Effects of Flooding and Anoxia on Activities of Alcohol Dehydrogenase and Lactate Dehydrogenase in Maize Seedlings

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(Received May 14, 1999)

To investigate the effects of flooding and anoxic stresses on respiratory metabolism in maize (*Zea mays* L.), the seedlings of two cultivars (cv. Jelly Bantam and Popcorn) were subjected to the stresses, and the activities of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) were determined. The relative importance of lactic and ethanolic fermentation was also characterized under such conditions. Flooding and anoxic stresses increased ADH activity in shoots and roots of two cultivars but the stresses did not increase LDH activity. The increases were greater in the roots than in the shoots of both cultivars under the flooding and the anoxic stresses, and greater in cv. Jelly Bantam than in cv. Popcorn under the flooding stress. Using polyacrylamide gel electrophoresis, only one ADH band was identified in extracts of the shoots and the roots of both cultivars under non-stress and three ADH isozymes were identified in the extracts under the stresses, which indicates that ADH subunit translation is increased by the stresses. These results suggest that the flooding and the anoxic stresses increase ADH activity in shoots and roots of two cultivars of maize but stresses do not increase LDH activity, and increased ADH activity may be due to increased synthesis of the enzyme.

INTRODUCTION

During the growing season, anoxia often occurs in field crop plants when they are exposed to flooded or waterlogged soil (Lin and Lin, 1992; Voesenek *et al.*, 1997). Anaerobic conditions in plants result in rapid changes in respiratory metabolism and in the pattern of related gene expression (Sachs and Ho, 1986; Millar *et al.*, 1994; Ricard *et al.*, 1994). Glycolytic metabolism in many plants is accelerated in such a condition and the increase in glycolytic flux is accompanied by the accumulation of glycolytic end products including ethanol and lactate (Roberts *et al.*, 1984; Kennedy *et al.*, 1992; Good and Muench, 1993). Ethanol is formed by reduction of acetaldehyde by alcohol dehydrogenase (ADH) and NADH, which is preceded by decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylase (PDC) in the ethanolic fermentation pathway. Lactate is formed by the reduction of pyruvate by lactate dehydrogenase (LDH) and NADH in the lactic fermentation pathway. Therefore, many plants have two simultaneous pathways competing for pyruvate and NADH under anaerobic condition (Davies, 1980; Kennedy *et al.*, 1992) and these alternative fermentation pathways allow glycolysis to continue owing to consumption of pyruvate and regeneration of NAD (Kennedy *et al.*, 1992; Ke *et al.*, 1994).

Different plant species show wide differences in their flooding tolerance and ADH is considered essential for anoxia survival since the ADH null mutants of plants are more

sensitive to flooding and concomitant anoxia (Davies, 1980; Ricard *et al.*, 1994; Zhang and Greenway, 1994). In addition, ADH was necessary for removal of acetaldehyde because of its phytotoxic effect (Perata and Alpi, 1991) and for cytoplasmic pH regulation (Roberts *et al.*, 1984). The objective of this study was to investigate the effects of flooding and anoxic stresses on two cultivars of maize seedlings and to characterize the relative importance of lactic and ethanolic fermentation under such conditions. Thus, the activities of LDH and ADH were determined in shoots and roots of the seedlings and ADH isozymes were characterized.

MATERIALS AND METHODS

Plant materials. Two cultivars of maize (*Zea mays* L.) were used: cv. Jelly Bantam and Popcorn. Maize seeds were sterilized in a 2% (w/v) solution of sodium hypochlorite for 15 min, rinsed in tap water for 2 h and germinated on two sheets of moist filter paper (No. 1; Toyo Ltd., Tokyo) at 25°C in daily cycles of 12-h-light and 12-h-dark in a growth chamber. Light was provided from above with a white fluorescent lamp (3.2 W m⁻² at plant level; FL40SSW, National, Tokyo). After 2 d, uniform seedlings were selected and transferred to 9-cm Petri dishes each containing two sheets of filter paper moistened with 10 mL distilled water. After being kept in the same daily cycle for one further day, the seedlings were used for flooding and anoxic treatments.

Flooding treatments. The Petri dishes were placed in plastic containers and the containers (30×60×45 (height) cm) were filled with 25°C-distilled water. The seedlings were completely submerged in the distilled water (about 3 cm below the water surface) and the containers were kept at 25°C in a growth chamber. For determination of enzyme activity, two–four Petri dishes were removed daily from the plastic containers and the seedlings were divided into shoots and roots using a razor blade and stored immediately at –80°C until extraction. Control seedlings were kept in air in the plastic containers at 25°C in the growth chamber and harvested as described above.

Anoxic treatments. The Petri dishes with the 3-d-old seedlings were placed into a 5-L gas changing desiccator at 25°C in a growth chamber. Distilled water (200 mL) was placed at the bottom of the desiccator to maintain a relative humidity and the Petri dishes were set above the water. Then, the desiccator was purged with nitrogen gas at a rate of 1 000 mL min⁻¹ for 20 min and the gas purge was performed every 12 h to maintain the anoxic condition. Control seedlings were purged with air at a rate of 1 000 mL min⁻¹ for 20 min at 12-h intervals. For determination of enzyme activity, the seedlings were harvested and stored as described above.

Extraction and assay of enzymes. Frozen shoots and roots of maize seedlings were homogenized in four volumes of ice-cold solution containing 100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 mM MgCl₂ and 5% (v/v) glycerol. The homogenate was centrifuged at 25 000×g for 30 min and the supernatant was used immediately for the measurements of ADH and LDH activities.

ADH and LDH activities were determined spectrophotometrically by monitoring the oxidation of NADH at 340 nm for 15 min at 30°C in the following 1-mL reaction mixture. The assay mixture for ADH contained 85 mM MES (pH 6.5), 0.15 mM NADH and 0.02 mL sample, and 5 mM acetaldehyde to initiate the reaction as described by Kato-Noguchi and Watada (1997). The assay mixture for LDH contained 100 mM Tris-HCl (pH 8.0), 0.24 mM NADH, 5 mM 4-methylpyrazole, 1.5 mM Na-pyruvate, and 0.2 mL of sample. 4-Methylpyrazole was used to inhibit interference from the coupled action of PDC and ADH (Hoffman *et al.*, 1986). The overall recovery of the enzyme activity through the quantification process was 87±5 and

84±7% for ADH and LDH, respectively, according to 5 repeated assays with pure enzyme in the extract.

Protein was determined by the method of Bradford (1976) using bovine gamma-globulin as a standard.

Polyacrylamide gel electrophoresis. Frozen shoots and roots of maize seedlings were homogenized and extracted as described in the above and applied to native slab polyacrylamide gel electrophoresis. Each gel track contained 10 μL of sample plus 10 μL of 90% glycerol containing 0.01% bromophenol blue. The running gel containing 14% (w/v) acrylamide, 0.42% bisacrylamide, 0.37 M Tris-HCl (pH 8.5) and 12% (w/v) glycerol, was polymerized with 0.9 $\mu\text{L mL}^{-1}$ TEMED and 0.4 mg mL^{-1} ammonium persulfate. The stacking gel containing 5% acrylamide, 0.2% bisacrylamide, 0.12 M Tris-HCl (pH 6.7) and 12% glycerol was polymerized with 0.6 $\mu\text{L mL}^{-1}$ TEMED and 0.3 mg mL^{-1} ammonium persulfate for 2 h. Gels were run with Tris-glycine buffer (pH 8.8, 10 mM Tris, 77 mM glycine) in both electrode tanks at 15 mA at 4°C. When the dye front migrated to the bottom of the running gel, the gel was stained in a 50 mL solution containing 0.1 M Tris-HCl (pH 8.0), 0.7 mM NAD, 1.2 μM phenazine methosulfate, 0.4 mM MTT and 3 mL ethanol (Arulsekar and Parfitt, 1986). ADH bands appeared within 20 min and development was allowed to proceed for an additional 30 min before the reaction was stopped by rinsing in water.

RESULTS AND DISCUSSION

Figures 1 and 2 show ADH and LDH activities in shoots and roots of two cultivars of maize seedlings under flooding. On day 0, the activities of ADH and LDH in the shoots of both cultivars were low. Under the stress, ADH activity in the shoots of both cultivars increased rapidly up to day 2 and leveled off, while LDH activity did not change for 3 d (Fig. 1). Increase in ADH activity was greater in cv. Jelly Bantam than in cv. Popcorn. The maximum ADH activity was 4.0 and 2.4 times that in the shoots of non-flooding controls for cv. Jelly Bantam and Popcorn, respectively.

Similarly, in the roots of both cultivars, ADH activity increased sharply and LDH activity remained unchanged (Fig. 2). Increase in ADH activity was greater in cv. Jelly Bantam than

Fig. 1 Changes in the level of ADH and LDH activity in shoots of cv. Jelly Bantam and Popcorn under flooding stress.

Control seedlings were kept in air. Means $\pm$ SE from three experiments with at least three assays for each determination.

Fig. 2 Changes in the level of ADH and LDH activity in roots of cv. Jelly Bantam and Popcorn under flooding stress.

Control seedlings were kept in air. Means $\pm$ SE from three experiments with at least three assays for each determination.

Fig. 3 Changes in the level of ADH and LDH activity in shoots of cv. Jelly Bantam and Popcorn under anoxic stress.

Control seedlings were kept in air. Means $\pm$ SE from three experiments with at least three assays for each determination.

in cv. Popcorn. By day 3, the ADH activity increased 7.3 and 4.5 times that in the roots of non-flooding controls for cv. Jelly Bantam and Popcorn, respectively. These results indicate that the flooding stress increased ADH activity in the roots and the shoots of cv. Jelly Bantam and Popcorn but did not increase LDH activity (Figs. 1 and 2). Induced ADH was 2 times more active in the roots than in the shoots of both cultivars and was more active in cv. Jelly Bantam than in cv. Popcorn.

Figures 3 and 4 show ADH and LDH activities in shoots and roots of cv. Jelly Bantam and Popcorn exposed to anoxia. The maximum ADH activity was 4.3 and 3.5 times that in the shoots of non-anoxic controls for cv. Jelly Bantam and Popcorn, respectively, and 10 and 6 times that in the roots of non-anoxic controls for cv. Jelly Bantam and Popcorn, respectively.

Fig. 4 Changes in the level of ADH and LDH activity in roots of cv. Jelly Bantam and Popcorn under anoxic stress.

Control seedlings were kept in air. Means $\pm$ SE from three experiments with at least three assays for each determination.

Fig. 5 Isozyme composition of ADH in shoots and roots of cv. Jelly Bantam and Popcorn exposed to anoxic stress for 2 d.

Control seedlings were kept in air. Lane 1: control, shoots; lane 2: control, roots; lane 3: anoxia, shoots; lane 4: anoxia, roots.

The pattern of changes in the activities of ADH and LDH in the seedlings exposed to the anoxic stress was similar to those in the seedlings exposed to the flooding stress (Figs. 1–4). The anoxic stress increased ADH activity but did not change LDH activity for 3 d. Induced ADH was 1.5 times more active in the roots than in the shoots of both cultivars. Plant ADH enzymes that have been examined are dimers and the two subunits of ADH are encoded by two unlinked genes (Gottlieb, 1982; Sachs and Ho, 1986). The products of these genes dimerize randomly to yield three electrophoretically distinct isozymes: ADH1-ADH1 homodimer, ADH1-ADH2 heterodimer, and ADH2-ADH2 homodimer (Gottlieb, 1982; Newman and VanToai, 1991). However, all isozymes could not be found in each plant tissue due to the limited expression of the specific ADH isozymes (Chourey and Widholm, 1980; Xie and Wu, 1989; Ke *et al.*, 1994). Using polyacrylamide gel electrophoresis, only one ADH band was identified in extracts of the shoots and the roots of both cultivars under non-anoxic stress and three ADH isozymes were identified in the extracts under anoxic stress (Fig. 5). These results indicate that the anoxic stress may increase in ADH activity due to activation of ADH subunit translation.

These results suggest that the flooding and the anoxic stresses increase ethanolic fermentation but do not increase lactic fermentation. Since the ADH induction was greater in the

roots than in the shoots of both cultivars, and greater in cv. Jelly Bantam than in cv. Popcorn, the ethanolic fermentation may be accelerated more in the roots than in the shoots of both cultivars, and more in cv. Jelly Bantam than in cv. Popcorn. This acceleration could allow glycolysis to continue due to consumption of pyruvate and regeneration of NAD (Davies, 1980; Kennedy *et al.*, 1992; Rivoal and Hanson, 1994). When oxygen becomes limiting, glycolysis has been shown to be accelerated in many plants and the glycolytic pathway was considered to replace the Krebs cycle as the main source of energy (Kennedy *et al.*, 1992). Thus, ethanolic fermentation could be essential for the plant's survival during oxygen limiting conditions (Davies, 1980; Ricard *et al.*, 1994; Zhang and Greenway, 1994). Maize seedlings showed an increase in ADH activity under the flooding and the anoxic stresses, which may allow to continue the glycolysis and to produce some ATP, permitting the seedlings to survive under the stresses.

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〈和文抄録〉

冠水と酸素欠乏ストレスがトウモロコシ芽生えのアルコール脱水素酵素と
乳酸脱水素酵素に与える影響

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研究の目的は、冠水と酸素欠乏ストレスがトウモロコシの呼吸代謝に与える影響を明らかにすることにある。そのため本研究では、トウモロコシの2品種（ジェリーバンタム、ポップコーン）の芽生えに冠水と酸素欠乏ストレスを与え、これらのストレスがアルコール脱水素酵素（ADH）と乳酸脱水素酵素（LDH）の活性に与える影響、およびアルコール発酵と乳酸発酵の重要性を調べた。冠水と酸素欠乏ストレスは、両品種の芽生えのシュートと根においてADH活性を増加させたが、LDH活性は増加させなかった。このADH活性の増加は、いずれのストレスでも、両品種ともに根でシュートより大きかった。また、冠水ストレスは、ジェリーバンタムでポップコーンよりADH活性を増加させたが、酸素欠乏ストレスでは、両品種間で差がなかった。また、電気泳動によるADHアイソザイム分析の結果、これらのストレスによって、対照区で発現しているADHアイソザイム（1種類）のバンドが著しく濃くなり、新たに2種類のADHアイソザイムバンドが両品種で確認できた。この結果は、これらのストレスはADHタンパク質の合成量を増加させたことを示唆している。以上の結果は、トウモロコシ芽生えにおいて、冠水と酸素欠乏ストレスは、ADH活性を増加させアルコール発酵を促進させること、LDH活性は増加させず乳酸発酵を促進しないことを示唆している。また、このADH活性の増加は、ADHタンパク質の合成量が増加したためであると考えられる。