BIOLOGIA PLANTARUM 42 (3): 445-449, 1999

BRIEF COMMUNICATION

Flooding induced increase in alcohol dehydrogenase activity in timothy and ryegrass seedlings

H. KATO-NOGUCHI

Department of Biochemistry and Food Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

Abstract

Two forage grasses, timothy (*Phleum pratense* L.) and ryegrass (*Lolium multiflorum* Lam.) were exposed to flooding, and activities of alcohol dehydrogenase (ADH) and their isozyme profiles were determined. The flooding stress increased ADH activities in both species. This increase was 2-times greater in timothy than in ryegrass. Only one ADH isozyme was found in non-flooded seedlings of both species, whereas two and four bands were identified in ryegrass and timothy seedlings, respectively, under flooding stress.

Additional key words: ADH isozymes, anaerobiosis, ethanolic fermentation, *Lolium multiflorum*, *Phleum pratense*.

Field plants suffer anaerobiosis if they are exposed to flooding or waterlogging during growing season. For survival many plants evolved a metabolic adaptation (*e.g.* Kennedy *et al.* 1992, Ricard *et al.* 1994, Vartapetian and Jackson 1997). Induction of alcohol dehydrogenase (ADH) during anaerobiosis has been observed in many plant species, *e.g. Arabidopsis* (Dolferus *et al.* 1997), maize (Paul and Ferl 1991, Good and Muench 1993), and rice (Rivoal *et al.* 1989, Muench *et al.* 1993). The advantage of ADH induction under the anaerobic conditions is acceleration of ethanolic fermentation pathway, which allows glycolysis to continue owing to consumption of pyruvate and regeneration of NAD (Kennedy *et al.* 1992, Good and Menuch 1993, Ke *et al.* 1994).

Received: 13 October 1998, *accepted* 21 January 1999.

Abbreviations: ADH - alcohol dehydrogenase; EDTA - ethylenediaminetetraacetic acid; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEMED - N,N,N',N'-tetramethylethylenediamine.

Fax: (+81) 87 891 3086, e-mail: hisashi@ag.kagawa-u.ac.jp

The objective of this study was to investigate the effect of flooding on ADH induction in forage grasses. Thus, activities of ADH in seedlings of timothy and ryegrass were determined under flooding stress and ADH isozymes were characterized.

Caryopses of timothy (*Phleum pratense* L.) and ryegrass (*Lolium multiflorum* Lam.) were sterilized in a 2 % (m/v) solution of sodium hypochlorite for 15 min, rinsed in tap water for 2 h and germinated on two sheets of moist filter paper at 25 °C, and 12-h photoperiod in a growth chamber. Light was provided with a white fluorescent lamp (3.2 W m^{-2} at plant level: *FL40SSW, National*, Tokyo). After 4 d, uniform seedlings were selected and transferred to 9-cm Petri dishes each containing two sheets of filter paper moistened with 10 cm^3 of distilled water. After 3 d, the Petri dishes were placed in the plastic containers and containers were filled with distilled water. The seedlings were held 3 cm below the water surface, which was covered a sheet of plastic film as described by Muench *et al.* (1993) and the containers were kept at 25 °C in a growth chamber.

For determination of ADH activity, the Petri dishes were removed from the containers, and the seedlings were harvested, frozen immediately with liquid nitrogen and stored at −80 °C until extraction. Frozen seedlings were homogenized in four volumes of ice-cold solution containing 100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 mM $MgCl_2$ and 5 % (v/v) glycerol. The brei was centrifuged at 25 000 *g* for 30 min and the supernatant was used immediately for the measurements of ADH activity. This was measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm. The 1 cm^3 of assay mixture contained 85 mM MES (pH 6.5), 0.9 mM NADH, 0.02 cm^3 sample, and 5 mM acetaldehyde (Kato-Noguchi and Watada 1997). The overall recovery of ADH activity for timothy and ryegrass through the quantification process was 84 ± 7 % and 82 ± 5 %, respectively, according to 5 repeated assays with pure enzyme in the extract. Protein was determined by the method of Bradford (1976) using bovine γ-globulin as a standard.

Frozen seedlings were homogenized and extracted as described above and run for 2 h through 1.5 mm nondenaturing gel at 15 mA at 4 °C. The running gel consisted of 9.0 % (m/v) acrylamide, 0.2 % bisacrylamide, 0.37 M Tris-HCl (pH 8.5) and 12 % (m/v) glycerol, which was polymerized with 0.08 % TEMED and 0.04 % ammonium persulfate. The stacking gel consisted of 5 % acrylamide, 0.2 % bisacrylamide, 0.12 M Tris-HCl (pH 6.7) and 12 % glycerol, which was polymerized with 0.06 % TEMED and 0.05 % ammonium persulfate. The running buffer consisted of 10 mM Tris and 77 mM glycine. 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 30 min before the reaction was stopped by rinsing in water.

The ADH activity in timothy and ryegrass seedlings was low at the beginning of experiment and did not change in both non-flooded controls (Fig. 1). Under flooding stress, the ADH activity in timothy seedlings increased over the course of 48 h to

6 times that in the non-flooded control. On the other hand, the ADH activity in ryegrass seedlings increased and leveled off after 24 h under the stress. The maximum activity in ryegrass seedlings was 3.5 times that in the non-flooded control. After 48 h, the activity was 0.9 and 0.4 μmol mg^{-1}(protein) min^{-1} for timothy and ryegrass, respectively, under flooding stress. The increases of ADH in both seedlings may accelerate ethanolic fermentation, which allows glycolysis to continue and to produce some ATP (Kennedy *et al.* 1992, Good and Menuch 1993, Ke *et al.* 1994). However, this acceleration may be greater in timothy than in ryegrass, since flooding induced 2 times higher increase in ADH activity in timothy than in ryegrass (Fig. 1). It was reported that maize mutants with zero ADH activity were more sensitive to anaerobic stress than their wild types and ADH activity in the seedlings showed the good correlation with their tolerance to the stress (Johnson *et al.* 1989, 1994).

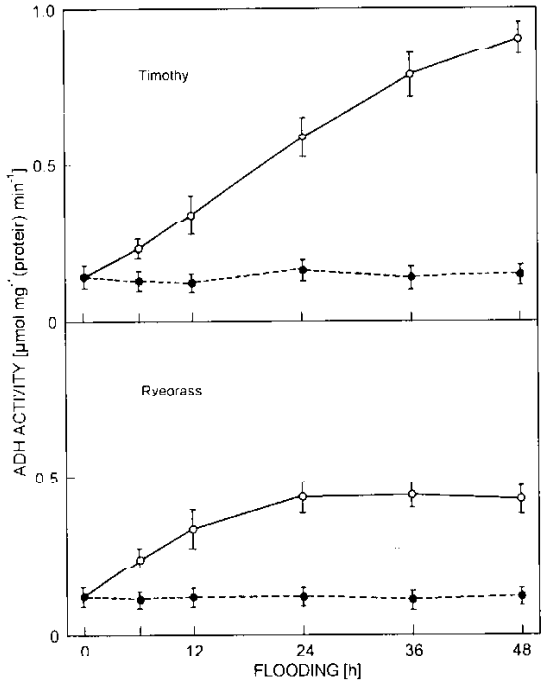

Fig. 1. Changes in the level of AHD activity in timothy and ryegrass seedlings under flooding stress (*open circles*) and in their non-flooded controls (*closed circles*). Means + SE from three experiments with at least three assays for each determination.

Most plant ADH enzymes that have been examined are dimers and the two subunits of ADH are encoded by two unlinked genes (Gottlieb 1982, Hanson *et al.* 1984, Sachs and Ho 1986). The products of these two 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). Some plants have more than two ADH genes and the products of these genes dimerize to more than three electrophoretically distinct isozymes, *e.g.*, barley (Hanson *et al.* 1984), *Acacia* (Small *et al.* 1990) and carrot (Chourey and Widholm 1980). However, all isozymes could not be found in each plant tissue because of the limited expression of the specific ADH isozymes (Chourey and Widholm 1980, Xie and Wu 1989, Ke *et al.* 1994).

Using polyacrylamide gel electrophoresis (Fig. 2), only single band of ADH isozyme was found in non-flooded seedlings of both species, whereas two and four bands were identified in seedlings of ryegrass and timothy exposed flooding, respectively. These results indicate that the flooding stress may increase ADH activity due to activation of ADH subunit translation and that timothy may have more than two ADH genes because four bands of ADH isozymes were found (Fig. 2).

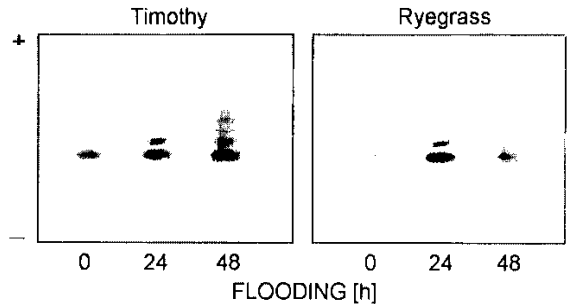

Fig. 2. Isozyme compositions of ADH in timothy and ryegrass seedlings exposed to flooding stress.

When oxygen becomes limiting, glycolysis has accelerated in many plants owing to induction of ADH, and the glycolysis and ethanolic fermentation were considered to replace the Krebs cycle as the main source of ATP supply. In addition, ADH was necessary for tight cytoplasmic pH regulation and for removal of acetaldehyde because of its phytotoxic effect (Kennedy *et al.* 1992, Rivoal and Hanson 1994). Thus, ethanolic fermentation considered to be essential for the plant's survival during anaerobic stress (Davies 1980, Ricard *et al.* 1994, Zhang and Greenway 1994).

References

Bradford, M.M.: A rapid and sensitive method for the quantification 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.

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.

Dolferus, R., Ellis, M., Bruxelles, G.De., Trevaskis, B., Hoeren, F.: Strategies of gene action in *Arabidopsis* during hypoxia. - Ann. Bot. **79**: 21-31, 1997.

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

Good, A.G., Muench, D.G.: Long-term anaerobic metabolism in root tissue: metabolic products of pyruvate metabolism. - Plant Physiol. **101**: 1163-1168, 1993.

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.

Johnson, J.R., Cobb, B.G., Drew, M.C.: Hypoxic induction of anoxia tolerance in root tips of *Zea mays*. - Plant Physiol. **91**: 837-841, 1989.

Johnson, J.R., Cobb, B.G., Drew, M.C.: Hypoxic induction of anoxia tolerance in roots of *Adh1* null *Zea mays* L. - Plant Physiol. **105**: 61-67, 1994.

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.

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.

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.

Rivoal, J., Ricard, B., Pradet, A.: Glycolytic and fermentative enzyme induction during anaerobiosis in rice seedlings. - Plant Physiol. Biochem. **27**: 43-52, 1989.

Rivoal, J., Hanson, A.D.: Metabolic control of anaerobic glycolysis. - Plant Physiol. **106**: 1179-1185, 1994.

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

Small, J.G.C., Burger, A.L., Botha, F.C.: Alcohol dehydrogenase in the desert species *Acacia erioloba*: ontogeny during germination and induction in seedling roots. - S. Afr. J. Bot. **56**: 403-408, 1990.

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

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.

Zhang, Q., Greenway, H.: Anoxia tolerance and anaerobic catabolism of aged beet root storage tissues. - J. exp. Bot. **45**: 567-575, 1994.