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RESEARCH PAPER

Interference with oxidative phosphorylation enhances anoxic expression of rice α-amylase genes through abolishing sugar regulation

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Abstract

Rice has the unique ability to express α-amylase under anoxic conditions, a feature that is critical for successful anaerobic germination and growth. Previously, anaerobic conditions were shown to up-regulate the expression of Amy3 subfamily genes (*Amy3B*/*C*, *3D*, and *3E*) in rice embryos. These genes are known to be feedback regulated by the hydrolytic products of starchy endosperm such as the simple sugar glucose. It was found that oxygen deficiency interferes with the repression of *Amy3D* gene expression imposed by low concentrations of glucose but not with that imposed by higher amounts. This differential anoxic de-repression depending on sugar concentration suggests the presence of two distinct pathways for sugar regulation of *Amy3D* gene expression. Anoxic de-repression can be mimicked by treating rice embryos with inhibitors of ATP synthesis during respiration. Other sugar-regulated rice α-amylase genes, *Amy3B*/*C* and *3E*, behave similarly to *Amy3D*. Treatment with a respiratory inhibitor or anoxia also relieved the sugar repression of the rice *CIPK15* gene, a main upstream positive regulator of *SnRK1A* that is critical for *Amy3D* expression in response to sugar starvation. SnRK1A accumulation was previously shown to be required for MYBS1 expression, which transactivates *Amy3D* by binding to a *cis*-acting element found in the proximal region of all Amy3 subfamily gene promoters (the TA box). Taken together, these results suggest that prevention of oxidative phosphorylation by oxygen deficiency interferes with the sugar repression of Amy3 subfamily gene expression, leading to their enhanced expression in rice embryos during anaerobic germination.

Key words: α-Amylase, anaerobic germination, anoxia, oxidative phosphorylation, sugar signalling.

Introduction

Rice (*Oryza sativa*) is the only cereal that has the ability to germinate and grow while submerged, a characteristic observed in only a few plant species (Perata *et al.*, 1997). Anaerobic tolerance in plants is known to be closely associated with the maintenance of energy metabolism (Webb and Armstrong, 1983; Ricard *et al.*, 1991). Therefore, a sufficient supply of metabolizable sugars is probably very important for the embryonic axis of submerged rice seedlings to overcome the low energetic efficiency of fermentation. Since only limited amounts of metabolizable sugars are present in pre-germinated seeds, mobilization of the starchy endosperm is critical to support the growth of non-photosynthetic embryonic tissues.

One of the features clearly distinguishing rice from other cereals is its ability to mobilize endosperm starch under anaerobic conditions (Guglielminetti *et al.*, 1995*a*, *b*). The importance of sugar availability has been demonstrated in anaerobic germination. For example, the inability of wheat seeds to undergo anaerobic germination and the very stunted anaerobic growth of rice *CIPK15* knockout mutants are related to their inability to mobilize endospermal starch under anoxia, because exogenous supplementation with glucose or sucrose rescues these plants under anoxic conditions (Perata *et al.*, 1993; Lee *et al.*, 2009). Therefore, even though the ability to undergo germination and post-germinative growth under anaerobic conditions is likely to be the result

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of many physiological and biochemical processes, the ability to mobilize starch anaerobically is essential for the growth of anoxia-tolerant cereals such as rice.

α-Amylase initiates the breakdown of intact starch granules from the endosperm, releasing glucose polymers in the form of amylose and amylopectin, which can be further digested by various hydrolases into soluble sugars. These sugars are mobilized to the embryonic axis, where they are utilized as carbon and energy sources for shoot and root apical meristems that differentiate and grow to become the seedling. Therefore, by controlling the rate of mobilization of the starchy endosperm, α-amylase plays a critical role in the germination process (Bewley and Black, 1994). Rice α-amylases are encoded by 10 separate genes belonging to three subfamilies (Amy1, 2, and 3) (Huang *et al.*, 1990). Rice α-amylase gene expression during aerobic germination has been well characterized. For example, the analyses of steady-state mRNA levels and *in situ* hybridization have shown that the expression of each member of the α-amylase multigene family is spatially and temporally regulated during aerobic germination and seedling growth (Karrer *et al.*, 1991; Hwang *et al.*, 1999). All genes of the Amy1 and 2 subfamilies of rice appear to be under phytohormonal control because the *cis*-acting gibberellic acid (GA) response element (GARE; 5′TAACAG/AA3′) is conserved in their promoters (unpublished data). In fact, transcription of *Amy1A*, a major α-amylase, is positively stimulated by GA, and this action is antagonized by abscisic acid (ABA) (O'Neill *et al.*, 1990; Itoh *et al.*, 1995). In contrast, all promoters of Amy3 subfamily genes lack the GARE, suggesting that their regulation is independent of GA/ABA control (unpublished data). In fact, *Amy3D* promoter activity in isolated embryos does not increase in response to exogenous GA (Karrer and Rodriguez, 1992). Instead, Amy3 subfamily genes are under sugar regulation in which their expression is strongly induced in the absence of sugars but is repressed by various sugars produced during endosperm mobilization (Karrer and Rodriguez, 1992; Yu *et al.*, 1992; Thomas and Rodriguez, 1994). This can explain the transitory expression pattern of Amy3 subfamily genes in scutellar tissue during aerobic germination (Hwang *et al.*, 1999). For example, in the initial stage of aerobic germination, *Amy3D* is expressed because no sugar is available around the embryo, but as endosperm starch is mobilized during the germination process, the increasing amount of sugars around the embryo inhibits *Amy3D* expression. Intriguingly, this transitory expression pattern of Amy3 subfamily genes disappears during anaerobic germination (Hwang *et al.*, 1999). For example, steady-state levels of *Amy3B*/*C*, *3D*, and *3E* increase by the fourth day and are sustained to the sixth day during anaerobic germination, instead of rapidly disappearing after 1 d in aerobic conditions. Sustained high expression of Amy3 subfamily genes during anaerobic conditions appears to be very important for anaerobic endosperm mobilization, since their relative contribution to α-amylase production becomes much greater (Perata *et al.*, 1997). In addition to rice seeds, rice anoxic coleoptiles also showed strong induction of the *Amy3D* gene in an RNA profiling study (Lasanthi-Kudahettige *et al.*, 2007). Very little is known, however, about the ability of rice to express α-amylase under anaerobic conditions.

In this study, the correlation between sugar regulation of Amy3 subfamily genes (including *Amy3D*) and their sustained high expression in a limited oxygen environment was investigated. The data demonstrate that sugar regulation of Amy3 subfamily gene expression is modulated by oxygen availability. For example, various respiration inhibitors as well as anaerobic growth conditions allow the expression of Amy3 subfamily genes even in the presence of glucose. These data indicate that inhibition of oxidative phosphorylation interferes with the repression of Amy3 subfamily gene expression by the sugars produced during germination, probably through de-repression of *CIPK15* expression, resulting in augmented expression during anaerobic germination

Materials and methods

Rice whole seed and rice embryo treatments

Whole rice seeds (*Oryza sativa L.* cv. Dongjin) or rice embryos manually dissected from the seed with a razor blade were surface sterilized as described in Hwang *et al.* (2005).

For aerobic germination, 70–100 sterilized whole seeds were placed on three layers of 3MM Whatman paper soaked with 10 mM $CaCl_2$ solution. For anoxic treatment, the same amount of seeds was submerged in 10 mM $CaCl_2$ solution under N_2 gas. Rice embryos were harvested from whole seeds on the indicated days, frozen with liquid N_2, and used for extraction of total RNA.

For embryo experiments, 70–100 manually dissected rice embryos were incubated on three layers of 3MM Whatman paper soaked with 10 mM $CaCl_2$ containing glucose or another sugar at the indicated concentration. For anoxic treatment, ~70–100 embryos were submerged in 10 mM $CaCl_2$ solution under N_2 gas, either with or without glucose at the indicated concentration as described above. The molarity of sugar was adjusted to be the same for all experiments by supplementing with mannitol.

Chemical treatments

Sodium azide (NaN_3) and 2,4-dinitrophenol (DNP) were purchased from Sigma-Aldrich Korea (Yongin, Korea).

Rice suspension cultures

Suspension-cultured rice cells (*O. sativa L.* cv. Dongjin) were kindly provided by Jong-Seong Jeon's lab in Kyung Hee University (Yongin, Korea). The suspension-cultured cells were maintained in AA2 culture medium (Thompson *et al.*, 1986) and subcultured every 10 d by transferring ~3–5 ml packed volume of the cells to 20 ml of fresh AA2 medium and actively shaking at 150 rpm, 28 °C in the dark. For any chemical treatment, 5-day-old cells of subcultures were washed twice with AA2 without glucose and then transferred to a flask containing 20 ml of AA2 medium with various sugars or other chemicals. Flasks were shaken at 150 rpm, 28 °C in the dark. For anoxic treatment, cells were transferred to flasks under N_2 gas containing 20 ml of AA2 medium with various sugars and other chemicals, and incubated at 28 °C without shaking in the dark.

RNA analysis

Total RNA was isolated from harvested rice embryos by grinding with a mortar and pestle in liquid N_2. The finely ground powder

was dissolved in 6 ml of TLE/SDS buffer (0.18 M TRIS, 0.09 M LiCl, 4.5 mM EDTA, 1% SDS, pH 8.2), vortexed vigorously, and incubated on ice for 10 min. The same volume of TLE-buffered phenol and chloroform was added and it was vigorously vortexed and placed on ice for 30 min. Nucleic acids were recovered by ethanol precipitation and then dissolved in 1 ml of RNase-free H_2O. Total RNA was specifically separated from DNA and other contaminants by the TRI Reagent from Molecular Research Center, Inc. (Cincinnati, OH, USA). RNA was precipitated with 100% ethanol and its concentration was determined spectrophotometrically. A 25 μg aliquot of total RNA was size-fractionated through a 1% formaldehyde–agarose gel, blotted onto a positively charged nylon membrane (Amersham, Piscataway, NJ, USA), and exposed to UV light of 1200 kJ/cm^2 to fix the RNA to the membrane.

A probe specific for the *Amy3D* gene was prepared by PCR using a primer set (Table 1) that primarily amplifies the 3′-untranslated region (UTR) of the *Amy3D* gene, using *Amy3D* cDNA (pOS137) as a template (O'Neill *et al.*, 1990). The probe was labelled non-radioactively using a digoxigenin (DIG) non-radioisotope labelling protocol (Roche Diagnostics, Mannheim, Germany). The specificity of this *Amy3D* probe has been previously demonstrated (Hwang *et al.*, 1999). PCR was carried out using a thermal controller (PTC-100; MJ Research, Waterdown, MA, USA) with a 2 min pre-denaturing step at 95 °C followed by 30 cycles of amplification, with a 30 s denaturing step at 95 °C, a 30 s annealing step at 55 °C, and a 2 min extension step at 72 °C. The final extension step was 5 min at 72 °C.

Pre-hybridization of the membrane was performed for 5 h in Ultrahyb solution (Ambion, Austin, TX, USA) at 42 °C. Hybridization was started by adding the heat-denatured DIG-labelled probe to the pre-hybridization solution and was performed overnight. Washing and detection were performed using the DIG Wash and Block buffer set from Roche Diagnostics (Mannheim, Germany) as described in the company's recommended protocol.

Quantitative real-time PCR

First-strand cDNA was synthesized with 1 μg of total RNA using a Maxim kit (iNtRON Biotechnology, Seongnam, Korea) and an oligo(dT) primer. Real-time quantitative reverse transcription-PCR (qRT-PCR) was carried out using the Mx3000P™ Real-time PCR system (Stratagene, La Jolla, CA, USA). Detection of real-time RT-PCR products was done by staining with SYBR Green (Takara Bio, Otsu, Japan) following the manufacturer's recommendations. A 1 μg aliquot of first-strand cDNA was used as the template for PCR. The PCR cycling conditions were 40 cycles after a 10 min pre-denaturing step at 95 °C, with a 30 s denaturing step at 95 °C, a 1 min annealing step at 60 °C, and a 1 min extension step at 72 °C. The final extension step was 5 min at 72 °C. The relative quantification method was used to evaluate quantitative variation between the triplicates examined. The relative amplification of the rice *actin* gene was used as an internal control to normalize all data. The gene-specific primers used for quantitative PCR are listed in Table 1.

Respiration rate measurement

The respiration rate of suspension-cultured rice cells was determined by an oxygen electrode (Rank Brothers, Cambridge, UK) using 1 ml of medium containing ~200 μl packed volume of cells. The total protein concentration was measured by the Bradford method (Bradford, 1976).

ATP measurement

The concentration of ATP present in each of the samples was determined using a bioluminescent detection reagent (ENLITEN rLuciferase/Luciferin; Promega). Suspension-cultured cells (200 μl packed volume) were ground with a mortar and pestle in liquid N_2, resuspended in 800 μl of grinding buffer (100 mm KH_2PO_4 at pH 7.8, 1 mm EDTA, 7 mm β-mercaptoethanol), vortexed, spun down quickly at 14 000 pm for 5 min, and the supernatant was used for the assay. A 100 μl aliquot of luciferase/luciferin reagent was added to 10 μl of the sample and luminescence was measured by a luminometer (Tuner Biosystems, Sunnyvale, CA, USA) using a 10 s integration. The amount of ATP present in the sample was calculated from the measured relative light units (RLU) using a standard curve. The total protein amount contained in the sample was determined by the Bradford method (Bradford, 1976).

Table 1. List of the primers used in this study

	Target gene	Primer sequence
DIG labelling	*Amy3D*	FW 5′-CGGGATAGTCATGCTCAAACCAG-3′
		RV 5′-GATTTTTTACTGCATCCTGAACCTG-3′
qPCR	*Amy3D*	FW 5′-GTAGGCAGGCTCTCTAGCCTCTAGG-3′
		RV 5′-GATTTTTTACTGCATCCTGAACCTG-3′
	Amy3B/C	FW 5′-AGCAAGCTGAAAATCCTTGCTGCTGA-3′
		RV 5′-TAATTGTTGCCGTGAGCAACGACATG-3′
	Amy3E	FW 5′-AGGAAGGCCTCAGGGTTCCTGCCGGT-3′
		RV 5′-TCTCGCAGCAAAATTGCATGAT-3′
	CIPK15	FW 5′-TAAGCCTTCAAAATTCTTCG-3′
		RV 5′-TATAAACAAAACCAGGACTC-3′
	Actin	FW 5′-ATGAAGATCAAGGTGGTCGC-3′
		RV 5′ GTACTCAGCCTTGGCAATCC-3′

Results

Anoxic conditions perturb the sugar regulation of Amy3D *in the embryo, causing a sustained increase in its expression*

Since there is some variation in the anoxic responses of different rice varieties (Magneschi *et al.*, 2009), the anoxic induction of *Amy3D* was first examined in the embryos of intact germinating seeds of Dongjin, a variety that was used in the experiments. *Amy3D* expression was examined in rice embryos dissected from aerobically or anaerobically germinating seeds on the indicated days of germination (Fig. 1A). In embryos from aerobically germinated seeds, *Amy3D* was weakly and transiently expressed. For example, its expression was detectable from day 1 of germination until day 2, and became undetectable by the fourth day of germination. The expression level of *Amy3D* from intact seeds was much lower than that from pre-isolated embryos without sugar (Fig. 1B), suggestive of tight sugar regulation by sugars newly released from endosperm digestion or pre-existing sugars around the embryo in intact seeds. In contrast, in the embryos of anaerobically germinated seeds, *Amy3D* expression increased prominently at day 1, and it continued at least to the fourth day of germination when no *Amy3D* transcripts were detectable from aerobically germinated seeds.

Since *Amy3D* expression, which is repressible by sugars, was enhanced and sustained in anaerobic conditions, experiments were carried out to investigate whether the sugar regulation of *Amy3D* gene expression was influenced by oxygen deficiency. Pre-isolated rice embryos were incubated in different amounts of sugar in the presence or absence of

oxygen for 1 d, and the steady-state levels of *Amy3D* mRNA were examined (Fig. 1B). In aerobic conditions, the expression of *Amy3D* was primarily controlled by the sugar level; it was strongly expressed in the absence of sugar and repressed in its presence. Surprisingly, anaerobic conditions were able to interfere significantly with this sugar regulation pattern. Anoxic conditions abolished the repression of *Amy3D* expression imposed by the presence of glucose at concentrations <80 mM, though it did not relieve *Amy3D* suppression resulting from glucose concentrations >165 mM. These results indicate that limited oxygen availability can counter the ability of lower concentrations of glucose to repress *Amy3D* gene expression, and they raise the possibility that sugar regulation of *Amy3D* can occur through two distinct pathways. The physiological concentrations of sugar in the scutellum tissues are considered in the Discussion.

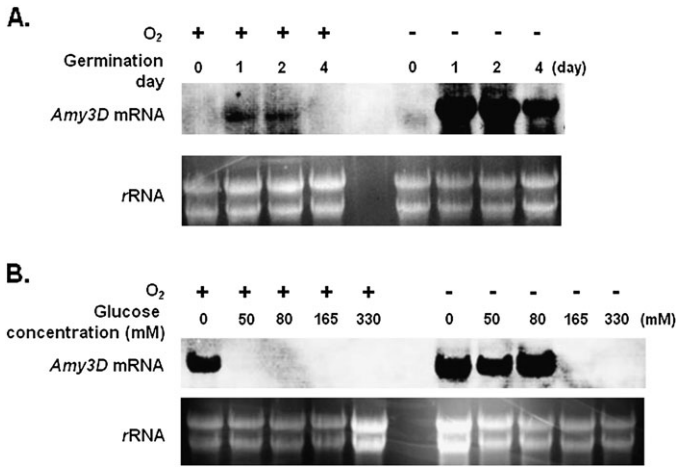

Fig. 1. Modulation of *Amy3D* expression by oxygen availability. (A) Expression of *Amy3D* in rice embryos during aerobic or anaerobic germination. For aerobic germination, rice seeds were placed on Whatman paper layers soaked with 10 mM $CaCl_2$ solution at 28 °C in the dark. For anoxic treatment, rice seeds were submerged in 10 mM $CaCl_2$ solution under N_2 gas at 28 °C in the dark. The presence or absence of O_2 is indicated as + or –. After germinating in the presence or absence of oxygen, rice embryos were harvested at the indicated times from whole seeds and subsequently frozen with liquid N_2. The transcript levels of *Amy3D* were examined from total RNA isolated from frozen tissues by using a probe specific to the 3′ UTR. The total amount of RNA loaded is indicated by rRNA stained with ethidium bromide. (B) Effect of anoxia on the sugar regulation of *Amy3D* expression in isolated rice embryos. *Amy3D* mRNA levels were examined in pre-isolated embryos incubated in various concentrations of glucose (0–330 mM) under aerobic and anaerobic conditions. For aerobic treatment, rice embryos were first manually dissected from whole seeds with a razor blade and incubated on Whatman paper layers soaked with 10 mM $CaCl_2$ containing glucose at the indicated concentrations. For anoxic treatment, rice embryos were submerged in 10 mM $CaCl_2$ solution at the same glucose concentrations as described above. The total sugar molarity was adjusted to be the same for all experiments by supplementing with mannitol. The presence or absence of O_2 is indicated as + or –. The transcript levels of *Amy3D* were detected as described above. The total amount of RNA loaded is indicated by rRNA stained with ethidium bromide.

Inhibition of oxidative phosphorylation perturbs sugar regulation of Amy3D *expression in the embryo*

To identify anoxic factors that alter sugar regulation of the *Amy3D* gene, it was determined whether respiratory inhibitors could mimic the anoxic effect on *Amy3D* gene expression. Respiratory inhibitors such as sodium azide (NaN_3) and potassium cyanide (KCN) inhibit cytochrome *c* oxidase by forming a complex with the iron ion in the cytochrome oxidase. As shown in Fig. 2, co-treatment with NaN_3 successfully de-repressed *Amy3D* expression in rice embryos incubated in 80 mM glucose solution for 1 d. KCN also showed the same de-repression effect (data not shown). Since these metabolic inhibitors interfere with the action of all different kinds of metal-containing oxidases (Beevers, 1961), another kind of respiratory inhibitor was employed to determine the effect of inhibiting oxidative phosphorylation. 2,4-Dinitrophenol (DNP) is an uncoupler that prevents the synthesis of ATP through the cytochrome *c* oxidase pathway by dissipating the proton gradient across mitochondrial cisternae. Like NaN_3 and KCN, DNP also effectively released glucose repression, indicating that de-repression of the *Amy3D* gene by metabolic inhibitors is due to inhibition of oxidative phosphorylation. These results suggest that the inhibition of oxidative phosphorylation can cross-talk with the sugar-dependent regulation of *Amy3D* expression.

Inhibition of oxidative phosphorylation also interferes with the sugar regulation of other Amy3 *subfamily genes, which show enhanced expression during anaerobic germination*

In addition to *Amy3D*, the expression of other Amy3 subfamily genes is known to be controlled by sugar levels

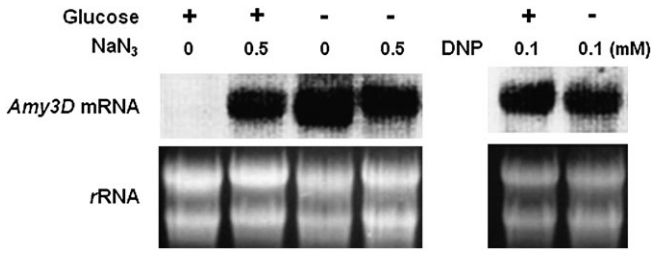

Fig. 2. Effects of oxidative phosphorylation inhibitors on the sugar regulation of the *Amy3D* gene in isolated rice embryos. Rice embryos were manually harvested from whole seeds and incubated on Whatman paper layers soaked with 10 mM $CaCl_2$ containing sugar (80 mM glucose or mannitol) with or without the respiratory inhibitors sodium azide (NaN_3) or 2,4-dinitrophenol (DNP) at the indicated concentrations for 1 d in the dark. The presence or absence of glucose is indicated as + or –. The transcript levels of *Amy3D* were detected as described in Fig. 1. The total amount of RNA loaded is indicated by rRNA stained with ethidium bromide.

(Karrer and Rodriguez, 1992; Thomas and Rodriguez, 1994). Therefore, experiments were performed to determine whether the sugar regulation of those genes would also be affected by anoxia or co-treatment with a respiration inhibitor (Fig. 3). Real-time quantitative PCR indicated that the expression of both *Amy3B/C* and *Amy3E* was repressed by glucose ~20-fold, but either oxygen deficiency or co-treatment with a respiratory inhibitor allowed those genes to be highly expressed even in the presence of glucose, as observed with *Amy3D*. These data indicate that de-repression of the glucose effect by the inhibition of oxidative phosphorylation is not unique to *Amy3D* gene regulation, and that this interference with the repression of Amy3 subfamily gene expression results in a prominently enhanced and sustained expression pattern during the anaerobic germination of rice seeds.

Prevention of oxidative phosphorylation abolishes the sugar regulation of CIPK15*, an upstream positive regulator of SnRK1A*

Activation of *Amy3D* expression under starvation conditions requires an accumulation of SNF1-related protein kinase (SnRK1A), a yeast SNF1 (sucrose non-fermenting-1) orthologue in rice. Recently, another rice protein kinase, CIPK15 (calcineurin B-like protein-interacting protein kinase), was demonstrated to be necessary for SnRK1A accumulation under starvation conditions. Intriguingly, the transcript levels of *CIPK15* are regulated by sugar similarly to those of *Amy3D*. Since CIPK15 is a more upstream signalling component than SnRK1A, and its expression itself is under the control of sugar, experiments were conducted to examine whether the sugar regulation of *CIPK15* expression is also affected by anoxia or inhibition of oxidative phosphorylation (Fig. 4). Both anoxic conditions and the respiratory inhibitor NaN_3 relieved the repressive effect of sugar on *CIPK15* transcription, as was observed for *Amy3D* expression, suggesting that anoxic de-repression of *Amy3D* expression may be a result of anoxic interference with the sugar regulation of *CIPK15*.

De-repression of Amy3D *by metabolic inhibitors is not due to inhibition of sugar utilization*

In addition to glucose, various other sugars are known to repress the expression of sugar-regulated genes (Sheen, 1990; Graham *et al.*, 1994; Umemura *et al.*, 1998). Some of these sugars (mannose, galactose, and fructose) were tested for their ability to repress *Amy3D* gene expression. All three sugars showed a repressive effect on *Amy3D* expression, and the repressive effect of 80 mM galactose and fructose could be prevented by co-treatment with NaN_3 (Fig. 5A). In contrast, the repression of *Amy3D* expression induced by 80 mM mannose could not be relieved by NaN_3 treatment. Previously, mannose was reported to be more effective at repressing sugar-regulated genes (Jang and Sheen, 1994). Similarly, the expression of the *Amy3D* gene was found to be much more sensitive to mannose than glucose in the present system. Since de-repression of the sugar effect by oxygen deficiency was sugar concentration dependent (as shown in Fig. 1B), the effect of oxygen deprivation on *Amy3D* repression imposed by a lower concentration of mannose was examined. Although 0.5 mM mannose was too low to repress the *Amy3D* gene, 1–5 mM mannose potently suppressed *Amy3D* expression. This repression by mannose over the lower concentration range (1–5 mM) was fully de-repressible by NaN_3, indicating that mannose-dependent repression works in the same manner as that of glucose (Fig. 5B).

Next, the respiration efficacy of these three sugars was compared to examine whether they could be efficiently

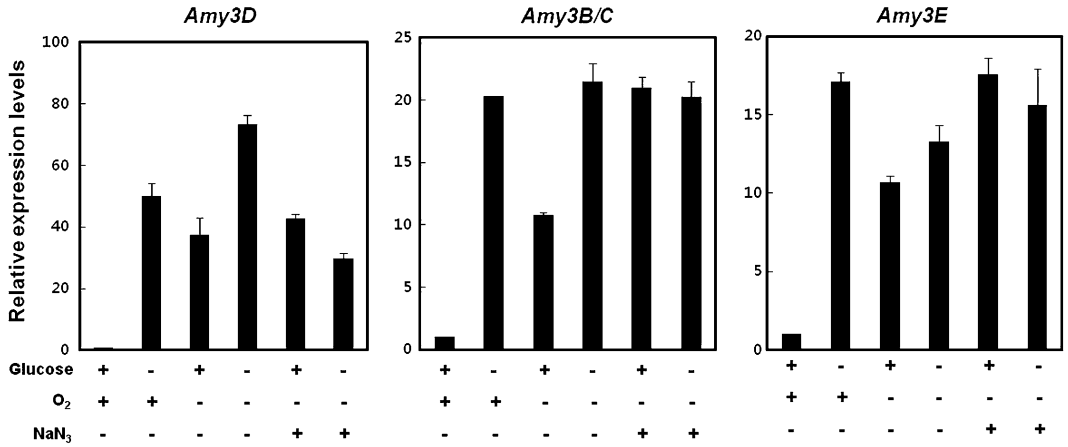

Fig. 3. Effects of respiratory inhibition on sugar regulation of Amy3 subfamily genes in isolated rice embryos. Rice embryos were incubated in 10 mM $CaCl_2$ solution containing 80 mM glucose or mannitol with or without 0.5 mM NaN_3. The presence or absence of glucose, O_2, and NaN_3 is indicated as + or –. Total RNA was isolated from rice embryos from each treatment and used for first strand synthesis. Real-time quantitative PCR was performed to measure the transcript levels of each Amy3 subfamily gene using the gene-specific primers described in Table 1. The expression ratio between *Amy3D*, *Amy3B/C*, or *Amy3E* and the rice *actin* gene in 80 mM glucose medium under aerobic conditions was set to 1 as a control. The expression ratios in other conditions are given relative to the control. The error bars represent the standard deviation of the mean (n=3).

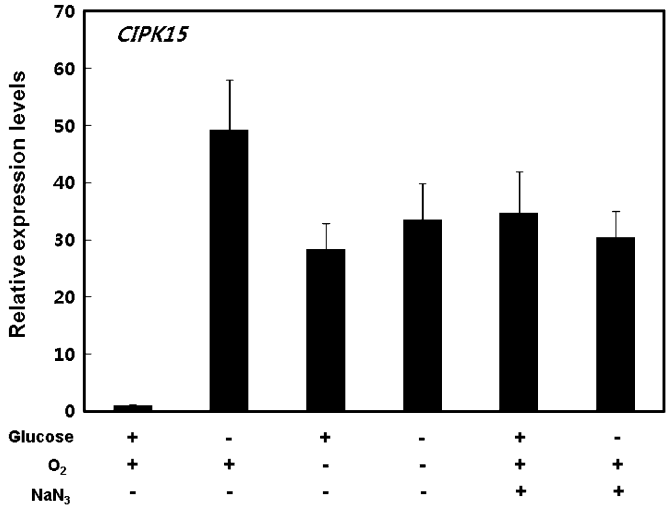

Fig. 4. Effects of respiratory inhibition on *CIPK15* expression. Rice embryos were incubated in 10 mM $CaCl_2$ solution containing 80 mM glucose or mannitol with or without 0.5 mM NaN_3 for 18 h. The presence or absence of glucose, O_2, and NaN_3 is indicated + or –. Total RNA was isolated from rice embryos from each treatment and used for first-strand synthesis. Real-time quantitative PCR was performed to measure the transcript levels of *CIPK15* using the gene-specific primers described in Table 1. The expression ratio between *CIPK15* and the rice *actin* gene in 80^mM glucose under aerobic conditions was set to 1 as a control. The expression ratios in the other conditions are given relative to the control. The error bars represent the standard deviation of the mean (n=3).

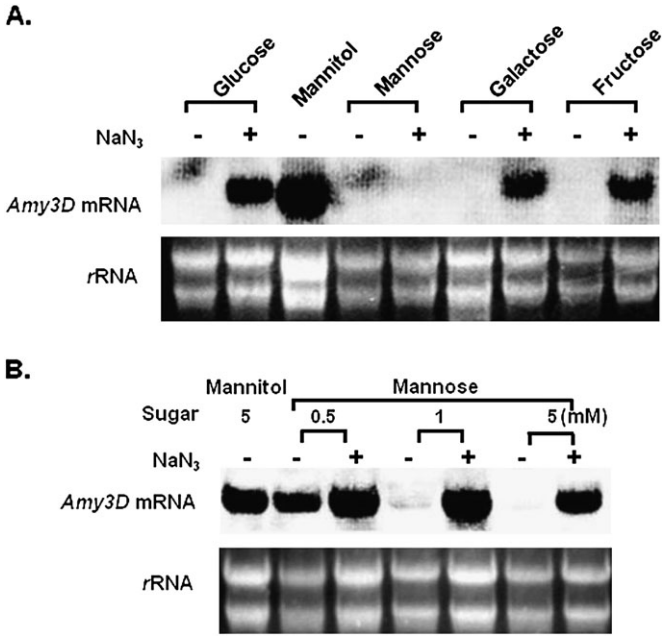

Fig. 5. Sugar concentration-dependent de-repression of mannose effects by respiratory inhibition. Rice embryos were manually harvested from whole seeds and incubated on Whatman paper layers soaked with 10 mM $CaCl_2$ containing (A) 80 mM of each indicated sugar or (B) mannose from 0.5 mM to 5 mM with or without 0.5 mM NaN_3 for 1 d in the dark. The presence or absence of NaN_3 is indicated as + or –. The transcript levels of *Amy3D* were detected as described in Fig. 1. The total amount of RNA loaded is indicated by rRNA stained with ethidium bromide.

metabolized by rice cells (Fig. 6). Suspension-cultured rice cells were starved for 3 d and then supplied with each sugar for 2 d. Then, the recovery of the respiration rate of the starved cells was examined using an oxygen electrode. In cells starved for 5 d, the respiration rate dropped to ~30% of the rate before starvation. As expected, cells fed with glucose showed full recovery of respiration within 2 d. Galactose and fructose, which repressed *Amy3D* expression, were also able to recover respiration rates to the level of non-starved cells, suggesting that they were actively utilized by the suspension-cultured cells. In contrast, mannose supplementation could not recover respiration in the starved cells at all, indicating that rice cells are incapable of respiring mannose as an energy source. Therefore, the de-repression effect induced by the respiratory inhibitors does not appear to be due to preventing rice cells from respiring the co-treating sugar. Instead, the metabolic inhibitors may exert their effect by perturbing the pre-existing cellular energy status.

Anoxia and respiratory inhibition rapidly alter cellular ATP levels

Next, experiments were conducted to determine whether the cellular ATP levels were perturbed in rice cells in which glucose repression was relieved by anoxia or respiratory inhibitor treatment. Since it is impossible to measure the ATP levels only in the epithelium tissue of the embryo, rice suspension cells derived from scutellar tissue were employed. Previously, it was demonstrated that these scutellum-derived rice suspension cells display the same sugar regulation of *Amy3D* gene expression as do intact seeds (Huang *et al.*, 1993). One day of treatment with anoxia or an oxidative phosphorylation inhibitor also de-represses the glucose effect on *Amy3D* gene expression in suspension-cultured rice cells (Fig. 7A). As shown in Fig. 7B, these treatments also lowered the ATP levels in the suspension cells to 30–60% of the glucose control.

Discussion

Phytohormonal control of α-amylase genes, which plays a central role in the endospermal digestion of cereal seeds during aerobic germination, has been extensively studied and remains one of the best examples of hormonal control of plant gene expression (Lovegrove and Hooley, 2000). However, despite the importance of anaerobic amylolytic activity during the anaerobic germination of rice seeds, not much is known about the anaerobic regulation of α-amylase gene expression. In this study, the anoxia-enhanced expression of Amy3 subfamily genes, which are under the control of sugar, was investigated. A previous study using *in situ* hybridization with an *Amy3D* antisense strand probe specifically localized *Amy3D* transcripts only to the scutellar epithelium of the embryo, which is a single layer of

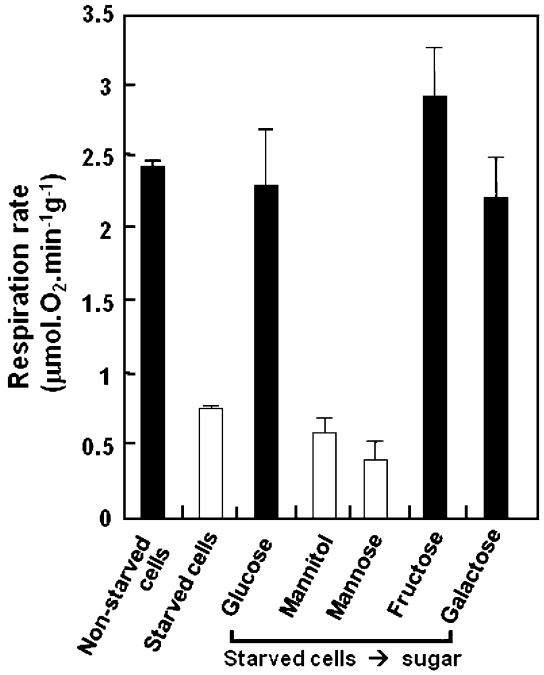

Fig. 6. Effects of various sugars on the respiration rate of suspension-cultured rice cells. Seven-day-old rice suspension-cultured cells were washed with sugar-free AA2 medium, incubated in sugar-free AA2 medium for 3 d, transferred to AA2 medium containing one of the indicated sugars, and then kept for 2 d in the dark with active shaking. 'Non-starved' and 'starved' indicate cells cultured in normal sucrose-containing medium or in sugar-free medium for 5 d, respectively. The respiration rate of the suspension-cultured cells was determined using an oxygen electrode. The error bars represent the standard deviation of the mean (n=3).

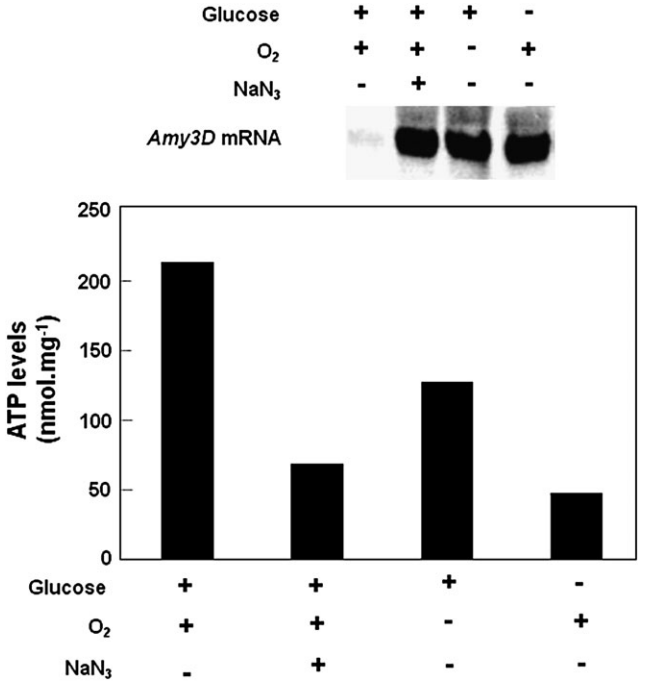

Fig. 7. Effects of respiratory inhibition on the sugar regulation of *Amy3D* expression and on the ATP levels in suspension-cultured cells. Suspension cells cultured for 5 d were incubated in AA2 medium containing 20 mM glucose or mannitol with or without 0.5 mM NaN_3 or O_2 for 1 d in the dark. The presence or absence of glucose, O_2, and NaN_3 is indicated as + or –. Total RNA was extracted from suspension-cultured rice cells of each treatment and used for northern analysis of *Amy3D* gene expression. The transcript levels of *Amy3D* were detected as described in Fig.1. Cell extracts from the same treatment were used to determine the ATP levels.

palisade-shaped cells in close contact with the starchy endosperm (Ranjhan *et al.*, 1992). Since the actual amount of sugars available in the scutellum during germination is unclear, a wide range of sugar concentrations (0–330 mM) in the incubation media were tested to examine the effect of oxygen deprivation on sugar regulation. It was found that oxygen deficiency released the repression of *Amy3D* expression due to glucose <80 mM (Fig. 1B). Because of the technical difficulty of specifically monitoring the local sugar content in a rice embryo, the steady-state levels of soluble sugars in the scutellum during germination can only be speculative. It is estimated that the amount of sugars there is unlikely to exceed 80 mM significantly during anaerobic germination. For example, total soluble sugar concentrations in rice seed endosperm during anoxic germination have been previously determined to be between 20 mM and 70 mM, depending on the rice variety (Huang *et al.*, 2003). Chen *et al.* (2006) also reported that the soluble sugar content in the endosperm was ~200 mM throughout the aerobically germinating period (up to 8 d). Since the anaerobic amylolytic activity of rice seed is <1/4 of the aerobic activity (Hwang *et al.*, 1999), the assumption for the sugar content is not unreasonable. Previously, Loreti *et al.* (2003) observed that *Amy3D* could not be expressed well in the presence of 100 mM glucose under anoxia. This observation led them to propose that rice embryos were able to express *Amy3D* highly in anaerobically germinated seeds because the amount of sugar produced during anaerobic germination was not sufficient to repress the gene. In the present test, it was observed that *Amy3D* expression was completely repressible by glucose concentrations of ~20 mM (data not shown). Therefore, considering this high sensitivity of *Amy3D* expression to low levels of sugar, de-repression of the sugar effect by oxygen deficiency probably leads to an increase in the expression of Amy3 subfamily genes (including *Amy3D*) during anaerobic germination.

The fact that anoxic de-repression of the glucose effect does not occur in a high-sugar environment (as shown in Fig. 2) suggests that sugar may regulate *Amy3D* expression via two distinct pathways. Actually, plant cells appear to have several different ways to sense the sugar available to them (Rolland *et al.*, 2006). Both biochemical and genetic evidence suggest that hexokinase plays the role of a sugar sensor and triggers the repression response in many sugar-regulated genes in higher plants (Jang *et al.*, 1997; Moore *et al.*, 2003; Cho *et al.*, 2007). Several lines of evidence imply that the sugar regulation of *Amy3D* expression also involves the rice hexokinase(s). For example, Umemura

et al. (1998) demonstrated that the *Amy3D* promoter was repressed only by the sugars that serve as a substrate for hexokinase. Also glucosamine, a hexokinase inhibitor, can alleviate the glucose repression of *Amy3D*. Recently, a transient expression study using rice protoplasts demonstrated that *OsHXK5* and *OsHXK6* (*Oryza sativa Hexokinase 5* and *6*), which are evolutionarily related to a well-characterized glucose sensor in *Arabidopsis* (*AtHXK1*), modulate the sugar regulation of the *Amy3D* gene (Cho *et al.*, 2009). Although the exact mode of action of hexokinase in sugar signalling is still not understood, the metabolic utilization of sugars is not likely to be involved in it (Rolland *et al.*, 2006). Recently, dual targeting of hexokinase to the nucleus has been demonstrated in *Arabidopsis* (Cho *et al.*, 2006) and rice (Cho *et al.*, 2009), suggesting that hexokinase-mediated sugar regulation of gene expression may occur directly through translocation of sugar-bound hexokinase to the nucleus.

On the other hand, cells are also likely to perceive the abundance of sugars by sensing the cellular energy status, since sugars serves as a major metabolic fuel (Halford *et al.*, 1999; Halford and Hey, 2009). For example, SnRK is a plant Ser/Thr protein kinase, similar to SNF1 in yeast and AMPK (AMP-activated protein kinase) in mammals, and has been suggested to act as a metabolic sensor in the global control of plant carbon metabolism (Halford *et al.*, 2003; Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008; Halford and Hey, 2009; Jossier *et al.*, 2009).

Extensive studies have revealed several signalling components involved in the sugar regulation of *Amy3D* gene expression (Lu *et al.*, 2002, 2007; Chen *et al.*, 2006; Lee *et al.*, 2009). Previously, Lu *et al.* (2007) demonstrated that SnRK1A serves as a positive regulator for *Amy3D* expression. For example, SnRK1A protein accumulation is required for the activation of *Amy3D* expression under starvation conditions. This kinase acts positively upstream of MYBS1, which binds to the TA box (5′TATCCA3′) of the *Amy3D* gene promoter and activates expression in response to sugar starvation. An RNA interference assay also indicated that rice SnRK1A is necessary for the expression of MYBS1. Therefore, SnRK1A accumulation under starvation conditions induces MYBS1 expression, resulting in *Amy3D* gene expression. Most importantly, transient expression assays in rice embryos demonstrated that SnRK1A expression was able to relieve the repression of *Amy3D* imposed by sugar (Lu *et al.*, 2007). Recently, another rice protein kinase, CBL (calcineurin B-like protein)-interacting protein kinase (CIPK15), was found to be required for the post-transcriptional accumulation of SnRK1A in response to starvation (Lee *et al.*, 2009). It is intriguing that the expression of rice *CIPK15* is under sugar regulation, which is also abolished by treatment with a respiratory inhibitor or anoxia (Fig. 4). Previously, transient expression assays have revealed that CIPK15 can effectively release the sugar-dependent repression of the *Amy3D* promoter (Lee *et al.*, 2009). Therefore, anoxic de-repression of *CIPK15* expression drives the accumulation of SnRK1A, which induces MYBS1 expression, allowing for the anoxic expression of *Amy3D* in the presence of sugar. The de-repression of other Amy3 subfamily genes by anoxia (Fig. 3) appears to work in the same way as that of *Amy3D*, since the proximal regions of all their promoters contain a TA box, which is a MYBS1-binding site under the control of SnRK1A.

It has been demonstrated that the anoxic de-repression of *Amy3D* is due to oxygen deficiency-induced interference with oxidative phosphorylation (Fig. 2). It is unknown how the inhibition of oxidative phosphorylation cross-talks with the sugar regulation of *Amy3D* expression. It is unlikely that the decrease in ATP production negatively affects hexokinase-mediated sugar signalling. Recent biochemical and genetic evidence strongly suggests that the catalytic activity of hexokinase is not necessary for sugar signalling in *Arabidopsis* (Moore *et al.*, 2003). In rice, catalytically inactive mutants of *OsHXK5* and *OsHXK6* were still able to rescue a glucose-sensitive seedling phenotype in the *Arabidopsis* glucose-insensitive *gin2-1* background and also allow sugar regulation of *Amy3D* gene expression (Cho *et al.*, 2009). This indicated that hexokinase is able to repress *Amy3D* expression without being able to phosphorylate glucose.

One possibility is that the inhibition of respiration perturbs energy metabolism, which prevents cells from sensing the abundance of sugars available to them. Such an energetic perturbation may be reflected in the transcriptional induction of *CIPK15*, which in turn drives a signalling cascade that induces *Amy3D* gene expression. SnRK1A may function in parallel or cooperatively with CIPK15 in response to energy disturbances, since this enzyme may have an evolutionarily conserved role as a sensor of cellular energy. In yeast and mammals, SNF1 and AMPK are activated by energy deficiencies represented by a high AMP/ATP ratio, and they act as metabolic sensors that re-adjust the energy homeostasis (Hardie and Hawley, 2001; Hardie *et al.*, 2006). Therefore, they are known to respond to various environmental stresses (such as sugar starvation and hypoxia, among other things) that can affect the metabolic status.

It is not clear why anoxia cannot abolish the repression of *Amy3D* expression induced by high levels of sugar. One possibility is a signalling competition between energy deficiency and sugar-bound hexokinase to promote and inhibit *Amy3D* transcription, respectively. Energy deficiency-driven signalling for *Amy3D* transcriptional activation may compete with repression signalling triggered by sugar-bound hexokinase. If one signal rules over the other, depending on the sugar environment, differential anoxic de-repression of *Amy3D* expression may take place. For example, if repression from sugar-bound hexokinase increases along with an increase in the amount of sugars, it may prevail against the activation signalling from a decrease in energy levels due to anoxia-induced oxidative phosphorylation inhibition. It was previously shown that as a phosphorylatable substrate of hexokinase, mannose much more effectively represses the expression of photosynthetic genes under control of hexokinase-mediated sugar regulation than glucose (Jang and Sheen, 1994). In *Amy3D* regulation, mannose repression appears to be much more difficult to counteract by respiratory inhibition (Fig. 5). For example, anoxia was able fully to abolish the repressive effect of 80 mM glucose on

Amy3D, but it was unable to counteract the effect of mannose even at 10 mM (data not shown).

In this study, it was demonstrated for the first time that inhibiting oxidative phosphorylation disrupts the sugar-dependent regulation of rice α-amylase gene expression, probably by de-repressing the sugar effect on *CIPK15* transcription, which leads to the accumulation of a positive regulator of *Amy3D* expression (SnRK1A). This explains the increased expression of Amy3 subfamily genes in embryo tissues during anaerobic germination, which is very important for rice to be able to grow in the underwater environment. Future studies are necessary to determine whether and how a rice cell is able to link changes in metabolic status to transcriptional changes in *CIPK15* and SnRK1A activity.

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