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Effect of Ethanol on the Expression of Two Fructokinases in Rice Seedlings

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Abstract: Germination of rice (*Oryza sativa* L.) caryopses in the presence of exogenous ethanol (the end-product of alcoholic fermentation under anoxia) results in a decrease of coleoptile and root growth. The ethanol level measured in rice caryopses incubated in 50 mM exogenous ethanol under aerobic conditions was similar to that in caryopses incubated under anoxia. Application of 50 mM ethanol under aerobic conditions induced the production of OsFK2, a fructokinase isoform having a central role in the response of rice to anoxia. The induction of OsFK2 by ethanol was organ-specific: OsFK2 was produced mainly in the embryo and partially in the coleoptile, but not in root tissues. The results of this research suggest that rice can sense ethanol under anoxia and shows a molecular response to oxygen deprivation.

Key words: Anoxia, Carbohydrate, Fermentation, *Oryza sativa* L., OsFK.

Ethanol is known to suppress rice seedling growth (Alpi and Beevers, 1983; Kato-Noguchi and Kugimiya, 2001). Ethanol also inhibits the growth of several tissues, modulates amylolitic enzymes in cereal seedlings (Perata et al., 1986; Perata and Alpi, 1991) and induces a family of heat shock proteins (high molecular weight) in etiolated soybean seedlings (Kuo et al., 2000). Cereal caryopses produce a high level of ethanol during anoxia. Rice is the only cereal species able to germinate under anaerobic conditions and it shows high tolerance to this alcohol (Perata et al., 1998) due to several adaptation mechanisms (Guglielminetti et al., 1995a, 1995b, 1997, 1999, 2000a, 2000b, 2001; Huang et al., 1999, 2000a, 2000b; Kato-Noguchi, 2000, 2001, 2002; Kato-Noguchi et al., 2011; Perata et al., 1996, 1998). One of the most important differences is its ability to degrade sucrose under anoxia utilizing the sucrose synthase (SS; EC 2.4.1.13) pathway (Guglielminetti et al., 1995a, 1997, 2001; Perata et al., 1996). This pathway represents an important mechanism in the adaptation to anoxia because the hydrolysis of one mole of sucrose by SS, instead of using invertase (EC 3.2.1.26), allows the net production of 6 moles of ATP against the 4 moles obtained through the invertase pathway (Perata et al., 1998).

Since sucrose hydrolysis by SS releases fructose and UDP-Glc, the role of fructokinases (EC 2.7.1.4) for driving this hexose into glycolysis and fermentation becomes crucial. Our previous work (Guglielminetti et al., 2006) demonstrated that a specific fructokinase isoform (OsFK2) was induced in anoxic rice seedlings at both transcriptional and translational levels while under aerobic conditions the predominant fructokinase was represented by OsFK1. The different presence of two isoforms in aerobic and anaerobic conditions is probably due to their different kinetics characteristic: OsFK2 is not strongly inhibited by substrate while OsFK1 is inhibited at a fructose concentration higher than 1 mM. Under anoxia, the re-oxidation of NADH by fermentation can efficiently take place only if hexoses are phosphorylated at a higher rate, and consequently, substrate-repression of fructokinases would be a disadvantage for survival under oxygen deprivation.

Some signals and mechanisms responsible for triggering the plant response to anoxia have been clarified in recent years. The stabilization of a N-terminal motif on some ethylene responsive factors (ERFs) under low oxygen activates these ERFs, which regulate low oxygen core genes that enable plants to tolerate abiotic stress such as flooding (Licausi et al., 2011). In addition, the production of reactive oxygen species (ROS) has been observed under hypoxia, suggesting that ROS might be part of the network involved in plant acclimation (Pucciariello and Perata, 2012 and references therein). On the other hand, to our knowledge, evidence that ethanol plays a role in the signalling of anoxia in higher plants has not been reported.

We present here data on the differential inductive effect of ethanol on two different rice fructokinases. We incubated young rice seedlings under an anaerobic

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Abbreviations: ANOVA, analysis of variance; ERFs, ethylene responsive factors; LSD, least significant difference; ROS, reactive oxygen species; SS, sucrose synthase.

condition (anoxia) or aerobic condition with or without exogenous application of ethanol and we measured the seedling growth, levels of ethanol and free sugars in the tissue, and the production of OsFK1 (mainly aerobic) and OsFK2 (induced during anoxia) with the main goal to evaluate a possible role of ethanol in the anoxia signalling.

Materials and Methods

1. Plant material

Caryopses of rice (*Oryza sativa* L., cv Nipponbare) were sterilized with 3% NaClO solution for 30 min, washed 10 times with sterile distilled water and sown in Petri dishes using 10 ml sterile distilled water for the aerobic control; and 50 and 100 mM ethanol solution for the aerobic-treated samples. Samples were incubated 5 days at 28ºC in the dark, either under aeration, or in an anaerobic chamber (Forma Scientifica, Canada).

2. Analysis of carbohydrates

Samples (0.1 – 1 g FW) were rapidly frozen in liquid nitrogen and ground to a powder, then extracted as described by Tobias et al. (1992) and assayed for glucose, fructose and sucrose content through coupled enzymatic assay methods as described by Damiani et al. (2012). The efficiency of the methods was tested by using known amounts of carbohydrates. Recovery experiments evaluated losses taking place during the extraction procedures. Two experiments were performed for each metabolite by adding known amounts of authentic standards to the sample prior to the extraction. The concentration of the standards added was similar to that estimated to be present in the tissues in preliminary experiments. The recovery ranged between 95 and 104%.

3. Analysis of ethanol

Samples were collected and extracted as described for carbohydrate analysis and assayed by coupled enzymatic assay methods, measuring the increase in A_{340} as described by Bernt and Gutman (1974) with minor modifications. The efficiency of the methods was tested by using known amounts of ethanol. The samples and standards were incubated at 25ºC for 70 min. The reaction mixtures (1 mL) were as follows: 72 mM $Na_4P_2O_7$, pH 8.7, 72 mM semicarbazide, 20 mM glycine, 0.5 mM NAD, 36 unit alcohol dehydrogenase; the A_{340} was recorded and compared with the standards.

Recovery experiments evaluated losses taking place during the extraction procedures. Two experiments were performed by adding known amounts of ethanol to the sample prior to the extraction. The concentration of ethanol added was similar to that estimated to be present in the tissues in preliminary experiments. The percentage recovery ranged between 94 and 107%.

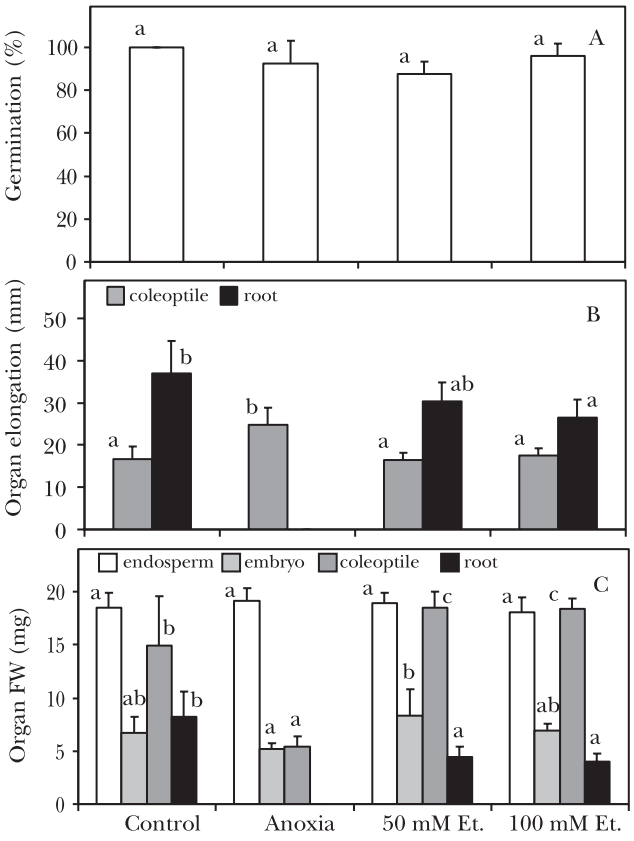

Fig. 1. Effect of ethanol on rice seedling growth. Percentage of germination (A), coleoptile and root elongation (B) and organ fresh weight (C) of 5-d old rice seedlings sowed under anoxia or under aerobic condition in the absence (Control) or presence of exogenous ethanol (50 or 100 mM). Each value represents the mean of three replicates (± SD). Within date, bars with similar letters are not statistically different from one another according to Fisher's protected LSD ($\alpha = 0.05$).

4. OsFK1 and OsFK2 protein levels

Dried caryopses and seedlings tissues were dissected and collected at the time reported in figures 4 and 5, extracted in 50 mM Tris-HCl buffer (pH 7.6 containing 10 mM DTT and 10% glycerol) and desalted on a Sephadex G25 column equilibrated with 20 mM Tris-HCl buffer (pH 7.6 containing 10 mM DTT). Protein quantification was performed with the kit purchased from Bio-Rad (Richmond, CA, USA). Equal amounts of protein (2 μg) were subjected to SDS-PAGE on 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose paper as previously described (Guglielminetti et al., 1995b). Analyses were performed using rice OsFK1 antibody (1/500) or rice OsFK2 antibody (1/500) as previously described (Guglielminetti et al., 2006). An alkaline phosphatase-labelled second antibody was used to detect immunoreactive bands. Band intensity was measured by ImageJ software.

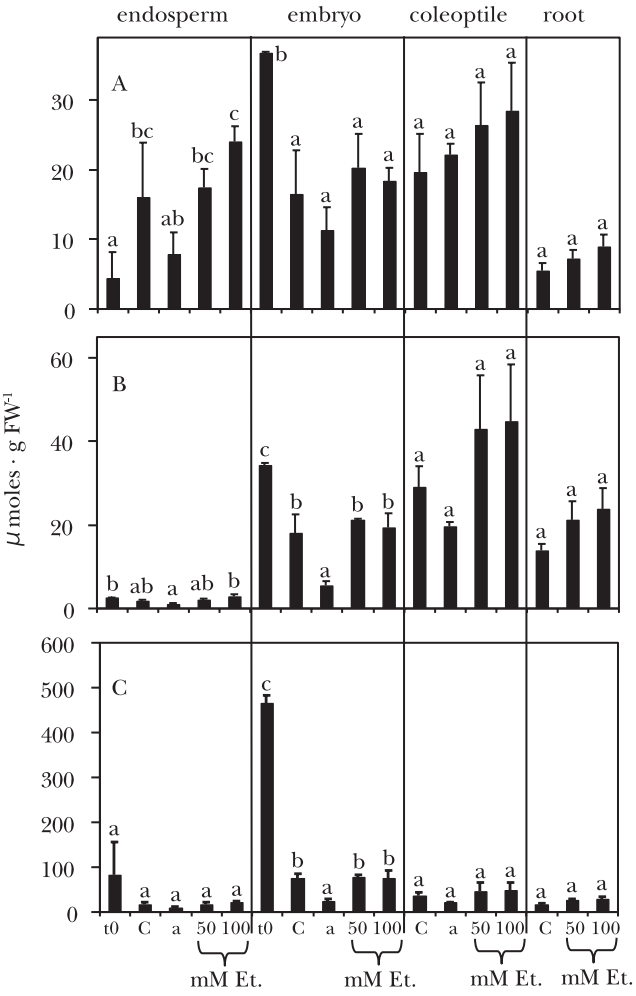

Fig. 2. Analysis of carbohydrates. Glucose (A), fructose (B) and sucrose (C) content in dry rice caryopses (t0) or 5-d old rice seedlings sowed under anoxia (a) or under aerobic condition in the absence (Control) or presence of exogenous ethanol (50 or 100 mM). Each value represents the mean of three replicates (± SD). Within date, bars with similar letters are not statistically different from one another according to Fisher's protected LSD ($\alpha = 0.05$).

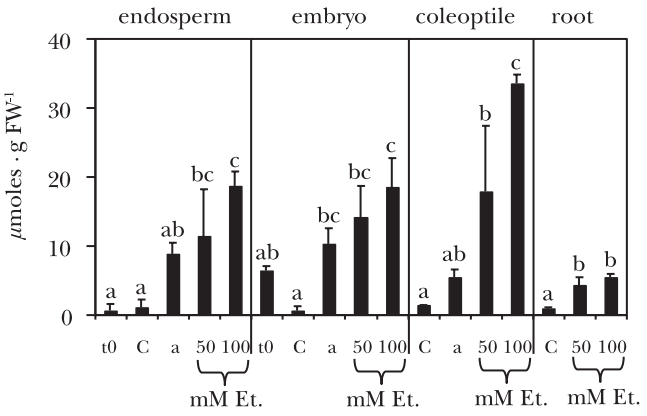

Fig. 3. Analysis of ethanol. Ethanol content in dry rice caryopses (t0) or 5-d-old rice seedlings sowed under anoxia (a) or under aerobic condition in the absence (C) or presence of exogenous ethanol (50 or 100 mM Et.). Each value represents the mean of three replicates (± SD). Within date, bars with similar letters are not statistically different from one another according to Fisher's protected LSD ($\alpha = 0.05$).

5. Statistical analysis

All determinations were replicated 3 times. Analysis of variance (ANOVA) of the data was evaluated and Least Significant Difference (LSD) was used to determine the statistical significance of the differences between the means ($P = 0.05$).

Results

1. Effect of ethanol on seedling growth.

Exogenous ethanol treatment on dry rice caryopses did not affect the germination. The germination percentage was close to 100% in all treatment groups (Fig. 1A). On the contrary, ethanol treatment affected root elongation in rice seedlings. In particular, root elongation (root is not produced under anoxia) was negatively affected by alcohol treatment (Fig. 1B). Ethanol treatment affected the root fresh weight. In fact, 100 mM ethanol significantly decreased the root fresh weight when compared with the control (Fig. 1C). On the other hand, coleoptile elongation was greater under anoxia than that observed under aerobic conditions, but ethanol treatment did not show any effect (Fig. 1B) while fresh weight was strongly reduced under anoxia. On the contrary, exogenous ethanol treatment significantly increased coleoptile fresh weight (Fig. 1C). Fresh weight of endosperms and embryos was not influenced by any treatment (Fig. 1C).

2. Analysis of carbohydrates.

Sucrose, fructose and glucose content were analyzed in dry caryopses and in 5-d-old seedlings treated as previously described. As shown in Fig. 2, the treatments, with the exception of anoxia, did not negatively affect the sugar content. Sucrose (Fig. 2C) is normally present at the same level in the control or ethanol-treated samples with the exception of dry embryos, while fructose (Fig. 2B) and glucose (Fig. 2A) were slightly higher in caryopses incubated in the presence of ethanol. On the contrary, under anoxia, fructose and sucrose levels in the embryo were lower in the ethanol-treated samples than in the control.

3. Analysis of ethanol.

Ethanol was analyzed in dry caryopses and in 5-d-old seedlings. As shown in Fig. 3, we observed a marked increase in ethanol content in rice caryopses under anoxia as well as under aerobic conditions in the presence of exogenous ethanol. In particular, ethanol content under anoxia was similar to that in the 50 mM ethanol-treated samples in all organs. The ethanol content in the 100 mM ethanol-treated

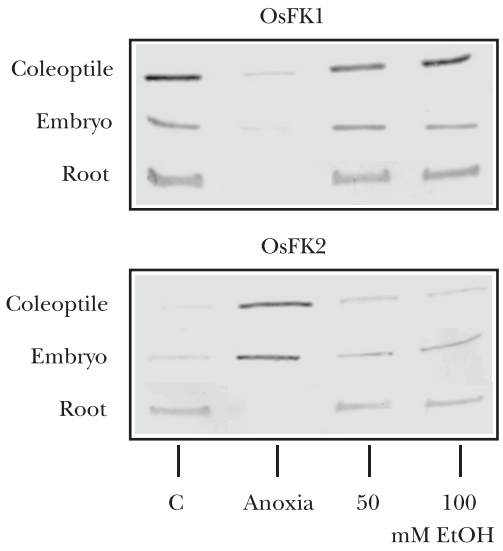

Fig. 4. Western blot analysis of OsFK1 and OsFK2. Proteins were extracted from tissues excised from 5-d-old seedlings sowed under anoxia or under aerobic conditions in the absence (Control) or presence of exogenous ethanol (50 or 100 mM). Each lane was loaded 2 μg of protein. The experiment was conducted in triplicate. Data in figure are representative of all the experiments.

samples was higher than that under anoxia In general, the ethanol content in the root was relatively low.

4. OsFK1 and OsFK2 protein levels.

To evaluate the level of OsFK1 and OsFK2 in rice seedlings, we performed immuno-blotting analysis using two specific polyclonal antibodies. Results of one representative replicate are shown in Fig. 4. These results demonstrated that anoxia strongly affects the reduction of OsFK1 levels in coleoptile and embryo (not produced in roots under anoxia) while ethanol did not show any significant effect on the OsFK1 level at any experimental concentration. The levels of OsFK2 were different from those of OsFK1. The anoxia-treated sample showed in a marked increase of OsFK2 signal in both coleoptile and embyo tissues while the aerobic control showed repressed production of this protein. Ethanol seemed to induce the production of OsFK2 in both coleoptile and embryo tissues. In the embryo, the level of OsFK2 was higher than in the control when caryopses were incubated in the presence of exogenous ethanol, moreover the effect was dose-dependent. In the coleoptile, the signal was very low under control conditions, but was present in the sample treated with exogenous ethanol. Fig. 5 shows the

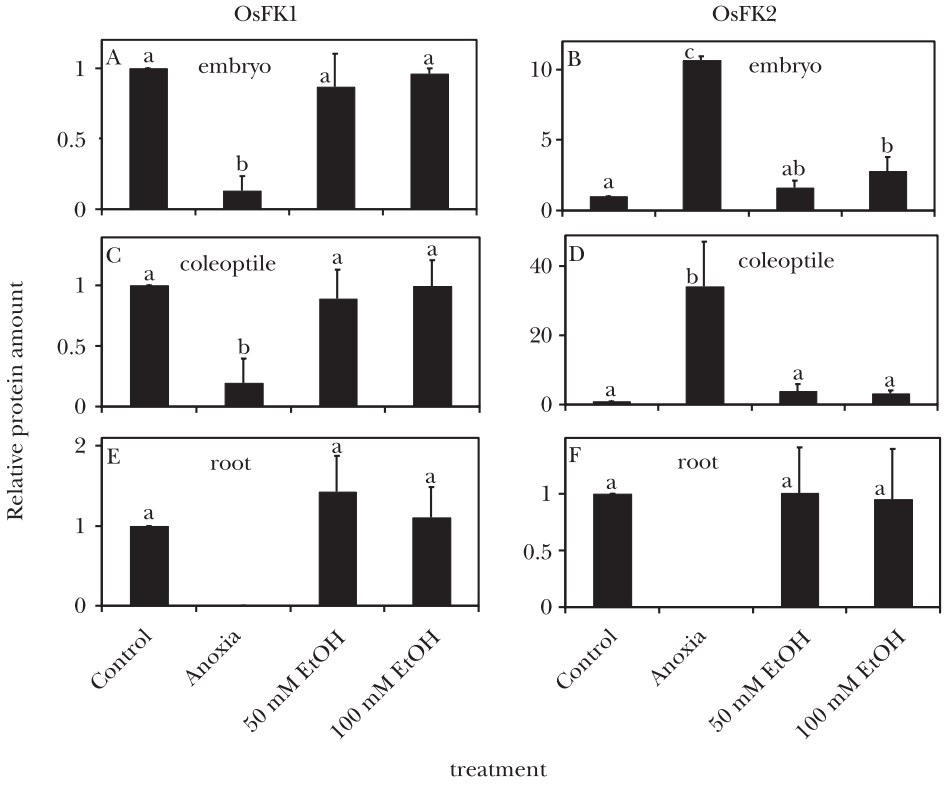

Fig. 5. OsFK1 and OsFK2 content in rice tissues. The results of the densitometric analysis (ImageJ software) of OsFK1 or OsFK2 western blot bands of embryo, coleoptile and root from seedlings sowed under anoxia or under aerobic condition in the absence (Control) or presence of exogenous ethanol (50 or 100 mM). Data are means of two replicates. Band intensity of Control was considered 1 arbitrary unit in each different panel.

densitometric value of both OsFK1 and OsFK2 in tissues under analysis. The level of OsFK2 was 3-fold higher in the embryo treated with 100 mM ethanol than that in the control and about 30% of the value in the anoxia-treated sample. In the coleoptile, the effect of exogenous ethanol on OsFK2 protein level was much lower than that under anoxia and the effect of ethanol was not significant compared with the control. Densitometric data of OsFK2 in root and OsFK1 in all tissues did not show any significant effect of ethanol on the protein level.

Discussion

Anoxic conditions have been reported to affect several glycolytic enzymes (Guglielminetti et al., 1995a; Bouny and Saglio, 1996; Fox et al., 1998) and various aspects of the molecular regulation of these induction have been recently clarified (Licausi et al., 2011; Pucciariello and Perata, 2012). Phosphorylation of fructose under anoxia has been suggested to support the energetic status of rice tissues (Perata et al., 1998).

Rice induces fructokinase activity under anoxia (Guglielminetti et al., 1995a) and a specific isoform (OsFK2) is induced under this stress condition at both transcriptional and translational levels (Guglielminetti et al., 2006) while the OsFK1 isoform is normally expressed under aerobic conditions and repressed under anoxia. The majority of green plants show activation of alcoholic fermentation when subjected to oxygen deprivation. Ethanol, at relatively low concentrations, is toxic to a large number of plants. On the contrary, rice can germinate in the presence of ethanol at high concentrations although the phenotype of rice seedlings is affected by the presence of ethanol. Recently, data and hypothesis on the mechanism by which plants can sense the absence of oxygen have been reported, but the role of ethanol is supposed to be involved in the modulation of the plant's response to anoxia (Pucciariello and Perata, 2012).

In the present paper we present data on the possible role of ethanol in the sensing of anoxia. In fact, using exogenous ethanol treatment in the samples incubated aerobically we obtained similar ethanol level to that obtained under anoxia. In this system, we demonstrated also that exogenous ethanol treatment did not interfere with primary metabolism. In fact the sugar level in rice caryopses incubated with exogenous ethanol did not change significantly in comparison with control samples. On the contrary ethanol treatment affects plant phenotype. In fact root elongation results repressed by ethanol. Under anoxia, rice seedlings did not protrude any roots and this phenomenom correlated with the reduced root elongation under ethanol treatment. Also OsFK2 production was related to ethanol perception. In the embryo, ethanol concentration was related to OsFK2 production and the anoxic physiological level of this alcohol showed a clear induction of this protein under air as compared with the control. On the contrary, no significant differences were observed in the ethanol-treated coleoptile suggesting that some factors other than ethanol may be largely implicated in the induction of OsFK2 expression under anoxia in this tissue.

In conclusion our data suggest that ethanol is not the only effector in the induction of OsFK2 and also that the mechanism in the coleoptile could be different from that in the embryo. On the other hand, ethanol did not repress OsFK1, which was typically repressed by anoxia, suggesting that this mechanism is driven by alternative ways.

To better understand the full mechanism modulating the OsFK2 production under anoxia, we are conducting further experiments using other possible effectors.

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