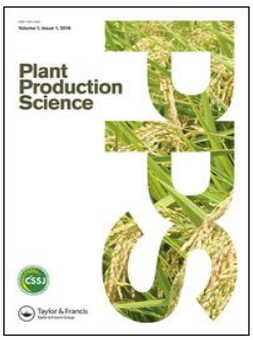

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α-Amylase Activity and Soluble Sugar Supply from Endosperm in Relation to Varietal Differences in Seedling Establishment under Low-Temperature Conditions in Rice (*Oryza sativa* L.)

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Abstract: We examined α-amylase(EC 3.2.1.1) activity in endosperm in 6 varieties of rice (*Oryza sativa* L.), which showed different seedling establishment traits in field experiments, using seedlings grown in sterilized agar-bed at 16°C. At the coleoptile elongation stage and the first leaf elongation stage, there were significant differences in α-amylase activity among the varieties investigated. However, the varietal difference in α-amylase activity at the coleoptile elongation stage did not correspond with that in coleoptile growth. Maltose, the immediate product of α-amylase activity, accumulated in the endosperm at the coleoptile elongation stage in a greater amount in Fukuhibiki, which has a poor seedling establishment trait, than in Arroz da Terra, which has a superior seedling establishment trait. The concentration of glucose detected in the exudate from the endosperm adjacent to the scutellum at the coleoptile elongation stage was also higher in Fukuhibiki than in Arroz da Terra. The results obtained in sterile agar-bed conditions clearly demonstrated that neither deficiency in α-amylase activity nor glucose production in the endosperm were responsible for coleoptile growth retardation at 16°C. Therefore, neither α-amylase activity nor sugar supply from the endosperm were responsible for the varietal differences seen in the rate of seedling establishment in paddy fields at around 16°C.

Key words: α-Amylase, Coleoptile, Direct seeding cultivation, Germination, Seedling establishment.

In direct-seeded rice (*Oryza sativa* L.) cultivation, seedling establishment traits show large varietal differences at low temperatures (Sasaki and Yamazaki, 1971; Jones and Peterson, 1976; Li and Rutgar, 1980; Amano et al., 1993; Redona and Mackill, 1996; Inoue et al., 1997; Ogiwara and Terashima, 2001; Ogiwara et al., 2003). Information on physiological characteristics relating to better seedling establishment is essential for *post-genome era* breeding programs. However, the physiological basis for these varietal differences is still unclear.

The coleoptile elongation rate is important for determining the percentage of established seedlings in submerged paddy fields at low temperatures: coleoptile growth measured in sterile agar-bed conditions was faster in varieties that showed superior seedling establishment in field experiments (Ogiwara and Terashima, 2001). Coleoptile growth depends on seed reservoir. Therefore, it is practical to estimate and compare the sugar supplying capability of endosperm during coleoptile elongation in varieties with different seedling establishment traits.

Estimation of the sugar supplying capability of endosperm, α-amylase (EC 3.2.1.1) is important since starch is a major component of cereal seed reserves (Beck and Ziegler, 1989), and α-amylase is the principal enzyme digesting starch macromolecules into smaller, soluble units (Sun and Henson, 1991). Many studies have been conducted on the relationship between α-amylase activity in the endosperm and seedling growth, but the results showed discrepancy.

Williams and Peterson (1973) and Sasahara and Ikarashi (1989) concluded that α-amylase in the endosperm is not a rate-limiting factor for seedling growth at low temperatures. Fukuda et al. (2008) found no correlation between coleoptile length and α-amylase activity in the endosperm. In contrast, Karrer et al. (1993) found that α-amylase activity and accumulation of *RAmy1A* mRNA strongly correlated with shoot weight at 16 days after sowing both at 15 and 30°C in 10 varieties.

A difference in growth stages might have caused these discrepancies, since α-amylase activity and its mRNA level

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Abbreviations : BPNPG7, blocked p-nitrophenyl maltoheptaoside; CES, coleoptile elongation stage; FLES, first leaf elongation stage; G50-E50, duration (hr) between 50% germination to 50% emergence to surface of agar-bed; LSD, least significant difference; PGS, pre-germination stage; PSSL, percentage of seedlings with second leaf.

Table 1. Seedling establishment rate (PSSL) of Arroz da Terra, Calrose, Haenuki, Fukuhibiki, S-201, and Blue Bonnet in field experiments under low temperatures.

Variety	1995	1996	1997		1998		1999	Average PSSL*
	May 15	May 25	April 26	May 8	April 27	May 7	April 28	
			%					% ± SE
Arroz da Terra	93.8	88.2	87.4	97.4	92.6	90.4	62.5	97.5 ± 8.1
Calrose	93.8	75.7	65.0	77.9	70.3	68.4	42.3	69.1 ± 7.7
Haenuki	71.0	75.4	55.6	68.8	65.3	71.7	37.1	63.6 ± 5.0
Fukuhibiki	54.8	36.4	35.9	50.9	58.5	52.9	39.7	47.0 ± 3.6
S-201	43.8	45.2	44.4	48.5	48.8	50.4	25.7	43.8 ± 4.8
Blue Bonnet	10.3	25.0	1.2	2.1	2.9	3.3	6.3	7.3 ± 4.8

PSSL: percentage of seedlings with second leaf at 1 month after sowing. Values for each experiment were the average of 4 replications. Date of sowing are shown at the top of each column. *) Average of 5 year experiments ± SE (n = 7). Average soil temperature during the experiments were 11.3 to 20.9ºC. For details, see Ogiwara et al., (2003).

in the endosperm vary with seedling age (Williams and Peterson, 1973; Itoh et al., 1995), and their time course vary with the ecotype and variety (Sasahara et al., 1986; Sasahara and Ikarashi, 1989).

A further complication is that, in these previous studies, seedling weight was measured to indicate seedling vigor (Williams and Peterson, 1973; Sasahara and Ikarashi, 1989; Karrer et al., 1993). From the agronomic point of view, the seedling density in the field is more important than the seedling size. Therefore, we examined the α-amylase activity in endosperm in varieties differing in seedling establishment rate.

In addition to α-amylase, the de-branching enzyme (R-enzyme), β-amylase, and α- and β-glucosidases, (Palmiano and Juliano, 1972; Dunn, 1974; Beck and Ziegler, 1989; Fincher, 1989; Yoon et al., 1997) play roles in the digestion of starch in the endosperm. Assays have been conducted on the R-enzyme (Toguri, 1991) and β-amylase (Yoon et al., 1997; Yamaguchi et al., 1999) and isozymes of α-amylase (Tanaka et al., 1970; Daussant et al., 1983; Ranjhan et al., 1992; Huang et al., 1999; Huang et al., 2000) in the early developmental stage of rice seedlings. However, it is difficult to estimate the capacity for sugar production from *in vitro* activity assays of each enzyme, and it is essential to describe the sugar composition and concentrations in the endosperm during coleoptile growth to discuss the relationship between sugar supply from the endosperm and seedling establishment.

In this study, soluble sugar composition and concentration in endosperm were determined by HPLC to reveal the soluble sugar status in the endosperm in addition to α-amylase activity, with a particular focus on the exudate collected from endosperm adjacent to the scutellum, through which soluble sugar is supplied to the seedling (Nomura et al., 1969; Nomura and Akazawa, 1973; Nomura and Akazawa, 1974; Matsukura et al., 2000).

Materials and Methods

1. Seeds and varieties

The seeds used in this study were harvested from experimental paddies at the Daisen Campus of the National Agricultural Research Center for the Tohoku Region (Daisen City, Akita Prefecture, Japan) 30 to 40 days after booting. Seeds with a specific gravity of greater than 1.13 were selected and stored in a refrigerator until use.

Varietal differences in seedling establishment traits at low temperatures were examined in submerged paddy fields for 5 years using seeds obtained in the preceding year, as described by Ogiwara and Terashima (2001) and Ogiwara et al. (2003). After soaked under flowing tap water (ca. 9ºC) for 36 hr, seeds were sown in nursery boxes with small compartments (“Kabumakipot”, Fujimoto Kagaku Kogyo Co., Tokyo Japan) and covered with 4-mm thick sieved paddy soil. The nursery boxes were placed in continuously irrigated paddy field. The water level was kept around 5 cm from the top of the boxes. Average soil temperature during the experiments was between 11.3ºC and 20.9ºC.

The percentage of seedlings with second leaf at one month after sowing (PSSL) was used to indicate the seedling establishment trait of each variety. Among 40 varieties used in the field experiment, 6 varieties with different seedling establishment traits (Arroz da Terra, Calrose, Haenuki, Fukuhibiki, S-201, and Blue Bonnet) were chosen for this study (Ogiwara et al. 2003). Table 1 shows their average PSSLs.

2. α-Amylase activity in endosperm at the early growth stages

(1) Plant material

Seeds were planted and grown in agar-beds under sterile conditions in polycarbonate pots, according to the method of Ogiwara and Terashima (2001). Agar beds (0.5% w/v,

Table 2. Sampling date of Arroz da Terra, Calrose, Haenuki, Fukuhibiki, S-201, and Blue Bonnet for the assay of α-amylase activity and sugar composition and concentration assays.

Variety	Sampling date for assays		
	16ºC		26ºC
	CES	FLES	CES
	days after imbibition		
Arroz da Terra	7	14	3
Calrose	8	15	3
Haenuki	8	15	4
Fukuhibiki	9	16	3
S-201	8	15	4
Blue Bonnet	11	17	3

purified agar powder for laboratory use grade) without nutrients were prepared in polycarbonate pots (Agripot), and sterilized by autoclaving. Seeds were sterilized for 4 hr in 50-fold diluted sodium hypochlorite solution with 100 ppm Triton X-100. After washing thoroughly with sterilized distilled water, seeds were imbibed in sterilized distilled water overnight at 16ºC, and then 25 seeds were placed embryo-side-up in the agar-bed adjusting the embryo of the seed to 7−8 mm below the surface of the agar-bed on a clean bench. A small amount of sterilized distilled water was added to the pots, which were then kept at 16ºC or 26ºC under continuous darkness until 80% of the coleoptiles elongated to the surface of the agar-bed (emergence). At that time, pots were moved to another incubator with the same temperature and irradiated with fluorescent lamps for 14 hr a day. For each pot, the numbers of germinated seeds, seedlings with emerged coleoptile, and 1st leaves appeared were counted daily. Coleoptile elongation duration was shown by the period (hr) from 50% germination to 50% emergence of coleoptile (G50-E50).

The planted seeds were harvested at three different growth stages, the pre-germination stage (PGS), coleoptile elongation stage (CES), and first leaf elongation stage (FLES). PGS was defined as 24 hr after imbibition, CES, as 24 hr after germination was observed in more than 85% of sown seeds, and FLES as 24 hr after the appearance of the first leaf in more than 60% of seedlings. Durations from imbibition to sampling were shown in Table 2. Coleoptile length was approximately 3 to 5 mm at CES, and first leaf length was 7 to 10 mm at FLES. Harvested samples were washed in distilled water and frozen immediately in liquid nitrogen, then stored at −80ºC until use.

(2) Measurement of α-amylase activity

Seeds were dehulled with forceps. The shoot and root were removed from the caryopsis, but the scutellum was left attached to the endosperm. Ten grains were ground in an ice-cooled glass mortar with 2 mL of the buffer containing 50 mmol L^{-1} malic acid, 50 mmol L^{-1} NaCl and 2 mmol L^{-1} $CaCl_2$ (pH 5.2). After centrifugation at 10,000×*g* for 10 min, the supernatant was diluted 10-fold with the same buffer.

A substrate purchased from Megazyme Co. Ltd. (Sydney, Australia) was used according to the method of Watanabe et al. (1994) and the instructions provided by the manufacturer with some alterations. This substrate contains a specific substrate for α-amylase (blocked p-nitrophenyl maltoheptaoside, BPNPG7) and excess glucoamylase and α-glucosidase. The substrate solution was prepared by dissolving the entire contents of the bottle provided by the manufacturer in 10 mL of distilled water. The sample solution was mixed with the same amount of substrate solution, 100 μL each, were mixed in an ice-cooled test tube, then put in a 40ºC hot water bath for 12 min. to determine the maximum potential activity or at 16ºC for 30 min to determine the activity at the growth temperature. After the reaction, the tubes were cooled immediately in wet ice. The reaction was terminated by adding 1.5 mL of a 1% Trizma base solution. OD at 410 nm was measured using a spectrophotometer (DU-640, Beckman Coulter, Inc., Fullerton, CA, USA). Unit values were calculated in comparison with standard malt flour purchased from Megazyme Co. Ltd. Two independent experiments were carried out, with at least 4 replicates each.

3. Sugar composition and concentration in the whole endosperm at early growth stages

(1) Plant material

Arroz da Terra and Fukuhibiki were chosen for further analysis, since these two varieties showed significant differences in both PSSL and α-amylase activity. Seeds were planted and grown in sterile conditions as in previous experiments at 16ºC or 25ºC. The seeds at CES and FLES were sampled in the same manner as for the α-amylase measurement. Dry seeds without imbibition were used as PGS samples. After freezing in liquid nitrogen, samples were vacuum-dried and stored in a refrigerator until use.

(2) Determination of sugar composition and concentration

The endosperm of dry seeds was obtained by removing the embryo from the seed with a scalpel under a microscope. The endosperm at CES and FLES was obtained by removing the shoot, root, and scutellum from freeze-dried samples with a scalpel under a microscope.

Ten endosperms were homogenized with 2 mL 50 mmol L^{-1} glycine-HCl buffer (pH3.5) containing 1 mmol L^{-1} EDTA. Buffer solution at this low pH was selected to reduce α-amylase activity as much as possible. As an internal standard, 5 μL of 100 mg mL^{-1} methyl-α-D-glucopyranoside was added. After centrifugation of the homogenate at 17,000 g for 10 min, acetonitrile was added

to the supernatant up to 60% (v/v). After filtration through Sample Prep C02-LH (0.5-μm pore, Millipore Co., Bedford, MA, USA), the sugar compositions and concentrations in the filtrate were determined by high performance liquid chromatography (HPLC. Waters 600 series, Waters Co. Milford, MA, USA) using a column for mono- and di-saccharide analysis (Waters High-performance Carbohydrate Column) and a differential refractometer (Waters 410). An acetonitrile:water (75:25, v:v) mixture was used as eluant at a flow rate of 1.2 ml min^{-1}. Data acquisition and analysis were done according to the internal standard method using the Millennium Chromatography Manager (Waters Co.). Three independent experiments were carried out for the endosperm at CES, and two for that at FLES. Each experiment had 4 replicates.

4. Evaluation of sugar composition and concentration in exudate from endosperm adjacent to the scutellum at the coleoptile elongation stage

Arroz da Terra and Fukuhibiki were grown in the same manner as in the previous experiments. Seeds were sampled at CES and hulled using forceps. After washing briefly in distilled water, the endosperm testa next to the scutellum was cut using a scalpel under a microscope, and the exudate from the cut end was collected using a micro-slide tube (0.1 mm×0.5 mm×30 mm inner radius, Iuchi Co., Osaka, Japan). After measuring the length of the filled part of the micro-slide tube, the tube was placed in a 1.5-mL plastic tube and frozen immediately on dry ice. After storing at −80ºC for several days, these samples were vacuum-dried and stored at 4ºC until use. The volume of the gathered exudate was estimated from cross-sectional area of the micro-slide tube multiplied by the length of the filled section.

After the addition of 200 μL of 10 mmol L^{-1} glycine-HCl (pH 3.5) buffer, the micro-slide tubes containing the samples were ground inside the 1.5 mL plastic tubes using a pestle. After centrifugation (8,000×*g* for 10 min) the sugar composition and concentration of the supernatants were determined by HPLC as described above. For the 16ºC samples, two independent experiments were carried out, with 3 and 4 replicates for Arroz da Terra and Fukuhibiki, respectively, in each experiment. For the sample grown at 26ºC, a single experiment with 3 replicates for each variety was carried out. For each replicate, the exudate was gathered from 10 to 20 grains. The average volume gathered from one grain was 0.20 μL.

Results

1. α-Amylase activity in endosperm at early growth stages

(1) Varietal differences

In the endosperm of rice seeds germinated and grown at 16ºC, α-amylase activity measured at 40ºC (potential maximum activity) increased 32- to 69-fold from PGS to CES (Fig. 1). From CES to FLES, the activity decreased by about 20 % in Arroz da Terra and Fukuhibiki, but it increased by 50 to 100% in the other 4 varieties (Fig. 1).

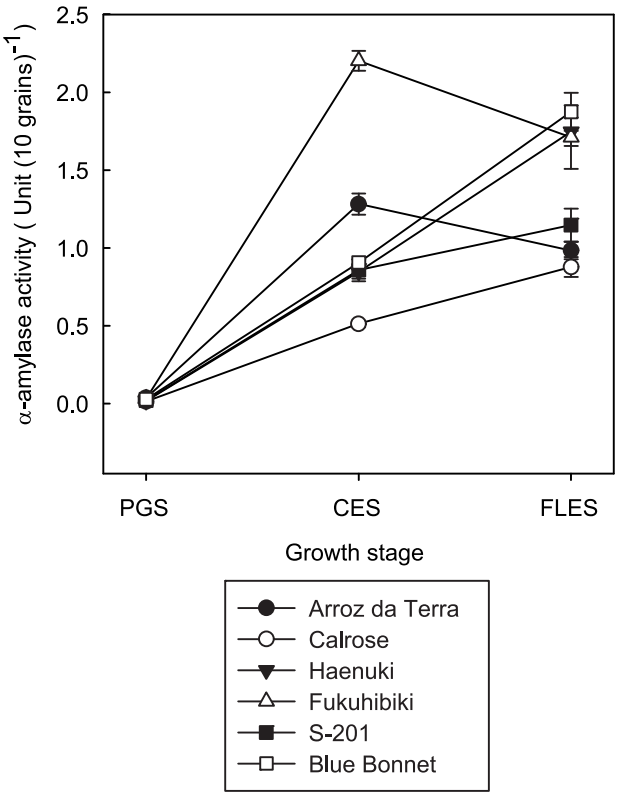

Fig. 1. Changes in α-amylase activity in endosperm of seedlings grown at 16ºC.
α-Amylase activity was estimated by digestion of a specific substrate, BPNPG7. Incubation temperature during digestion was 40ºC. Bars show± SE (n=8). PGS: pre-germination stage (1 day after imbibition), CES: coleoptile elongation stage, FLES: first leaf elongation stage. ● : Arroz da Terra, ○ : Calrose, ▼ : Haenuki, △ : Fukuhibiki, ■ : S-201, □ : Blue Bonnet.

Varietal differences in endosperm α-amylase activity were significant at the 1% level at both CES (LSD=0.182) and FLES (LSD=0.403). The activity in Fukuhibiki at CES was 2.20 units $(10 grains)^{-1}$ and was the highest among the 6 varieties (Fig. 1). On the other hand, the activity in Arroz da Terra, which showed the fastest coleoptile growth (Ogiwara ant Terashima, 2001) and superior PSSL at low temperatures (Table 1), was 1.28 units $(10 grain)^{-1}$. Although significant varietal differences were observed in α-amylase activity, seedling establishment rate (PSSL) and coleoptile elongation period G50-E50, α-amylase activity at CES correlated with neither PSSL nor with G50-E50 (Fig. 2). At FLES, α-amylase activity in Arroz da Terra and Calrose were 0.98 and 0.88 units $(10 grain)^{-1}$, respectively, and significantly lower than that in Haenuki, Fukuhibiki, and Blue Bonnet (Fig. 1).

Using the same samples, α-amylase activity was measured

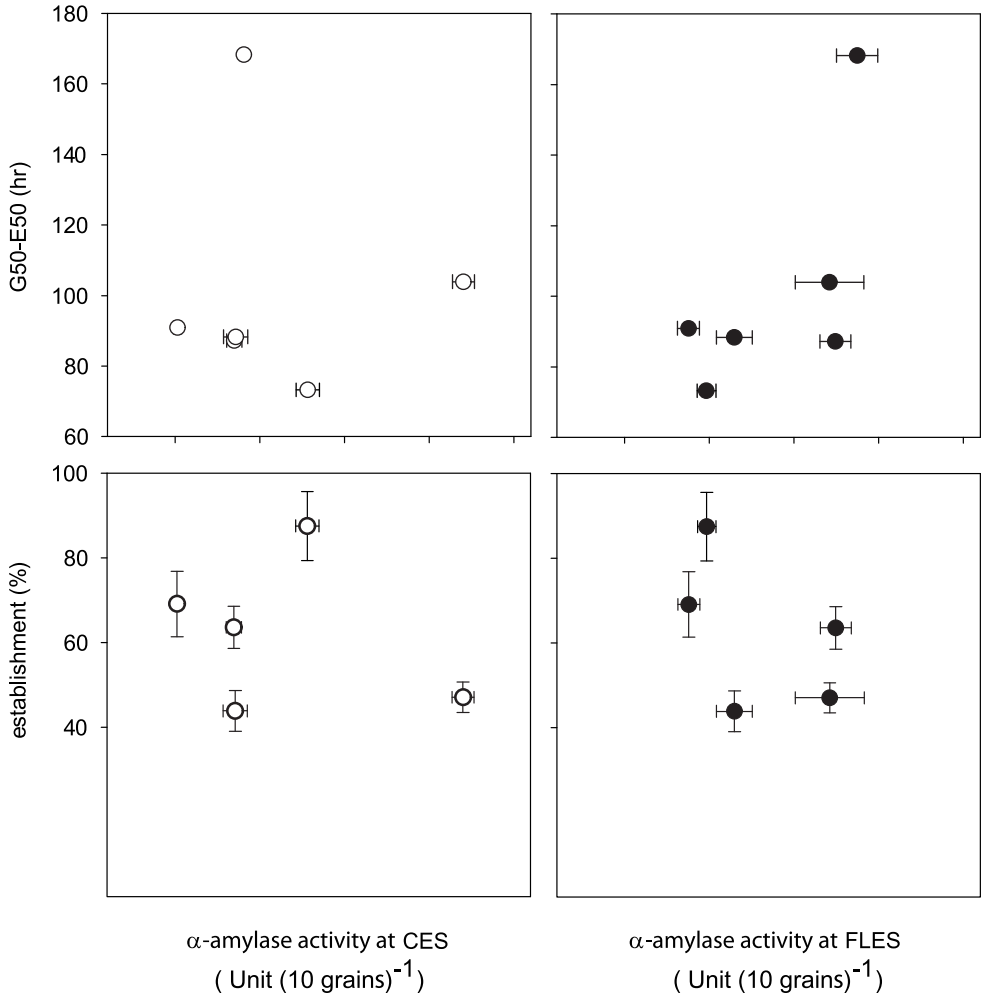

Fig. 2. Correlations of α-amylase activity with coleoptile elongation period (top) and seedling establishment rate (bottom) at low temperatures.
α-Amylase activities assays were carried out at the coleoptile elongation stage (CES) and the first leaf elongation stage (FLES), using seedlings grown in sterilized agar-bed at 16ºC. Coleoptile elongation period was shown as duration (hr) from 50% germination to 50% emergence from agar-bed (G50-E50), in sterilized agar-bed experiment at 16ºC (Ogiwara and Terashima 2001). Seedling establishment at low temperatures was estimated in submerged paddy fields from 1995 to 1999, according to our previous report (Ogiwara et al. 2003). Average soil temperature during the experiments was between 11.3ºC and 20.9ºC. Bars show±SE. Varieties were Arroz da Terra, Calrose, Haenuki, Fukuhibiki, S-201, and Blue Bonnet.

at 16ºC (equal to growth temperature). Although the activity was 5- to 10-fold lower than that measured at 40ºC, Fukuhibiki had the highest α-amylase activity among the 6 varieties at CES. At both CES and FLES, the correlation between the activities measured at 16ºC and 40ºC were obvious (r^2=0.907 and 0.661 at CES and FLES, respectively) (Fig. 3).

(2) Effect of growth temperature

The α-amylase activity in the endosperm measured at 40ºC (simply called α-amylase activity, hereafter) at the coleoptile elongation stage was examined in the seedlings grown at 16ºC and 26ºC using 4 varieties. The activities in seedlings grown at 16ºC were significantly higher than those in the seedlings grown at 26ºC in Arroz da Terra and Fukuhibiki, but not in Haenuki and Blue Bonnet, (Fig. 4).

In the seedlings grown at 26ºC, the α-amylase activity in Fukuhibiki was 1.83 units (10 grains)$^{-1}$, and this activity was significantly higher than that in Arroz da Terra, Haenuki, or Blue Bonnet grown at 26ºC (Fig. 4).

2. Sugar content of the endosperm at the early seedling growth stage

(1) Composition and concentration of sugars in the whole endosperm

In the endosperm of pre-germination dry seeds (PGS), sucrose was the only detectable sugar (Fig. 5). In the

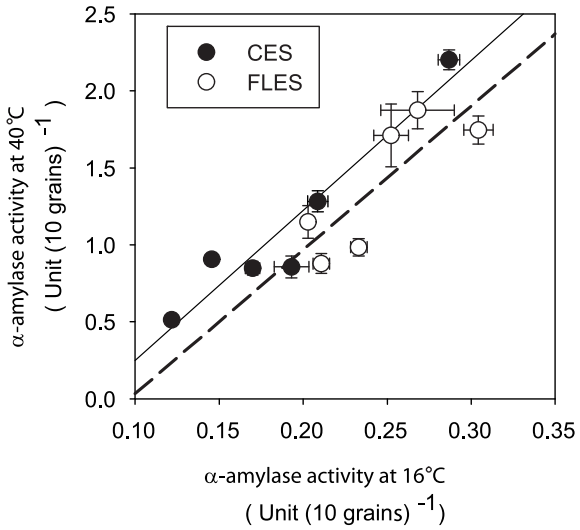

Fig. 3. Relationship between α-amylase activity measured at reaction temperatures of 16°C and 40°C using BPNPG7. Seedlings were grown at 16°C. Bars show±SE (n=6). Varieties were Arroz da Terra, Calrose, Haenuki, Fukuhibiki, S-201, and Blue Bonnet.

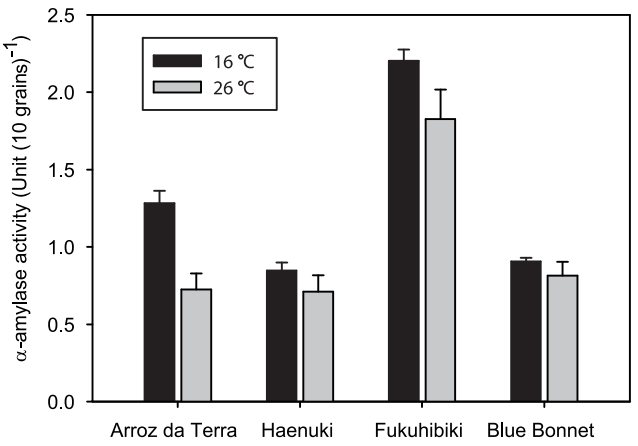

Fig. 4. Effect of growth temperature on α-amylase activity in endosperm at the coleoptile elongation stage (CES) in Arroz da Terra, Haenuki, Fukuhibiki, and Blue Bonnet. Seedlings were grown at 16°C and 26°C. Incubation temperature during the digestion of the substrate (BPNPG7) was 40°C. Bars show±SE (n=4 to 8).

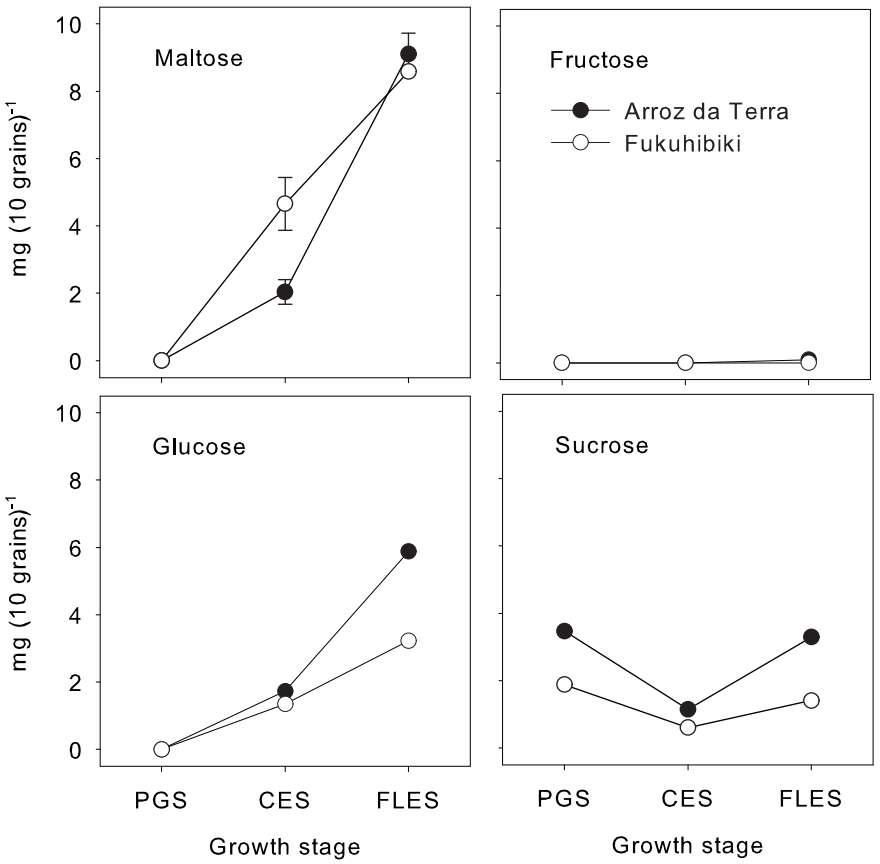

Fig. 5. Maltose, glucose, fructose, and sucrose concentrations in whole endosperm of Arroz da Terra and Fukuhibiki seedlings grown at 16°C. PGS: pre-germination stage (dry mature seeds), CES: coleoptile elongation stage, FLES: first leaf elongation stage. Sugar compositions and concentrations were determined by HPLC using whole endosperm of Arroz da Terra (closed circles) and Fukuhibiki (open circles). Bars show±SE (n=5).

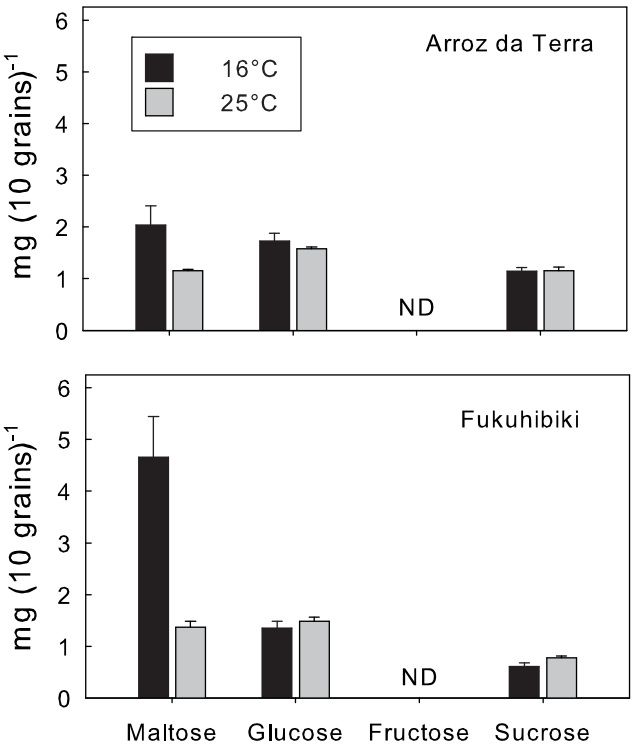

Fig. 6. Effect of growth temperature on maltose, glucose, fructose, and sucrose concentrations in whole endosperm of Arroz da Terra and Fukuhibiki at the coleoptile elongation stage (CES). Sugar compositions and concentrations were determined by HPLC. ND: not detected. Bars show±SE (n=5).

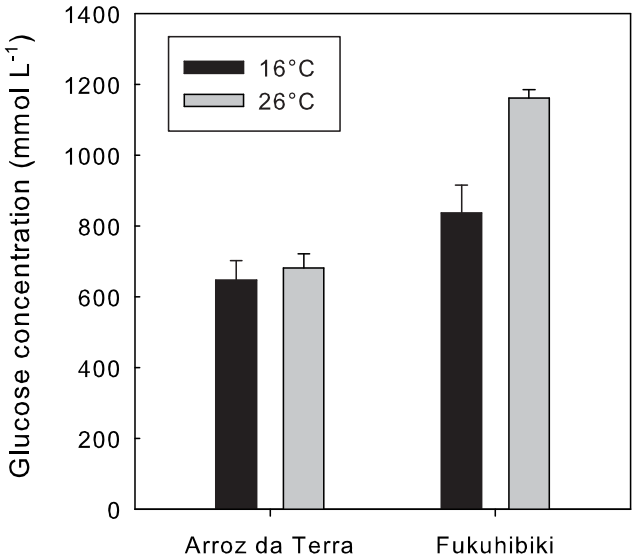

Fig. 7. Glucose concentration in exudate from endosperm adjacent to the scutellum at the coleoptile elongation stage (CES) in Arroz da Terra and Fukuhibiki grown at 16 and 26ºC. Glucose concentration was determined by HPLC. Bars show±SE (n=4 to 6).

endosperm after germination, glucose, maltose, and sucrose were abundant, but only trace amounts of fructose and lactose were detected at FLES (data not shown).

Maltose and glucose accumulated continuously during early seedling growth (Fig. 5), but the sucrose content decreased from PGS to CES, and then increased from CES to FLES (Fig. 5). In the endosperm, maltose was more abundant than glucose or sucrose at CES and FLES, irrespective of variety.

The concentration of maltose, the immediate product of α-amylase, concentration at CES Fukuhibiki was higher (4.7 mg (10 grains)$^{-1}$) than in Arroz da Terra (Fig. 5), consistent with Fukuhibiki's higher α-amylase activity (Fig. 1). On the other hand, there was no significant difference in glucose or sucrose content between these varieties. On the other hand, at FLES, glucose and sucrose contents in Arroz da Terra were 5.9 and 3.3 mg (10 grain)$^{-1}$, respectively, and higher than those in Fukuhibiki, while maltose content was almost the same.

(2) Effects of growth temperature on sugar contents at the coleoptile elongation stage

Glucose content of the endosperm at CES was 1.4–1.7 mg (10 grain)$^{-1}$ in both Fukuhibiki and Arroz da Terra at both growth temperatures, 16 and 25ºC (Fig. 6). On the other hand, maltose content was higher in Fukuhibiki grown at 16ºC. The maltose content of endosperm in Fukuhibiki grown at 16ºC was as high as 4.7 mg (10 grains)$^{-1}$, and that grown at 25ºC was only 2.0 mg (10 grains)$^{-1}$.

3. Composition and concentration of sugar in the exudate from endosperm adjacent to the scutellum

Glucose was the dominant sugar in the exudate collected from the endosperm adjacent to the scutellum at the coleoptile growth stage regardless of variety and growth temperature. Concentrations of maltose and sucrose were less than 1% of that of glucose in all samples (data not shown). Only a trace amount of fructose was detected.

In Fukuhibiki, the glucose concentrations were 838 and 1162 mmol L^{-1} in seedlings grown at 16ºC and 26ºC, respectively, while in Arroz da Terra, the concentration was 630–670 mmol L^{-1} at either growth temperature (Fig. 7). Thus the glucose concentration in the exudate was significantly higher in Fukuhibiki than in Arroz da Terra grown at either 16 or 26ºC .

Discussion

To survive anaerobic conditions, rice coleoptiles play a critical role as a "snorkel". When rice seeds are sown in submerged conditions, the coleoptile elongates after germination and grows until it reaches the air; while growth of leaves and roots is suspended (Wada, 1961; Ogiwara and Terashima, 2001). Coleoptile growth of rice seedling is enhanced under anaerobic conditions (Kordan, 1976; Kordan, 1977), and the complete set of starch-degrading enzymes in endosperm, including α-amylase,

is also induced under anaerobic conditions (Perata et al., 1992; Perata et al., 1993; Guglielminetti et al., 1995). Ogiwara and Terashima (2001) found a significant correlation between the duration from 50% germination to 50% emergence (G50-E50) in the agar-bed experiment and PSSL in field experiments. G50-E50 indicates the time it took for the coleoptile to reach the surface of the agar-bed.

At the coleoptile elongation stage, the difference in α-amylase activity was apparent among the 6 rice varieties (Fig. 1). However, the varietal difference in α-amylase activity did not correspond with the difference in the coleoptile elongation period (G50-E50) in the agar-bed or with the seedling establishment rate (PSSL) in the submerged and low-temperature conditions (Fig. 2). For example, Fukuhibiki endosperm had much higher enzyme activity than the other varieties at CES, despite of its slower coleoptile growth and poorer seedling establishment than Arroz da Terra, Calrose, and Haenuki (Table 1, Fig.1).

On the other hand, a significant correlation was found between the α-amylase activity at 40ºC and 16ºC in the 6 varieties (Fig. 3). This result eliminated the possibility that varieties showing superior seedling establishment under low temperature conditions such as Arroz da Terra had an α-amylase that shows higher activity at low temperatures than the others.

In addition, α-amylase activity at CES in the seedlings grown at 16ºC was not greatly different from that in the seedlings grown at 26ºC in any of the 4 varieties tested (Fig. 4). In rice seeds, α-amylase is synthesized *de novo* after imbibition (Perata et al., 1993; Guglielminetti et al., 1995), this result strongly suggests that a reduction in temperature from 26ºC to 16ºC has no significant effect on α-amylase synthesis.

From the above results, it is presumed that α-amylase activity at CES is not a limiting factor for the survival of seedlings at low temperatures, in accordance with Fukuda et al. (2008), supporting the conclusions of Williams and Peterson (1973), Sasahara and Ikarashi (1989).

Williams and Peterson (1973) concluded that α-amylase is not a rate-limiting factor in seedling development at low temperatures. This conclusion was based on the experiment using only one variety, Calrose, in which α-amylase activity dropped sharply when temperatures were reduced from 30ºC to 18ºC. However, the same treatment caused an even more drastic reduction in seedling weight. Sasahara and Ikarashi (1989) also reported that differences in α-amylase activity did not correspond with the varietal differences in shoot and root fresh weights at 3, 6 or 9 days after sowing at 18ºC.

On the other hand, Karrer et al. (1993) found that α-amylase activity and accumulation of *RAmy1A* mRNA in the endosperm strongly correlated with shoot weight at 16 days after sowing at both 15 and 30ºC in 10 varieties. Although Karrer et al. (1993) did not state at which growth stages they harvested the samples, it was presumably well after emergence and might have been at the two-to-three leaf stage, since 16 days is long enough to develop several leaves at 30ºC. Williams and Peterson (1973) also found a significant correlation between α-amylase activity and shoot weight at 5, 7 and 9 days after sowing at 30ºC in a single variety, Calrose. It is evident that α-amylase activity has a significant influence on seedling weight after development of several leaves. However, it has less effect on seedling survival, since the growth of leaves has less effect on seedling establishment (Ogiwara and Terashima, 2001), and coleoptile weight does not correlate with the coleoptile length (Wada, 1961).

The present study also revealed the *in vivo* sugar compositions and concentrations in the endosperm of Arroz da Terra and Fukuhibiki during coleoptile elongation, in addition to the *in vitro* α-amylase activity. When sugar compositions and concentrations were determined using the whole endosperm, maltose accumulation was observed both at CES and FLES (Fig. 4). In the endosperm of seedlings grown at 16ºC, maltose concentration was significantly higher in Fukuhibiki than in Arroz da Terra (Fig. 5) in accordance with the higher α-amylase activity in Fukuhibiki (Fig. 1). Since maltose is an intermediate metabolite in starch digestion, these results suggest that maltose production proceeds faster than its conversion into glucose by α-glucosidase or via other pathways at 16ºC (Konishi et al., 1994).

On the other hand, glucose was the dominant sugar in the exudate collected from the endosperm adjacent to the scutellum (Fig. 7). Glucose is the end product of starch digestion in endosperm (Beck and Ziegler, 1989) and is absorbed by the embryo through the scutellum (Murata et al., 1968; Nomura et al., 1969). Thus it is assumed that the sugar content of this exudate reflects the actual sugar availability to the embryo. In the seedlings grown at 16ºC, the glucose concentration in this exudate was higher in Fukuhibiki than in Arroz da Terra (Fig. 7) in spite of slower coleoptile growth (Ogiwara and Terashima 2001) and poorer performance in seedling establishment in Fukuhibiki (Table 1).

The glucose concentration in the exudate from the endosperm adjacent to the scutellum was extremely high. It was 838 mmol L^{-1} in Fukuhibiki grown at 16ºC, for example (Fig. 7). This glucose concentration is four-fold higher than the sucrose concentration in phloem sap in leaf sheaths at the 7th to 8th leaf stage in rice plants (Fukumorita and Chino, 1982; Hayashi and Chino, 1990). At a much lower concentration of glucose (90 to 250 m mol L^{-1}), repression of α-amylase gene expression in rice scutellum was observed (Karrer and Rodriguez, 1992; Toyofuku and Yamaguchi, 1998). In addition, sucrose transporter in phloem companion cells in rice embryo was

induced at eight-fold lower glucose concentration than that observed in Fukuhibiki (Matsukura et al., 2000). Although there is no information about the turnover rate of glucose in this exudate, it is obvious that the scutella is in contact with the glucose-rich exudate during coleoptile growth. This is direct evidence against the hypothesis that a deficiency in α-amylase activity or glucose production in the endosperm are the cause of growth retardation at around 16ºC. This result strongly suggests that further utilization of carbohydrates by embryo, for example, sucrose synthase activities (Fukuda et al., 2008) is more significant for seedling growth.

Estimation of α-amylase activity in endosperm and subsequent assays revealed the presence of not only adequate α-amylase activity (Fig.1) but also rather excessive amounts of glucose in the endosperm (Fig.7) during coleoptile growth at 16ºC. Since the varietal differences in coleoptile growth strongly correlated with the seedling establishment rate in rice in a submerged field under low-temperature conditions (Ogiwara and Terashima 2001), and the coleoptile has a vital function as "snorkel", it is plausible that α-amylase activity and glucose production in the endosperm are not relevant to the varietal difference in seedling establishment trait under low temperature conditions.

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* In Japanese with English abstract.
** In Japanese with English summary.
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