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Characterization of β-amylase and its deficiency in various rice cultivars

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Abstract β-Amylase deficiency in various cultivars of rice was examined at the molecular level. Using an antibody against β-amylase purified from germinating seeds of rice, we were able to demonstrate the expression and organization of the β-amylase gene in normal and deficient cultivars. Although β-amylase is a starch-hydrolyzing enzyme, as is α-amylase, the β-amylase protein/gene is expressed differently from the α-amylase protein/gene; i.e. (1) β-amylase is synthesized only in aleurone cells, (2) the enzyme production in the embryo-less half-seeds is not under hormonal control. We identified some cultivars of rice that are deficient for β-amylase activity. We present new evidence that synthesis is blocked at the level of mRNA synthesis in the deficient cultivars. The usefulness of β-amylase as a crop trait is also discussed.

Key words β-amylase-deficient mutant · *Oryza sativa* · Germination · Direct seedling

Introduction

Starch breakdown during the germination of cereal seeds results from the action of hydrolytic enzymes, and it is generally accepted that phosphorylases are not involved in this process. While α-Amylase plays a major role during the degradation of native starch granules (Beck and Ziegler 1989; Perata et al. 1992), the concerted action of α-amylase, β-amylase, a debranching enzyme and α-glucosidase is essential for the complete hydrolysis of starch (Sun and Henson 1991; Guglielminetti et al. 1995).

β-Amylase (1,4-α-glucan maltohydrolase; EC 3.2.1.2) catalyzes the liberation of α-maltose from the non-reducing ends of starch-related 1,4-α-glucans. In cereals, β-amylase takes part in the mobilization of starch in germinating grains. In barley, the enzyme is already present in the dry seeds, where it accumulates during the process of grain development and is mainly bound to the starch granules. The bound form of the enzyme has a molecular mass of 64 kDa, and the process of in vivo release is mediated by a protease leading to the free form with a molecular mass of 59 kDa (Sopanen and Lauriere 1989; Loreti et al. 1998). Although most studies deal with the "endosperm" form of the enzyme, a distinct type of β-amylase gene, designated "ubiquitous" form, has been reported and its translation product is also present in vegetative tissues such as leaves and roots (Shewry et al. 1988; Lauriere et al. 1992). Wang et al. (1995) recently reported the localization of another type of the enzyme in the phloem of dicots.

In rice, β-amylase is synthesized de novo during seed germination and is almost absent in dry seeds (Okamoto and Akazawa 1980; Guglielminetti et al. 1995; Wang et al. 1996). Wang et al. (1996) recently found phytohormone-mediated β-amylase gene expression in rice and reported that the exogenous addition of gibberellin does not enhance that β-amylase activity, while that of abscisic acid inhibits the enzyme activity and mRNA accumulation. However, detailed analyses on expression in other tissues have not yet been performed.

Some inbred lines of mutants in barley (Kreis et al. 1987) and rye (Rorat et al. 1994) display a markedly

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reduced or null β-amylase activity in the mature grains. Deficiency of the enzyme activity in these lines or mutants results from a lack of synthesis of the "endosperm" form during seed development. Previously, we observed a similar deficiency of β-amylase activity in some rice cultivars. Since dry seeds of rice lack the "endosperm" form, the deficiency of the "ubiquitous" form of β-amylase might be observed in some rice cultivars. Further studies at the molecular level are necessary to clarify the nature of these phenomena: whether the deficiency results from synthesis of an unstable abnormal protein, from a defect in mRNA stability, from the absence of the transcription of the gene or from a default in the gene itself.

To determine the molecular aspects of the β-amylase mutation in rice, we prepared an antibody against β-amylase purified from germinating seeds of rice, and examined the expression and organization of β-amylase genes in normal and deficient cultivars.

Materials and methods

Plant materials

Seeds of rice (*Oryza sativa* L.; Japonica-type cultivars: 'Taichung 65', 'Nipponbare', 'Aoinokaze' and others) were obtained from the Nagoya University farm. These were sterilized in 1% NaClO for 1 h and thoroughly rinsed in sterile distilled water. Rice seeds were dehulled prior to sterilization.

Enzyme purification

Seeds of rice (Aoinokaze) were imbibed and incubated in a dark chamber at 30°C for 8 days. The germinating seeds (330 g) were homogenized with a mixer and a polytron in 800 ml of 50 m*M* TRIS/HCl buffer (pH 8.5) (buffer A). The homogenate was squeezed through two layers of nylon mesh (88 μm), and the crude filtrate obtained was centrifuged at 15,000 *g* for 20 min. The supernatant was used as the crude enzyme preparation. This crude enzyme preparation was then precipitated with $(NH_4)_2SO_4$ (25–60%). The precipitate obtained was dissolved in 10 ml of buffer A and centrifuged at 15,000 *g* for 20 min. After dialysis of the concentrated supernatant against buffer A, the preparation was applied to a column of DEAE Cellulose, DE 52 (Whatman), which had been equilibrated with buffer A; elution being carried out using a linear gradient of NaCl (0–0.5 *M*). The solution was dialyzed and applied to an α-cyclodextrin (CHA) affinity column (Vretblad 1974), which has specific affinity for α-amylase but not for β-amylase. After the fractions passing through the column were collected, they were applied to a Superose 12 column chromatography (Pharmacia). Those fractions showing activity were collected for further experiments.

Enzyme assay

The activity of β-amylase was assayed by measuring the release of reducing sugars from soluble starch (Guglielminetti et al. 1995). The assay buffer consisted of 50 m*M* sodium acetate, pH 5.2, and 10 m*M* $CaCl_2$. The substrate was 4.2% boiled soluble potato starch. The mixture of assay buffer (30 μl) and crude extracts (20 μl) was incubated for 15 min at 37°C and treated with 37.5 μl of 3,5-dinitrosalycilic acid (DNS) solution at 105°C. After the addition of the sample to distilled water (1 ml), the A_{530} was measured and the reducing power evaluated using a standard curve obtained with glucose (0–10 μ*M*).

Isoelectric focusing gel

The crude extract was examined by isoelectrofocusing using Pharmacia broad range (pH 3.5–9.5) Ampholine Pageplates. Isoelectric focusing was performed for 1.5 h according to the manufacturer's instruction manual. Samples (15 μl crude extract) were applied to application paper placed 3 cm from the cathode. The application paper was removed after the first 45 min of electrophoresis and the run was continued for an additional 45 min. The bands of amylolytic activities were visualized by incubating the gel for 1 h in 50 m*M* sodium acetate buffer (pH 5.2) containing 10 m*M* $CaCl_2$ and 1% boiled soluble starch. After washing with distilled water, the gel was stained with a 0.6% I_2 and 6% KI solution.

Antibody preparation and immunoblotting

The band on the SDS-PAGE that showed β-amylase activity following elution from a Superose 12 column was collected, ground and mixed with Freund's complete adjuvant to immunize rabbits. The procedure was repeated four times every 7 days. Five days following the last immunization, the rabbit's blood was collected and antiserum obtained. We confirmed that the antiserum specifically contained anti-rice β-amylase antibody.

The proteins to be examined were separated by SDS-PAGE or isoelectric focusing, transferred onto nitrocellulose membrane using the Novablot Protein Transfer Kit apparatus and analyzed using the anti-β-amylase antibody. An alkaline-phosphatase-labeled second antibody was used to detect the immunoreactive band.

Northern and Southern blotting

RNA was extracted by the aurintricarboxylic acid method, as described by Skadsen (1993). Northern blotting was performed by the standard method described by Perata et al (1997). The rice β-amylase gene probe was prepared by polymerase chain reaction (PCR) amplification for exon 2 (403 bp) of rice β-amylase gene (L10345, GenBank accession number) using genomic DNA from leaves (Aoinokaze). Equal loading was checked by reprobing with an rRNA cDNA probe and ethidium bromide staining. Blots were exposed using a Fujix BAS2000 Bio-Imaging analyzer (Fuji Photo Film Co, Tokyo, Japan).

In situ hybridization

Seeds were fixed in a mixture of 3% paraformaldehyde and 0.25% glutaraldehyde in 0.1 *M* sodium phosphate buffer (pH 7.2) for 20 h at 5°C and then dehydrated in a graded ethanol series. They were then embedded in Paraplast Plus (Fisher Scientific), sectioned (8-μm thickness) by a rotary microtome and applied to slide glasses treated with Vectabond (Vector Lab). A digoxygenin-labeled RNA probe of rice was prepared from a cDNA clone of β-amylase gene. Probes were degraded to a mean length of 100 bp by incubation in alkali at 60°C. In situ hybridization and the detection of hybridization signals were performed as reported by Kouchi and Hata (1993).

Results

Purification of rice β-amylase and its antibody preparation

To purify rice β-amylase we subjected the $(NH_4)_2SO_4$-precipitated fraction obtained from the germinated rice seeds to three distinct chromatographies, i.e. ion exchange (DE52), affinity (α-cyclodextrin column) and gel filtration (Superose 12 HR 10/30) chromatographies. The profile of the 53-kDa protein on SDS-PAGE coincided with that of β-amylase activity (data not shown). The antibody raised against the 53-kDa protein demonstrated a good degree of cross-reactivity for the β-amylase protein which was separated by isoelectric focusing gel electrophoresis (Fig. 1). The purified protein sample showed similar characters [molecular size, 53 kDa; isoelectric point, 4.8; a loss of activity at 70°C for 15 min (see heat treatment in Fig. 6); no hydrolytic activity for β-limit dextrin] to those reported previously (Matsui et al. 1977; Okamoto and Akazawa 1978). These results indicated that the antibody raised against the 53-kDa protein was a specific anti-rice β-amylase antibody.

De novo synthesis of β-amylase in aleurone cells of germinating rice seeds

Immunoblot analysis showed no signal of β-amylase protein in the embryos, plumules or radicles (Fig. 2). However, β-amylase protein was detected in 6-day and 8-day germinating seed and endosperm, although the protein was not detected in dry mature and 2-day germinating seeds. These results suggest that the β-amylase detected in the germinating seeds was synthesized de novo in the endosperm (Fig. 2). To examine the expression site in the endosperm, we performed in situ mRNA detection for β-amylase in the 4-day germinating seeds using the specific DNA probe (Fig. 3).

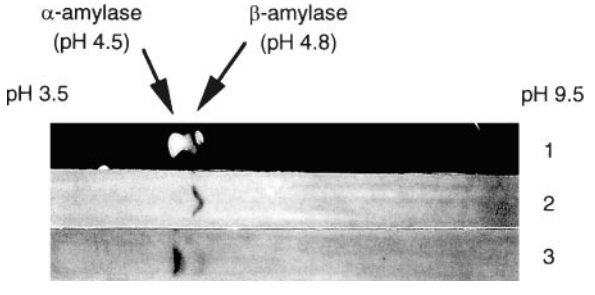


Fig. 1 Identification of β-amylase protein on isoelectric focusing gel using antibody raised against 53-kDa protein. *Lane 1* Activity staining of amylases from the germinating seeds of rice. The gel was stained with an I_2-KI solution after incubation in soluble starch. *Lanes 2* and *3* Immunoblots of β-amylase and α-amylase, respectively, detected by their specific antibodies

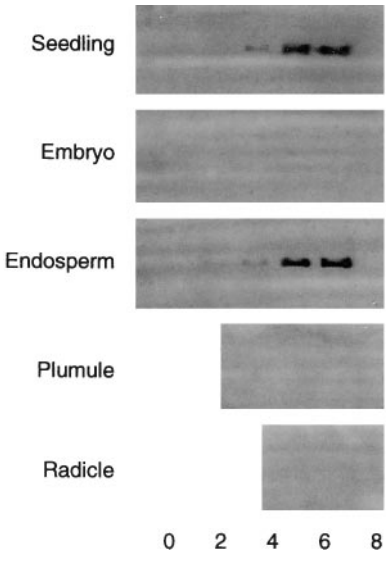


Fig. 2 Immunochemical detection of tissue-specific β-amylase in the germinating seeds of rice. Rice seeds were imbibed and incubated in a dark chamber at 30°C for 0 (dry seed), 2, 4, 6 and 8 days. The germinating seeds were separated into embryo, endosperm, plumule and radicle. Conditions for the sample preparation and immunoblotting of extracts from each tissue are those of Guglielminetti (1995) except that the homogenizing buffer contains 100 m*M* Hepes/KOH (pH 7.6), 10 m*M* $CaCl_2$ and 1% Triton X-100. Equal amounts of protein (7 μg) were loaded on each lane

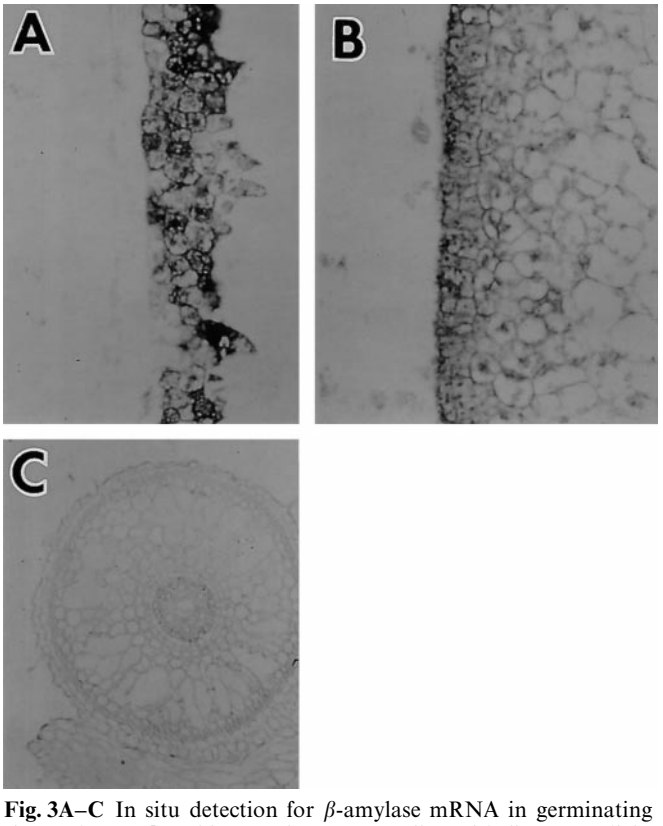


Fig. 3A–C In situ detection for β-amylase mRNA in germinating rice seeds. **Panels A** Aleurone layer, **B** scutellar epithelium, **C** radicle. Rice seeds were germinated for 4 days. Conditions for in situ hybridization are described in the text

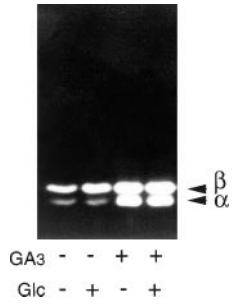


Fig. 4 Effects of gibberellin and glucose on β-amylase production in embryo-less half-seeds of rice. Six embryo-less half-seeds were incubated with 1 ml of culture medium and allowed to germinate at 30°C in darkness according to the method of Mitsunaga et al. (1994). The culture medium was supplemented with 1 μM gibberellic acid (GA_3) or 90 mM glucose (*Glc*). Amylase activities were visualized after isoelectric focusing

The hybridization signal was detected in cells of the aleurone layers (panel A), but not in the epithelium cells of either the embryos (panel B) or roots (panel C), indicating that β-amylase mRNA was produced in the aleurone cells.

Cereal α-amylase is known to be induced by gibberellin (Itoh et al. 1995) and β-amylase in sweet potato to be induced by sugar (Nakamura et al. 1991). We examined the effect of gibberellin (GA_3) and glucose on the production of α- and β-amylases in embryo-less half-seeds of rice (Fig. 4). α-Amylase was produced in only small amounts in the aleurone layer of the endosperm in the absence of applied GA_3, but it was produced in large quantities in the presence of GA_3. β-Amylase was produced even in the absence of GA_3, although production was slightly enhanced by GA_3. These results indicated that de novo induction of the β-amylase was not strictly controlled by gibberellin. Glucose slightly promoted the production of β-amylase. Thus, the mode of induction of β-amylase is not similar to that of cereal α-amylases. Indeed, β-amylase mRNA was detected in only the aleurone cells (Fig. 3), while α-amylase mRNA was found in both aleurone and epithelium cells (Sugimoto et al. 1998)

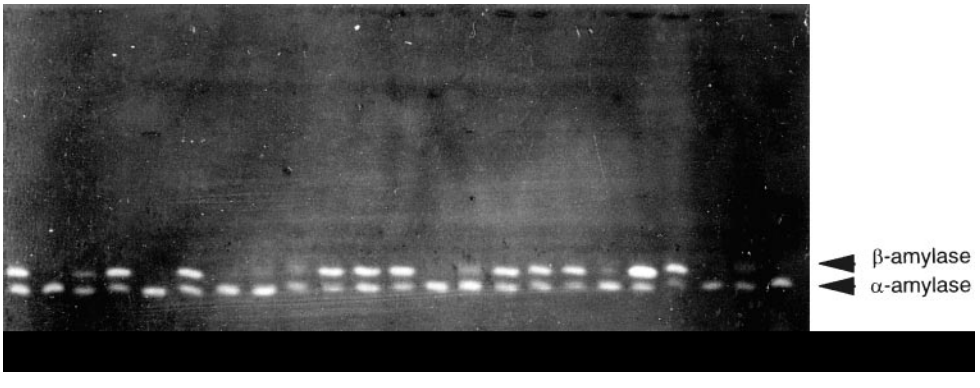
Fig. 5 Amylase patterns in the germinating seeds of various rice cultivars. Amylases were separated by isoelectric focusing [pH range: 3.5 (bottom) to 9.5 (top)], and the gel was stained with an I_2-KI solution after incubation in soluble starch. *Lanes 1* 'Tsukinohikari', *2* 'Koganebare', *3* 'Ozora', *4* 'Tachikaze', *5* 'Nipponbare', *6* 'Akibare', *7* 'Sachikaze', *8* 'Shinyamabuki', *9* 'Koganenishiki', *10* 'Hatsushimo', *11* 'Kinmaze', *12* 'Tokaisenbon', *13* 'Chukyoasahi', *14* 'Tokaiasahi', *15* 'Senbonasahi', *16* 'Aichiasahi', *17* 'Sakaeshinriki', *18* 'Kyotonishiki', *19* 'Takenari', *20* 'Omachi', *21* 'Kiryoyoshi', *22* 'Mikawanishiki', *23* 'Shinriki'. *Lanes 5*, *7*, *13*, and *23* are typical β-amylase deficient cultivars

β-Amylase-deficient cultivars in rice

Rice cultivars deficient in β-amylase activity in the germinating seeds were identified by amylolytic activity staining after the separation of β-amylase from α-amylase by isoelectric focusing (Fig. 5). At least 4 cultivars ('Nipponbare', lane 5; 'Sachikaze', 7; 'Chukyoasahi', 13; 'Shinriki', 23) of the 23 tested were identified as a β-amylase-deficient phenotype. We tested β-amylase activity in the germinating seeds of 60 cultivars and at least 17 cultivars were identified as having the deficient phenotype (data not shown).

Analysis on β-amylase deficiency in 'Nipponbare' cultivars

To clarify the mechanisms of β-amylase deficiency in the deficient rice cultivars, we examined 2 rice cultivars, 'Nipponbare' (deficient type) and 'Taichung 65' (normal type). Amylolytic activity patterns during the germination clearly revealed that 'Taichung 65' has β-amylase activity but that 'Nipponbare' does not (Fig. 6), and that α-amylase activity is present in both cultivars. "Endosperm" β-amylase, which is bound to starch granules in the cereal endosperm, is known to be released from the insoluble precipitate after reducing reagent treatment during protein extraction. No activity was observed in the protein extract from the 'Nipponbare' seedling after mercapthoethanol (reducing reagent) treatment (data not shown), indicating that the β-amylase in rice is a "ubiquitous" form and that the



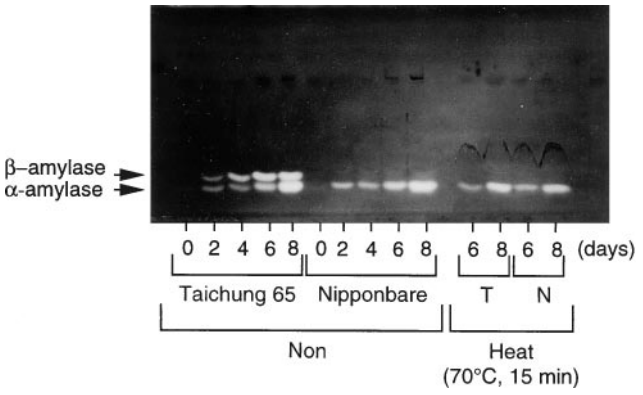


Fig. 6 Changes in the amylase pattern in germinating seeds of 'Taichung 65' and 'Nipponbare'. Rice seeds were imbibed and incubated in darkness at 30°C for 0 (dry seed), 2, 4, 6 and 8 days. Conditions for the sample preparation are described in the legend of Fig. 2. Amylases were separated by isoelectric focusing [pH range: 3.5 (bottom) to 9.5 (top)], and the gel was stained with an I_2-KI solution after incubation in soluble starch. *N* Nipponbare, *T* Taichung 65

deficiency in 'Nipponbare' is due to a lack of the "ubiquitous" form. Immunoblot and Northern analyses showed that both protein and mRNA for β-amylase are present in the germinating seeds of 'Taichung 65' but not in those of 'Nipponbare' (Fig. 7). These results indicate that the deficiency of β-amylase activity in 'Nipponbare' is due to lack of mRNA for the enzyme.

The organization of the β-amylase structural genes was investigated in a normal ('Taichung 65') and deficient rice cultivar ('Nipponbare') using Southern blot hybridization and four different restriction enzymes (Fig. 8). Most of the digests contained a single hybridizing fragment, suggesting that the β-amylase locus is composed of a single coding unit and that the coding region is mostly conserved even in the deficient-type cultivar, 'Nipponbare'.

β-Amylase in shoot

We examined β-amylase protein in the shoots of rice. The protein was not detectable in the shoots of 'Nipponbare' (Fig. 9A). However, the protein signal was detected in the shoots of slender rice, a tall mutant derived from 'Nipponbare' (Fig. 9A). The immunoblot for the protein extract from each shoot unit of the slender mutant revealed that the fourth and fifth units had the strongest signal in the seventh leaf-stage shoots. The slender mutant develops internode elongation even at the seedling-stage while normal rice does not (characters of the slender rice will be reported elsewhere), indicating that the immunoreactive band might result from the presence of the internode-specific β-amylase. During elongation of the leaf sheath in rice, starch granules disappear (Matsukura et al. 1998), and β-amylase might play a role in the degradation of the starch.

Discussion

β-amylase is distinct from α-amylase with respect to its manner of expression during seed germination in rice

α-Amylase is well characterized by its manner of expression during seed germination. The enzyme is not present in the dry cereal seeds, but is rapidly induced by the action of gibberellins (GAs), which are produced in the embryo, triggering α-amylase gene regulation in the aleurone layers. The induction of α-amylase by gibberellin in cereal grains and the counteractive role of abscisic acid on this process represents a classical model system for studying the mode of action of gibberellin. Besides the aleurone layers, the scutellum also plays an important role in the production of α-amylase (Perata et al. 1997; Sugimoto et al. 1998). The modulation of α-amylase genes by sugars has also been reported (Karrer and Rodriguez 1992; Umemura et al. 1998; Morita et al. 1998). Although β-amylase is a starch-hydrolyzing enzyme, as is α-amylase, the expression behavior of β-amylase is distinct from that of α-amylase; i.e. (1) β-amylase is synthesized only in aleurone cells (Figs. 2 and 3), while α-amylase is synthesized in both the aleurone and epithelium, (2) β-amylase production from the embryo-less

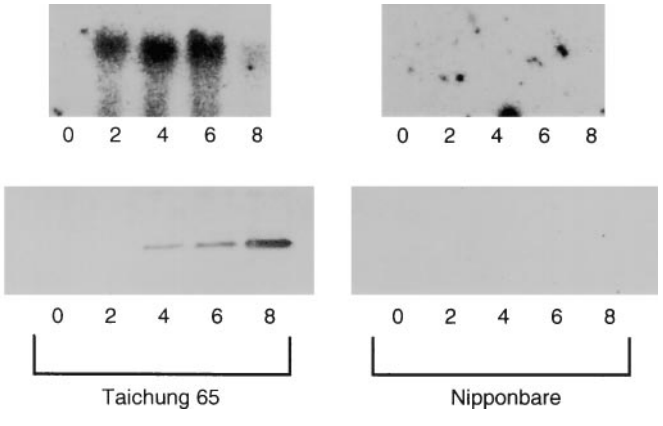


Fig. 7 Immunoblot and Northern analyses of the germinating seeds of 'Taichung 65' and 'Nipponbare'. Conditions for the sample preparation, immunoblot and Northern analysis are described in the legend of Fig. 6 and Materials and methods. Equal amounts of total RNA (20 μg) and proteins (20 μg) were loaded onto each lane

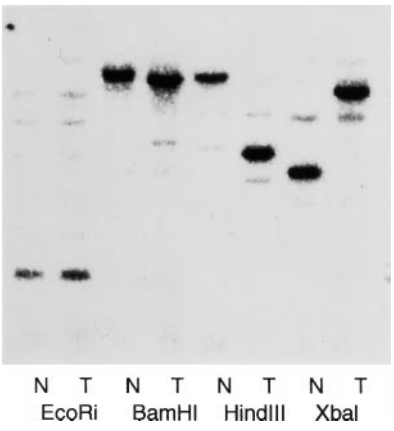


Fig. 8 Southern blot analysis of the genomic DNA from 'Taichung 65' and 'Nipponbare'. Genomic DNA of 'Taichung 65' and 'Nipponbare' were digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Xba*I. *N* 'Nipponbare', *T* 'Taichung 65'

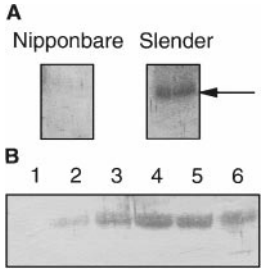


Fig. 9A, B Immunoblot of β-amylase in the shoots of 'Nipponbare' and slender rice. Seeds were germinated and grown for 1 month at 30°C under fluorescent light. Each leaf unit was extracted with SDS-sample buffer at extraction ratios (w/v) of 1 : 3. Procedures for immunoblotting are described in the Materials and methods. Twenty microliters of protein extract was loaded in each lane. **A** Whole shoots. **B** Each leaf unit; *lanes 1* whole shoots from 'Nipponbare', *2* third leaf-unit, *3* fourth leaf-unit, *4* fifth leaf-unit, *5* sixth leaf-unit, *6* seventh leaf-unit from slender rice. Each leaf-unit consists of the leaf blade, leaf sheath and internode

half-seeds is not under hormonal control (Fig. 4), while α-amylase is under the control of gibberellin. Further experiments will be needed to identify the inducer molecules for β-amylase production in the seeds.

β-Amylase deficiency in several rice cultivars is due to a lack of the mRNA

Some plant species deficient in β-amylase activity have been reported in rye (Rorat et al. 1994), barley (Kreis et al. 1987) and sweet potato (Kumagai et al. 1990). We identified some cultivars of rice as being deficient in β-amylase activity (Figs. 5 and 6). In the present experiment, no signal of β-amylase was detected either by Northern blot hybridization or by immunoblot in the deficient cultivar 'Nipponbare' (Fig. 7). This suggests that either transcription of the gene is blocked or that the mRNA is highly unstable in the deficient cultivar. To characterize the deficiency further, we analyzed Southern blot patterns with four different restriction enzyme (Fig. 8). In most cases, a single restriction fragment hybridized with the probes in stringent conditions, indicating that the protein is most likely coded by a single gene. There was a different restriction fragment length polymorphism between the deficient and normal cultivars (Fig. 8). As mentioned earlier, our Southern blot results are consistent with a deletion or mutation in the promoter region which can affect the transcription event of the gene. However, the possibility of a mutation of the gene for the *trans*-acting factors that promote the β-amylase gene transcription cannot be ruled out. Further genetic analysis and functional promoter analyses for the gene will be needed to clarify that deficient event.

Chen et al. reported the sequences for two rice genomic DNA clones encoding the β-amylase gene (unpublished but sequence data available from GenBank accession numbers L10345 and L10346). The peptide sequences derived from the DNA sequences indicate that the carboxyl terminal lacks the glycine-rich repetitive sequence which is present in "endosperm" β-amylase. This result is consistent with our assumption that the β-amylase synthesized during the germination is the "ubiquitous" form.

β-Amylase as a useful crop trait

β-Amylase occurs in plants and in certain bacteria; however, its physiological function is still uncertain because the enzyme and its substrates are spatially separated in some plants (Monroe and Preiss 1990). For example, in leaves, β-amylase is located outside the chloroplast, while starch granules, the expected substrate for the enzyme, is found exclusively in the chloroplast. In cereal seeds, except for rice, β-amylase exists in the endosperm and is believed to catalyze degradation of endosperm starch through the concerted action of α-amylase, debranching enzyme and α-glucosidase, although no significant growth retardation was observed in the mutant seeds with the β-amylase-deficient phenotype. In rice, β-amylase is de novo synthesized in the aleurone; that is, the enzyme and its substrates seem to be also spatially separated.

Yoon et al. (1998) demonstrated the correlation between seedling emergence and amylolytic activity under low temperatures and submerged soil conditions in 25 rice cultivars. A significant correlation between β-amylase activity and emergence coefficients and seedling length was observed under submerged soil conditions at 18°C. These results indicate that β-amylase might be useful for direct seedling cultivation of rice in temperate regions such as Japan. Nielsen et al. (1997) also reported the presence of cold-inducible β-amylase during the cold-sweetening process in potato

tubers. Such a β-amylase might play a specific role in the vigor of plant adapted to cold temperature.

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References

Beck E, Ziegler P (1989) Biosynthesis and degradation of starch in higher plants. Annu Rev Plant Physiol Plant Mol Biol 40: 95–117

Guglielminetti L, Yamaguchi J, Perata P, Alpi A (1995) Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. Plant Physiol 109: 1069–1076

Itoh K, Yamaguchi J, Huang N, Rodriguez RL, Akazawa T, Shimamoto K (1995) Developmental and hormonal regulation of rice α-amylase (*RAmy1A*)-*gusA* fusion genes in transgenic rice seeds. Plant Physiol 107: 25–31

Karrer EE, Rodriguez RL (1992) Metabolic regulation of rice α-amylase and sucrose synthase genes in planta. Plant J 2: 517–523

Kouchi H, Hata S (1993) Isolation and characterization of novel nodulin cDNA representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238: 106–119

Kreis M, Williamson M, Boxton B, Pywell J, Hejgaard J, Svendsen I (1987) Primary structure and differential expression of β-amylase in normal and mutant barleys. Eur J Biochem 169: 517–525

Kumagai T, Umemura Y, Baba T, Iwanaga M (1990) The inheritance of β-amylase null in storage roots of sweet potato, *Ipomoea batatas* (L.) Lam. Theor Appl Genet 79: 369–376

Lauriere C, Doyen C, Thevenot C, Daussant J (1992) β-Amylase in cereals: a study of the maize β-amylase system. Plant Physiol 100: 887–893

Loreti E, Guglielminetti L, Gonzali S, Yamaguchi J, Alpi A, Perata P (1998) Effect of anoxia on gibberellic acid-induced protease and β-amylase processing in barley seeds. J Plant Physiol 152: 44–50

Matsui H, Chiba S, Shimomura T (1977) Purification and some properties of active β-amylase in rice. Agric Biol Chem 41: 841–847

Matsukura C, Itoh S, Nemoto K, Tanimoto E, Yamaguchi J (1998) Promotion of leaf sheath growth by gibberellic acid in a dwarf mutant of rice. Planta 205: 145–152

Mitsunaga S, Tashiro T, Yamaguchi J (1994) Identification and characterization of gibberellin-insensitive mutants selected from among dwarf mutants of rice. Theor Appl Genet 87: 705–712

Monroe JD,Preiss J (1990) Purification of a β-amylase that accumulates in *Arabidopsis thaliana* mutants defective in starch metabolism. Plant Physiol 94: 1033–1039

Morita A, Umemura T, Kuroyanagi M, Futsuhara Y, Perata P, Yamaguchi J (1998) Functional dissection of a sugar-repressed α-amylase gene (*RAmy1A*) promoter in rice embryos. FEBS Lett 423: 81–85

Nakamura K, Ohoto M, Yoshida N, Nakamura K (1991) Sucrose-induced accumulation of β-amylase occurs concomitant with the accumulation of starch and sporamin in leaf-petiole cuttings of sweet potato. Plant Physiol 96: 902–909

Nielsen TH, Deiting U, Stitt M (1997) A β-amylase in potato tubers is induced by storage at low temperature. Plant Physiol 113: 503–510

Okamoto K, Akazawa T (1978) Purification of α- and β-amylase from endosperm tissues of germinating rice seeds. Agric Biol Chem 42: 1379–1384

Okamoto K, Akazawa T (1980) Enzymic mechanism of starch breakdown in germinating rice seeds. 9. *De novo* synthesis of β-amylase. Plant Physiol 65: 81–84

Perata P, Pozueta-Romero J, Akazawa T, Yamaguchi J (1992) Effect of anoxia on starch breakdown in rice and wheat seeds. Planta 188: 611–618

Perata P, Matsukura C, Vernieri P, Yamaguchi J (1997) Sugar repression of gibberellin-dependent signaling pathway in barley embryos. Plant Cell 9: 2197–2208

Rorat T, Sadowski J, Grellet F, Daussant J, Deseny M (1994) Characterization of cDNA clones for rye endosperm β-amylase deficiency in rye mutant lines. Theor Appl Genet 83: 257–263

Shewry PR, Parmar S, Buxton B, Gale MD, LIu CJ, Hejgaard J, Kreis M (1988) Multiple molecular forms of β-amylase in seeds and vegetative tissues of barley. Planta 176: 127–134

Skadsen RW (1993) Aleurones from a barley with low α-amylase activity become highly responsive to gibberellic acid when detached from the starchy endosperm. Plant Physiol 102: 195–203

Sopanen T, Lauriere C (1989) Release and activity of bound β-amylase in a germinating barley grain. Plant Physiol 89: 244–249

Sugimoto N, Takeda G, Nagato Y, Yamaguchi J (1998) Temporal and spatial expression pattern of α-amylase gene during germination in rice and barley. Plant Cell Physiol 39: 323–333

Sun Z, Henson CA (1991) A quantitative assessment of the importance of barley seed α-amylase, β-amylase, debranching enzyme, and α-glucosidase in starch degradation. Arch Biochem Biophys 284: 298–305

Umemura T, Perata P, Futsuhara Y, Yamaguchi J (1998) Sugar sensing and α-amylase gene repression in rice embryos. Planta 204: 420–428

Vretblad P (1974) Immobilization of ligands for biospecific affinity chromatography their hydroxyl groups. The cyclohexaamylose-β-amylase system. FEBS Lett 47: 86–89

Wang Q, Monroe J, Sjolund RD (1995) Identification and characterization of a phloem-specific β-amylase. Plant Physiol 109: 743–750

Wang S-M, Lue WL, Eimert K, Chen J (1996) Phytohormone-regulated β-amylase gene expression in rice. Plant Mol Biol 31: 975–982

Yoon B-S (1998) Seedling emergence and β-amylase activity of rice cultivars under the low temperature and submerged soil condition. PhD thesis, University of Tokyo, Tokyo, Japan