Differential Anoxic Expression of Sugar-Regulated Genes Reveals Diverse Interactions between Sugar and Anaerobic Signaling Systems in Rice

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The interaction between the dual roles of sugar as a metabolic fuel and a regulatory molecule was unveiled by examining the changes in sugar signaling upon oxygen deprivation, which causes the drastic alteration in the cellular energy status. In our study, the expression of anaerobically induced genes is commonly responsive to sugar, either under the control of hexokinase or non-hexokinase mediated signaling cascades. Only sugar regulation via the hexokinase pathway was susceptible for O₂ deficiency or energy deficit conditions evoked by uncoupler. Examination of sugar regulation of those genes under anaerobic conditions revealed the presence of multiple paths underlying anaerobic induction of gene expression in rice, subgrouped into three distinct types. The first of these, which was found in type-1 genes, involved neither sugar regulation nor additional anaerobic induction under anoxia, indicating that anoxic induction is a simple result from the release of sugar repression by O₂-deficient conditions. In contrast, type-2 genes also showed no sugar regulation, albeit with enhanced expression under anoxia. Lastly, expression of type-3 genes is highly enhanced with sugar regulation sustained under anoxia. Intriguingly, the inhibition of the mitochondrial ATP synthesis can reproduce expression pattern of a specific set of anaerobically induced genes, implying that rice cells may sense O₂ deprivation, partly via perception of the perturbed cellular energy status. Our study of interaction between sugar signaling and anaerobic conditions has revealed that sugar signaling and the cellular energy status are likely to communicate with each other and influence anaerobic induction of gene expression in rice.

INTRODUCTION

Sugars were recently shown to play a pivotal role in cellular signaling. They are able to affect many vital processes by regulating expression of various genes and enzyme activities through specific sugar signaling pathways (Hanson and Smeekens, 2009;

Ramon et al., 2008; Rolland et al., 2006). Cellular energy homeostasis is very susceptible to various environmental changes, which disrupt the cellular sugar status or metabolism itself. Upon sensing an energy deficit caused by an environmental perturbation(s), plants re-establish homeostasis by down-regulating growth-related energy-consuming processes and promoting nutrient mobilization, defense, acclimation, and ultimately, adaptation. The orchestrated re-allocation of energy use mediated by energy signaling ensures better tolerance of stress, allowing for more successful survival in fluctuating environments (Baena-Gonzalez, 2010; Baena-Gonzalez and Sheen, 2008).

Sugar signaling is likely to interact with various other developmental programs and environmental responses, thereby extending the complexity of the cellular signaling network (Sheen, 2010). Due to its bi-functional nature of sugar, both energy and sugar signaling must be adjusted appropriately upon altering cellular sugar status. Moreover, such adjustments should be closely coordinated to maintain harmonious physiology (Koch et al., 2000). Many stress-related genes were found to be coordinately expressed under sugar starvation, either via hexokinase-dependent or independent pathways in rice (Ho et al., 2001). Such sugar regulated expression of many stress-related genes suggested two signaling cascades of the nutritional and environmental stress would be converged at certain point of the pathway.

One of the most obvious stresses caused by anaerobic conditions is the perturbation of metabolic status. Under O₂-deficient conditions, cellular energy cannot be retrieved from the mitochondrial respiration of sugar owing to the absence of the last electron acceptor in the oxidative phosphorylation chain. Instead, fermentation-mediated sugar metabolism provides cellular energy, albeit with very low efficiency. The resultant change in basic energy metabolism causes cellular ATP levels drop to at least 30% of those under aerobic conditions (Greenway and Gibbs, 2003; Mustroph et al., 2006; Raymond et al., 1985). Rice is one of only a few plant species that survive the energetically unfavorable consequences of submergence. This suggests a strong likelihood that rice plants have evolved mechanisms to

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establish more adaptive and harmonious interactions between energy and sugar signaling than most plant species. Previously, a large number of anaerobically inducible genes were identified in rice through RNA profiling experiment (Lasanthi-Kudahettige et al., 2007). In the current study, we selected those metabolic genes for which expression is highly induced under anaerobic conditions in this published microarray data, and examined their responsiveness to sugar to assess the possible interaction between sugar signaling and anaerobic conditions. Our analyses revealed that under anaerobic conditions, rice can induce the expression of various sugar-regulated genes via multiple paths, including interference with sugar signaling.

MATERIALS AND METHODS

Rice suspension cell cultures

Suspension-cultured rice cells (*O. sativa* L. cv. Dongjin) were established as described by Huang et al. (1993). The cells were maintained in AA2 culture medium (Thompson et al., 1986), and subcultured every 10 days by transferring a 3-5 ml (packed volume) of the cells to 20 ml of fresh AA2 medium and actively shaking at 150 rpm and 28°C in the dark. In our preliminary experiment, the time-course experiment was performed to determine the best treatment time for the survey of gene expression patterns. Because treatment of cells with sugar or anoxia for 12 to 36 h gave the similar result pattern (Supplementary Fig. S2A and S2B), 24 h treatment of cells was employed in our analyses. The simple CaCl₂ solution was used for treating samples in current study, because no difference was found in the response pattern of gene expression between cells incubated in the CaCl₂ solution and AA2 medium for 1 day. Prior to chemical and anaerobic treatments, 7-d-old subcultured cells were washed twice with 10 mM CaCl₂ solution. For anaerobic treatment, about 200 µl of suspension-cultured cells were submerged in 10 mM CaCl₂ solution containing either glucose or mannitol, and then flushed with N₂ gas for 24 h without shaking at 28°C in the dark. For all other experiments, including treatment with 2,4-dinitrophenol, the cells were kept under active shaking at 150 rpm and 28°C in the dark.

Chemicals

The 2,4-dinitrophenol was purchased from Sigma-Aldrich Korea (USA).

Quantitative real-time RT-PCR

Total RNA isolation, first-strand cDNA synthesis, and real-time quantitative reverse transcription-PCR (RT-qPCR) using the Thermal Cycler Dice Real Time System (Takara Shuzo, Japan) were carried out as described by Park et al. (2010). Detection of the accumulation of RT-qPCR products was monitored by staining with SYBR Green (Takara Bio, Japan) following the manufacturer's instruction. The relative amplification of the rice actin gene (*Act1*) was used as an internal control to normalize all data. Triplicates of each sample were examined to evaluate the quantitative variation in each sample, and each experiment was repeated at least 3 times. The gene-specific primers used for RT-qPCR are listed in Supplementary Table S1.

Semi-quantitative RT-PCR

Total RNA isolation, and first-strand cDNA synthesis were performed as described by Park et al. (2010). To examine the expression pattern of genes in the control and treated cells, RT-PCR analysis was performed using gene-specific primer sets (Supplementary Table S1), and the ubiquitin gene (*UbQ5*) was

used as a loading control. The PCR mix consisted of 0.25 mM dNTPs, 0.5 µM each of sense and antisense primers, 2.5 U of EX Taq polymerase (Takara Shuzo, Japan) and 1× PCR buffer supplied with the Taq polymerase. The thermal cycling conditions involved incubation at 95°C for 1 min; 20-35 cycles at denaturing at 95°C for 10 s, annealing at 58°C for 30 s, extension at 72°C for 1 min; and final elongation at 72°C for 5 min, with use of TPC-100™ (MJ Research, USA). The numbers of PCR cycles in the linear range of DNA amplification were empirically determined for each gene, which were between 20 to 35 cycles. All PCR products were examined on a 1% (w/v) agarose gel containing 0.01% (v/v) ethidium bromide, and documented by Bio-Rad gel Dac (USA).

RESULTS

Many anaerobically induced genes are also regulated by sugar

Lasanthi-Kudahettige et al. (2007) profiled changes in the transcriptome caused by anoxia by comparative microarray analysis of total RNA isolated from 4-d old rice coleoptiles grown aerobically or anaerobically. A total of 3,134 probe sets were differentially expressed in coleoptiles grown under anaerobic and aerobic conditions. The transcriptomic analysis revealed that expression of many metabolic genes was induced by anaerobic conditions. Among those genes, we selected 12 genes induced more than 8-fold by anoxia (Supplementary Fig. S1). These genes are involved in various metabolic aspects related to starch degradation and synthesis [rice α-amylase, (*Amy3D*, Os08g36910); ADP-glucose pyrophosphorylase (*ADPG-PPase*, Os07g13980)], glycolytic flux [Hexokinase 7 (*OsHxk7*, Os05g09500)], pyruvate and pyrophosphate metabolism [PPI-dependent phosphofructokinase (*PPI-PFK*, Os05g010650); glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*, Os08g34210); pyruvate kinase (*PK*, Os09g022410); phosphoenolpyruvate carboxykinase (*PCK*, Os10g13700); pyruvate orthophosphate dikinase (*PPDK*, Os03g31750); PPI-dependent vacuolar H⁺-translocase (*H⁺-PPase*, Os02g55890)], acetaldehyde detoxification [acetaldehyde dehydrogenase (*ALDH2a*, Os02g49720)], and alcohol fermentation [pyruvate decarboxylase 1 (*PDC1*, Os05g39310); alcohol dehydrogenase 2 (*ADH2*, Os11g10510)]. Although expression of *Amy3D* and *CIPK15* genes was previously analyzed (Park et al., 2010; Yim et al., 2012), they were included in our survey as the positive control for our treatment. Rice suspension cultured cells were treated with glucose or mannitol for 24 h to examine expression of anoxia-inducible genes for their responsiveness to sugar by RT-qPCR and semi-quantitative RT-PCR. Whereas the expression of the house-keeping genes that encode ubiquitin (*UBQ5*) and elongation factor (*REFA4*) was not influenced by sugar, 10 of the pre-selected 13 genes displayed various degrees of sugar regulation (Figs. 1A and 1B). A more than 100-fold increase in expression was observed for *Amy3D*, *OsHxk7*, and *CIPK15* genes upon deprivation of exogenous sugar, with an approximately 50-fold change for *H⁺-PPase*. Moderate levels of gene induction between 2- to 5-fold occurred for the *PCK*, *PPDK*, *PK*, *ADPG-PPase*, and *ALDH2a* genes. Expression of *PDC1* increased at least 5-fold following exposure to sugar, whereas expression of *PPI-PFK*, *G3PDH*, and *ADH2* was not affected by the presence or absence of sugar. Taken together, anaerobically induced genes commonly display sugar responsiveness in their expression, implying that both signaling systems related to sugar and anaerobic conditions are highly likely to communicate with each other.

Fig. 1. Responsiveness of anaerobically induced metabolic genes to sugar. The transcript levels of each gene were measured by real-time quantitative PCR (RT-qPCR), using the gene-specific primers described in Supplementary Table S1 (A). *Amy3D* and *CIPK15* were also included in our survey as the positive control for our treatment. The expression ratio between each gene and the rice actin gene (*Act1*) in cells incubated in glucose (control) was set to 1, and the expression ratio following incubation in mannitol is given relative to the control. The error bars represent the standard deviation of the mean ($n = 3$). Semi-quantitative RT-PCR was performed to examine the expression pattern of genes in the control and treated cells (B). An image of ethidium bromide stained agarose gel is presented as an inverted tone.

Various sugar-regulated genes are differentially expressed under anaerobic conditions in the presence or absence of sugar

Given that many anoxia-inducible genes turn out to be sugar-regulated, it was examined how anaerobic conditions affect sugar regulation of those genes. Our RT-qPCR and semi-quantitative RT-PCR analyses revealed that sugar-regulated genes can be categorized into 3 groups, based on their expression patterns in response to O₂ deprivation (Figs. 2A and 2B). For instance, the first of these, which was found in type-1 genes (*OsHxk7*, *Amy3D*, and *CIPK15*), involves anoxic release of sugar repression, which is mainly responsible for anaerobic increase in their gene expression. In the case of *H⁺-PPase* and *PDC1* genes (type-2 genes), sugar regulation of gene expression is also abolished by anoxia, but the anaerobic induction levels of this type of genes are much higher than those that can be attained by anoxic interference of sugar regulation. For example, *H⁺-PPase* and *PDC1* genes displayed additional ~10- and 100-fold induction of expression upon O₂ deficiency, respectively, as compared to the levels of expression achieved by abolishment of sugar regulation. The remaining genes, which belong to the type-3 category (*PCK*, *PPDK*, *PK*, *ADPG-PPase*, and *ALDH2a*) are expressed in much higher levels under anoxic conditions than under aerobic conditions, but retain sugar-regulation in their expression even under anaerobic conditions. Therefore, O₂ deficiency does not affect regulation of type-3 genes by sugar. As a result, the anaerobic expression pattern of type-3 genes is simply an amplified version of the aerobic expression pattern.

Hexokinase triggers sugar signaling of specific types of genes, which can be affected by anoxic conditions

Of the multiple sugar-sensing systems identified in plants, the most extensively studied sugar sensor is hexokinase. Hexokinase isoforms have been proposed to have regulatory functions in addition to catalyzing the phosphorylation of glucose to enable its entry into the glycolytic pathway. A range of evidence suggests that the hexose phosphorylating activity itself is corre-

lated with the initiation of sugar signaling. Various glucose analogs and sugars that can be phosphorylated but not metabolized have been used to investigate the involvement of hexokinase in sugar signaling (Jang et al., 1997). For example, the analogs 3-O-methylglucose and 6-deoxyglucose are neither metabolized nor phosphorylated by hexokinase. By contrast, the glucose analog 2-deoxyglucose and a glucose isomer, mannose can be phosphorylated by hexokinase, but do not undergo additional glycolysis, because of the absence of the 2-hydroxyl group in 2-deoxyglucose and the inefficient catalytic activities of both hexose phosphate isomerase and hexose 6-phosphate dehydrogenase in plants (Loughman et al., 1989; Salas et al., 1965; Sheu-Hwa et al., 1975). We used the same strategy of feeding experiments that involved glucose analogs and different kinds of sugars, categorizing those 3 classes of genes into 2 groups that rely on either hexokinase-dependent or -independent sugar signaling. As shown in Fig. 3A, sugar-regulated expression of type-1 and -2 genes was effectively exerted by all tested sugars such as mannose, galactose and fructose, which act as a substrate for hexokinase. However, suppressive effect of mannose was not prominent at all for type-3 genes, as compared to that of glucose. To further confirm the importance of the role of hexokinase in sugar regulation of type-1 and -2 genes, we examined the effects of various glucose analogs on expression of those genes. Sugar regulation of type-1 and -2 genes was successfully triggered by 2-deoxyglucose, while not by 3-O-methylglucose or 6-deoxyglucose. Again, expression of type-3 genes was affected neither by 2-deoxyglucose, nor by 3-O-methyl glucose, nor by 6-deoxyglucose (Fig. 3B). Previously, Ho et al. (2001) found that treatment of 2-deoxyglucose up to 0.5 mM was toxic to suspension cultured rice cells. However, no significant deterioration of total RNA isolated from 2-deoxyglucose treated cells was observed in our examination of ethidium bromide staining of rRNA. Above all, repression observed in type-1 and -2 gene expression by 2-deoxyglucose cannot be ascribed to non-specific toxic effect, because induction of type-3 gene expression normally occurred in the same sample. The discrepancy of

Fig. 2. Differential expression of sugar-regulated genes under anaerobic conditions. The transcript levels of each gene were measured by RT-qPCR, using the gene-specific primers described in Supplementary Table S1 (A). *Amy3D* and *CIPK15* were also included in our survey as the positive control for our treatment. The ratio between expression of each gene and the rice actin gene (*Act1*) in glucose (control) was set to 1, and the expression ratios of other conditions are given relative to the control. The error bars represent the standard deviation of the mean ($n = 3$). Semi-quantitative RT-PCR was performed to examine the expression pattern of genes in the control and treated cells (B). An image of ethidium bromide stained agarose gel is presented as an inverted tone.

sensitivity of cultured cells to toxicity of this glucose analog may be due to the difference of rice cultivars from which the suspension cultured cells were derived or due to the conditions of suspension cultured cells which may vary. Thus, distinct susceptibility to 2-deoxyglucose and mannose suggests that sugar-regulated expression of type-1 and -2 categories of genes appears to be under the hexokinase-dependent sugar signaling pathway, whereas that of type-3 genes appears to be mediated by hexokinase-independent pathway.

Uncoupling of oxidative phosphorylation differentially affects expression of sugar-regulated genes of types-1, -2, and -3

Demonstration that mitochondrial respiration inhibitors could mimic the effects of anoxia on sugar repression of *Amy3D* and *CIPK15* suggested that changes in the levels of cellular ATP might affect the regulation of these genes by sugar (Park et al., 2010; Yim et al., 2012). Actually, treatment of rice suspension cultured cells with the respiratory inhibitor decreased the cellular levels of ATP by 80% with within 24 h, as compared to that of non-treated cells (Park et al., 2010). Therefore, we examined whether anoxic effects on the expression of genes of the type-1, -2, and -3 categories could be reproduced by inhibition of cellular ATP synthesis. Figure 4 shows expression of sugar-regulated genes of all 3 types in rice suspension cultured cells that were incubated in glucose or mannitol medium with or without 2,4-DNP. This uncoupler of oxidative phosphorylation abolishes the proton gradient, thereby uncoupling ATP synthesis and electron transport in the mitochondrial cytochrome pathway (Beevers, 1961). The uncoupling of oxidative phosphorylation abolished the sugar-dependent differential expression of both type-1 and type-2 genes. For example, upon uncoupling of oxidative phosphorylation, sugar-repressible genes of types 1 and 2 were successfully expressed even in the presence of glucose to the levels of the starved cells. Also, 2,4-DNP could induce expression of the sugar-inducible *PDC1* gene in the absence of glucose, suggesting that a perturbed cellular energy status by itself is able to activate *PDC1* gene expression. As exemplified by differences in the levels of ex-

pression of *H⁺-PPase* and *PDC1* genes between cells co-treated with 2,4-DNP and those subjected to anoxia, 2,4-DNP failed to increase expression of type-2 genes to levels as high as those observed under anoxic conditions. In addition, sugar regulation of type-3 genes was not affected by uncoupling of oxidative phosphorylation. This is consistent with the absence of any effect of anaerobic conditions on sugar regulation of type-3 genes (Fig. 2). However, the overall levels of expression of type-3 genes were still apparently enhanced by treatment with the uncoupling agent, causing them to increase to levels similar to those observed following anoxic treatment.

DISCUSSION

Rice is one of only a few plant species that survive the energetically unfavorable consequences of submergence. This suggests a strong likelihood that rice plants have evolved mechanisms to establish more adaptive and harmonious interactions between energy and sugar signaling than most plant species. In this study, we have investigated the potential cross-talking between the dual roles of sugars as metabolic fuels and signaling molecules, by probing the responsiveness of various anaerobically inducible genes to sugars, and conversely, the effects of anaerobiosis on sugar-regulated gene expression in rice.

Anaerobically induced genes commonly display sugar responsiveness

Ten out of 13 anaerobically induced genes we have examined turn out to be responsive to sugar in their expression, as well (Fig. 1). Previously, a genome wide analysis for gene expression profiling upon low oxygen stress was carried out using Arabidopsis seedlings under normoxic and hypoxic conditions over 24 h (Liu et al., 2005). In this public data, we collected total 652 genes that were differentially regulated more than 2-fold by hypoxic conditions at any time point over 24 h. Next, we determined the possible sugar responsiveness of those genes by examining the data of a DNA microarray analysis by Price et al. (2004) to determine the effects of glucose on gene expression in Arabidopsis seedlings. In this experiment, 2,564 genes

Fig. 3. Effects of various sugars and glucose analogs on expression of selected sugar-regulated genes. Rice suspension cultured cells were incubated in the medium containing different types of sugars (A) [60 mM for glucose (glc), mannitol (mtl), galactose (gal), or fructose (fru); or 5 mM for mannose (man)] or glucose analogs (B) [4 mM for 2-deoxyglucose (2-dG), and 9 mM for 3-O-methyl-glucose (3-OMG) and 6-deoxyglucose (6-dG)] for 24 h. The transcript levels of each gene were determined and expressed as described in Fig. 2. The error bars represent the standard deviation of the mean (n = 3).

were identified to be differentially expressed more than 2-fold in response to glucose 3%. In our comparative analysis of these two public microarray data, significant amount of hypoxia responsive genes as much as 24.2% (158/652) were found to be also responsive to glucose in their expression (Supplementary Fig. S3A). In case of rice, total 2,648 genes were identified to be differentially expressed more than 2-fold depending on O₂ deprivation in rice coleoptile in global transcriptomic analysis of Lasanthi-Kudahettige et al. (2007). Currently, genome-wide scale data for glucose response is not available in rice. Therefore, we have checked the potential sugar responsiveness of those selected genes by examining their occurrence in genomic pro-

filings data for sucrose starvation in rice suspension cells (Wang, 2007). More than 32% of anoxia-responsive genes (848/2648) were found to be sugar-responsive in rice, too (Supplementary Fig. S3B). These comparative analyses of the microarray data of mono- and di-cots strongly suggest that interconnection of sugar and anaerobic signaling systems are highly conserved.

The cross-talk between sugar and energy signaling

Hexokinases (HXKs) are the most evolutionarily conserved sugar sensors in a broad range of organisms from bacteria to mammals (Rolland et al., 2006). Genetic, phenotypic and biochemical analyses using Arabidopsis hexokinase signaling mutants

Fig. 4. Expression of selected sugar-regulated genes under anoxia or following co-treatment with an uncoupler of oxidative phosphorylation. Rice suspension cultured cells were incubated in the medium containing glucose or mannitol for 24 h with or without 2,4-DNP (0.08 mM). For anaerobic treatment, the cells were incubated in the same solution with N₂ gas flushing without shaking. The transcript levels of each gene were determined and expressed as described in Fig. 2. The error bars represent the standard deviation of the mean (n = 3).

Fig. 5. A model proposing interactions between sugar signaling and anaerobic conditions. Many anaerobically induced genes also share sugar responsiveness in their expression. These anoxia- and sugar-responsive genes are subgrouped into 3 types, based on their expression pattern with or without sugar and oxygen. The specific susceptibility to the type of sugars and glucose analogs indicates that sugar regulation of type-1 and type-2 genes is regulated by hexokinase-dependent sugar signaling, whereas that of type-3 genes is not. Sugar signaling responsible for the regulation of type-1 and type-2 genes is disrupted by O₂ deficiency or the energy deficits evoked by the uncoupler 2,4-dinitrophenol, whereas that of type-3 genes was not affected. This suggests that only a branch of sugar signaling cascades is specifically affected by the perturbed cellular energy status, which is possibly sensed by energy sensing system of

SnRK1. The failure of treatment with the uncoupler to enhance the levels of expression of type-2 genes to the levels triggered by exposure to anaerobic conditions suggests that an independent additional anaerobic induction mechanism is likely to account for the anaerobic expression of the type-2 genes. The perturbed cellular energy status can enhance the expression of type-3 genes regardless of the presence of sugar, possibly by affecting mRNA stability.

intriguingly have suggested that glucose signaling function of HXK1 can be uncoupled from its catalytic activity and that glucose signaling *via* hexokinase does not result from either the accumulation or depletion of downstream metabolic products or from changes in the ATP:ADP ratio. Especially, two hexokinases of rice (*OsHXK5* and 6) also have been demonstrated to

serve as glucose sensor and also their catalytically inactive forms still successfully delivered the sugar regulation, too [Cho et al. (2009a), see Cho (2009b) for review]. However, the finding of the uncoupling of the metabolic and signaling activities of hexokinase unwittingly led to underscore the possible interaction of sugar signaling with the cellular metabolic status. Our

results suggest that the signaling effects of sugars could be disrupted following inhibition of cellular ATP synthesis. For example, the repressive effects of sugars on the expression of type-1 and type-2 genes that disappeared under anaerobic conditions could be mimicked by 2,4-DNP, which uncouples ATP synthesis from electron transport in the mitochondrial cytochrome pathway (Fig. 4). This indicated that the factor responsible for interfering with the signaling effects of sugar on type-1 and type-2 genes is the perturbation of cellular ATP synthesis that occurs under anaerobic conditions when the oxidative phosphorylation process is inhibited by inadequate levels oxygen, which is the final electron acceptor. However, in contrast to type-1 and type-2 genes, sugar-mediated regulation of type-3 genes is neither triggered by hexokinase nor affected by the same conditions. This suggests that at least hexokinase-mediated sugar signaling is affected by the energy deficit caused by anaerobiosis. Therefore, the perturbed energy status of cells appears to be able to compete with the signaling elicited by hexokinase. Plants have SNF1-related protein kinase 1 (SnRK1) members, which have activities similar to those of yeast SNF1 and mammalian AMPK, the master metabolic regulators that control responses to a variety of stresses that affect the cellular energy supply (Baena-Gonzalez, 2007; Hardie, 2007; Hedbacker and Carlson, 2008; McGee and Hargreaves, 2008). In particular, rice SnRK1 served as a positive regulator of the regulation of *Amy3D* by sugar, and can induce its own expression following sugar starvation, which increases the AMP:ATP ratio (Lu et al., 2007). Therefore, the SnRK1-mediated energy signaling system may participate in the crosstalk between sugar signaling and the system that signals conditions of energy deficit.

Anaerobiosis affects sugar-regulated gene expression in rice through different signaling pathways activated by anoxia

No difference was observed between levels of expression of type-1 genes under O_2 deficiency and their abundances after perturbation of cellular ATP synthesis (Fig. 4). This means that the levels of expression of type-1 genes under anaerobic conditions result from elimination of sugar repression following inhibition of oxidative phosphorylation. Although regulation of type-2 genes by sugars is also disrupted by anaerobic conditions, this cannot explain their highly enhanced levels of expression caused by anoxia. For example, the levels of expression of the type-2 genes, *H⁺-PPase* and *PDC1* are approximately 10- and 800-fold higher under anaerobic conditions, respectively than following treatment of cells with respiratory inhibitors (Fig. 2). This suggests that an independent mechanism for anaerobic induction in addition to abolishment of sugar regulation must contribute to the levels of expression of type-2 genes under O_2 deficiency. One of mechanisms that underlie the anaerobic induction of type-2 genes would be transcriptional activation through specific *cis*-regulatory elements as seen in many anaerobic genes (Hoeren et al., 1998; Mohanty et al., 2012; Olive et al., 1991). Previous molecular characterization of rice knockout mutant for *CIPK15* gene suggested that this protein may serve as a positive key regulator to direct the O_2 deficiency signal to sugar sensing cascade via activating SnRK1 (Lee et al., 2009). Therefore, the interference with sugar signaling by O_2 deprivation observed in type-1 and -2 genes may be mediated by this CIPK15-SnRK1 pathway. Modulation of RNase activities has been expected to play a role in the regulation of RNA turnover in response to certain abiotic and biotic cues (Green, 1993; Wilson, 1975). Especially, Fennoy et al. (1997) has demon-

strated that a specific set of mRNAs could be conserved during O_2 deficiency through modulation of specific RNase activities in response to O_2 deprivation. If specific group of mRNAs is preferentially stabilized under anoxia by such a decrease in specific RNase activity, the resultant pattern of response would resemble that observed for the type-3 genes in Fig. 2. Therefore, as a future study, it needs to examine if type-3 genes have the differential mRNA stability upon O_2 deprivation.

Recently, two independent studies showed evidence that the N-end rule pathway of protein degradation serves as a homeostatic oxygen sensor in plants (Gibbs et al., 2011; Licausi et al., 2011); Abundance of key hypoxia response transcription factors is regulated by oxygen availability because their destabilization occurs through O_2 -dependent modification of N-terminus, followed by ubiquitin-dependent proteasomal degradation, which in turn leads to switching on and off downstream hypoxia signaling cascade. In addition to such a direct sensing mechanism, several other possible mechanisms also have been proposed to indirectly detect changes in oxygen availability in plants, such as sensing the redox status, ROS, the energy status, etc (Sasidharan and Mustroph, 2011). Our result supports the proposal that changes in oxygen availability may be sensed by cells through monitoring the cellular energy status in anaerobic conditions. As shown in Fig. 5, uncoupling of oxidative phosphorylation is able to perfectly reproduce such an anaerobic response pattern of type-3 genes. Therefore, rice cells are likely to transduce the signal to regulate the expression of a specific set of anaerobic genes, adjusting the anaerobic physiology, upon perceiving the perturbed energy status caused by anaerobic conditions. To our knowledge, this is the first report that the perturbed cellular energy status can serve as a specific signal to switch on expression of anaerobic genes in plants.

Taken together, our current study suggests that anaerobically induced genes commonly share the sugar responsiveness in their expression, which is often altered in anaerobic conditions. O_2 deprivation, more specifically, the energy deficit caused by inhibition of oxidative phosphorylation, compete with sugar signaling triggered by hexokinase, but not with hexokinase-independent sugar signaling. Perturbation of cellular energy status also could be perceived as an anaerobic signal by rice cells, switching on expression of a specific set of anaerobically induced genes. As a result, anaerobic induction of gene expression in rice is actually contributed by various independent mechanisms, including the release from sugar regulation, independent anaerobic inductions, the combination of them, etc. Figure 6 summarizes the different sites of interactions between two signaling systems of sugar and anaerobic conditions in rice. Global transcriptome analysis for sugar-regulated gene expression under anaerobic conditions will be needed to investigate whether the multiple anoxic response patterns of sugar-regulated genes observed in this study can be extrapolated more broadly. Since OsHXK5 and 6 have been demonstrated to play as a glucose sensor in rice, cell lines of mutant alleles for those genes will also provide valuable information about interaction between hexokinase mediated sugar signaling and anoxic signaling.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

- Baena-Gonzalez, E. (2010). Energy signaling in the regulation of gene expression during stress. *Mol. Plant* 3, 300-313.
- Baena-Gonzalez, E., and Sheen, J. (2008). Convergent energy and stress signaling. *Trends Plant Sci.* 13, 474-482.
- Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signaling. *Nature* 448, 938-942.
- Cho, J.I., Ryoo, N., Eom, J.S., Lee, D.W., Kim, H.B., Jeong, S.W., Lee, Y.H., Kwon, Y.K., Cho, M.H., Bhoo, S.H., et al. (2009a). Role of the rice hexokinases OshXK5 and OshXK6 as glucose sensors. *Plant Physiol.* 149, 745-759.
- Cho, J.I., Ryoo, N., Hahn, T.R., and Jeon, J.S. (2009b). Evidence for a role of hexokinases as conserved glucose sensors in both monocot and dicot plant species. *Plant Signal. Behav.* 4, 908-910.
- Beevers, H. (1961). *Respiratory metabolism in plants* (New York: Row-Peterson and Company).
- Fennoy, S.L., Jayachandran, S., and Bailey-Serres, J. (1997). RNase activities are reduced concomitantly with conservation of total cellular RNA and ribosomes in O₂-deprived seedling roots of maize. *Plant Physiol.* 115, 1109-1117.
- Gibbs, D.J., Lee, S.C., Isa, N.M., Gramuglia, S., Fukao, T., Bassel, G.W., Correia, C.S., Corbineau, F., Theodoulou, F.L., Bailey-Serres, J., et al. (2011). Homeostatic response to hypoxia is regulated by the N-end rule pathway in plants. *Nature* 479, 415-418.
- Green, P.J. (1993). Control of mRNA stability in higher plants. *Plant Physiol.* 102, 1065-1070.
- Greenway, H., and Gibbs, J. (2003). Review: mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Funct. Plant Biol.* 30, 999-1036.
- Hanson, J., and Smeekens, S. (2009). Sugar perception and signaling—an update. *Curr. Opin. Plant Biol.* 12, 562-567.
- Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat. Rev. Mol. Cell Biol.* 8, 774-785.
- Hedbacker, K., and Carlson, M. (2008). SNF1/AMPK pathways in yeast. *Front. Biosci.* 13, 2408-2420.
- Ho, S.L., Chao, Y.C., Tong, W.W., and Yu, S.M. (2001). Sugar coordinately and differentially regulates growth- and stress-related gene expression via a complex signal transduction network and multiple control mechanisms. *Plant Physiol.* 125, 877-890.
- Hoeren, F.U., Dolferus, R., Wu, Y., Peacock, W.J., and Dennis, E.S. (1998). Evidence for a role for AtMYB2 in the induction of the Arabidopsis alcohol dehydrogenase gene (*ADH1*) by low oxygen. *Genetics* 149, 479-490.
- Huang, N., Chandler, J., Thomas, B.R., Koizumi, N., and Rodriguez, R.L. (1993). Metabolic regulation of alpha-amylase gene expression in transgenic cell cultures of rice (*Oryza sativa* L.). *Plant Mol. Biol.* 23, 737-747.
- Jang, J.C., Leon, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. *Plant Cell* 9, 5-19.
- Koch, K., Ying, Z., Wu, Y., and Avigne, W. (2000). Multiple paths of sugar-sensing and a sugar/oxygen overlap for genes of sucrose and ethanol metabolism. *J. Exp. Bot.* 51, 417-427.
- Lasanthi-Kudahettige, R., Magneschi, L., Loreti, E., Gonzali, S., Licausi, F., Novi, G., Beretta, O., Vitulli, F., Alpi, A., and Perata, P. (2007). Transcript profiling of the anoxic rice coleoptile. *Plant Physiol.* 144, 218-231.
- Lee, K.W., Chen, P.W., Lu, C.A., Chen, S., Ho, T.H., and Yu, S.M. (2009). Coordinated responses to oxygen and sugar deficiency allow rice seedlings to tolerate flooding. *Sci. Signal.* 2, ra61.
- Licausi, F., Kosmacz, M., Weits, D.A., Giuntoli, B., Giorgi, F.M., Voesenek, L.A., Perata, P., and van Dongen, J.T. (2011). Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilization. *Nature* 479, 419-422.
- Liu, F., Van Toai, T., Moy, L.P., Bock, G., Linford, L.D., and Quackenbush, J. (2005). Global transcription profiling reveals comprehensive insights into hypoxic response in Arabidopsis. *Plant Cell* 17, 1115-1129.
- Loughman, B.C., Ratcliffe, R.G., and Southon, T.E. (1989). Observations on the cytoplasmic and vacuolar orthophosphate pools in leaf tissues using *in vivo* ³¹P-NMR spectroscopy. *FEBS Lett.* 242, 279-284.
- Lu, C.A., Lin, C.C., Lee, K.W., Chen, J.L., Huang, L.F., Ho, S.L., Liu, H.J., Hsing, Y.I., and Yu, S.M. (2007). The SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling growth of rice. *Plant Cell* 19, 2484-2499.
- McGee, S.L., and Hargreaves, M. (2008). AMPK and transcriptional regulation. *Front. Biosci.* 13, 3022-3033.
- Mohanty, B., Herath, V., Wijaya, E., Yeo, H.C., de Los Reyes, B.G., and Lee, D.Y. (2012). Patterns of *cis*-element enrichment reveal potential regulatory modules involved in the transcriptional regulation of anoxia response of japonica rice. *Gene* 511, 235-242.
- Mustroph, A., Boamfa, E.I., Laarhoven, L.J., Harren, F.J., Albrecht, G., and Grimm, B. (2006). Organ-specific analysis of the anaerobic primary metabolism in rice and wheat seedlings. I: Dark ethanol production is dominated by the shoots. *Planta* 225, 103-114.
- Olive, M.R., Peacock, W.J., and Dennis, E.S. (1991). The anaerobic responsive element contains two GC-rich sequences essential for binding a nuclear protein and hypoxic activation of the maize *Adh1* promoter. *Nucleic Acids Res.* 19, 7053-7060.
- Park, M., Yim, H.K., Park, H.G., Lim, J., Kim, S.H., and Hwang, Y.S. (2010). Interference with oxidative phosphorylation enhances anoxic expression of rice alpha-amylase genes through abolishing sugar regulation. *J. Exp. Bot.* 61, 3235-3244.
- Price, J., Laxmi, A., St. Martin, S.K., and Jang, J.C. (2004). Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. *Plant Cell* 16, 2128-2150.
- Ramon, M., Rolland, F., and Sheen, J. (2008). Sugar sensing and signaling. *The Arabidopsis book/American Society of Plant Biologists* 6, e0117.
- Raymond, P., Al-Ani, A., and Pradet, A. (1985). ATP production by respiration and fermentation, and energy charge during aerobiosis and anaerobiosis in twelve fatty and starchy germinating seeds. *Plant Physiol.* 79, 879-884.
- Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Ann. Rev. Plant Biol.* 57, 675-709.
- Salas, J., Salas, M., Vinuela, E., and Sols, A. (1965). Guco kinase of rabbit liver. *J. Biol. Chem.* 240, 1014-1018.
- Sasidharan, R., and Mustroph, A. (2011). Plant oxygen sensing is mediated by the N-end rule pathway: a milestone in plant anaerobiosis. *Plant Cell* 23, 4173-4183.
- Sheen, J. (2010). Discover and connect cellular signaling. *Plant Physiol.* 154, 562-566.
- Sheu-Hwa, C.S., Lewis, D.H., and Waker, D.A. (1975). Stimulation of photosynthetic starch formation by sequestration of cytoplasmic orthophosphate. *New Phytol.* 74, 383-392.
- Thompson, J., Abdullah, R., and Cocking, E. (1986). Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Sci.* 47, 123-133.
- Wang, H.J., Wan, A.R., Hsu, C.M., Lee, K.W., Yu, S.M., and Jauh, K.W. (2007). Transcriptomic adaptations in rice suspension cells under sucrose starvation. *Plant Mol. Biol.* 63, 441-463.
- Wilson, C. (1975). Plant nucleases. *Annu. Rev. Plant Biol.* 26, 187-208.
- Yim, H.K., Lim, M.N., Lee, S.E., Lim, J., Lee, Y., and Hwang, Y.S. (2012). Hexokinase-mediated sugar signaling controls expression of the *calcineurin B-like interacting protein kinase 15* gene and is perturbed by oxidative phosphorylation inhibition. *J. Plant Physiol.* 169, 1551-1558.