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Induction of alcohol dehydrogenase in lettuce seedlings by flooding stress

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Abstract

Alcohol dehydrogenase (ADH), its isozyme profiles and ethanol concentration in lettuce (*Lactuca sativa* L.) seedlings subjected to flooding stress were determined. Flooding stress caused increases in ADH activity and ethanol concentration. By 48 h, ADH activity and ethanol concentration in the flooded seedlings increased 3.2- and 7.0-fold, respectively, in comparison with those in non-stressed seedlings. Five electrophoretically separable ADH bands were found in extract of the flooded seedlings, whereas only two or three ADH bands were found in extract of non-stressed seedlings. These results indicate that lettuce ADH may have a system of three-gene and six-isozyme, and the increase in ADH activity in the flooded seedlings may be due to increased synthesis of the enzyme.

Additional key words: *Lactuca sativa*, ethanol, isozymes, low oxygen, waterlogging.

Introduction

Flooding and waterlogging evoke hypoxic soil conditions, which leads to rapid changes in respiratory metabolism and in the pattern of protein synthesis in plants (Crawford 1982, Sachs and Ho 1986, Paul and Ferl 1991). The switching from oxidative respiration to fermentative glycolysis enables to produce the metabolic energy and to survive in low oxygen tensions for several days (Waters *et al.* 1991, Drew 1997).

The activation of the fermentative glycolysis is accompanied by induction of alcohol dehydrogenase (ADH; Davies 1980, Kennedy *et al.* 1992). The advantage of ADH induction in the low oxygen condition allows to continue the glycolysis for production of methabolic energy owing to pyruvate comsumption and recycling NAD^+ (Kennedy *et al.* 1992, Ricard *et al.* 1994), and permits cytoplasmic pH regulation (Davies 1980, Roberts *et al.* 1984). Therefore, induction of ADH and activation of ethanolic fermentation was considered one of strategies for plants to survive in the low oxygen condition (Drew 1997, Vartapetian and Jackson 1997, Kato-Noguchi 1999).

Although ADH has been extensively studied, information has come from relatively few species (Ricard *et al.* 1994, Vartapetian and Jackson 1997, Kato-Noguchi 1999). Lettuce seedlings were often used for physiological experiments, but little is known about the induction of ADH in low oxygen condition. The objective of this study was to investigate the effect of hypoxia on ADH in the lettuce seedlings. Thus, ADH activity and ethanol level, and ADH isozyme composition were determined in the seedlings subjected to flooding stress.

Materials and methods

Plants and flooding treatment: Seeds of lettuce (*Lactuca sativa* L. cv. Cisco) were germinated on two sheets of moist filter paper (No 1; *Toyo Ltd*, Tokyo, Japan) at temperature of 25 °C in a growth chamber. Light was provided in a 12-h photoperiod by a white fluorescent lamp (*FL-40SSW National*, Tokyo, Japan; irradiance, 3.2 W m^{-2} at a plant level). After 3 d, uniform seedlings were selected and transferred to 9-cm Petri dishes each containing two sheets of filter paper moistened with 10 cm^3 distilled water, and grown in the same condition.

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Abbreviations: ADH - alcohol dehydrogenase; EDTA - ethylenediaminetetraacetic acid; DTT - dithiothreitol; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PVP - polyvinylpyrrolidone.
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After 3 d, the Petri dishes were placed into the plastic containers (30 × 60 × 45 cm) with distilled water. The seedlings were completely submerged and the water surface was covered with a sheet of plastic film as described by Muench *et al.* (1993) and the containers were kept at 25 °C in the growth chamber. Non-stressed seedlings were not submerged. The seedlings were harvested 12, 24, 36 and 48 h after onset of the flooding stress, frozen immediately in liquid N_2 and stored at -80 °C until extraction.

Extraction and assay of ADH: Frozen seedlings were homogenized in four volume of ice-cold solution containing 100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10 mM DTT, 1mM $MgCl_2$, 5 % (m/v) glycerol and 2.5 % (m/v) PVP. The homogenate was centrifuged at 25 000 *g* for 30 min and the supernatant was used for ADH assay.

Activity of ADH was measured spectrophotometrically by monitoring NADH oxidation at 340 nm in the following 1-cm^3 reaction mixture as described by Kato-Noguchi and Watada (1997): 85 mM MES (pH 7.0), 0.15 mM NADH and 0.02 cm^3 sample, and 10 mM acetaldehyde to initiate the reaction. The overall recovery of ADH activity through the quantification process was 86 ± 5 % according to 5 repeated assays with pure enzyme added to the extraction medium before sample homogenation. Protein was determined by the method of Bradford (1976) using bovine γ-globulin as a standard.

Quantification of ethanol: Quantification was carried out by gas chromatography according to the method of Kato-Noguchi and Watada (1997). Frozen seedlings (2 g fresh mass equivalent) were homogenized with 10 cm^3 of 0.1 M HCl. A 2-cm^3 aliquot of extract was incubated in Teflon sealed 5-cm^3 screw-cap test tube at 70 °C. After 20 min incubation, a 1-cm^3 sample of head space gas was analyzed by gas chromatography equipped with a flame ionization detector and a packed column (3 mm × 3 m; *Porapac Q*, *GL Science*, Tokyo, Japan). Internal standards for ethanol added to the extraction medium before sample homogenation showed 79 ± 6 % recovery as calculated from five replications.

Polyacrylamide gel electrophoresis: Frozen seedlings were homogenized and extracted as described above and applied to native slab polyacrylamide gel electrophoresis as described previously (Kato-Noguchi and Watada 1997). 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 cm^3 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 cm^3 ethanol (Good and Crosby 1989). ADH bands appeared within 20 min and development was allowed to proceed an additional 20 min before the reaction was stopped by rinsing in water.

Results and discussion

The optimum pH for ADH in crude extract of lettuce seedlings was 7.0 in the acetaldehyde to ethanol direction under standard assay condition (Fig. 1). The Km values of ADH in the extract for acetaldehyde and NADH were about 0.9 mM and 18 μM, respectively. These characteristics are similar to those reported for ADH from other plant sources (Ke *et al.* 1994, 1995).

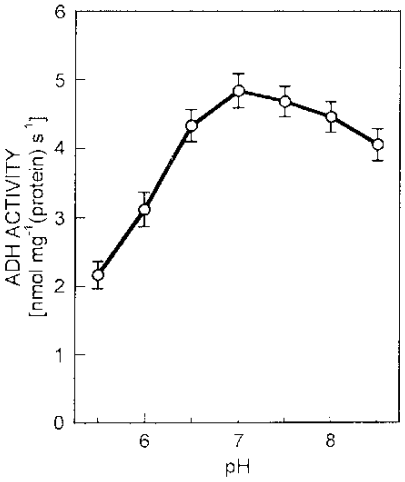

Fig. 1. Effect of pH on the activity of ADH catalysing the reduction of acetaldehyde to ethanol. Means ± SE from three experiments with at least four assays for each determination.

At the beginning of experiment, ADH activity was 1.5 nmol mg^{-1}(protein) s^{-1} and did not change in non-stressed seedlings. In response to flooding stress, the activity increased rapidly during first 12 h and slowly thereafter. The activity in the flooded seedlings was 2.8- and 3.2-fold greater than that in non-stressed seedlings after 24 and 48 h, respectively.

Concentration of ethanol in lettuce seedlings was 1.4 μmol g^{-1}(f.m.) at the beginning of the experiment, and it remained unchanged in non-stressed seedlings (Fig. 3). After onset of flooding stress, ethanol accumulated rapidly. The average accumulation rate of ethanol during 48 h was about 0.2 μmol g^{-1}(f.m.) h^{-1}. After 48 h, the concentration in the flooded seedlings was 7.0-fold greater than that in non-stressed seedlings.

To investigate ADH isozyme composition, native slab polyacrylamide gel electrophoresis was used and five separable bands having ADH activity were identified in the extracts of flooded seedlings, while two or three ADH bands were identified in extracts of non-stressed seedlings (Fig. 4). Thus, two or three additional bands were found

and the others were stained more intensely in the flooded seedlings than in non-stressed seedlings. Changes in staining intensity of the isozymes indicated that the increase in ADH activity in the flooded seedlings may be due to increased synthesis of the enzyme (Sachs and Ho 1986).

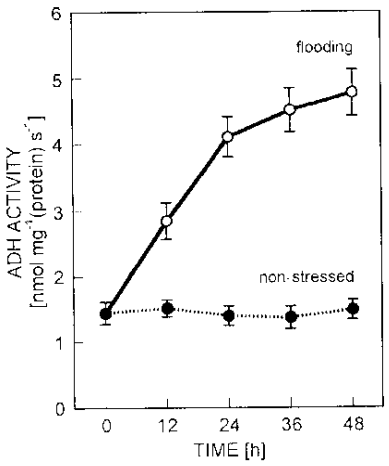

Fig. 2. Changes in the activity of ADH in lettuce seedlings under flooding stress and in the non-stressed seedlings. Means ± SE from three experiments with at least four assays for each determination.

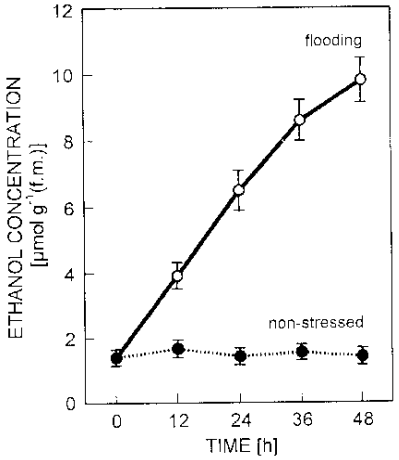

Fig. 3. Changes in the concentration of ethanol in lettuce seedlings under flooding stress and in the non-stressed seedlings. Means ± SE from three experiments with at least four assays for each determination.

Diploid plants have multiple ADH genes, at which products associated randomly to be dimer (Gottlieb 1982, Sachs and Ho 1986). Most plants have a two-gene system and the gene products dimerize to yield three electrophoretically distinct isozymes: ADH1-ADH1 homodimer, ADH1-ADH2 heterodimer, and ADH2-ADH2 homodimer (Xie and Wu 1989, Newman and VanToai 1991). Several plants have a three-gene system in which the gene products associated randomly to form six isozymes: ADH1-ADH1, ADH1-ADH2, ADH1-ADH3, ADH2-ADH2, ADH2-ADH3, ADH3-ADH3 (Hanson *et al.* 1984, Chourey and Widholm 1980). Although only five ADH bands were identified and one band was missing (Fig. 4), lettuce ADH may have six isozymes randomly associated and encoded by three different *Adh* genes (Fig. 4).

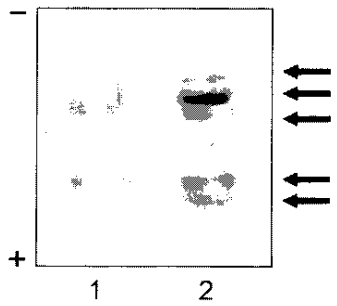

Fig. 4. Isozyme composition of ADH in lettuce seedlings subjected to flooding stress for 24 h and that in non-stressed seedlings. *Lane 1* - non-stressed seedlings. *lane 2* - flooded seedlings.

In summary, flooding stress increased the activity of ADH (Fig. 2) and the concentrations of ethanol in the lettuce seedlings (Fig. 3). Five electrophoretically separable ADH bands were found in extract of the seedlings subjected to flooding, which indicates lettuce ADH may be three-gene, six-isozyme system.

References

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chourey, P., Widholm, J.: Tissue specific alcohol dehydrogenase isozyme variation in carrot: Whole plant versus *in vitro* cultured cells. - *In Vitro* **16**: 571-574, 1980.

Crawford, R.M.M.: Physiological responses to flooding. - In: Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (ed.): Physiological Plant Ecology II. Water Relations and Carbon Assimilation. Pp. 453-477. Springer-Verlag, New York 1982.

Davies, D.D.: Anaerobic metabolism and the production of organic acids. - In: Davies, D.D. (ed.): The Biochemistry of Plants. Vol. 2. Pp. 581-611. Academic Press, New York 1980.

Drew, M.C.: Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. - Annu. Rev. Plant. Physiol. Plant. mol. Biol. **48**: 223-250, 1997.

Good, A.G., Crosby, W.L.: Anaerobic induction of alanine aminotransferase in barley root tissue. - Plant Physiol. **90**: 1305-1309, 1989.

Gottlieb, L.P.: Conservation and duplication of isozymes in plants. - Science **216**: 373-380, 1982.

Hanson, A.D., Jacobsen, J.V., Zwar, J.A.: Regulated expression of three alcohol dehydrogenase genes in barely aleurone layers. - Plant Physiol. **75**: 573-581, 1984.

Kato-Noguchi, H., Watada, A.E.: Effects of low-oxygen atmosphere on ethanolic fermentation in fresh-cut carrots. - J. amer. Soc. hort. Sci. **122**: 107-111, 1997.

Kato-Noguchi, H.: Flooding induced increases in alcohol dehydrogenase acivity in timothy and ryegrass seedlings. - Biol. Plant. **42**: 445-449, 1999.

Ke, D., Yahia, E., Mateos, M., Kader, A.A.: Ethanolic fermentation of 'Bartlett' pears as influenced by ripening stage and atmospheric composition. - J. amer. Soc. hort. Sci. **119**: 976-982, 1994.

Ke, D., Yahia, E., Hess, B., Zhou, L., Kader, A.A.: Regulation of fermentative metabolism in avocado fruit under oxygen and carbon dioxide stresses. - J. amer. Soc. hort. Sci. **120**: 481-490, 1995.

Kennedy, R.A., Rumpho, M.E., Fox, T.C.: Anaerobic metabolism in plants. - Plant Physiol. **100**: 1-6, 1992.

Muench, D.G., Archibold, O.W., Good, A.G.: Hypoxic metabolism in wild rice (*Zizania palustris*): Enzyme induction and metabolite production. - Physiol. Plant. **89**: 165-171, 1993.

Newman, K.D., VanToai, T.T.: Cell biology and molecular genetics: Developmental regulation and organ-specific expression of soybean alcohol dehydrogenase. - Crop Sci. **31**: 1253-1257, 1991.

Paul, A.-L., Ferl, R.J.: *In vivo* footprinting reveals unique *cis*-elements and different models of hypoxic induction in maize Adh1 and Adh2. - Plant Cell **3**: 159-168, 1991.

Ricard, B., Couée, I., Raymond, P., Saglio, P.H., Saint-Ges, V., Pradet, A.: Plant metabolism under hypoxia and anoxia. - Plant Physiol. Biochem. **32**: 1-10, 1994.

Roberts, J.K.M., Callis, J., Wemmer, D., Walbot, V., Jardetzky, O.: Mechanism of cytoplasmic pH regulation in hypoxic maize roots tips and its role in survival under hypoxia. - Proc. nat. Acad. Sci. USA **81**: 3379-3383, 1984.

Sachs, M.M., Ho, T.-H.D.: Alteration of gene expression during environmental stress in plants. - Annu. Rev. Plant Physiol. **37**: 363-376, 1986.

Vartapetian, B.B., Jackson, M.B.: Plant adaptations to anaerobic stress. - Ann. Bot. **79**: 2-20, 1997.

Waters, I., Morrell, S., Greenway, H., Colmer, T.D.: Effects of anoxia on wheat seedlings. II. Influence of O_2 supply prior to anoxia on tolerance to anoxia, alcoholic fermentation, and sugar levels. - J. exp. Bot. **42**: 1437-1447, 1991.

Xie, Y., Wu, R.: Rice alcohol dehydrogenase genes: Anaerobic induction, organ specific expression and characterization of cDNA clones. - Plant mol. Biol. **13**: 53-68, 1989.