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Patterns of cis-element enrichment reveal potential regulatory modules involved in the transcriptional regulation of anoxia response of japonica rice

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ABSTRACT

Unlike other cereal species, rice is able to germinate and elongate under anoxia. The regulatory mechanism that configures the transcriptome of rice during anaerobic germination is yet to be established. In this study, the major regulatory modules among anoxia-responsive genes in rice identified from published microarray data were predicted by *ab initio* analysis of cis-regulatory information content. Statistically overrepresented sequence motifs were detected from *bona fide* promoter sequences [−1000 to +200], revealing various patterns of cis-element enrichment that are highly correlated with bZIP, ERF and MYB types of transcription factors. As implied by the cis-element enrichment patterns, combinatorial mechanisms configure the overall changes in gene expression during anoxic germination and coleoptile elongation. High enrichment of cis-elements associated with ARF, bZIP, ERF, MYB and WRKY (SUSIBA2) transcription factors was also detected among the glycolytic and fermentative associated genes that were upregulated during anoxia. The patterns established from the global analysis of cis-element distribution for upregulated and downregulated genes and their associations with potential cognate regulatory transcription factors indicate the significant roles of ethylene and abscisic acid mediated signaling during coleoptile elongation under anoxia. In addition, the regulation of genes encoding enzymes in the glycolytic and fermentative metabolism could be associated with abscisic acid and auxin in rice coleoptiles to maintain sugar and ATP levels for longer survival.

1. Introduction

Rice is the most important cereal crop and staple food for more than half of the world's population. It is also regarded as a reference species for the monocotyledonous group due to its relatively small diploid genome and well established genetic and genomic resources (Yuan et al., 2001; Goff et al., 2002). Although some rice varieties exhibit some degree of tolerance to anaerobic stress that normally occurs during partial (hypoxia) or complete submergence (anoxia), it remains a major factor that limits productivity specially for the rainfed lowland cropping systems in Southeast Asia, where an average family relies on subsistence rice farming (Jackson and Ram, 2003; Xu et al., 2006). However, our understanding of the regulatory mechanism controlling its unique behavior is very limited. In this regard, current resources for genetic analysis of complex traits at the global scale provide an opportunity to unravel the complex network of genes involved in the expression of stress tolerance potential. Furthermore, the availability of genomic resources for rice provides the foundation for comparative studies at the systems-level, specifically in the context of understanding the fundamental molecular differences responsible for the responses to anoxia during germination. Central to this goal is the ability to dissect the regulation of anaerobic metabolism by transcriptome analysis.

Plants switch from aerobic to anaerobic respiration as a consequence of low levels or complete absence of oxygen during prolonged submergence. For many years, studies have focused primarily on fermentative pathways as a major mechanism under anaerobic respiration (Dennis et al., 2000). Several enzymes involved in the glycolytic pathway, fermentation and sucrose breakdown are involved in cellular processes triggered by hypoxia (Vartapetian and Jackson, 1997; Subbaiah

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Abbreviations: ABA, abscisci acid; ADH, alcohol dehydrogenase; BLAST, basic local alignment search tool; GA, gibberellic acid; IAA, auxin/indole 3-acetic acid; PDC, pyruvate decarboxylase; TSS, transcription start site.

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and Sachs, 2003). It has been shown that hypoxia strongly induces relevant genes in glycolysis and fermentation in *Arabidopsis*, as well as in root tips of maize and during anoxic germination of rice (Chang et al., 2000; Klok et al., 2002; Paul et al., 2004; Liu et al., 2005; Lasanthi-Kudahettige et al., 2007; Shingaki-Wells et al., 2011). Sucrose synthase genes have been shown to be preferentially expressed over invertase genes (Liu et al., 2005). These trends indicated that enzymes involved in the breakdown of sucrose, glycolysis and fermentation have major roles in the metabolic shift from aerobic to anaerobic respiration. In addition, Mustroph et al. (2006) have demonstrated that the high fermentation rate in rice seedlings, together with a higher content of soluble carbohydrate and an optimized ATP use during anoxia could explain the ability of rice to withstand such stress better than other intolerant cereals.

Besides glycolysis and fermentation, several other metabolic proteins and signal transduction components were also shown to be induced during hypoxia and anoxia (Dennis et al., 2000; Liu et al., 2005; Lasanthi-Kudahettige et al., 2007; Shingaki-Wells et al., 2011). Recently, transcriptome data have provided a global picture of differentially expressed transcripts, hence more comprehensive picture of the changes in cellular status during hypoxia in *Arabidopsis* and anoxia in rice and wheat (Klok et al., 2002; Liu et al., 2005; Loreti et al., 2005; Lasanthi-Kudahettige et al., 2007; Shingaki-Wells et al., 2011). Microarray analysis was used to investigate the global pattern of gene expression in seedlings of *Arabidopsis* without oxygen for 6 h (Klok et al., 2002; Gonzali et al., 2005; Liu et al., 2005). Results indicate that 1600 genes were affected by anoxia, many of which were linked to fermentation and sucrose related pathways. An independent study conducted by Lasanthi-Kudahettige et al. (2007) reported a high induction of pyruvate decarboxylase (PDC) genes in response to anoxia. While alcohol dehydrogenase (ADH) genes were also up regulated at very high levels, induction was significantly less than the PDC. It is evident from these results that a complex metabolic network involving multiple pathways operates in synergy under such stress condition. The molecular basis of the adaptation to anaerobic condition and the role of the enzymes involved in other pathways and signal transduction has not yet been fully investigated.

Gene expression under stress conditions involved the specific interaction of trans-acting factors (transcription factors) and their cognate cis-elements located in the upstream regulatory region of a gene. Transcription factors recognize and bind to specific cis-elements to facilitate activation or repression of transcription in coordination with other regulatory proteins (*i.e.*, both basal and specialized components). In plants, the nature of transcription factors with important roles in anaerobic stress response is just beginning to be uncovered from detailed analysis of the overall composition and regulatory information content of the transcriptome. For instance, the *AtMYB2* transcription factor has been shown to be critical for the regulation of hypoxia-related genes in *Arabidopsis* (Dolferus et al., 2003). Recently, detailed dissection of the promoter structures of hypoxia-related genes in *Arabidopsis* revealed that besides *AtMYB2*-associated cis-element, other putative regulatory elements also play crucial roles in the regulation of hypoxia-related genes including G-box-like elements associated with certain classes of bZIP transcription factors and a sugar-response-like element associated with certain types of WRKY transcription factors. In another study involving a genome-wide survey of gene expression in *Arabidopsis* seedlings exposed to 12 h of hypoxia, Branco-Price et al. (2005) showed that a low GC nucleotide content in the 5′-UTR of a small group of mRNA provides a selective advantage for translation under hypoxia. However, the patterns of cis-element distribution and enrichment that define anoxia-regulated expression at the level of the entire transcriptome have not been critically examined. In this study, we have used an *ab initio* approach to reveal meaningful patterns of cis-element distribution with potential significance to the understanding of the nature of transcriptional modules involved in the regulation of enzymes associated with anoxia as well as glycolysis and fermentative pathways in rice. Given the unique ability of rice to withstand anoxic conditions better than most other cereal crops, our specific aim was to establish a good picture of transcriptional regulatory mechanisms by investigating the occurrence of common and/or unique transcriptional modules that differentiate the glycolysis and fermentative pathways. Additionally, trends established from global analysis of transcriptional modules were used to establish testable hypotheses on the most critical transcription factors that may function as major regulators of rice germination under anoxic condition.

2. Materials and methods

2.1. Selection of anoxia-responsive genes and extraction of promoter sequences

Upregulated or downregulated genes by anoxia were identified based on published microarray data of *Oryza sativa ssp. japonica cv. Nipponbare* on elongating coleoptiles (Lasanthi-Kudahettige et al., 2007). Exact location of transcription start site (TSS) was determined by mapping full-length cDNAs (with full-length 5′ UTR) to the genomic sequences by BlastN analysis (Altschul et al., 1997). The sequences of full-length cDNAs were obtained from RIKEN (Kikuchi et al., 2003; http://cdna01.dna.affrc.go.jp/cDNA). The genomic sequences for rice were obtained from the MSU Rice Genome Annotation (http://rice.plantbiology.msu.edu/, http://www.tigr.org/tdb/e2k1/osa1/). Sequences with 100% identity on the 5′ end over the length of 25 nucleotides and having an e-value less than 0.001 were considered as reliable matches. The promoter sequences for each cDNA were extracted covering [−1000, +200] relative to the mapped TSS.

Promoter sequences covering the [−1000, +200] regions relative to the *bona fide* (experimentally validated) TSS, were extracted from the genomic loci corresponding to genes that were either upregulated or downregulated by anoxia. Promoter sequences of 842 genes that were upregulated by anoxia in rice coleoptiles were extracted from our in-house promoter sequence database. Similarly, the promoter sequences for downregulated genes (1794 genes) were also extracted from our in-house database. Promoter sequences of genes that were not affected by anoxia were used as background sequences for cis-element analysis.

2.2. Ab initio promoter motif detection and transcription factor identification

Upstream sequence motifs corresponding to previously identified or putative (uncharacterized) cis-elements were detected by the Dragon Motif Builder (DMB) Algorithm with EM2 option (Huang et al., 2005). Thirty motifs were detected each time having length 8–10 nucleotides per detection with threshold values of 0.875 similar to the previous detection (Meier et al., 2008; Yun et al., 2010; Park et al., 2010). Motifs occurrence in over 50% of the sequences at a threshold e value of $\leq 10^{-3}$ were considered as statistically overrepresented. The biological significance of these motifs was verified by their significant matches with entries at the transcription factor binding databases such as TRANSFAC (Matys et al., 2003; www.gene-regulation.com), PLACE database (Higo et al., 1999; http://www.dna.affrc.go.jp/PLACE/) and AGRIS (Davuluri et al., 2003; Yilmaz et al., 2011; http://arabidopsis.med.ohio-state.edu/). Total motif enrichment score was calculated by adding the percentage occurrences of all motif species belonging to the same transcription factor family in the upregulated (UR), downregulated (DR) and glycolytic upregulated genes. Motifs detected for upregulated, downregulated and glycolytic genes were grouped based on predicted associations with known families of transcription factors. Significantly upregulated transcription factors were identified from the rice microarray data and annotated based on the most recent genome annotations (Itoh et al., 2007; Tanaka et al., 2008; http://rapdb.dna.affrc.go.jp/). The methods used for the whole analysis has been summarized in Fig. 1.