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Transcriptomic adaptations in rice suspension cells under sucrose starvation

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Abstract Sugar is an important resource for energy generation and developmental regulation in plants, and sucrose starvation causes enormous changes in cellular morphology, enzyme activities and gene expression. Genome-wide gene expression profiling provides a comprehensive knowledge of gene expression under nutrient depletion and senescence; however, that of a monocot model plant, rice, under sucrose depletion is still under investigation. Here, the time-course monitoring of gene expression profiles in sucrose-starved rice (*Oryza sativa* cv Tainung67) suspension cells was investigated by 21495 probes contained in Agilent rice chip. In sucrose-starved cells, the induced vacuolar biogenesis coincided with significantly upregulated transcripts of H^+-pyrophosphatase, δ-TIP, one putative α-TIP, several vacuolar proteases and proteinase inhibitors, and one OsATG3. To survey the overall metabolic adaptations under sucrose depletion, the genes with significantly altered expression level were incorporated into multiple metabolic pathways. Most genes encoding enzymes involved in biosynthesis and degradation pathways of various macromolecules were comprehensively down- and upregulated, respectively, with sucrose starvation. Transcriptional regulation of gene expression is important for physiological adaptations to environmental stress, and many transcription factors, including bZIPs, NACs, and WRKY, showed significant increase in transcriptional level under sucrose starvation. Concurrently, statistical analysis revealed that their corresponding consensus *cis*-elements, such as ABA-responsive element, CACG, ACI, ACII and CTTATCC, were frequently found in the promoter regions of many sucrose starvation-upregulated genes. Particle bombardment-mediated and luciferase activity-based transient promoter assays revealed the CTTATCC, derived form TATCCA, and the AC motifs to be promising sucrose-starvation responsive activators in rice suspension cells.

Keywords *cis*-elements · Metabolic pathways · Rice (*Oryza sativa* cv Tainung 67) suspension cells · Sucrose starvation · Transcriptome · Transcription factor

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Introduction

In plants, sugars synthesized from photosynthesis are essential for cell vitality as the raw substrates for energy generation by respiration and for the metabolic intermediates in biosynthesis of other macromolecules such as proteins, lipids, DNA and RNA (Plaxton 1996). Carbohydrate deficiency induced by extended darkness and drought is a frequently confronted stress

during the plant life cycle (Brouquisse et al. 1998; Rose et al. 2005). Immobile plants have evolved several elaborate cellular and molecular mechanisms to endure this adverse circumstance. For example, various physiological responses, including arrest of cell growth, rapid consumption of cellular-stored carbohydrates, decrease in respiration rate, and degradation of lipids and proteins, are adapted to overcome the insufficient supply of sugars (Aubert et al. 1996b; Brouquisse et al. 1992a; Contento et al. 2004; Koch 1996; Yu 1999a). Modification of enzymatic activities and/or gene expression profiles of catabolic pathways is another strategy to acclimatize carbohydrate deprivation, through a decrease in enzymatic activities in glycolysis, respiration and protein synthesis or increase in gene expression and enzymatic activities related to fatty acids and protein catabolism (Contento et al. 2004; Dieuaide et al. 1993; Koch 1996; Lin and Wu 2004; Scheible et al. 2004; Tassi et al. 1992; Thimm et al. 2004).

Nutrient starvation also accompanies modification in subcellular organization, and the best-characterized phenomenon is the induction of autophagic machinery, whereby authophagic vacuoles/autolysosomes degrade bulk cytoplasmic components for nutrient recycling (Moriyasu and Klionsky 2004). In yeast and mammalian cells, as autophagy is triggered, a double membranous structure, an isolation membrane, forms and sequesters a portion of cytoplasm and encloses it to form a double membrane vesicle, the autophagosome. The autophagosome transports and fuses with tonoplast to deliver its inner cytoplasmic components into the vacuole/lysosome for degradation (Klionsky 2004). Carbon starvation-induced autophagy and vacuolar enlargement have also been observed in maize root tip (Brouquisse et al. 1991) and suspension cells of rice (Chen et al. 1994), sycamore (Aubert et al. 1996b), tobacco (Moriyasu and Ohsumi 1996), and Arabidopsis (Ketelaar et al. 2004; Rose et al. 2005; Slavikova et al. 2005; Thompson et al. 2005; Yoshimoto et al. 2004). In yeast, genetic studies have revealed a group of genes involved in the induction of autophagy, formation of autophagosome, and control of autophagosome size (Noda and Ohsumi 2004). In Arabidopsis, null mutations of yeast autophagic ortholog genes (e.g., *AtATG4*, *AtATG7* and *AtATG9*) showed similar phenotypes and accelerated starvation-induced chlorosis and senescence in leaves, which suggests that autophagy plays similar roles in nutrient recycling and re-allocation in plants (Doelling et al. 2002; Hanaoka et al. 2002; Yoshimoto et al. 2004).

The expression of genes involved in several metabolic pathways is greatly altered under Suc (sucrose) starvation. For instance, the expression of genes for rice α-amylase (Sheu et al. 1996; Yu et al. 1991), maize sucrose synthase (*Sh1*) (Brouquisse et al. 1992b), cucumber malate synthase and isocitrate lyase (Graham et al. 1994), vacuolar invertase, processing enzyme-γ (VPE-γ), aleurain (Ahmed et al. 2000; Kinoshita et al. 1999; Tymowska-Lalanne and Kreis 1998) and Suc remobilization/transport are upregulated during Suc starvation, but that of genes encoding the proteins for storage and utilization of Suc are downregulated (Koch 1996). Recently, genome-wide transcriptomic profiling rendered a comprehensive knowledge of gene expression and more insights into the diverse cellular responses to Suc starvation in Arabidopsis suspension cells (Contento et al. 2004) and senescent leaves (Buchanan-Wollaston et al. 2005). The genome of the monocot model plant, rice, has been completely sequenced (Project 2005), and recent transcriptomic studies have thus been able to identify genes involved in anther development (Wang et al. 2005), pollination (Lan et al. 2005), and phosphate starvation (Yi et al. 2005). However, the gene expression profile of rice cells in response to Suc depletion is still under investigation.

The rice embryo scutellum-induced suspension cells with a uniform cell type show molecular and cellular hypersensitive response to Suc starvation (Chen et al. 1994; Yu 1999a, b). Here, we describe the cellular, transcriptomic, potential metabolic modifications and regulation of gene expression of rice suspension cells under Suc starvation. Ultrastructural observation showed progressive induction of vacuolar biogenesis, which was associated with induced expression of genes involved in vacuolar biogenesis, such as several tonoplast-integrated proteins, vacuolar proteases and one OsATG3. Agilent rice chips containing 21495 probes were used to monitor the gene expression profiling during Suc starvation. BLASTX search and metabolic pathway analyses offered molecular insights into the gene expression profile in Suc-starved cells: (1) downregulated gene expression in metabolic pathways such as glycolysis, the citrate cycle, oxidative phosphorylation reaction, and the biosynthetic processes of many macromolecules; (2) upregulated transcripts for proteins involved in degradation of various molecules such as sucrose, polysaccharides, fatty acids, and branched-chain amino acids; and (3) upregulated genes encoding for transcription factor families of bZIP, NAC and WRKY. In addition, statistical analysis of promoter sequences of Suc-starved upregulated genes revealed several putative Suc-starvation responsive elements, such as ABA-responsive

element, CACG, ACI, ACII and TATCCA-derived CTTATCC. Particle bombardment-mediated and luciferase activity-based transient promoter assay further verified the CTTATCC and AC elements as the promising Suc-starvation responsive activators in rice suspension cells. The potential coordinated roles of these transcription factors (TFs) and their corresponding *cis*-elements in regulating gene expression under Suc starvation are discussed.

Materials and methods

Rice cell culture and Suc-starved treatment

The suspension cell culture of rice (*Oryza sativa* cv Tainung 67) was established as described previously (Yu et al. 1991). About 0.5 ml cells were subcultured every 7 days in 50 ml of fresh liquid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% (w/v) Suc, 2.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in a 250-ml flask on a reciprocal shaker at 100 rpm and incubated at 28°C in the dark. Suc starvation was performed on fourth day after subculture. An amount of 3 ml cells was rinsed with ddH_2O twice and transferred into 50 ml fresh MS media containing 3% Suc (control cells) or no Suc (starved cells) to culture for 12, 24 and 48 h. For osmotic control, ddH_2O-rinsed cells were transferred into 50 ml fresh MS media containing 88 mM sorbitol (osmotic control cells) without Suc to culture for 12 h. Finally, cells were collected for tissue preparation or quickly frozen in liquid nitrogen at desired time points and stored at –80°C for analyses.

Tissue preparation for transmission electron microscopy

Cells freshly collected from different time intervals and treatments were fixed in 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 100 mM phosphate buffer (pH 7.0) for 4 hr at room temperature. Fixed cells were then rinsed with phosphate buffer (3×15 min), post-fixed in 1% OsO_4 in the buffer again, rinsed with the buffer (3×15 min), and dehydrated by use of a graded acetone series before being embedded in Spurr's resin. Ultra-thin sections (70 nm) cut by a Leica Reichert Ultracut S were collected onto nickel grids, post-stained with 6% uranyl acetate and 0.4% lead citrate, rinsed for 6×15 s with ddH_2O, and examined under a Phillips CM100 electron microscope (Eindhoven, The Netherlands).

Total RNA extraction, mRNA purification, Northern blotting and semi-quantitative RT-PCR

Total RNA for RT-PCR, northern blotting and GeneChip microarray analysis was extracted by use of the Tripure™ Kit according to the manufacturer's manual (Genesis Biotech, Taipei, Taiwan) and respective mRNA were purified from 200 μg total RNA by use of Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 3 μg total RNA with oligo(dT) primer and random primers according to the manufacturer's protocol (M-MLV Reverse Transcriptase; Invitrogen Life Technologies, Carlsbad, CA, USA). Northern blotting was performed by following standard procedures (Sambrook et al. 1989) with the radiolabeled probe made from a cDNA fragment of *OsHSP70* (AK109747). To examine the expression pattern of genes in the control and Suc-starved cells and the efficacy of cDNA synthesis by PCR amplification, RT-PCR analysis was performed with the gene-specific primer sets (Supplemental Table 3), and the *OsHsp* gene (AK071176) was used as a loading control. The PCR mix consisted of 1.5 mM magnesium chloride, 0.2 mM dNTPs, 0.5 μM each of sense and antisense primers, 2.5 U of *Taq* polymerase (MDBio, Taipei, Taiwan) and 1× PCR buffer supplied with the *Taq* polymerase. The reaction was as follows: 94°C for 10 min; 25 to 28 cycles at 94°C for 1 min, annealing at 55°C for 1 min, 72°C for 1 min; and final elongation at 72°C for 10 min with use of a Biometra" T3 Thermocycler (Whatman Biometra, Goettingen, Germany). PCR products were examined on a 1% (w/v) agarose gel containing 0.01% (v/v) ethidium bromide and their intensities were determined by Kodak 1D Image Analysis Software 3.6 (Eastman Kodak, NY, USA).

Agilent rice DNA microarray hybridization and expression data analyses

Agilent rice DNA chips (G4138A, Agilent Technologies, Palo Alto, CA, USA) were used to study the transcriptomic profile of Suc starvation. Total RNA extraction and mRNA isolated from treated suspension cells cultured for different times were used for cDNA probe preparation and microarray experiments. The labeling of fluorescent cDNA probes was performed at the Institute of Plant and Microbial Biology DNA microarray core facility as described at http://www.botany.sinica.edu.tw/microarray/protocols.htm. The hybridizations were conducted as suggested by the manufacturer (Agilent Technologies). Three independent biological replicates of hybridizations were

performed for all treatments as follows: 3 hybridizations of 12-h starved versus control, 3 hybridizations of 24-h starved versus control, 3 hybridizations of 48-h starved versus control and 3 hybridizations of 12-h starved versus osmotic control. The hybridization signals for each DNA element were detected and analyzed by Axon GenePix 4000B and GenePix 4.0 (Axon Instruments, Inc., Union City, CA, USA), respectively, then exported as the GenePix Result (GPR) files that were imported into GeneSpring 7.2 (Silicon Genetics, Redwood, CA, USA) for data normalization by ''LOWESS Normalization''. Before being imported into SAM program, the normalized expression data sets of three biological replicates had to pass the following 2 quality control categories, the hybridization results not flagged as bad and the net intensities of both channels equal to or greater than 200. Under these criteria, about 46% genes present in the chips were detected and their expression signals were higher than background. The ''one-class'' analysis was used to retract the genes showing statistically significant change in expression with three biological repeats at each time point with the criteria of FDR <5%. From osmotic control experiments, 5990 genes showed significant change by osmotic stress and 5845 genes were mainly found with amount of change ranging between 0.5 and 2-fold. To avoid the osmotic interference on alternation in gene expression specifically responding to Suc starvation, all altered genes between 0.5 and 2-fold were deduced. The remaining 145 of 5990 osmotic stress-induced genes with change greater than 2-fold were removed individually from the pool of Suc starvation-altered genes. The resulting genes significantly up- and downregulated by Suc starvation at any time point examined were used for serial analyses. The genes were classified into five groups by K means cluster program, and each group was presented by use of the Gene Tree program in Gene Spring 7.2. The Gene Ontology Slim classification in GeneSpring 7.2 was used for the functional category classification in different K-means cluster-classified groups. For pathway organization, biochemical pathways in Arabidopsis were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg/) and imported into GeneSpring 7.2. The BLASTX program in SWISSPROT protein database was used to obtain the enzymatic EC number of genes presented our array chip, and approximately 20% of them with assigned EC number were mapped to Arabidopsis KEGG metabolic pathways by use of GeneSpring 7.2. For transcription factor and *cis*-element analyses, the gene lists of rice TFs obtained from the Rice Transcription Factor Database (http://ricetfdb.bio.uni-potsdam.de/) and rice genomic sequences obtained from TIGR Rice Genomic Annotation (http://www.tigr.org/tdb/e2 k1/osa1/index.shtml), respectively, were imported to and analyzed by use of GeneSpring 7.2. ''Find potential regulatory sequence'' in GeneSpring 7.2 program was applied to identify and localize the potential *cis*-elements in the selected promoter regions (the net expression intensities were either >200 or >500 and amount of change more than 2- or 3-fold) of all Suc-starvation up-regulated genes. The selected criteria in ''Find potential regulatory sequence'' was: from 5- to 8-nucleotide length without any point discrepancies within 0–500 bases upstream of the TSS of each gene. In all statistical analysis relative to the promoter region of other genes, we used $P < 0.01$. To obtain the distribution of the selected *cis*-elements in promoters, the *cis*-elements in the upregulated genes and the number of genes containing the *cis*-elements were used to calculate the overall distribution rate.

Vector construction and transient promoter activity assays

Plasmid p3Luc.18 and p3Luc.34 containing the promoter region (–186 to –82) of *OsAmy3D* (AK073487) with or without 2 adjacent TATCCA boxes, respectively, were fused to a *CaMV35S* minimal promoter (–46 bp from the TSS)-*Adh1* intron-*Luc* coding sequence-nopaline synthase gene (*Nos*) terminator (Lu et al. 1998). Rice genomic DNA extracted by Plant Genomic DNA Mini Kit (Geneaid, Taipei, Taiwan) was used as the template to obtain the promoter fragment (~50 to~–500 bp from the TSS) of selected genes by PCR amplification with sequence-specific primer sets (Supplemental Table 3) containing PstI and XhoI sites. PCR product was cloned into T-easy vector (Promega, Madison, WI, USA) and the promoter fragment was confirmed by nucleotide sequencing. Each promoter fragment lacking a *cis*-element was created by megaprimer-based PCR method. To obtain the megaprimer, the T-easy vector containing a promoter fragment was used as a template, a 5′ primer lacking a *cis*-element sequence and a sequence-specific 3′ primer were used to generate the megaprimer fragment and was purified by use of a gel elution kit (Geneaid, Taipei, Taiwan). The full-length promoter fragment with the deleted *cis*-element was amplified with a megaprimer and a sequence-specific 5′ primer by use of a T-easy vector containing a promoter fragment as a template. The PCR product of the promoter fragment without the *cis*-element was separated by electrophoresis, purified by use of a gel elution kit,

cloned it into a T-easy vector and confirmed by nucleotide sequencing. The promoter region of *OsAmy3D* in the plasmid p3Luc.18 was replaced by the above PstI and XhoI-fragments from the individual T-easy vector. All constructs were amplified by *E. coli*, purified by use of a plasmid midi kit (Qiagen, Valencia, CA, USA) and 1 μg/μl of each plasmid was used for transient promoter activity assay. The transient promoter activity assay was conducted by use of particle bombardment-mediated transient expression assay (Lee et al. 2003; Lu et al. 2002; Umemura et al. 1998). Briefly, approximately 1 ml of rice suspension cells were sub-cultured in MS medium containing 3% (w/v) Suc and 2.4 mg/l 2,4-D in a 250-ml flask for 4 days. Then, 1.5 ml cells plated on MS medium solidified with 0.3% (w/v) Phytagel (Sigma-Aldrich, St. Louis. MO, USA) were used for bombardment, with three individual replicates. Plasmid pUG containing the *Ubiquitin*::*GUS* gene was used as an internal control for promoter activity assay, and the ratio of test DNA to internal control plasmid DNA was 2:1. After bombardment, cells on each plate were divided equally into two parts; one half was incubated in liquid MS medium containing 3% (w/v) Suc and the other half was grown in liquid Suc-free MS medium with shaking at 100 rpm at 28°C for 18 h. Total proteins were extracted from bombarded rice suspension cells with 0.5 ml of CCLR buffer [100 mM $KH_2(PO_4)$, pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 7 mM β-mercaptoethanol], and 100 and 50 μl of extracted samples were used for luciferase and GUS activity assays, respectively. Luciferase activity was normalized by dividing it against GUS activity.

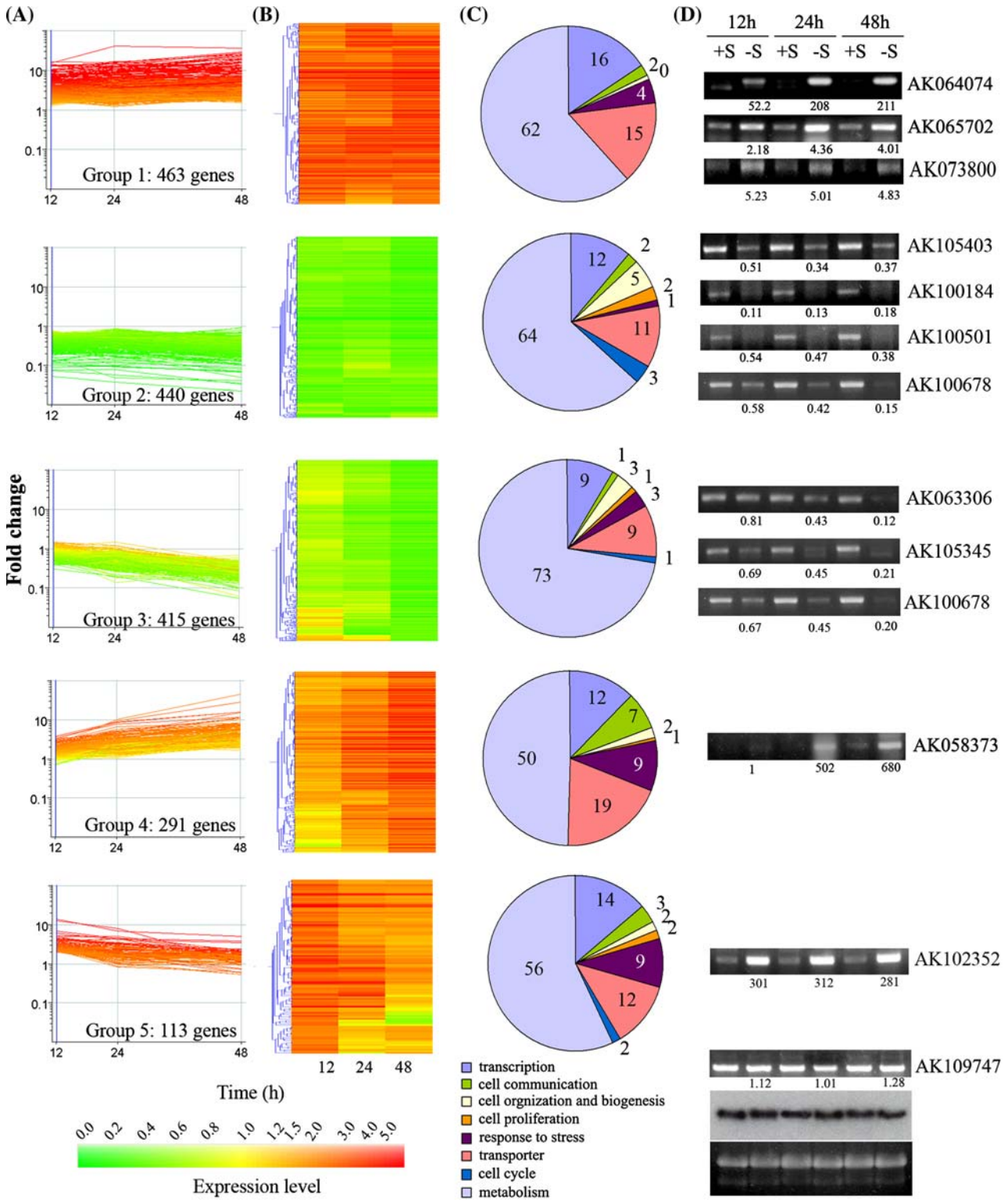
Results and discussion

Morphology and transcriptome in Suc-starved rice suspension cells

Suspension cells induced from rice embryos cultured in Suc-containing (control) or -free (Suc starvation) media for 12, 24 and 48 h were first used for ultrastructural observation to confirm the cellular responses to Suc starvation. Compared to control cells (Fig. S1 A, C, E), Suc-starved cells gradually formed larger vacuoles in the cytoplasm, as was found by Chen et al. (1994). Portions of cytoplasm engulfed by tonoplast and retained inside the vacuolar lumen were observed in 12-h starved cells (arrows in Fig. S1B). In 24-h starved cells, the lumens of the enlarged vacuoles contained cytoplasmic debris (arrows in Fig. S1D). The extremely large vacuole in 48-h-starved cells occupies the entire intracellular space, with the cytoplasm and nucleus confined to a narrow area (Fig. S1F). Physiological analysis in Suc-starved rice (Chen et al. 1994) and Arabidopsis suspension cells (Contento et al. 2004) suggested that most of the irreversible cellular apoptosis induced by Suc starvation occurred after 48-h starvation. Therefore, the condensed nucleus found in 48-h Suc-starved cells may reflect the irreversible lost of cell viability after long periods of Suc depletion.

We used 2 color-based Agilent rice gene chips to investigate the gene expression profiling in rice cells Suc starved for 12, 24 and 48 h. To eliminate the effect of osmotic stress on alteration of gene expression during sample processing, we used cells cultured in Suc-free medium compared with 88 mM sorbitol in Suc-free MS medium for 12 h as an osmotic control in our array analysis. The full raw data sets from 3 independent biological repeats at each time point (total of 12 hybridization analyses including 3 of osmotic control), which have been deposited in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), were subjected to GeneSpring 7.2 analysis. Among 21495 genes represented in the Agilent chips, 9865 transcripts (~46%) were quantitatively detectable, and approximately 60% showed no amino acid sequence homology to any known protein in the available database. The detected hybridization signals normalized by GeneSpring 7.2 were used to retract the genes with significant alteration in gene expression at each time point by use of Significance Analysis of Microarrays program (SAM, http://www-stat.stanford.edu/~tibs/SAM/), with the false discovery rate (FDR) of genes set to lower than 5%. After deducing the osmotic stress-induced genes from Suc starvation-induced genes at all time points, the resulting 867 and 855 genes significantly up- and down-regulated by Suc starvation at any time point underwent K means cluster, KEGG pathway analysis, transcription factor classification and *cis*-element prediction.

The up- and down-regulated genes were classified into five groups by K means cluster analysis (Fig. 1A, B). Groups 1 and 2 represent 463 and 440 genes exhibiting early and constant up- and downregulation, respectively, during Suc starvation. Group 3 represents 415 genes with gradual declining expression during Suc starvation, but 291 genes in Group 4 showed a progressively upregulated tendency after 12-h starvation. Group 5, with 113 genes, shows significantly enhanced expression at the early stage but less upregulated expression at a late stage of Suc starvation. In general, Groups 1, 4, and 5 (867 genes) and Groups 2 and 3 (855 genes) represent significantly up- and downregulated genes under Suc starvation. In terms of biological functions, all genes in each group were sorted into 8



different categories (Fig. 1C). Most genes in both the up- and downregulated categories are involved in metabolism: the 250 upregulated (62%, 50%, and 56% in Groups 1, 4, and 5) and 340 downregulated (64% and 73% in Groups 2 and 3) genes are involved in catabolic and biosynthetic pathways, respectively (see below). The genes responsible for transcription and transportation are the second and third major categories of genes in each group significantly affected by Suc starvation. Nevertheless, ''response-to-stress'' genes are the fourth largest group of genes (28 genes in Groups 1, 4, and 5; 10 genes in Groups 2 and 3), which

◀**Fig. 1** Clustered expression profiles, functional categories and verification of the transcript levels of diverse genes in response to Suc starvation. After Significance Analysis of Microarrays (SAM) analysis, the transcripts on microarray chips showing significant up- and downregulated by Suc starvation were classified by K means cluster analysis into five groups, each with genes showing similar expression pattern upon Suc starvation. The degree of change was color coded (yellow, red and green represent no change, up- and downregulation, respectively). **(A)** indicates the expression tendency and number of genes in each individual group. The expression of each group at 3 time points is shown by the gene tree in (**B**). (**C**) is the gene functional categories of each group analyzed by Gene Ontology Slim classification in the GeneSpring 7.2 algorithm; the unspecified and unknown categories are not included. The accuracy of the microarray data was verified by semi-quantitative RT PCR with transcripts selected from each group (**D**). The expression ratio for each gene responding to Suc starvation (–S) validated by RT-PCR was shown as setting the intensity of gene under normal culture condition (+S) as 1. The *OsHSP70* (AK109747) that showed no significantly change during Suc starvation as evidenced by northern blotting was used as the loading control

suggests that most of the stress-associated genes are also significantly upregulated by Suc starvation. Semi-quantitative RT-PCR of genes selected from each group was used to cross-validate the microarray expression data (Fig. 1D).

Suc starvation simultaneously induces vacuolar biogenesis and its functionally related genes

In rice suspension cells, the remarkable increase in vacuolar size occurred as early as 12 h after Suc starvation (Fig. S1). To sustain proper vacuolar biogenesis and function, the cell has to generate large amounts of tonoplast, tonoplast proteins and proteases. From our array data, many genes encoding for the tonoplast-integrated proteins, receptors and vacuolar luminal proteins were significantly induced upon Suc starvation (Table 1). Tonoplast intrinsic proteins (TIPs), the plant aquaporins, consist of α-TIP, β-TIP, γ-TIP and δ-TIP families, and each family defines different types of vacuoles (Jauh et al. 1999). The δ-TIP (AK073531), one H^+-pyrophosphatase (AK099807) and one nonclassified ZmTIP3-1 (AK111931) were the highest expressed genes induced as early as 12 h after Suc starvation and persistently increased their expression levels under starvation. For the nonclassified ZmTIP3-1 gene, BLASTX search revealed its high homology to Arabidopsis α-TIP (CAA45114, 63% identity and 80% similarity), a marker for autophagic vacuole-like organelle in the cells of aleurone and barley root tip (Moriyasu et al. 2003). The early and significantly induced expression of ZmTIP3-1 and other tonoplast-integrated proteins implies their potential roles in autophagic vacuole biogenesis in Suc-starved rice cells. In addition to several vacuolar lumen proteases, 1 genes encoding for cysteine proteinase inhibitors and 2 for Bowman-Birk-type trypsin inhibitor and subtilisin-chymotrypsin inhibitor were also quickly and significantly upregulated by Suc starvation (Table 1). Many plant proteinase inhibitors function as defensive molecules against phytophagous

Table 1 Vacuole-related genes show notably stimulated expression levels during Suc starvation period

Accession number	12[a]	24	48	Annotation	k means cluster
Tonoplast-integrated proteins					
AK111931	6.81 (1.20)	12.4 (4.68)	14.07 (2.66)	*Zea mays* tonoplast membrane integral protein ZmTIP3-1	1
AK099807	6.77 (3.18)	8.47 (3.06)	15.36 (7.18)	*Oryza sativa* vacuolar H^+-pyrophosphatase	1
AK073531	5.27 (5.47)	7.95 (7.08)	11.26 (10.36)	*Triticum aestivum* δ-TIP	1
AK072531	0.82 (0.31)	1.64 (1.07)	4.25 (2.66)	*Zea mays* tonoplast membrane integral protein ZmTIP4-2	4
AK098846	2.02 (0.30)	2.30 (0.93)	3.69 (1.37)	*Triticum aestivum* vacuolar targeting receptor BP-80	4
AK063178	1.00 (0.29)	1.65 (0.64)	2.32 (0.33)	*Oryza sativa* vacuolar H^+-ATPase (vatp-P1)	4
AK072667	2.66 (0.52)	2.24 (0.29)	2.20 (0.31)	*Triticum aestivum* vacuolar targeting receptor BP-80	1
Vacuolar proteinases					
AK064050	7.74 (3.13)	8.99 (0.87)	24.60 (1.50)	*Oryza sativa* Bowman-Birk type trypsin inhibitor	1
AK063833	7.16 (5.11)	4.75 (0.68)	16.70 (3.52)	*Zea mays* subtilisin-chymotrypsin inhibitor	1
AK073275	1.88 (0.47)	2.79 (1.41)	4.32 (1.20)	*Glycine max* cysteine proteinase inhibitor	4
AK066453	1.27 (0.29)	1.95 (0.51)	3.63 (1.96)	*Oryza sativa* aspartic protease	4
AK100749	2.06 (0.28)	2.32 (0.62)	3.58 (1.83)	*Oryza sativa* aspartic protease	1
AK065206	2.45 (0.84)	2.54 (0.42)	3.44 (1.27)	*Oryza sativa* aspartic proteinase	1
AK061947	1.41 (0.29)	2.04 (0.50)	3.39 (1.30)	*Zea mays* cysteine proteinase, clone CCP	4
AK067257	3.16 (3.10)	3.11 (0.64)	3.01 (1.84)	*Oryza sativa* Bowman-Birk proteinase inhibitor	1
AK064771	1.83 (0.29)	1.78 (0.28)	2.18 (0.31)	*Zea mays* nucellin-like aspartic protease	1
AK106147	2.02 (0.60)	2.03 (0.54)	1.91 (0.36)	*Oryza sativa* putative hydrolase	1
AK061670	2.36 (2.20)	1.96 (0.28)	1.91 (0.84)	*Oryza sativa* Lon protease (Lon1)	1

[a] Fold change between the normalized intensities of starved and control cells at the time point indicated. The fold change values shown in this table were calculated using the mean ratio of three sets of biological replicates. The number in the parentheses is referred to standard error

insects (Ryan 1990) and fungal pathogens (Qu et al. 2003). Nevertheless, they are also synthesized in response to wounding and to developmental switches that affect carbon and nitrogen sinks and function as storage proteins inside the lumen of vacuoles on tonoplast labeling with δ-TIP alone (Jauh et al. 1999). Their physiological roles in rice suspension cells under Suc starvation are still unclear. Since the rice suspension cells we used originated from embryo scutellum and recent study showed that induction of Bowman-Birk-type trypsin inhibitor was an embryonic defense mechanism (Qu et al. 2003), the high induction of this gene could be a generic response to various stresses, including Suc starvation.

In rice cells, the vacuolar biogenesis triggered during the onset of Suc starvation is probably via a putative autophagic machinery as is found in Arabidopsis (Rose et al. 2005; Yoshimoto et al. 2004). Several autophagy-related transcripts of *AtATG3*, *AtATG4*, *AtATG7* and *AtATG8* genes were immediately induced in Suc-starved Arabidopsis cells, but they were greatly repressed after 24-h Suc starvation (Contento et al. 2004). However, our array analysis showed that only the *ATG3* (*OsATG3*, AK067737) among 8 *OsATGs* present on the gene chip was significantly upregulated in 12-h Suc-starved cells. The early induced expression of *OsATG3* may coordinate with other vacuole-related genes in vacuolar biogenesis during the early stage of Suc starvation. Since only 21495 genes were represented in the Agilent rice gene chips and the annotation of all rice genes is still in progress, other unidentified or unannotated *OsATGs* may play important roles in starvation-induced autophagy formation and sustenance.

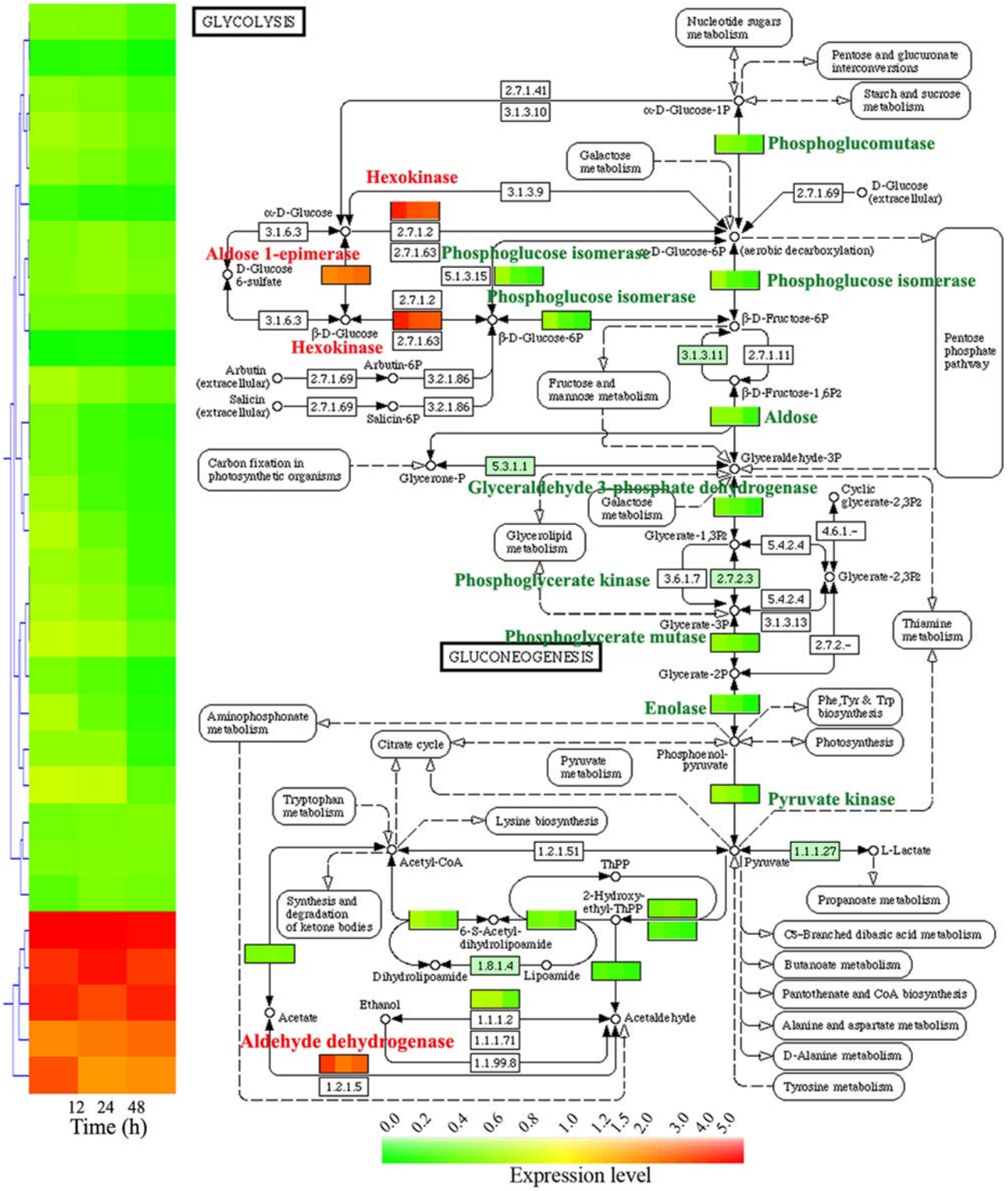
Suc starvation decreases the expression of genes involved in sugar metabolic pathways

In plant cells, Suc starvation-induced decrease in enzymatic activities of sugar metabolism and protein synthesis but increase in enzymatic activities related to catabolism of fatty acids, amino acids, and proteins were reported previously. Such a change can presumably protect cells against nutrient stress by switching off biosynthesis to conserve energy and by substituting protein and lipid catabolism for sugar catabolism to sustain respiration and metabolic processes (Yu 1999a). Actually, the genes participating in metabolism are the major group of genes showing significant alteration of expression in response to Suc starvation (Fig. 1C). To explore the overall metabolic pathways potentially affected in Suc-starved cells, we incorporated each annotated gene showing significant change in expression level into multiple metabolic pathways.

Fig. 2 Downregulation of genes encoding for most enzymes involved in glycolysis and gluconeogenesis during Suc starvation. The average expression level of glycolysis-related genes obtained and sorted by SAM analysis and the gene tree, respectively, are shown in left panel. Only transcripts showing significant up- and downregulation after SAM analysis were incorporated into each metabolic pathway. The right panel shows the pathways in which the expression profile of all genes represented on the array color coded in each pathway. Every color code (yellow, red and green represent no change, up- and downregulation, respectively) shows the ratio of total expression intensity of the genes on the array chip encoding for the same enzymes under control and Suc starvation conditions at 3 time points, 12, 24 and 48 h from left to right. Selected EC numbers of genes are listed for references. The average expression levels of most transcripts for the enzymes involved in both pathways, except for the hexokinase, aldehyde dehydorgenasae and aldose 1-epimerase, are significantly downregulated

Glycolysis is the central pathway to convert glucose and other sugar derivatives (e.g. glycerol, sucrose, ribose, starch and cellulose) into pyruvate, which is further oxidatively phsphorylated to produce ATP for normal cellular metabolism. The intermediates of glycolysis may also enter other pathways to synthesize components essential for biological and cellular functions. Hence, we first surveyed the expression of genes involved in glycolysis, gluconeogenesis, the citrate cycle and oxidative phosphorylation. As shown in Fig. 2, genes corresponding to most of the glycolytic enzymes, except hexokinase, aldehyde dehydrogenase and aldose 1-epimerase, were downregulated gradually during Suc starvation. Seven hexokinase genes represented on the Agilent gene chip showed antagonistic expression patterns, with only AK067988 markedly upregulated under Suc depletion. In Arabidopsis, the AK067988 ortholog encodes hexokinase 1 (AtHXK1), but its expression did not significantly change under Suc starvation (Contento et al. 2004). Since hexokinase 1 functions as a glucose sensor and triggers complicated signaling processes to maintain normal growth and development (Rolland et al. 2002), its rice ortholog may play analogous roles in Suc-starved cells. The transcriptional repression of multiple sugar metabolic enzymes caused by Suc starvation may be due to decreased cellular glucose level. To survive, the starved cells need to efficiently use the limited resources of glucose derived from stored Suc or the hydrolyzed products from starch and autophagy for ATP production.

The pyruvate produced from glycolysis is eventually oxidized to CO_2 for ATP production through the citrate cycle and oxidative phosphorylation. Most genes encoding for enzymes involved in the citrate cycle (Fig. S2A) and oxidative phosphorylation (Fig. S2B) were significantly downregulated by Suc starvation. The downregulation of genes encoding for enzymes for



glycolysis, the citrate cycle and oxidative phosphorylation may account for the decreased activities of those enzymes found in maize root tips during glucose starvation (Brouquisse et al. 1991). In the citrate cycle, only the gene encoding for phosphoenolpyruvate carboxykinase (AK102392) was highly induced by Suc starvation (Fig. S2A). Actually, this enzyme is also involved in gluconeogenesis. In animals, gluconeogenesis provides an alternative pathway to regenerate glucose from pyruvate and oxaloacetate, which are

derived from several amino acids (such as alanine, cysteine, serine and aspartate) under starvation; phosphoenolpyruvate carboxykinase may play a similar role to sustain Suc depletion in rice cells. The pentose phosphate pathway produces the raw substrate ribose phosphates for the biosynthesis of DNA and RNA, and the genes encoding for the enzymes involved in the pathway are all downregulated (Fig. S3).

Sucrose and starch are the major stored carbohydrates in plants. They are used for normal cellular metabolisms and production of glucose and fructose as well as phosphorylated monosaccharide derivatives (e.g., glucose-6-phosphate) for aerobic respiration through the glycolysis pathway. Figure 3 shows the expression of genes encoding for enzymes involved in starch and Suc metabolism, most genes being downregulated. Three genes for major starch-hydrolyzed α-amylase, AK073487 (*OsAmy3D*), AK064071 (*OsAmy3E*), and AK064300, were significantly activated by Suc starvation, as was found by Sheu et al. (1996). A similar upregulation was found in genes encoding for catabolic enzymes for trehalose degradation. This alternative degradation pathway may compensate for the Suc starvation. Trehalose, a disaccharide composed of 2 α-1,1-linked glucoses, and its derivative trehalose 6-phosphate (T6P) can modulate carbon flux into glycolysis and growth in plants (Schluepmann et al. 2003). Overexpression of T6P synthase (TPS) in Arabidopsis resulted in the accumulation of T6P and enhanced seedling growth on the sucrose-containing medium. In contrast, seedling growth was significantly inhibited in transgenic plants overexpressing the T6P degradation enzyme trehalose phosphate hydrolase (TPH) (Schluepmann et al. 2003). All these studies indicate the crucial role of T6P in regulating carbohydrate metabolism. In Arabidopsis suspension cells, the expression of 3 of 11 *TPS* transcripts was increased under Suc starvation (Contento et al. 2004). In rice

Fig. 4 Significant downregulation of enzymes involved in the biogenesis and degradation pathways of fatty acids during Suc starvation. At the onset of Suc starvation, the expression of genes involved in the biosynthesis of fatty acids are totally inhibited (**A**), but those involved in the degradation of fatty acids are gradually enhanced (**B**). The expression change of each gene is color-coded as described in Fig. 2

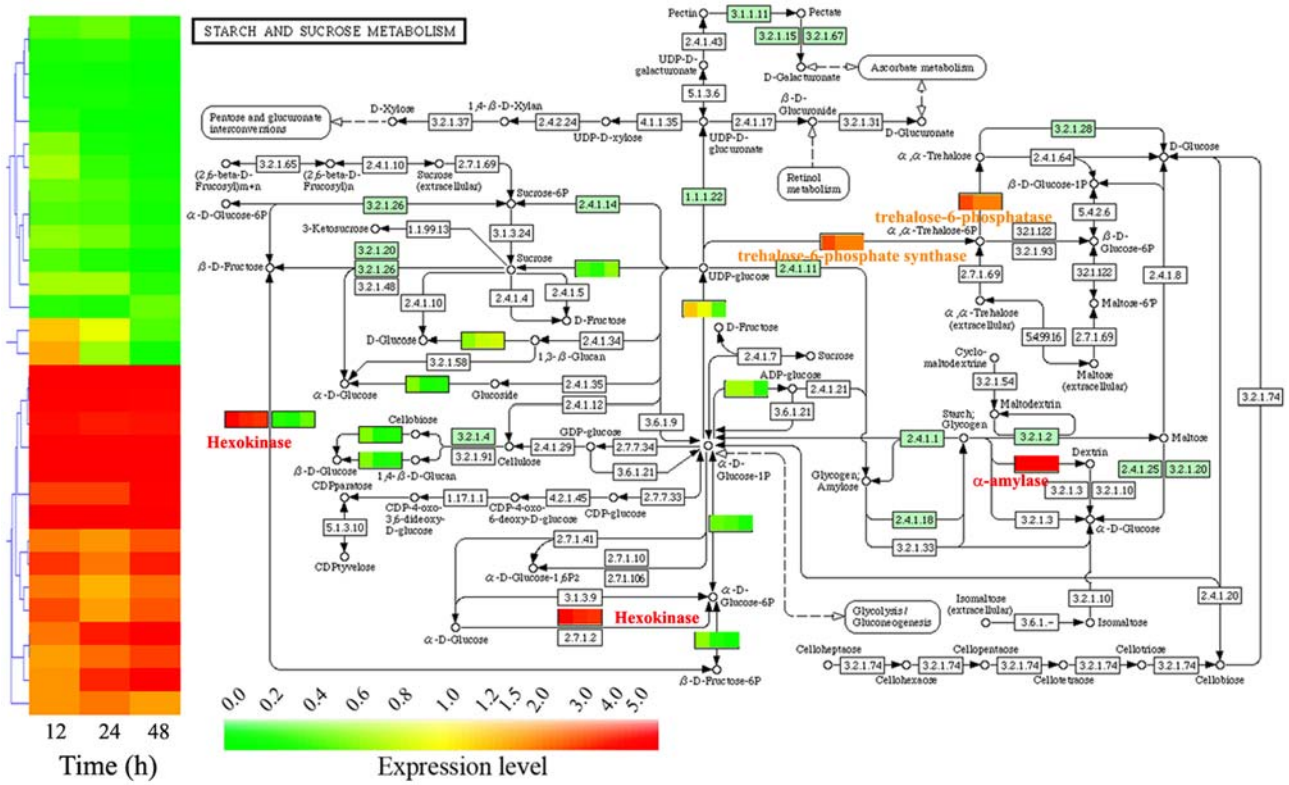
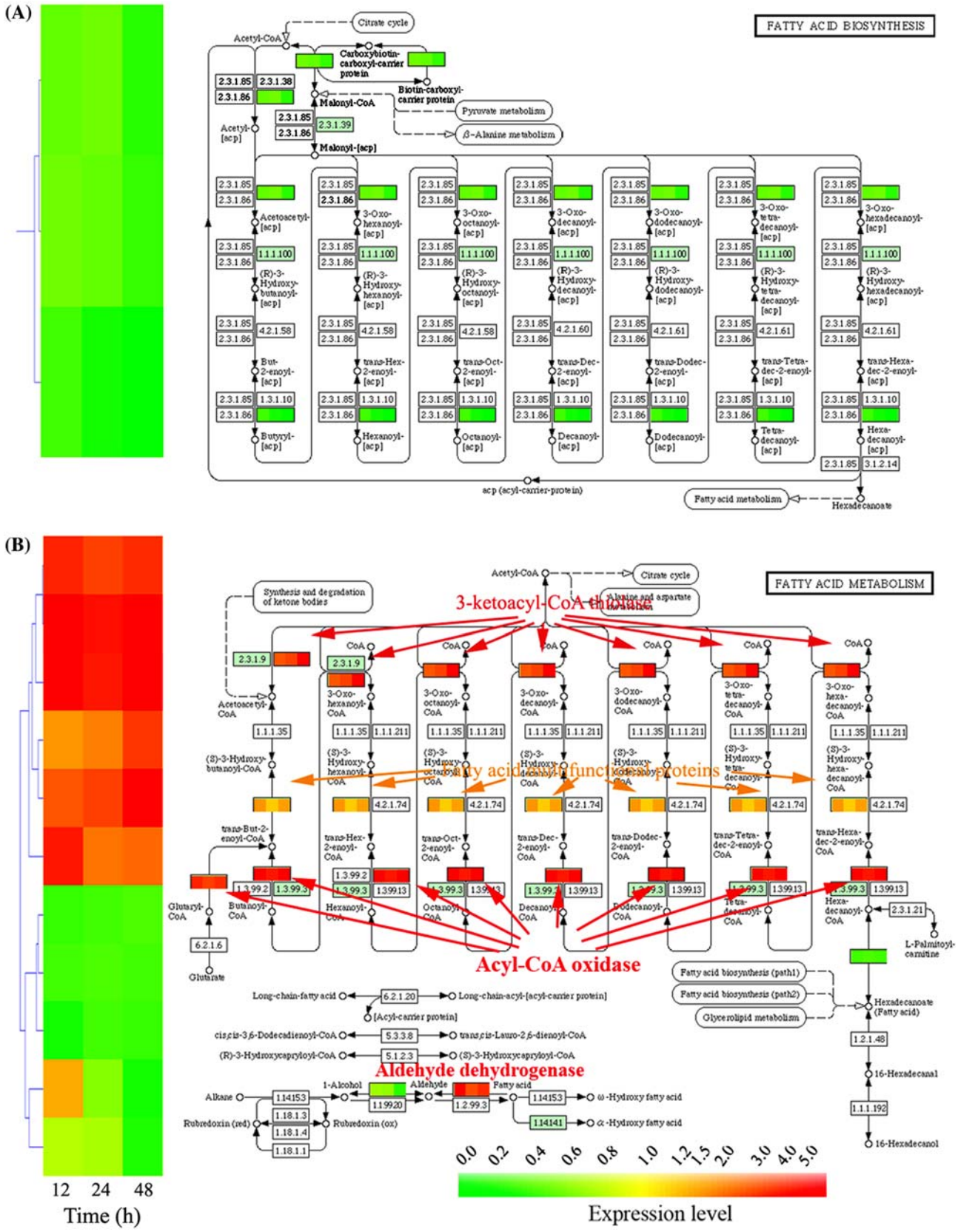


Fig. 3 Significant upregulated expression genes encoding for enzymes participating in starch and sucrose metabolism. The expression levels of genes encoding for enzymes involved in starch and sucrose metabolic pathways were sorted by gene tree. Genes encoding for the enzymes for starch and sucrose biosynthetic processes are exclusively downregulated by Suc starvation, but transcripts for the enzymes involved in the degradation of sucrose, starch and in the metabolism of trehalase are significantly induced during Suc starvation. The expression change of each gene at 3 time points is color coded as described in Fig. 2


(A)
FATTY ACID BIOSYNTHESIS
Citrate cycle
Acetyl-CoA
Carboxybiotin-carboxyl-carrier protein
Biotin-carboxyl-carrier protein
Malonyl-CoA
Pyruvate metabolism
β-Alanine metabolism
Malonyl-[acp]
Acetoacetyl-[acp]
(R)-3-Hydroxybutanoyl-[acp]
But-2-enoyl-[acp]
Butyryl-[acp]
3-Oxohexanoyl-[acp]
(R)-3-Hydroxyhexanoyl-[acp]
trans-Hex-2-enoyl-[acp]
Hexanoyl-[acp]
3-Oxooctanoyl-[acp]
(R)-3-Hydroxyoctanoyl-[acp]
trans-Oct-2-enoyl-[acp]
Octanoyl-[acp]
3-Oxodecanoyl-[acp]
(R)-3-Hydroxydecanoyl-[acp]
trans-Dec-2-enoyl-[acp]
Decanoyl-[acp]
3-Oxododecanoyl-[acp]
(R)-3-Hydroxydodecanoyl-[acp]
trans-Dodec-2-enoyl-[acp]
Dodecanoyl-[acp]
3-Oxotetradecanoyl-[acp]
(R)-3-Hydroxytetradecanoyl-[acp]
trans-Tetradec-2-enoyl-[acp]
Tetradecanoyl-[acp]
3-Oxohexadecanoyl-[acp]
(R)-3-Hydroxyhexadecanoyl-[acp]
trans-Hexadec-2-enoyl-[acp]
Hexadecanoyl-[acp]
acp (acyl-carrier-protein)
Fatty acid metabolism
Hexadecanoate
(B)
FATTY ACID METABOLISM
Synthesis and degradation of ketone bodies
3-ketoacyl-CoA thiolase
Fatty acid beta-oxidation multifunctional proteins
Acyl-CoA oxidase
Aldehyde dehydrogenase
Acetoacetyl-CoA
(S)-3-Hydroxybutanoyl-CoA
trans-But-2-enoyl-CoA
Glutaryl-CoA
Glutarate
Butanoyl-CoA
Hexanoyl-CoA
Octanoyl-CoA
Decanoyl-CoA
Dodecanoyl-CoA
Tetradecanoyl-CoA
Hexadecanoyl-CoA
L-Palmitoyl-carnitine
Fatty acid biosynthesis (path1)
Fatty acid biosynthesis (path2)
Glycerolipid metabolism
Hexadecanoate (Fatty acid)
16-Hexadecanal
16-Hexadecanol
Long-chain-fatty acid
Long-chain-acyl-[acyl-carrier protein]
[Acyl-carrier protein]
cis,cis-3,6-Dodecadienoyl-CoA
trans,cis-Lauro-2,6-dienoyl-CoA
(R)-3-Hydroxycapryloyl-CoA
(S)-3-Hydroxycapryloyl-CoA
Alkane
1-Alcohol
Aldehyde
Fatty acid
ω-Hydroxy fatty acid
α-Hydroxy fatty acid
Rubredoxin (red)
Rubredoxin (ox)
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.5 2.0 3.0 4.0 5.0
Expression level
12 24 48
Time (h)

suspension cells, Suc starvation also significantly upregulated the expression of 4 of 11 *TPS* genes (AK100050, AK072066, AK067731 and AK105382) and 1 of 3 *TPP* genes (T6P phosphatase; AK069361) (Fig. 4; Supplemental Table 1). Since T6P might function as a starvation signal, as suggested by Thimm et al. (2004), the enhanced expression of TPS may provide an alternative route to redress the sugar imbalance caused by Suc starvation.

In addition to the enzymes involved in carbohydrate degradation, the expression of the sucrose transporter (AK100027), hexose transporter (AK065247) and monosaccharide transporter (*OsMST3*, AK099079) was significantly upregulated after 12-h Suc starvation. Similar results were reported for the sucrose transporters from *Arabidopsis AtSUC1* (Contento et al. 2004) and citrus roots *CitSUC1* (Li et al. 2003) under Suc starvation. The significant activation of genes encoding for sugar transporters and degradative enzymes that accompany the repression of genes involved in biosynthetic pathways under Suc starvation may efficiently facilitate the starved cells to scavenge glucose/sucrose from various sources for survival purposes.

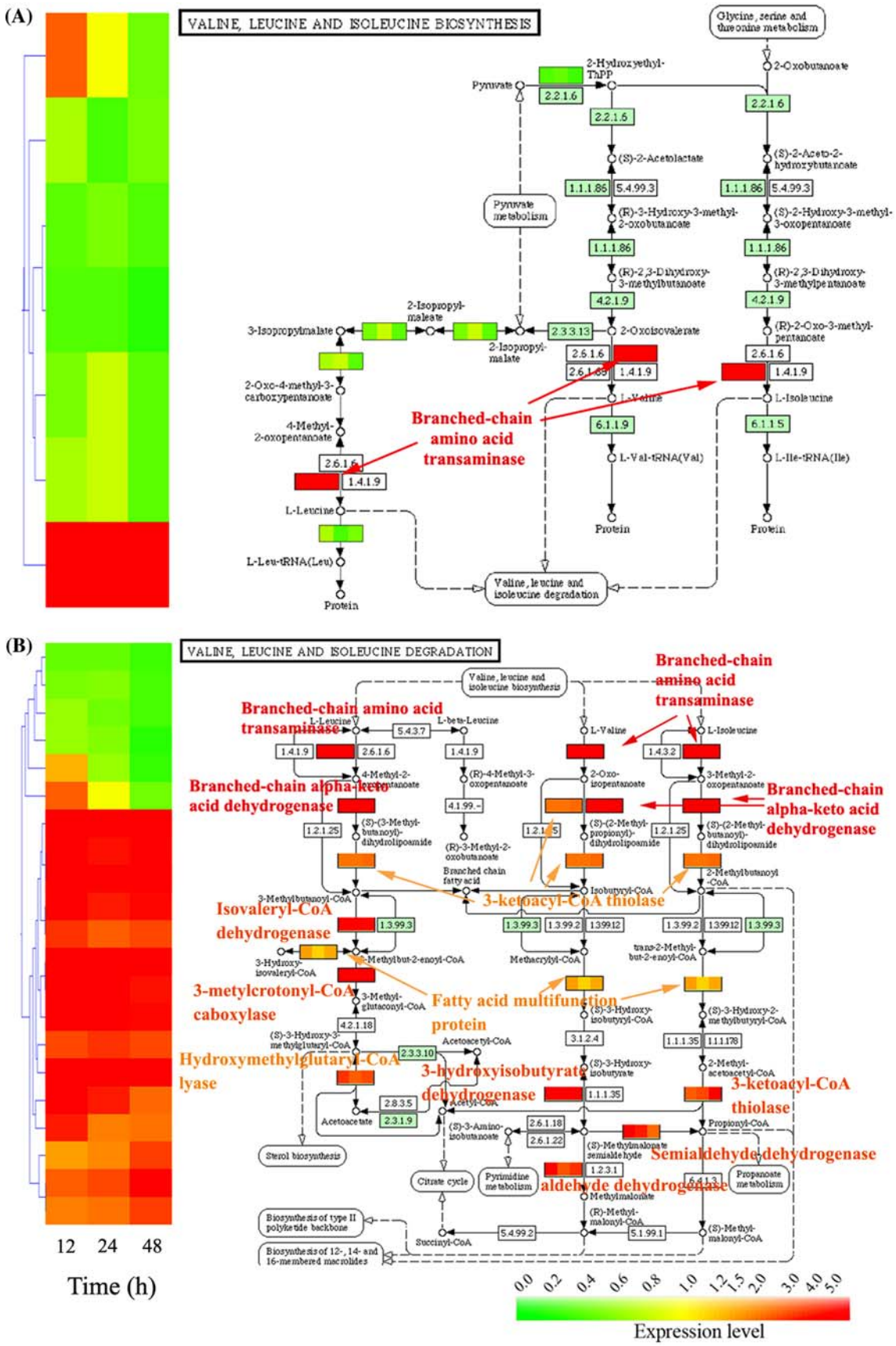
Effect of Suc starvation on the metabolism of other macromolecules

Fatty acids and amino acids not only provide the construction materials of numerous intracellular components but also function as the energy source used by the cells to overcome unfavorable situations such as nutrient deficiency. Many senescence- and starvation-activated genes encode enzymes participating in degradation of macromolecules (such as lipid and proteins) and mobilization of metabolites (Buchanan-Wollaston et al. 2005; Contento et al. 2004; Thimm et al. 2004). In glucose-starved maize root tip, the activity of fatty acid β-oxidation increased 2.5 times (Dieuaide et al. 1992). The expression of genes involved in fatty acid degradation was also induced on Suc starvation and dark-induced senescence (Dieuaide et al. 1993; Thimm et al. 2004). In our study, Suc starvation repressed the expression of transcripts involved in fatty acid synthesis but induced those involved in fatty acid degradation, such as 3-ketoacyl-CoA thiolase, fatty acid multifunctional proteins and acyl-CoA oxidase (Fig. 4). Buchanan-Wollaston et al. (2005) found similarly enhanced gene expression, except for that of 3-ketoacyl-CoA thiolase, in senescent or dark-induced senescent leaves and Suc-starved Arabidopsis suspension cells. These results suggest that the enhanced gene expression patterns and enzymatic activities involved in fatty acid β-oxidation facilitate the production of acetyl-CoA from β-oxidation of fatty acid, which may be directly utilized by respiration for energy production under Suc starvation.

Fig. 5 Significant upregulated expression of genes encoding for ▶ enzymes involved in branched-chain amino acid metabolism. The expression of enzymes involved in biogenesis and degradation pathways of branched-chain amino acids are shown in **A** and **B**, respectively. The expression of genes for biogenetic pathways of branched-chain amino acids is downregulated, except for the branched-chain amino acid transaminase, which is also involved in the degradative process. Genes involved in degradative pathways of branched-chain amino acids are all upregulated during Suc starvation. The expression change of each gene are color coded as described in Fig. 2

Branched-chain amino acids composed of a long hydrocarbon chain (e.g., valine, leucine and isoleucine) with the equivalent properties of energy generation as fatty acids, play a critical role in biosynthesis of proteins and other secondary metabolites in plants. In castor bean and maize, leucine participates in gluconeogenesis (Stewart and Beever 1967) and in conversion of leucine to starch, sugars, or organic acids (Sodek and Wilson 1973). Aubert et al. (1996a) observed the induction of leucine catabolism in Suc-starved sycamore cells. Recent studies by Schuster and Binder (2005) showed that Arabidopsis mitochondrial branched-chain amino acid transaminases (BCATs) participate in degradation of leucine, isoleucine and valine. Here, we found that 2 rice BCAT genes (AK062472 and AK105963, Supplemental Table 1) and nearly all genes involved in the degradation pathway were remarkably upregulated upon Suc starvation, whereas the genes encoding for enzymes of the branched-chain amino acid biogenesis pathway were downregulated (Fig. 5). Recently, Ishizaki et al. (2005) found that the expression of electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO), a nuclear-encoded mitochondrial protein involved in the catabolism of leucine and potentially of several aromatic amino acids, was essential for plant survival under long-term dark-induced senescence. In our array data, rice ETFQO (AK065934) was upregulated by 3-, 1.8- and 1.5-fold after 12-, 24-, and 48-h Suc starvation, respectively. All these results suggest an important role of degradation of branched-chain amino acids in plants for competitive survival under adverse conditions.

Suc starvation also represses the expression of genes for biosyntheses of DNA, RNA (purine and pyrimidine, Fig. S4) and protein (aminoacyl-tRNA and ribosome biosynthesis, Fig. S5). A similar downregulated pattern under Suc starvation was found in the Arabidopsis orthologs (Contento et al. 2004; Fujiki et al. 2001; Thimm et al. 2004). Reduction of these biosynthetic pathways may preserve the chemical energy and carbon/nitrogen sources, which are ultimately used for survival strategy.


(A)
VALINE, LEUCINE AND ISOLEUCINE BIOSYNTHESIS
Glycine, serine and threonine metabolism
Pyruvate
2-Hydroxyethyl-ThPP
2-Oxobutanoate
(S)-2-Acetolactate
(S)-2-Aceto-2-hydroxybutanoate
Pyruvate metabolism
(R)-3-Hydroxy-3-methyl-2-oxobutanoate
(S)-2-Hydroxy-3-methyl-3-oxopentanoate
(R)-2,3-Dihydroxy-3-methylbutanoate
(R)-2,3-Dihydroxy-3-methylpentanoate
3-Isopropylmalate
2-Isopropyl-maleate
2-Isopropyl-malate
2-Oxoisovalerate
(R)-2-Oxo-3-methyl-pentanoate
2-Oxo-4-methyl-3-carboxypentanoate
4-Methyl-2-oxopentanoate
L-Valine
L-Isoleucine
Branched-chain amino acid transaminase
L-Val-tRNA(Val)
L-Ile-tRNA(Ile)
L-Leucine
L-Leu-tRNA(Leu)
Protein
Valine, leucine and isoleucine degradation
(B)
VALINE, LEUCINE AND ISOLEUCINE DEGRADATION
Valine, leucine and isoleucine biosynthesis
Branched-chain amino acid transaminase
L-beta-Leucine
Branched-chain alpha-keto acid dehydrogenase
3-Methyl-2-oxopentanoate
(R)-4-Methyl-3-oxopentanoate
2-Oxo-isopentanoate
(S)-(3-Methyl-butanoyl)-dihydrolipoamide
(S)-(2-Methyl-propionyl)-dihydrolipoamide
(S)-(2-Methyl-butanoyl)-dihydrolipoamide
Branched chain fatty acid
2-Methylbutanoyl-CoA
3-Methylbutanoyl-CoA
Isobutyryl-CoA
3-ketoacyl-CoA thiolase
Isovaleryl-CoA dehydrogenase
3-Hydroxy-isovaleryl-CoA
2-Methylbut-2-enoyl-CoA
Methacrylyl-CoA
trans-2-Methyl-but-2-enoyl-CoA
3-methylcrotonyl-CoA caboxylase
3-Methyl-glutaconyl-CoA
Fatty acid multifunction protein
(S)-3-Hydroxy-isobutyryl-CoA
(S)-3-Hydroxy-2-methylbutyryl-CoA
(S)-3-Hydroxy-3-methylglutaryl-CoA
Acetoacetyl-CoA
Hydroxymethylglutaryl-CoA lyase
3-hydroxyisobutyrate dehydrogenase
(S)-3-Hydroxy-isobutyrate
2-Methyl-acetoacetyl-CoA
3-ketoacyl-CoA thiolase
Acetyl-CoA
Acetoacetate
(S)-3-Amino-isobutanoate
Propionyl-CoA
(S)-Methylmalonate semialdehyde
Semialdehyde dehydrogenase
Sterol biosynthesis
Citrate cycle
Pyrimidine metabolism
aldehyde dehydrogenase
Propanoate metabolism
Methylmalonate
(R)-Methyl-malonyl-CoA
(S)-Methyl-malonyl-CoA
Biosynthesis of type II polyketide backbone
Biosynthesis of 12-, 14- and 16-membered macrolides
Succinyl-CoA
12 24 48
Time (h)
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.5 2.0 3.0 4.0 5.0
Expression level

The target of rapamycin (TOR), a protein kinase found in all eukaryotic cells, acts as an energy sensor in governing protein and ribosome biosynthesis, transcription regulation and autophagy (Dennis et al. 2001; Hay and Sonenberg 2004; Schmelzle and Hall 2000). In animals and yeast, nutrient deficiency inhibits TOR activity, which in turn decreases ribosome synthesis and eventually triggers autophagic machinery (Schmelzle and Hall 2000). The TOR and the regulatory-associated protein for TOR (RAPTOR) orthologs have been found in Arabidopsis and are necessary for embryonic development (Deprost et al. 2005; Menand et al. 2002). In addition, the TOR-RAPTOR interaction is involved in the regulation of cellular metabolic adjustment to osmotic stress through modulating the activity of S6 kinase in Arabidopsis (Mahfouz et al. 2006). Downregulation of S6 kinase activity under stress slows protein synthesis and ribosome biogenesis. Coupled with the induction of autophagic machinery, a putative rice TOR kinase signaling pathway may also be responsible for downregulation of protein/ribosome biosynthesis in Suc-starved rice cells.

Trans-factors and *cis*-elements in response to Suc starvation

Suc starvation causing multiple metabolic pathway changes at transcription levels suggests the involvement of complicated but coordinated transcriptional regulation by different TFs. Identifying the potential *trans*-factors and their responsible *cis*-elements in the promoter regions of Suc starvation-induced upregulated genes is crucial. We analyzed the genes encoding for potential TFs from our DNA microarray data to survey the potential Suc starvation-responsive TFs. Among 1145 TFs genes, 66 and 37 showed significant up- and downregulation, respectively, during Suc starvation and were further classified into 32 TF families (Fig. 6). Although the downregulated TFs certainly played important roles in response to Suc starvation, we focused on the upregulated TF families in this study. Ten TF families, members in each family shown totally upregulated by Suc starvation, were SBP, TAZ, bZIPs, GRAS, ZIM, EIL, NAC, HSF, TUB and WRKY (Fig. 6 and Supplemental Table 2).

To investigate the putative Suc-starvation responsive elements existing in the promoters of all upregulated genes, we analyzed their 500-bp upstream genomic sequences of the transcription start site (TSS) by ''Find Potential Regulatory Sequence'' in GeneSpring 7.2, using the criteria $P < 0.01$ and net expression intensity >200. Several motifs predominant in the promoter regions of upregulated genes showed enhanced expression greater than 2- and 3-fold (Table 2-I). Coordinately, the potential TFs binding to these consensus *cis*-elements, such as the MYB, NAC and bZIP, were also significantly upregulated under Suc depletion (Fig. 6; Table 2-I). Among these *cis*-elements, the ACGTG motif contains

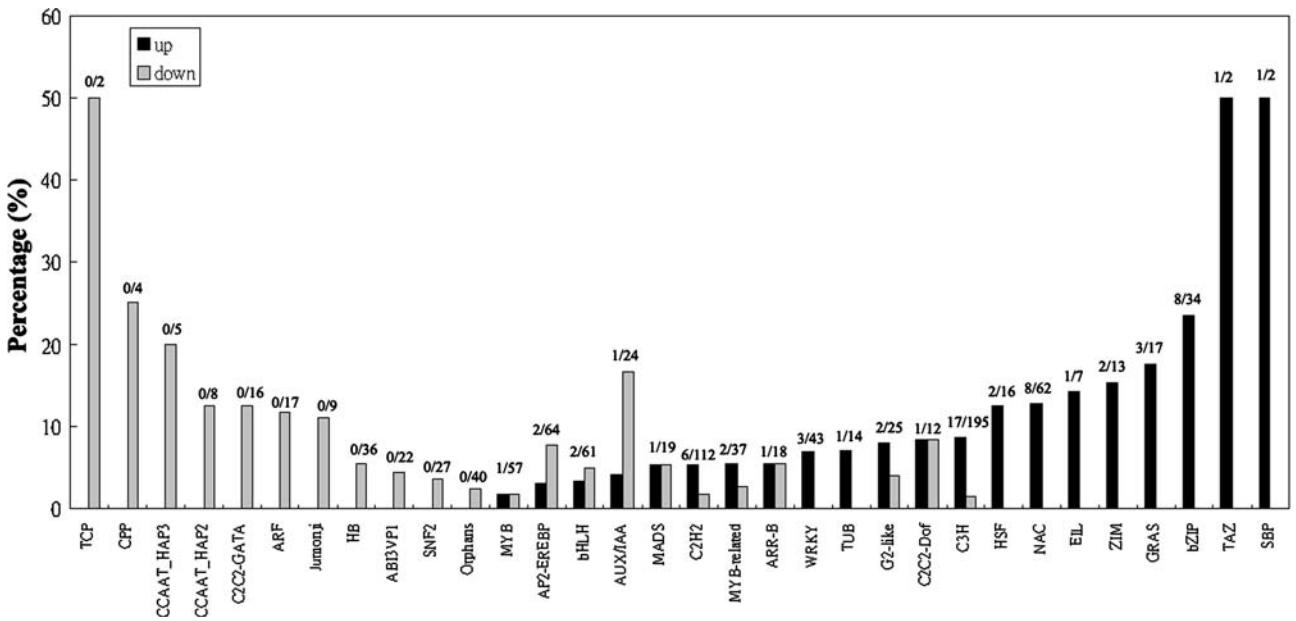


Fig. 6 Classification and expression of transcription factor families in response to Suc starvation. Considerably up- and downregulated genes encoding for dozens of transcription factors after SAM analysis were chosen for investigation. To present the expression behavior of individual gene family, the percentage of upregulated (black bar) or downregulated (gray bar) genes was calculated on the basis of total number of genes in each family member. In each family, only the significantly upregulated gene numbers versus the total gene numbers are indicated on the top of bars

Table 2 Putative Suc starvation elements presented in the promoter regions of Suc-starvation upregulated genes[a]

Sequence [b]	Frequency (%)[c]	*P*-value[d]	*cis*-Element core	Potential TFs	Reference
I. 851 genes with enhanced expression greater than 2-fold and intensity >200					
ACGTG	340/851 (40)	6.91E–04	ABRE	b-ZIP	Izawa et al. (1993)
CACGC	385/851 (45)	1.26E–07	CACG	NAC	Tran et al. (2004)
CACGCA	146/851 (17)	3.63E–03	CACG	NAC	Tran et al. (2004)
CACGCG	160/851 (19)	2.26E–04	CACG	NAC	Tran et al. (2004)
CCACGC	180/851 (21)	9.60E–03	CACG	NAC	Tran et al. (2004)
CGCACG	139/851 (16)	9.56E–03	CACG	NAC	Tran et al. (2004)
GCACGC	143/851 (17)	2.50E–03	CACG	NAC	Tran et al. (2004)
414 genes with enhanced expression greater than 3-fold and intensity >200					
ACGTG	181/414 (44)	1.76E–03	ABRE	b-ZIP	Izawa et al. (1993)
ATCCA	252/414 (61)	4.46E–03	TATCCA	MYB	Lu et al. (2002)
CACGC	139/414 (34)	4.30E–03	CACG	NAC	Tran et al. (2004)
CTTATCC	43/414 (10)	1.06E–03	TATCCA	MYB	Lu et al. (2002)
II. 515 genes with enhanced expression greater than 2-fold and intensity >500					
CCCCAC	153/515 (30)	1.24E–03	ACI, ACII	MYB	Sablowski et al. (1994)
CCCCCC	106/515 (21)	4.11E–03	n/a	n/a	
CGCCCC	93/515 (18)	8.06E–03	n/a	n/a	
TCCTCC	189/515 (37)	1.05E–03	n/a	n/a	
TTCCTC	157/515 (30)	2.21E–03	n/a	n/a	
CTTATCC	50/515 (10)	2.75E–03	TATCCA	MYB	Lu et al. (2002)
CCTTATCC	30/515 (6)	9.68E–06	TATCCA	MYB	Lu et al. (2002)
252 genes with enhanced expression greater than 3-fold and intensity >500					
CTTATCC	34/252 (13)	1.11E-04	TATCCA	MYB	Lu et al. (2002)
CCTTATCC	19/252 (8)	8.43E-04	TATCCA	MYB	Lu et al. (2002)

[a] Consensus sequences exist in the 500-bp upstream relative to TSS of genes upregulated more than 2- and 3-fold under Suc starvation at least in one time point examined

[b] Underline sequences are the conserved motifs revealed from the literature

[c] The frequency was calculated as the number of genes containing indicated *cis*-elements among the total upregulated genes

[d] $P < 0.01$ was considered significant for presence of sequences as consensus *cis*-elements in the promoter regions

the central core sequences for the well-known *cis*-element ABRE (Hobo et al. 1999a; Shen and Ho 1997; Zhang et al. 2005). The ABRE motif is prominently located in the promoter regions of ABA- and abiotic stress-induced genes (Kim et al. 2004). The TFs often associated with this module are bZIPs, including ABF2, HvABI5, and TRAB1 (Casaretto and Ho 2003; Hobo et al. 1999b; Kim et al. 2004). Interestingly, their orthologs in rice, such as AK107021 (Arabidopsis *bZIP*), AK072062 (*TRAB1*) and AK105312 (*HvABI5*), were simultaneously and significantly upregulated by Suc starvation (Supplemental Table 2). In Arabidopsis, Suc starvation and dark-induced senescence also induce the expression of several *bZIP* genes (Contento et al. 2004; Lin and Wu 2004). All these results suggest that the interaction of the consensus ABRE and bZIP may transactivate the expression of the ABRE-containing genes under Suc starvation.

CACG, the core DNA binding site for the NAC (NAM, ATAF1, 2, CUC2), is another consensus *cis*-element in the promoters of genes upregulated greater than 2- and 3-fold under Suc starvation. Simultaneously, 8 rice *NACs* showed significant induction by Suc starvation (Fig. 6), and similar enhanced expression of 24 *NACs* has been reported in Arabidopsis senescent leaves (Buchanan-Wollaston et al. 2005). The binding of upregulated NAC proteins to the consensus CACG core in the target genes may also transactivate the expression of the downstream gene under Suc starvation. NACs are plant specific, and abundant TFs with more than 100 members are present in the Arabidopsis genome (Olsen et al. 2005). They play diverse roles in the plant life cycle: defense (Collinge and Boller 2001; Ren et al. 2000; Xie et al. 1999), abiotic stress response (Fujita et al. 2004; Hegedus et al. 2003; Tran et al. 2004) and normal developmental programs, including the formation of the shoot apical meristem, floral organ, lateral shoot and secondary cell wall thickening in various tissues (Sablowski and Meyerowitz 1998; Souer et al. 1996; Takada et al. 2001; Vroemen et al. 2003; Xie et al. 2000).

To precisely identify the promising Suc-starvation responsive elements responsible for high gene expression, we used higher selection criteria for gene expression, $P < 0.01$ and net expression intensity

>500, to identify another set of potential *cis*-elements by Gene Spring 7.2 analysis (Table 2-II). Several are uncharacterized, such as CCCCCC, CGCCCC, TTCCTC and TCCTCC, but one (CCCCAC) belongs to the AC-elements, ACI and ACII, and their putative responsible TFs are MYBs (Sablowski et al. 1994). The AC elements were first identified in the promoter of bean phenylalanine ammonia-lyase gene *PAL2*, in the phenylpropanoid biosynthetic pathway, which plays diverse functions during plant development (Hatton et al. 1995). Another set of interesting *cis*-elements is the CTTATCC/CCTTATCC sequences, which are the only motifs found in the promoter regions of genes with >3-fold enhanced expression and net expression intensity >200 (Table 2-I) and > 2- and 3-fold enhanced expression and net expression intensity >500 (Table 2-II). The sequences of CTTATCC/CCTTATCC are similar to the TATCCA motif, a component of the GA response complex (GARC) and sugar response sequence (SRS) localized inside the promoters of all α-amylase genes isolated from rice, barley and wheat (Yu 1999a, b). The TATCCA motif was first identified in the promoter regions of *OsAmy3C* and *OsAmy3D* (AK101358 and AK073487), which were recognized by OSMYBS1, OSMYBS2 and OSMYBS3, but only OSMYBS1, OSMYBS2 TFs transactivated the expression of *OsAmy3D* during Suc starvation (Lu et al. 1998; 2002).

Members of several TF families involved in biotic and abiotic stress responses, also notably upregulated by Suc deficiency, are heat shock transcription factors (HSFs) and WRKY genes. HSFs are significantly activated under heat shock and drought stress to activate target genes that trigger several resistant responses such as anti-oxidation, Ca^{2+} influx and refolding of denatured proteins to enhance thermotolerance (Lee et al. 1995; Panchuk et al. 2002; Prandl et al. 1998). Our array results showed that 2 *HSF* genes were upregulated under Suc starvation. Simultaneously, several prospective targeted genes of HSFs were also significantly upregulated, including the transcripts for glutathione peroxidase (AK062772 and AK101583), which may scavenge ROS (reactive oxygen species) produced under Suc starvation. In Arabidopsis, Suc starvation and dark-induced senescence also induce the expression of several *HSF* genes (Contento et al. 2004; Lin and Wu 2004). Recently, a transcriptomic analysis from the Arabidopsis *hsfA1a/hsfA1b* double-mutant line showed that HSFs regulate a number of genes involved in protein biosynthesis, protein processing and carbohydrate metabolism (Busch et al. 2005), which correlates well with our metabolic results mentioned above. These results suggest the important regulatory roles of these HSFs in Suc-starved rice suspension cells. The WRKY proteins mediating many cellular events in response to biotic and abiotic stresses, Suc starvation and senescence (Contento et al. 2004; Laloi et al. 2004; Li et al. 2004; Lin and Wu 2004; Mare et al. 2004; Miao et al. 2004) were also preferably upregulated under Suc starvation. In our promoter analysis, the responsible *cis*-elements for HSFs and WRKYs could not be identified, possibly because the numbers of their target genes are too few to show statistical significance by the selection thresholds used. Although the mechanisms of regulation of these TFs in Suc-starved cells are still unclear, our gene expression profile provides tremendous information regarding the complicated cross-talk and/or coordinated networks of these TFs in response to Suc starvation and other stress at the transcriptional level.

Characterization of promising Suc-starvation responsive elements under Suc starvation

To explore the role of the CTTATCC motif functioning as the promising Suc-starvation responsive element, we surveyed the top 20 Suc starvation-upregulated genes containing this element in their promoters. As shown in Table 3, CTTATCC, not TATCCA, is the most conserved motif found in all Suc starvation-upregulated genes, including 3 *OsAmy3s*. Surprisingly, *OsAmy3D* (AK073487) and 3 genes for unknown proteins all have 2 copies of CTTATCC elements in their promoter regions. In fact, these three major CTTATCC motif-containing genes shown in Table 3 fall into the top-10 highest expressed genes among all Suc starvation-upregulated genes we found (Supplementary Table 1). All these results imply that CTTATCC may function as a promising *cis*-element in response to Suc starvation.

Proper and defined locations of *cis*-elements within the promoter region are important for their functions in regulating gene expression. For example, ABRE are usually located 200 bases upstream from the TSS of targeted genes (Zhang et al. 2005). Therefore, we analyzed the distribution of several consensus *cis*-elements shown in Table 2 in the selected promoter regions (~50 to ~500 bp from TSS) of all Suc starvation-upregulated genes by ''Find potential regulatory sequence'' in GeneSpring 7.2. As shown in Fig. 7, the *cis*-elements acquired with less rigorous criterion (Fig. 7A, net expression intensity >200, Table 2-I) showed a more broad distribution pattern, within 400 bases of TSS and within 5′ UTRs, but *cis*-elements acquired from a more rigorous criterion (Fig. 7B, net expression intensity >500, Table 2-II) were predominantly

Table 3 List of top 20 genes significantly upregulated under Suc starvation that contain the CTTATCC Suc-starvation response elements in their promoter regions

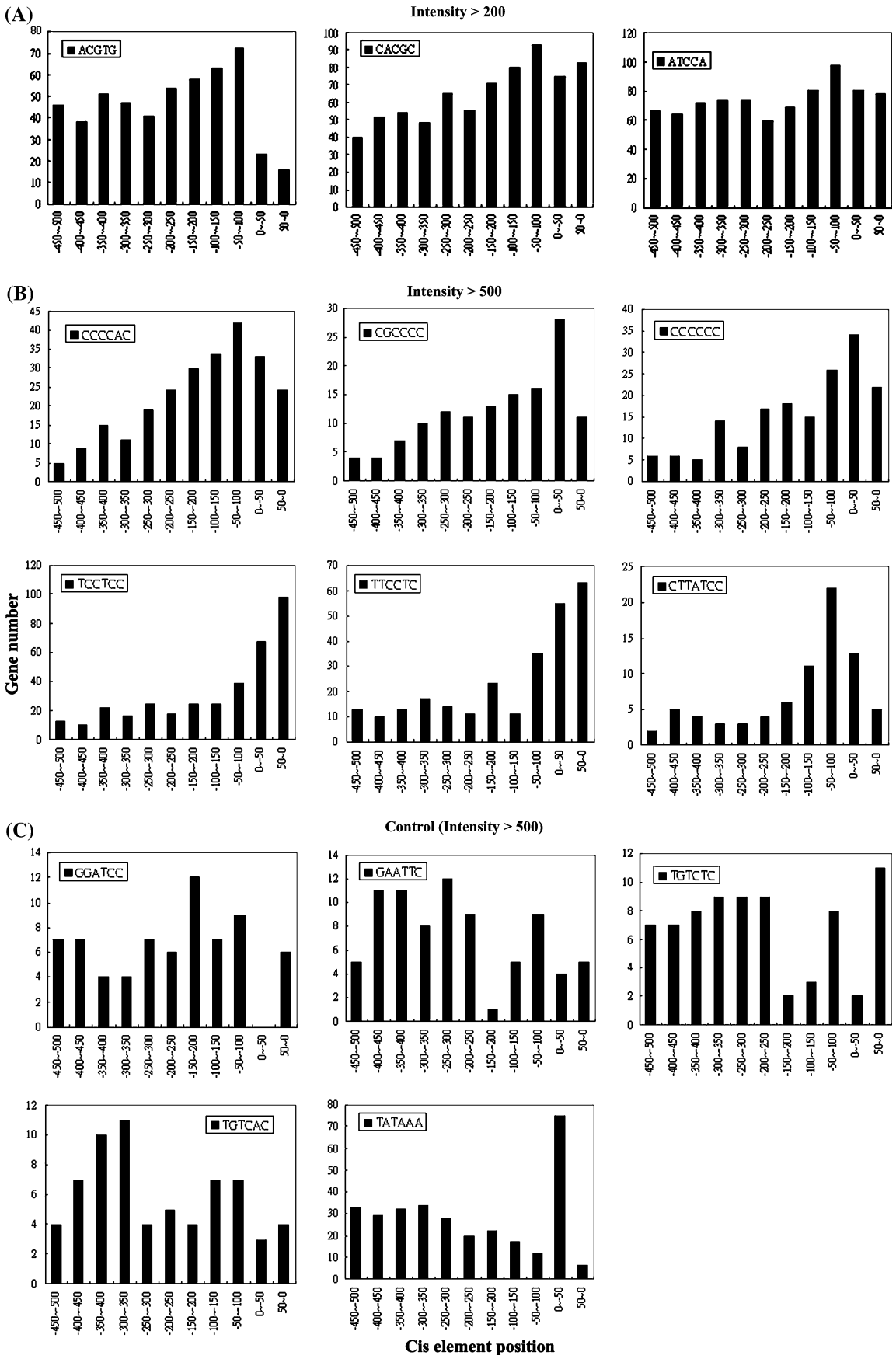
Accession number	12[a]	24	48	Annotation	Position in promoter[b]	*cis*-Element sequence
AK073487	16.46	15.94	29.41	Rice α-amylase (OsAmy3D)	–195	AAATGCTCGC CTTATCC AAGCCGGCGA
					–50	GCTTTATTGC CTTATCC ATATCCACGC
AK064071	7.12	9.42	27.43	Rice α-amylase (OsAmy3E)	–118	ATCCATGTTG CTTATCC GTGAATTGCA
AK064300	8.13	8.66	25.88	Rice α-amylase	–118	ATCCATGTTG CTTATCC GTGAATTGCA
AK064050	7.74	8.99	24.60	Rice Bowman-Birk type trypsin inhibitor	–99	GCCACGTGCT CTTATCC ACACAATTAA
AK061614	4.42	6.60	7.40	Unknown expressed protein	–17	TCCCCACCTC CTTATCC AATCCCAACT
AK102516	2.30	3.80	7.40	Unknown expressed protein	–43	CCTACCCATC CTTATCC TCTCATTCCT
AK060977	4.16	5.18	6.67	Unknown expressed protein	–126	CCTCATTGCT CTTATCC GATCACCATT
					–75	TGCTGGTGAT CTTATCC CCATAGTTTG
AK062480	4.54	4.42	5.79	Unknown expressed protein	–350	GATGGACTCA CTTATCC AAAACAAAAA
					–76	CATATACGGC CTTATCC ACTCCTACCG
AK063701	3.22	5.24	5.40	Unknown expressed protein	–150	TAAACATTGC CTTATCC ATCCATCCAC
AK065103	5.66	3.74	4.99	Unknown expressed protein	–427	ATGTGGAGGC CTTATCC ACTCTCTCCC
					–164	CTCGCTCGTT CTTATCC TCCTCTGCCA
AK069768	2.27	2.58	4.73	Rice 3-ketoacyl-CoA thiolase	–54	AAGTGGGCAG CTTATCC GGTAGTGGCG
AK065906	2.08	4.76	4.51	Unknown expressed protein	–70	CTCCATCGTT CTTATCC TCTCCACCAC
AK068593	1.52	2.85	4.37	Arabidopsis DNA-binding protein	–87	GCAACATGTC CTTATCC CTCTATGTCA
AK058349	2.12	3.28	4.19	Unknown expressed protein	–136	CCCACCTGAC CTTATCC CTTCGCCCCC
AK059150	4.05	3.63	4.09	Rice LIP5	–89	TCTTTTCCTC CTTATCC GATGGGCCTT
AK072062	1.46	2.22	4.09	Rice (japonica cultivar-group) TRAB1	–269	ATTAAAATAC CTTATCC CACTCGACCC
AK102970	1.55	2.61	4.02	Rice PR thaumatin-like protein	–422	TAGCTCCTTT CTTATCC TGGGTTGAAC
AK107771	2.33	3.59	3.94	Unknown expressed protein	–58	CATGCACATC CTTATCC TCTCTACAGT
AK105218	2.56	3.80	3.71	Unknown expressed protein	–90	GGTCAACATC CTTATCC ACTCGCTTCT
AK068392	1.92	1.76	3.68	Rice OsNAC6 protein	–434	AGACCGCATC CTTATCC CACCACGTCC

[a] Fold change between the normalized intensities of starved and control cells at the time point indicated. The fold change values shown in this table were calculated using the mean ratio of three sets of biological replicates

[b] The positions of the CTTATCC *cis*-element refer to the upstream of the TSS

located 150–200 bases from TSS and within 5′ UTRs. For the positive control, the well-known TATA box was chosen, and most were within 50 bases of TSS; for the negative controls, DNA sequences of 2 auxin response elements [ER7 (TGTCTC) and ER9 (TGTCAC)] and 2 restriction enzymes sites [*Bam*HI (GGATCC) and *Eco*RI (GAATTC)] were randomly distributed within the promoter regions (Fig. 7C).

The above results suggest that the *cis*-elements listed in Table 2-II are the promising elements involved in upregulated gene expression under Suc starvation. Particle bombardment-mediated and luciferase activity-based transient promoter functional assays were conducted to examine the functions of these elements in upregulating gene expression under Suc starvation. Since only a small proportion of the CTTATCC and AC elements have been well characterized to date, we chose the region ~ –50 to ~ 500 bp from the TSS promoter portion containing these elements from several genes highly upregulated on Suc starvation, except for *OsAmy3s*, for further investigation. For the CTTATCC *cis*-element, we selected AK064050 (Bowman-Birk-type trypsin inhibitor, pA1), AK102516 (unknown expressed protein, pA2), AK063701 (unknown expressed protein, pA3) and AK069768 (3-ketoacyl-CoA thiolase, pA4). A luciferase reporter gene driven by the portion of promoter containing the intact (pAsLuc in Fig. 8A) or deleted CTTATCC motifs (pAsLucΔ in Fig. 8A) was bombarded into rice suspension cells, followed by Suc depletion for 18 h; then the relative luciferase activity was measured. The *OsAmy3D* promoter containing (p3Luc.18 in Fig. 8B) and lacking (p3Luc.34 in Fig. 8B) the 2×TATCCA elements fused to luciferase reporter genes were used as positive and negative controls, respectively. As shown in Fig. 8B,


(A)
Intensity > 200
ACGTG
CACGC
ATCCA
(B)
Intensity > 500
CCCCAC
CGCCCC
CCCCCC
TCCTCC
TTCCTC
CTTATCC
Gene number
(C)
Control (Intensity > 500)
GGATCC
GAATTC
TGTCTC
TGTCAC
TATAAA
Cis element position

◀**Fig. 7** Position statistics of putative Suc-starvation responsive elements present in all Suc-starvation upregulated genes. The promoter regions (500-bp upstream relative to TSSs) of Suc starvation upregulated genes were used to investigate the distribution of the start positions of 9 *cis*-elements selected from Table 2. The *cis*-elements located in the promoter regions of upregulated genes selected by two different thresholds of expression levels are shown; the net expression intensity is >200 (**A**) and > 500 (**B**). (**C**). The TATA box (TATAAA) as well as the DNA sequences of 2 auxin response elements (ER7,TGTCTC, and ER9, TGTCAC) and 2 restriction enzymes (*Bam*HI, GGATCC, and *Eco*RI, GAATTC) were included as positive and negative controls, respectively, to specify the distribution of these putative Suc-starvation responsive elements in the promoter regions of upregulated genes

Suc starvation strikingly enhanced luciferase activity driven by the intact but not mutated *OsAmy3D* promoter, as was described by Lu et al. (1998). Similar Suc starvation-enhanced luciferase activity, but to a lesser extent as compared with that of p3Luc.18, was found in all tested intact promoters, except pA4 and pB2. As expected, the luciferase activity was reduced as the CTTATCC motif was removed from the examined promoters. For AC elements, the promoters of AK068638 (glycolate oxidase, pB1) and AK073181 (unknown expressed protein, pB2) showed similar tendencies as the CTTATCC motif in response to Suc starvation. Nevertheless, the luciferase activities in pA2LucΔ, pA4LucΔ and pB2LucΔ were repressed, even under normal growth conditions which suggests the essential role of these motifs in basal gene expression.

The promoter activity assay results show that both CTTATCC and AC elements are functional *cis*-activators in response to Suc starvation in rice suspension cells; however, their effect in enhancing luciferase activity does not completely reflect their comparable upregulation found in array data. For example, from the array data, the gene expression of Bowman-Birk-type trypsin inhibitor (pA1) enhanced by Suc starvation was much higher than that of the unknown expressed protein (pA3) (Table 3), but results of promoter activity assays of these 2 promoter portions showed their similar luciferase activity in response to Suc starvation (Fig. 8B) The possible explanations for such controversial results is that other important *cis*-elements present in or upstream of the selected promoter region are also required to coordinate the regulation of native gene expression under Suc starvation. The promoter of *OsAmy3D* contains additional ABRE and GC boxes located upstream near the 2×TATCCA box, and they function coordinately and result in extremely high upregulation of *OsAmy3D* in both degree of change and net expression intensity under Suc starvation (Lu et al. 1998; Fig. 8B). In addition, Lu et al. (2002) showed that OSMYBS1 and OSMYBS2 bind to 2 adjacent TATCCA boxes in a much tighter manner than to a single copy of a TATCCA box. Another possibility for conflicting results is due to the mRNA stability of the selected genes under Suc starvation, which is not truly reflected by the reporter gene, the luciferase. For example, the mRNA of *OsAmy3D* was significantly stabilized to increase the total amount of transcripts under Suc starvation (Sheu et al. 1996). Further identification of other functional *cis*-elements and corresponding *trans*-factors for these upregulated genes will provide a comprehensive understanding in the transcriptional regulation of gene expression in response to Suc starvation.

Conclusions

In this study, we provided cellular and transcriptomic results to reveal the potential mechanisms involved in rice suspension cells in response to nutrient, sucrose, depletion. To adapt to this nutrient stress, cells first undergo morphological modification by increasing vacuolar biogenesis, as suggested by the potential autophagy-like machinery, and enhance the expression of genes encoding for the vacuolar tonoplast proteins and lumen proteases to recycle degraded nutrients for survival. As the nutrient deficiency advances, the entire metabolism in the cells shows adverse regulation in gene expression profile; all of the genes encoding for enzymes involved in biosynthetic pathways for macromolecules are significantly downregulated, but those involved in the catabolic pathways to degrade macromolecules and sucrose for ATP production are comprehensively upregulated. To regulate the expression of those genes in response to Suc starvation, various kinds of TFs are induced coordinately, as evidenced by the presence of several *cis*-element cores for these TFs in the promoter regions of upregulated genes. Among the putative Suc-starvation responsive elements identified in this study, promising elements are the CTTATCC and AC elements recognized by putative OSMYBs predominately located 150–200 bases from TSS regions of several highly upregulated genes. Our transient promoter activity assay also revealed that they are functional Suc-starvation responsive *cis*-activators. Nevertheless, further experimental examination of the function of individual TFs, *cis*-elements of targeted genes and unidentified *OsATG* genes are required to explore the fundamental strategies and mechanisms the cells use to survive under Suc starvation.

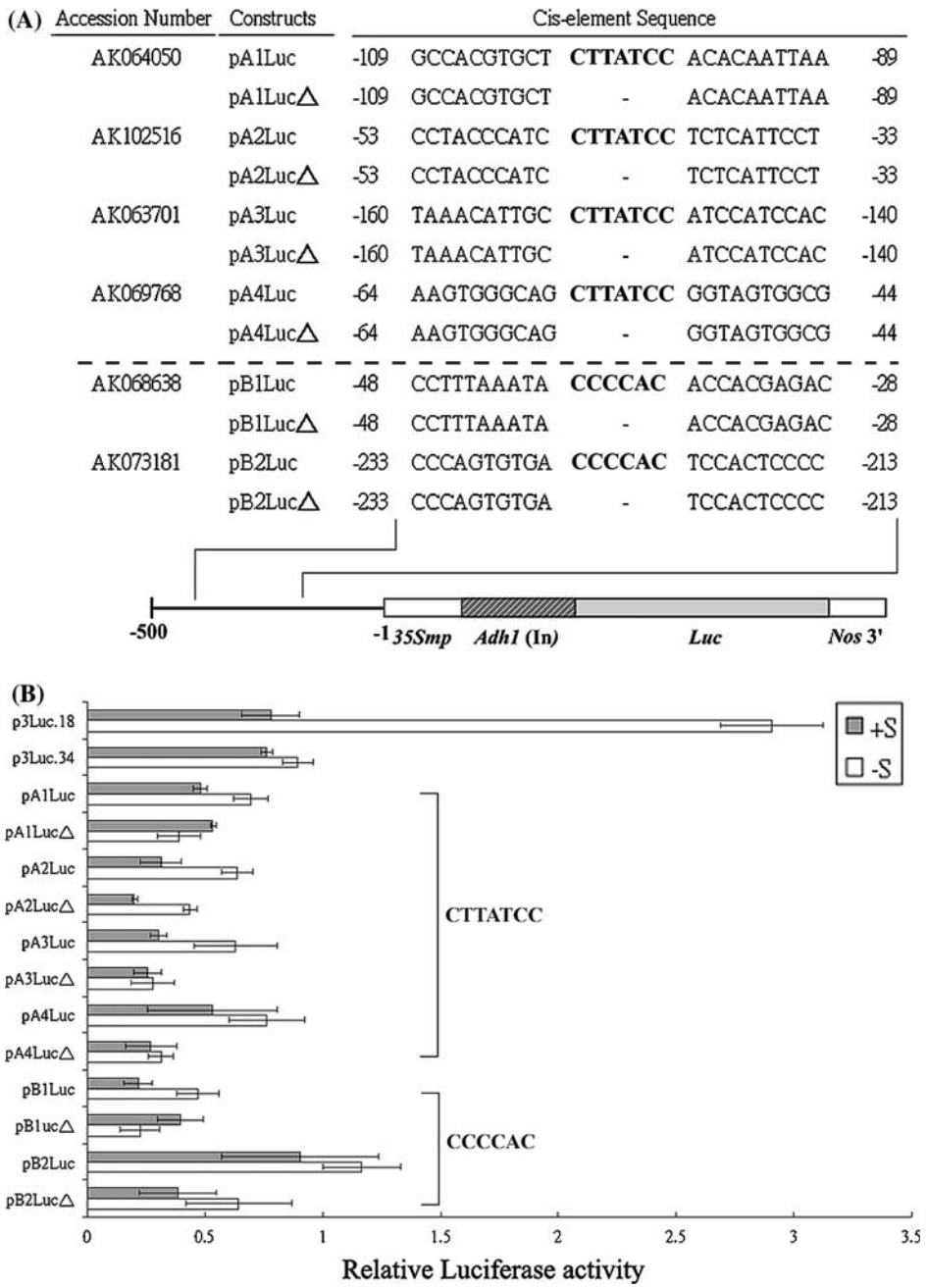


Fig. 8 Transient promoter activity assay reveals 2 promising candidates of Suc-starvation responsive elements, CTTATCC and CCCCAC, present in the promoters of Suc-starvation upregulated genes. Bombardment-mediated transient promoter activity assay in rice suspension cell was used to characterize the capability of 2 putative *cis*-elements mediating the expression of luciferase activity in response to Suc starvation. (**A)** Promoters, corresponding assay constructs, *cis*-element sequences and the schemes of luciferase expression vector are shown. For the CTTATCC element, promoters of genes encoding for Bowman-Birk-type trypsin inhibitor (AK064050, pA1), unknown expressed protein (AK102516, pA2), unknown expressed protein (AK063701, pA3) and 3-ketoacyl-CoA thiolase (AK069768, pA4) were chosen; for the CCCCAC element, the promoters of genes encoding for glycolate oxidase (AK068638, pB1) and an unknown expressed protein (AK073181, pB2) were chosen. A luciferase reporter gene driven by a promoter containing either an intact (such as pAsLuc and pBsLuc) or deleted motif (such as pAsLucΔ and pBsLucΔ) was used for bombardment and the *OsAmy3D* promoters with (p3Luc.18) or without (p3Luc.34) the 2×TATCCA elements fused to luciferase reporter genes were used as a positive and negative controls, respectively. (**B**) The results of transient-expressed luciferase activity analysis driven by intact and mutated promoter fragments. After bombardment, the transformed rice suspension cells were cultured in medium containing Suc (+S) or lacking Suc (–S) as indicated for 18 h before luciferase activity analysis as described in Materials and methods. Three individual replicates were conducted for all experiments to obtain statistical significance. Two independent experiments were conducted and showed similar results

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