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Differential Transcript Levels of Genes Associated with Glycolysis and Alcohol Fermentation in Rice Plants (*Oryza sativa* L.) under Submergence Stress[1]

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Expression of genes encoding enzymes involved in specialized metabolic pathways is assumed to be regulated coordinately to maintain homeostasis in plant cells. We analyzed transcript levels of rice (*Oryza sativa* L.) genes associated with glycolysis and alcohol fermentation under submergence stress. When each transcript was quantified at several times, two types (I and II) of mRNA accumulation were observed in response to submergence stress. Transcripts of type I genes reached a maximum after 24 h of submergence and were reduced by transfer to aerobic conditions or by partial exposure of shoot tips to air. In a submergence-tolerant rice cultivar, transcript amounts of several type I genes, such as glucose phosphate isomerase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, and enolase, increased significantly compared to an intolerant cultivar after 24 h of submergence. This suggests that the mRNA accumulation of type I genes increases in response to anaerobic stress. mRNA accumulation of type II genes, such as aldolase and pyruvate kinase, reached a maximum after 10 h of submergence. Following transfer to aerobic conditions, their transcript levels were not so rapidly decreased as were type I genes. These results suggest that the mRNA levels of genes engaged in glycolysis and alcohol fermentation may be regulated differentially under submergence stress.

Environmental stresses can cause severe effects on plant cells sufficient to cause cell death. To overcome such constraints, recognition and transduction of external signals and regulatory mechanisms that underlie specialized gene expressions are essential requirements for the sustainable living of plants (for a review, see Sachs and Ho, 1986). Expression of plant genes related to homeostasis is likely to be controlled in a coordinated manner under stressed conditions. It is, therefore, worthwhile to analyze gene expressions of enzymes engaged in a specific metabolic pathway.

Although rice (*Oryza sativa* L.) plants are known to be tolerant of anaerobiosis, most cultivars cannot survive complete submergence of the shoot and root system for very long (Taylor, 1942; Vergara et al., 1976). A comparison by Mazaredo and Vergara (1982) of tolerance to 6 d of total submergence revealed marked differences between cultivars. Fatality in the most susceptible cultivar (IR42) was 75%, whereas only 16 to 17% of the most tolerant cultivars (FR13A and Kurkaruppan) were killed. Although numerous studies of submergence tolerance have been carried (Karin et al., 1982; Mazaredo and Vergara, 1982), the molecular aspects have not been clearly understood.

It has been demonstrated that the synthesis of several proteins engaged in the glycolysis and fermentation processes is induced in plants exposed to anaerobic conditions. These anaerobic polypeptides include aldolase (Kelley and Freeling, 1984b; Kelley and Tolan, 1986), pyruvate decarboxylase (Wignarajah and Greenway, 1976; Laszlo and St. Lawrence, 1983; Kelley, 1989), and alcohol dehydrogenase (Sachs and Freeling, 1978; Ferl et al., 1979; Dennis et al., 1984, 1985). Alcohol dehydrogenase 1 transcripts and alcohol dehydrogenase enzyme activity were analyzed intensively in maize seedlings (Andrews et al., 1993, 1994). The results indicated that each tissue at a different age showed differential induction of alcohol dehydrogenase by anoxia or hypoxia. Bailey-Serres et al. (1988) reported general enzyme activities in glycolysis and alcohol fermentation pathways in maize roots and characterized their expressions under low oxygen conditions. In rice plants, anaerobic response was analyzed in the two enzymes, alcohol dehydrogenase (Cobb and Kennedy, 1987; Xie and Wu, 1989) and glyceraldehyde phosphate dehydrogenase (Ricard et al., 1989), at the mRNA and protein levels. Understanding of the molecular response to submergence stress has been restricted to several genes because of the limited number of genes available that are engaged in the energy-producing pathway in any one plant species. Recently, the expressions of genes encoding pyruvate decarboxylase, alcohol dehydrogenase, and Fru-1,6-bisphosphate aldolase were investigated in the amphibious plant *Acorus calamus* L., and a complex anaerobic response was suggested in terms of differential regulation of transcription, translation, and posttranslational processes (Bucher and Kuhlemeier, 1993).

Here, we carried out transcript analyses of many genes engaged in glycolysis and alcohol fermentation in rice seedlings subjected to complete or partial submergence. We report also the transcript levels in a submergence-tolerant rice cultivar compared with those in an intolerant cultivar. The results suggest that there are two distinct types of mRNA accumulation under submergence stress.

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MATERIALS AND METHODS

Plant Material and Growth Conditions

Rice plants (*Oryza sativa* L. var Yamahoushi) were grown under 12 h of dark and 12 h of light (10,000 lux) in an incubator at 28°C. Two rice varieties, FR13A and IR42, which are tolerant of and susceptible to complete submergence, respectively (Mazaredo and Vergara, 1982; Jackson et al., 1987; Setter et al., 1989), were also used. For all experiments, 7-d-old seedlings were used. Under aerobic conditions, only roots were submerged in distilled water. For treatment of submergence stress, whole seedlings were submerged in distilled water without aeration on a 12-h dark and 12-h light schedule.

Northern and Slot Blot Analyses

Total RNA was isolated by the phenol-SDS method from approximately 300 seedlings including both shoots and roots (Palmiter, 1974). Yield was determined by spectrophotometric A_{260}. After electrophoresis of 10 μg of total RNA per lane in a 1.2% formaldehyde agarose gel, RNA was transferred to a Hybond-N^+ membrane (Amersham) according to the manufacturer's specifications. For slot blot analysis, 10 μg of total RNA were blotted to a Hybond-N^+ membrane using Bio-Dot SF (Bio-Rad). The membrane was then incubated at 65°C for 1 h in 5 mL/100 cm^2 of hybridization solution, which consisted of 1% (w/v) SDS, 1 M NaCl, 10% (w/v) dextran sulfate, and 0.1 mg/mL heat-denatured salmon sperm DNA. A ^{32}P-labeled DNA probe was then added to the solution, and the filter was incubated at 65°C for 12 to 15 h. After hybridization, the filter was washed in 2× SSC for 10 min at room temperature with vigorous shaking and then twice in 2× SSC and 1% SDS for 30 min at 65°C. The filter was dried and exposed to x-ray film. Quantities of northern or slot blots were measured in photo-stimulated luminescence value using an Imazing Plate Scanner BAS2000 (Fuji Film Co., Tokyo, Japan). Backgrounds were assigned for each lane and subtracted from the radioactivities of corresponding bands. Multiple blots were conducted for each probe using RNA from the same lot of rice seedlings. A 0.24- to 9.5-kb RNA ladder (GIBCO BRL) was used as a size marker. cDNA of 25S rRNA was used as a probe to confirm that there were equal amounts of RNA in each lane.

Preparation of Probes

Plasmid DNAs carrying rice cDNA inserts were obtained from random cDNA sequencing (Uchimiya et al., 1992; Umeda et al., 1994). Nucleotide sequences of each cDNA clone were deposited in DDBJ, EMBL, and GenBank data bases under the following accession numbers: Glc phosphate isomerase, D10411; phosphofructokinase, D17765; aldolase, D10419; triose phosphate isomerase, D17766; glyceraldehyde phosphate dehydrogenase, D10409; phosphoglycerate kinase, D10408; enolase, D21278; pyruvate kinase, D17768; pyruvate decarboxylase, D10413; alcohol dehydrogenase, D10412; ribosomal protein YS25 (Nishi et al., 1993), D12633; and 25S rRNA, D17770. PCR was performed using 3 units of Tth DNA polymerase (Toyobo Co., Osaka, Japan), 50 ng of plasmid DNA, and 1 μM each of two universal primers that flank the insert DNA fragment. The PCR products were then purified by electrophoresis in 1.2% low-gelling-temperature agarose. The purified DNA fragments were labeled with [α-^{32}P]dCTP (110 TBq/mmol; ICN, Costa Mesa, CA) using a random primer DNA-labeling kit (Takara, Kyoto, Japan).

RESULTS

mRNA Accumulation Associated with Glycolysis and Alcohol Fermentation Shows Two Types of Response to Submergence Stress

For analysis of mRNA amounts during submergence stress, 7-d-old aerobically grown seedlings of rice (*O. sativa* L. var Yamahoushi) were submerged in water, and total RNA was isolated at several times. Northern blot hybridization was conducted using cDNA clones of genes engaged in glycolysis and alcohol fermentation as shown in Figure 1. A single band with the expected size was observed for each gene examined. The mRNA accumulation patterns were reproducible through multiple blots using RNA from the same lot of seedlings in a series of stress treatments.

The northern analyses revealed that the genes could be classified into two groups concerning the mRNA accumulation under submergence conditions; type I genes (Glc phosphate isomerase, phosphofructokinase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, enolase, pyruvate decarboxylase, and alcohol dehydrogenase) showed the maximum level of transcript after 24 h of submergence, whereas the mRNA of type II genes, such as aldolase and pyruvate kinase, reached a maximum after 10 h of submergence (Fig. 2). When a ribosomal protein YS25 gene was used as a probe on the same filter, the maximum level of transcript was seen after 10 h of submergence (Fig. 2), indicating that the pattern of mRNA accumulation was the same as those of type II genes. After

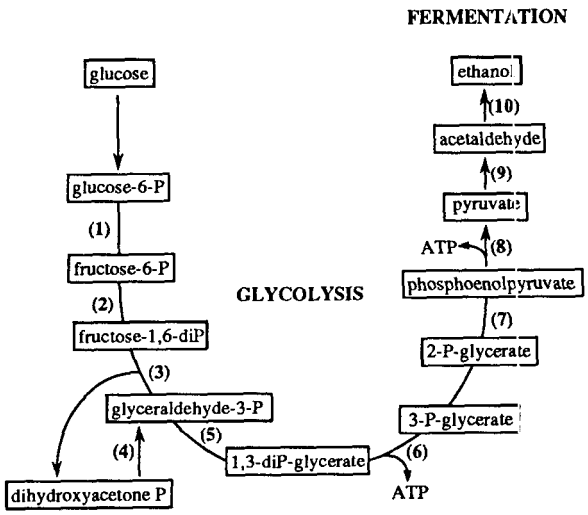


Figure 1. The ATP-generating pathway of glycolysis and alcohol fermentation. Numbers in parentheses correspond to those in Figures 2 to 5. P, Phosphate.

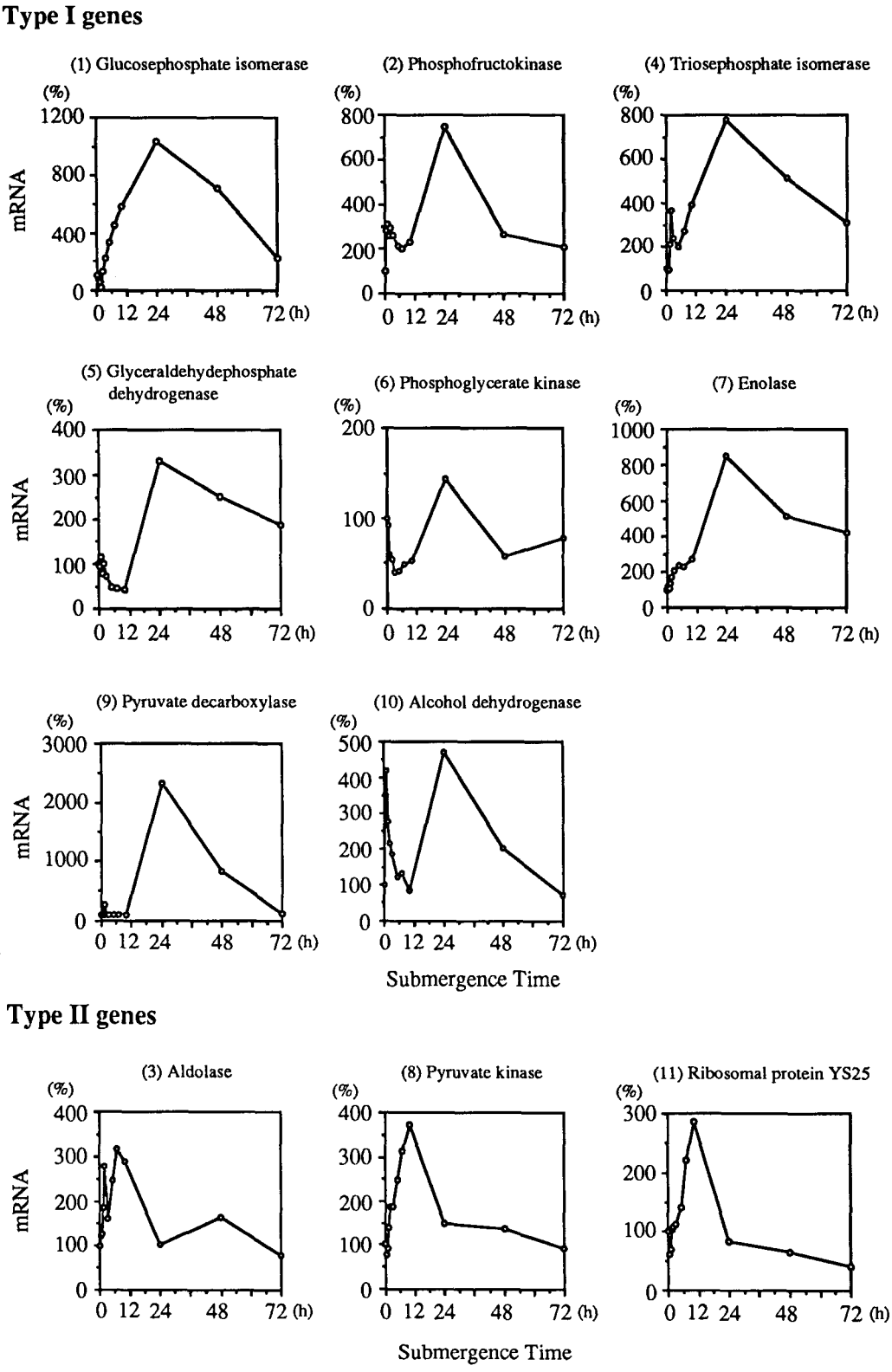


Figure 2. mRNA accumulation after various times of submergence stress. Relative amounts of mRNA are represented in percentages on the ordinate, taking the amounts of transcript at the initial time (0 h) as 100%. The abscissa indicates time (h) under submergence stress. Numbers in parentheses correspond to those in Figure 1.

48 and 72 h of submergence, mRNA levels were reduced for almost all type I and type II genes (Fig. 2).

We also found variation of mRNA accumulation patterns within type I genes: (a) transcripts of Glc phosphate isomerase, triose phosphate isomerase, and enolase genes increased gradually up to 24 h and were maintained at 48 h, (b) those of glyceraldehyde phosphate dehydrogenase, pyruvate decarboxylase, and alcohol dehydrogenase genes dramatically increased between 12 and 24 h and were maintained at 48 h, and (c) those of phosphofructokinase and phosphoglycerate kinase genes showed a transient peak at 24 h (Fig. 2).

Transcript Levels following Transfer from Submergence to Aerobic Conditions

We then analyzed mRNA levels after transfer of rice seedlings submerged in water for 24 h to aerobic conditions. The results of northern blots showed distinct differences in mRNA levels between type I and type II genes (Fig. 3). Transcripts of type I genes were rapidly reduced to less than 50% in 5 h after transfer to aerobic condition, although they were maintained at different levels after long exposure to air compared with 0 time. Among type II genes, the aldolase gene showed an increase in mRNA after 2 h, whereas the pyruvate kinase gene decreased its transcripts gradually (Fig. 3). These results suggest that mechanisms regulating the transcript levels of type I and type II genes are distinct from each other.

Transcript Levels under Partial Submergence Conditions

Submergence of rice seedlings in water creates an environment deficient in air supply. Transcript amounts in whole seedlings whose shoot tips were partially exposed to air by 1 cm on an average for 24 h were also analyzed by northern blot hybridization (Fig. 4A). Experimental conditions other than exposed shoot tips were the same as those for complete submergence. When the mRNA levels were compared with those in seedlings submerged completely, type I and type II genes showed a clear difference in relative amounts of transcripts (Fig. 4B). mRNA levels of type I genes decreased by exposure of shoot tips to air. Among them, genes encoding glyceraldehyde phosphate dehydrogenase, enolase, pyruvate decarboxylase, and alcohol dehydrogenase showed a larger reduction in mRNA amounts. On the other hand, transcripts of type II genes increased more than 2-fold (Fig. 4B), indicating that partial submergence of rice seedlings increased the mRNA levels of type II genes after 24 h.

mRNA Accumulation in Submergence-Tolerant and Submergence-Intolerant Rice Cultivars

Whole seedlings of the submergence-tolerant rice cv FR13A and submergence-intolerant cv IR42 were used for RNA analysis to determine whether different patterns of mRNA accumulation could be seen. Since northern blot analysis showed that a single band was detected for each gene, transcript amounts in FR13A and IR42 were compared by slot blot hybridization. RNAs isolated from both FR13A and IR42 were blotted to the same filter and hybridized with each probe to compare the absolute levels of transcripts between the two cultivars. Note that there was a little difference in mRNA amounts of each gene at the initial time (0 h) (Fig. 5).

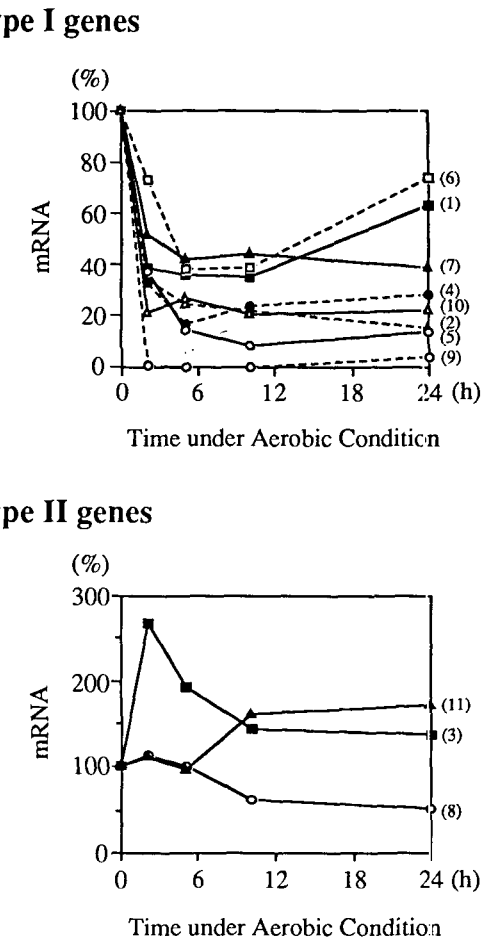
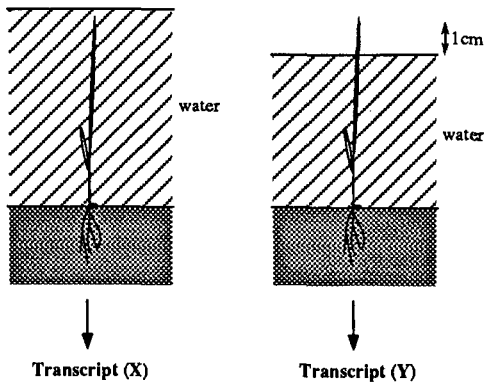


Figure 3. mRNA accumulation following transfer of submerged seedlings to aerobic conditions. Relative amounts of mRNA are represented in percentages on the ordinate, taking the amounts of transcript at the initial time (0 h) as 100%. The abscissa indicates time (h) following transfer of rice seedlings submerged in water for 24 h to aerobic conditions. Numbers in parentheses correspond to those in Figures 1 and 2.

Several type I genes, such as Glc phosphate isomerase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, and enolase, showed higher transcript levels in FR13A than in IR42 after 24 h of submergence (Fig. 5). The other type I genes, however, showed higher transcript levels at early times in IR42 rather than in FR13A. These results were reproducible through multiple blots. Surprisingly, the maximum mRNA levels of pyruvate decarboxylase and alcohol dehydrogenase genes after 24 h of submergence were much the same in the two cultivars, although the timing of increase in transcript amounts was different. Note that the transcripts of type I genes increased from 10 to 24 h more dramatically in FR13A than in IR42. The mRNA accumulation of type II genes was similar in FR13A and IR42 and did not show any notable increase under submergence conditions (Fig. 5).

A



B

Gene	Relative amounts of transcript [Y/X(%)]
Type I	
(1) Glucosephosphate isomerase	87.7
(2) Phosphofructokinase	51.6
(4) Triosephosphate isomerase	97.3
(5) Glyceraldehydephosphate dehydrogenase	29.3
(6) Phosphoglycerate kinase	82.5
(7) Enolase	37.5
(9) Pyruvate decarboxylase	12.7
(10) Alcohol dehydrogenase	35.7
Type II	
(3) Aldolase	264.2
(8) Pyruvate kinase	283.5
(11) Ribosomal protein YS25	251.5

Figure 4. Transcript levels in rice seedlings whose shoot tips were partially exposed to air. mRNA amount in seedlings submerged completely in water for 24 h is shown by X and that in seedlings whose shoot tips were partially exposed to air for 24 h is indicated by Y. A, Schematic representation of stress treatments of complete or partial submergence. B, Relative mRNA amounts of each gene represented by Y/X in percentages. Numbers in parentheses indicating each enzyme correspond to those in Figures 1 and 2.

DISCUSSION

We described changes in transcript levels in response to submergence stress in rice plants. We focused on genes engaged in glycolysis and alcohol fermentation. Submergence of rice seedlings caused mRNA accumulation of glycolysis- and alcohol fermentation-related genes. Transcripts of the pyruvate decarboxylase gene increased dramatically when compared with the aerobic condition, in which a very low amount of transcripts was detected in northern blot analysis. Similar results have also been obtained in *Zea mays* (Kelley, 1989; Peschke and Sachs, 1993). Since pyruvate decarboxylase catalyzes the first reaction of alcohol fermentation, which determines whether pyruvate is metabolized through alcohol fermentation or the tricarboxylic acid cycle, its high induction under submergence conditions may be important for activation of the anaerobic metabolic pathway.

Submergence of rice seedlings in water causes an oxygen deficit and gives rise to anaerobic metabolism of carbohydrate and protein (for a review, see Kennedy et al., 1992). The effects of anaerobiosis on gene expression have been studied mainly in maize. Transcriptional induction under anaerobic conditions has been investigated in alcohol fermentation- and glycolysis-related enzymes; pyruvate decarboxylase (Kelley, 1989; Peschke and Sachs, 1993), alcohol dehydrogenase (Gerlach et al., 1982; Rowland and Strommer, 1986; Andrews et al., 1993, 1994), Glc phosphate isomerase (Kelley and Freeling, 1984a), aldolase (Kelley and Freeling, 1984b), and glyceraldehyde phosphate dehydrogenase (Martinez et al., 1989; Russell and Sachs, 1989). In rice plants, studies of anaerobic induction are limited to a few enzymes, such as alcohol dehydrogenase (Cobb and Kennedy, 1987; Xie and Wu, 1989) and glyceraldehyde phosphate dehydrogenase (Ricard et al., 1989). In this paper, we analyzed changes in transcript amounts of 10 enzymes engaged in anaerobic metabolism and found that two types of mRNA accumulation were observed under submergence conditions. Although parallel induction under anoxic conditions has been reported in several anaerobic proteins (Laszlo and St. Lawrence, 1983; Hake et al., 1985), no information has been obtained so far about differential transcript levels of genes encoding enzymes involved in the entire metabolic pathway.

Under complete submergence, mRNA reached a maximum after 24 h for type I genes and after 10 h for type II genes. The general similarity of the patterns among type I genes and the difference with type II genes suggest that there are obviously different regulatory mechanisms involved. When seedlings grown on a 12-h light and 12-h dark schedule were transferred to a totally dark condition, the same patterns were observed in response to submergence stress. Moreover, seedlings grown aerobically under 12-h light and 12-h dark conditions showed no increase in mRNA amounts of either type I or type II genes (data not shown). These results indicate that the illumination conditions used in the present study could not cause the increase of mRNA amounts of type I and type II genes as described in this paper. In most cases, transcript levels decreased after 48 and 72 h of submergence. Since rice is capable of germinating under anaerobic conditions and growing under anoxia, it is likely that mRNAs of fermentation-related genes are kept at higher levels for several days under submergence stress. In this respect, the observed decrease may be due to the submergence conditions used in this study.

Several genes, such as phosphofructokinase, triose phosphate isomerase, alcohol dehydrogenase, and aldolase, showed transient increase of mRNA within 2 h of submergence. Transcripts of the alcohol dehydrogenase gene increased dramatically after 1 h of submergence. The transient increase observed here was reproducible through multiple blots. Their constitutive mRNA levels were considerable under aerobic conditions, suggesting that decreased turnover of a constitutively expressed mRNA might play an important role in the transient increase at early times of submergence. In this respect, it is also possible that the different mRNA accumulation patterns within type I or type II genes may reflect the mRNA turnover of each gene. Activation or repression of different members of a gene family can cause differential expression of an enzyme as reported in glyceraldehyde phosphate dehydrogenase and pyruvate decarboxylase genes

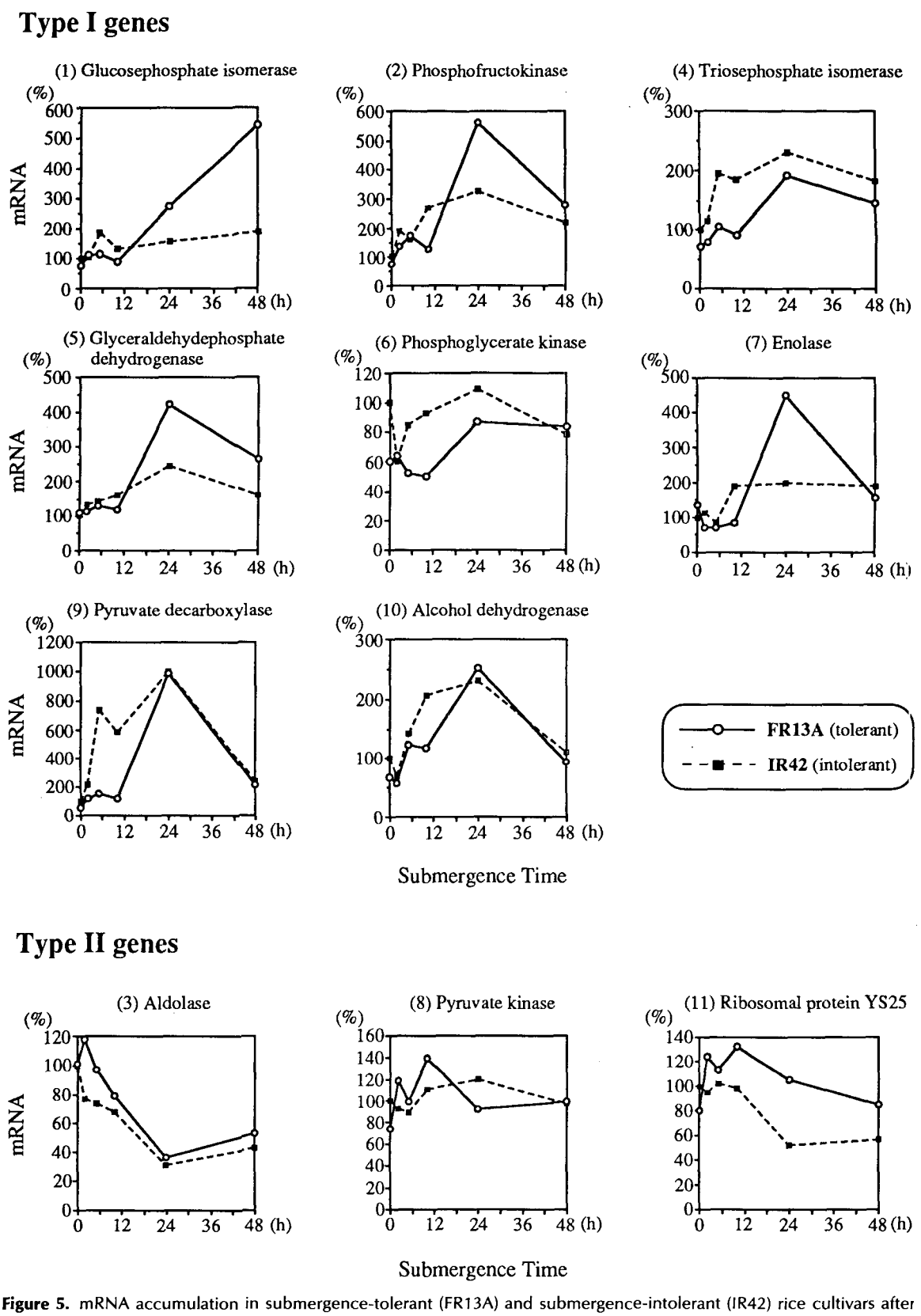


Figure 5. mRNA accumulation in submergence-tolerant (FR13A) and submergence-intolerant (IR42) rice cultivars after various times of submergence stress. Relative amounts of mRNA are represented in percentages on the ordinate, taking the amounts of transcript at the initial time (0 h) in IR42 as 100% for both FR13A and IR42. The abscissa indicates time (h) under submergence stress. Relative amounts of transcript in FR13A and IR42 are shown by open circles and closed squares, respectively. Numbers in parentheses correspond to those in Figures 1 and 2.

in maize and *Arabidopsis* (Russell and Sachs, 1989; Peschke and Sachs, 1993; Yang et al., 1993). Therefore, the complex response of a gene family to submergence stress might result in different accumulation patterns of transcripts within each group.

When rice seedlings submerged in water were transferred to normal aerobic conditions, transcripts of type I genes were reduced rapidly. Type I mRNAs were maintained at different levels after long exposure to air. This may reflect a physiological change caused by 24 h submergence followed by normal aerobic conditions. Partial exposure of shoot tips to air caused a decrease of type I mRNAs negatively correlated to the mRNA accumulation by complete submergence. Rice plants are known to develop aerenchyma to efficiently take air into anaerobic tissues such as roots, indicating that oxygen can be provided by partial exposure of shoot tips to air (Justin and Armstrong, 1987; Hoshikawa, 1989). We assumed that the mRNA levels of type I genes were reduced by air supply. These results strongly support the idea that an oxygen deficit increases the transcripts of type I genes coordinately. Transcripts of type II genes increased more than 2-fold after 24 h of partial submergence. The reason for this conspicuous response remains unknown.

RNA analysis using submergence-tolerant rice cv FR13A and submergence-intolerant cv IR42 suggested another aspect of regulatory mechanisms that underlie expression of anaerobically induced genes. Several type I genes, Glc phosphate isomerase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, and enolase, showed higher transcript levels in FR13A after 24 h of submergence. Glc phosphate isomerase transcripts in FR13A continued to increase up to 48 h. mRNA accumulation of the other type I genes was also different between FR13A and IR42. On the other hand, type II genes, aldolase and pyruvate kinase, did not show any notable increase of transcripts in either FR13A or IR42. These results indicate that the genetic background of FR13A changed the transcript levels of type I genes. Since in FR13A the increase in mRNA amounts was observed not only in a single gene but in several genes, it is likely that some element unlinked to the structural genes may be involved in acquisition of submergence tolerance. In maize, DNA sequences required for anaerobic expression of the alcohol dehydrogenase gene have been identified (Dennis et al., 1984, 1985; Walker et al., 1987). Therefore, it is possible that a factor that binds to these sequences and regulates their gene expressions may be altered in FR13A to increase transcripts of type I genes. In yeast, the expression of glycolytic genes is activated by the transcriptional activators named Gcr1 and Gcr2 (Kawasaki and Fraenkel, 1982; Baker, 1986; Holland et al., 1987; Uemura and Fraenkel, 1990; Uemura and Jigami, 1992). It is therefore probable that *trans*-acting factors homologous to Gcr1 and Gcr2 are present in plants and regulate the expressions of glycolysis- and alcohol fermentation-related genes.

The regulatory mechanism of anaerobic metabolism is complex, involving substrate and product concentration, enzyme synthesis and degradation, and enzyme activation or repression, in addition to transcript levels, which are the result of transcription rates and transcript stability. In this respect, transcript levels alone could not explain the activation or repression of the whole metabolic pathway. However, the different transcript levels described here are important for understanding the molecular mechanisms involved in the anaerobic response of rice genes. Isolation and characterization of a transcriptional activator/repressor will further facilitate the understanding of a gene network under oxygen stress.

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