM5 belong to subfamily II and have a possible chloroplast-targeted signal peptide as predicted by analysis using the ChloroP program (Table 2). Furthermore, OsBAM2, OsBAM3, OsBAM4 and OsBAM5 genes were mainly expressed in leaf blades, leaf sheaths and internodes after heading (Fig. 2), all of which transiently accumulate starch and subsequently serve as source organs. Thus, we noted the genes encoding OsBAM2, OsBAM3, OsBAM4 and OsBAM5 proteins as candidates of β-amylase isozymes functioning in plastids of leaves. In the present study, we analyzed OsBAM2 and OsBAM3 in detail, because the genes encoding these proteins were expressed more clearly in leaf blades, leaf sheaths and internodes after heading than the OsBAM4 and OsBAM5 genes (Fig. 2).

In Arabidopsis, analyses of recombinant proteins expressed in E. coli revealed that AtBAM1, AtBAM2 and AtBAM3 have measurable β-amylase activity (Lao et al., 1999; Sparla et al., 2006; Fulton et al., 2008). On the other hand, AtBAM4 lacks β-amylase activity and amino acid residues of the key active site, despite the fact that the bam4 mutant has elevated leaf starch (Fulton et al., 2008; Li et al., 2009). In the present study, β-amylase activities were detected in soluble protein fractions prepared from E. coli expressing either the OsBAM2 or OsBAM3 fusion protein (Fig. 3b, c, d), indicating that OsBAM2 and OsBAM3 proteins possess β-amylase activity. The reaction with PNPβ-G3 progressed more rapidly in recombinant OsBAM3 than in recombinant OsBAM2 (Fig. 3d). This result could, at least in part, be attributed to a relatively low level of recombinant OsBAM2 expressed in the E. coli cells (Fig. 3b, c). In order to analyze the subcellular localization of OsBAM2 and OsBAM3, we introduced chimeric constructs of OsBAM2 and OsBAM3 with GFP fused to their C termini into epidermis of A. cepa scaly bulb. The fluorescence of green GFP and red DsRed2 was observed with a fluorescence microscope. In the merged images (c, f), the colocalized signals of GFP and DsRed2 appear yellow. Scale bars, 50 μm.
been reported to be deficient in germinating seeds of the rice cultivar Nipponbare (Yamaguchi et al., 1999) owing to the insertion of a Mutator-like transposon in the promoter region (Saika et al., 2005). Likewise, in our study, the OsBAM1 transcript was detected in germinating seeds at much lower levels as determined from the number of PCR cycles required for detection (Fig. 2). In addition, the expression of β-amylose genes other than OsBAM1 was also low level in germinating seeds. These results suggest that the major β-amylose genes primarily function in vegetative organs such as leaf blades and sheaths rather than in germinating seeds, especially in cv. Nipponbare.

In the present study, OsBAM2 and OsBAM3 have been characterized as β-amylose proteins targeted to plastids in rice. However, it remains unclear whether the other β-amylose isoforms are localized in plastids. Further investigation is needed to determine whether OsBAM2 and OsBAM3 actually function to degrade starch in leaves. Chen and Wang (2008) reported that the β-amylose gene, which we designated OsBAM3, was highly expressed in leaf sheaths at the post-heading stage. Hereafter, analysis of physiological function of OsBAM2 and OsBAM3 may help elucidate a pathway of starch degradation in rice leaf sheaths. In Arabidopsis leaves, besides β-amylose, a debranching enzyme isoamylose 3 is implicated in the hydrolysis of glucans in the starch granule (Delatte et al., 2006). In rice leaves, it has been reported that α-amylose I-1 targeted to chloroplasts is involved in starch breakdown (Asatsuma et al., 2005). Studies focusing on the interaction between β-amylose and other starch-metabolizing enzymes are also needed to elucidate the mechanism of starch degradation inside plastids in rice.

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