Submergence Acclimation to Low-Temperature Stress in Rice Roots

Hisashi Kato-Noguchi

(Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan)

Abstract: A low temperature (10°C, 48 h) inhibited primary root growth of rice seedlings (Oryza sativa L.). However, the inhibition was significantly mitigated by submergence for 24 h before the exposure to low temperatures, which induced alcohol dehydrogenase and increased the ethanol concentration in roots. Exogenous application of ethanol also had a similar mitigating effect. These results suggest that submergence pretreatment increases the tolerance to low temperature in rice roots due to ethanol accumulation in the roots.

Key words: Acclimation, Alcohol dehydrogenase, Ethanol, Ethanolic fermentation, Low temperature tolerance, Rice.

Most plants are often under various environmental stresses; for example, low and high temperatures, high and low irradiation, excess and insufficient water, low oxygen, and salinity. For survival under these stress conditions, many plants have evolved a series of adaptive physiological and morphological changes which enhance their ability to survive the adverse conditions (Crawford and Braendle, 1996; Vartapetian and Jackson, 1997). The exposure to mild environmental stress conditions has also been reported to greatly improve the viability of the plant during subsequent periods of severe environmental stress conditions (Drew, 1997; Thomashow, 1999).

Chilling injury occurs in many plants of tropical and subtropical origin when exposed to low temperature, non-freezing temperatures below 10-15°C (Saltveit and Morris, 1990). Rice seedlings also suffer low-temperature stress, which is a major constraint to its growth and development (Levitt, 1980). Low temperature strongly suppresses many proteins and genes of rice seedlings, but also induces several genes and proteins (Graham and Patterson, 1982; Hahn and Walbot, 1989; Christie et al., 1991; Minhas and Grover, 1999; Sung et al., 2003). These proteins have been considered to be involved in the adaptation to low-temperature stress conditions (Guy, 1990; Thomashow, 1999; Sung et al., 2003).

Low temperature has been found to rapidly induce gene expression of alcohol dehydrogenase (ADH) in rice seedlings (Christie et al., 1991; Minhas and Grover, 1999). ADH is an enzyme involved in ethanolic fermentation and is the most studied enzyme in relation to anaerobiosis (Kennedy et al., 1992; Ricard et al., 1994; Tadege et al., 1999). However, the biological role of ADH is so far apparent only under anaerobiosis, and activity of ethanolic fermentation and ethanol concentration in the seedlings under low-temperature conditions has not yet been determined.

In this study, we examined the effects of submergence pretreatment on the viability of rice seedlings during the subsequent period of low-temperature stress. To examine the possible involvement of ethanolic fermentation in the mechanism of submergence-induced tolerance to low-temperature stress, we also investigated the ADH activity and ethanol concentration in rice seedlings subjected to submergence followed by low-temperature stress.

Materials and Methods

1. Plant material and treatment with submergence and low temperature

Seeds of rice (Oryza sativa L. Nipponbare) were surface sterilized in a 25 mM sodium hypochlorite for 15 min and rinsed four times in distilled water. The seeds were germinated on two sheets of moist filter paper (No 1; Toyo Ltd., Tokyo, Japan) at 25°C with a 12-h photoperiod in a growth chamber for three days. Light was provided from above with a white fluorescent tube (irradiance, 2.9 W m⁻² at plant level; FL40SBR, National, Tokyo). Then, uniform seedlings were transferred to plastic containers (10 × 10 × 15 (height) cm) and completely submerged in water (submergence pretreatment). 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 with a 12-h photoperiod for one day.

After the submergence pretreatment or without the submergence pretreatment, the seedlings were transferred to the Petri dishes containing two sheets of filter paper moistened with 10 mL distilled water,
and grown at 10°C with a 12-h photoperiod for two days (low temperature treatment). After the above treatment (6 days after seeding), the length of primary roots of the seedlings was measured with a ruler.

Control seedlings were grown at 25°C with a 12-h photoperiod in Petri dishes containing two sheets of filter paper moistened with 10 mL distilled water. Fig. 1 shows the experimental design. These experiments were repeated five times.

2. Extraction and assay of ADH

For determination of ADH in primary roots of rice seedlings, 30 primary roots were powdered in a mortar containing liquid N₂ using a pestle and homogenized with 3 mL of ice-cold solution containing 100 mM Tris-HCl (pH 8.0), 10 mM Na-ascorbate, 10 mM DTT, 50 mg bovine serum albumin and 15% (w/v) glycerol (Hanson et al., 1984). The homogenate was centrifuged at 30,000 x g for 20 min and the supernatant was used immediately for enzyme assay. ADH activity was measured in the acetaldehyde to ethanol direction in the 1 mL reaction mixture containing 85 mM MES (pH 6.5), 0.15 mM NADH, 0.02 mL sample, and 5 mM acetaldehyde by monitoring NADH oxidation at 340 nm as described by Kato-Noguchi (2000). These experiments were repeated three times.

3. Extraction and determination of acetaldehyde and ethanol

Rice primary roots were powdered as described above and homogenized with 5 mL of 0.1 M HCl. A 2-mL aliquot of extract was incubated in a Teflon-sealed 5 mL screw-cap test tube at 70°C. After 20 min incubation, a 1 mL sample of headspace gas was analyzed in a gas chromatograph, and acetaldehyde and ethanol were determined according to the method of Kato-Noguchi (2000). These experiments were repeated three times.

4. Ethanol treatment

Rice seedlings were germinated and grown as described above. Uniform 3-day-old seedlings were then transferred to Petri dishes containing two sheets of filter paper moistened with 10 mL of 100 mM ethanol, and incubated at 25°C. After 24 h, the seedlings were rinsed five times with distilled water, transferred to the Petri dishes containing two sheets of filter paper moistened with 10 mL distilled water, and grown at 10°C for 48 h. Fig. 4 shows the experimental design. The length of primary roots of the seedlings was measured with a ruler at the end of the treatment (6 days after seeding). These experiments were repeated.
Results

1. Low-temperature tolerance

Low temperature treatment inhibited primary root growth of rice seedlings to 34% of the control (Fig. 1). The low temperature after submergence pretreatment, although submergence itself also inhibited root growth, the growth inhibition was mitigated and the root length became 73% of the control. These results indicate that the submergence pretreatment increases low-temperature tolerance of the roots of rice seedlings.

2. ADH activity

ADH activity in rice primary roots measured two

| Treatment            | Acetaldehyde concentration (nmol g⁻¹ fresh weight) |
|----------------------|---------------------------------------------------|
| Non-stressed         | 18 ± 1.7                                          |
| Submergence          | 24 ± 2.1                                          |
| Low temp.            | 20 ± 1.9                                          |
| Submergence + Low temp. | 29 ± 2.5                                       |

Experimental design is as shown in Fig. 1. Means ± SE from 3 independent experiments with 4 assays for each determination are shown.
days after submergence (day 6) was 475 nmol g\(^{-1}\) fresh weight min\(^{-1}\), which was 4.9-fold greater than that in control roots (Fig. 2). Just after the submergence (day 4), however, ADH activity was 763 nmol g\(^{-1}\) fresh weight min\(^{-1}\). Thus, ADH activity, which increased by submergence, decreased somewhat after submergence by incubation at 25\(^\circ\)C for two days. Induction of ADH during submergence has been reported in many plant species (Setter et al., 1997; Vartapetian and Jackson, 1997; Kato-Noguchi, 2001).

Low temperature increased ADH activity in rice primary roots, to 2.6-fold that in the control (Fig. 2). ADH activity in the roots of seedlings subjected to low temperature after submergence pretreatment, increased to 8.4-fold that in the control. Low temperature treatment also induces ADH gene and protein in *Arabidopsis* and maize seedlings (Christie et al., 1991; Jarillo et al., 1993; Dolferus et al., 1997) and rice seedlings (Christie et al., 1991; Minhas and Grover, 1999).

3. **Ethanol production**

Since ADH is the main enzyme in ethanolic fermentation, ethanol concentration was determined in rice primary roots in order to examine if low-temperature stress accelerates ethanolic fermentation and increases ethanol production. Just after submergence pretreatment (day 4), ethanol concentration was 11.7 \(\mu\)mol g\(^{-1}\) fresh weight, and after incubation for two days at 25\(^\circ\)C, the concentration decreased to 9.7 \(\mu\)mol g\(^{-1}\) fresh weight (Fig. 3; submergence). Submergence stress has been reported to increase ethanol fermentation in many plant species including rice (Setter et al., 1997; Vartapetian and Jackson, 1997; Kato-Noguchi, 2001).

Low temperature increased the ethanol concentration in rice primary roots to 7.6-fold that in the control (Fig. 3). Exposure to low temperature after submergence pretreatment increased the ethanol concentration in the roots to 35-fold that in the control. These results indicate that low-temperature stress increased ethanol production in the roots, and the submergence pretreatment increased the ethanol production in the low temperature condition.

4. **Acetaldehyde production**

Acetaldehyde concentration was much lower than ethanol concentration in rice primary roots (Table 1), and was not increased significantly by either low temperature or submergence. The concentration in the roots was increased slightly when the seedlings were subjected to low-temperature stress after submergence pretreatment. The roots and shoots of all rice seedlings did not show any symptoms of toxicity.
5. Effect of exogenously applied ethanol on low temperature tolerance

Exogenously applied 100 mM ethanol did not significantly affect the growth of primary roots of rice seedlings (Fig. 4). Ethanol has been reported not to inhibit the growth of rice seedlings at the concentration up to 200 mM (Kato-Noguchi, 2002) and to be less toxic than previously stated (Perata and Alpi, 1991; Kennedy et al., 1992). Low-temperature treatment for 2 days decreased the primary root length to 34% of the control, but when the seedlings were pretreated with ethanol the root length was 65% of the control (Fig. 4). These results suggest that ethanol increases low-temperature tolerance in the roots of rice seedlings.

Discussion

Submergence pretreatment increased the low-temperature tolerance of rice roots (Fig. 1), accompanied by an increase in the ethanol concentration in rice roots (Fig. 3). One of the primary effects of low-temperature stress is the alteration of membrane fluidity properties in plant cells. Low temperature induces lipid peroxidation in plasma membrane resulting in the phase transition of the membrane from liquid to gel (Uemura and Yoshiida, 1986; Feng et al., 2000). Ethanol increases membrane fluidity and prevented its phase transition from liquid to gel (Saltveit, 1994; Frenkel and Erez, 1996; Saltveit et al., 2004). Thus, increase the low-temperature tolerance by increasing the fluidity of plant cell membranes.

The phase change of plant cell membranes by low-temperature stress directly affects membrane-bound metabolic processes, such as plasma membrane ATPase activity and cellular calcium gradients (Levitt, 1980; Guy, 1990; Thomashow, 1999). The limited activity of the ATPase led to cytoplasmic acidosis, which is correlated with plant cell death (Yoshida, 1994). Ethanol treatment also increases ATPase activity in plasma membranes (Peters and Frenkel, 2004). In addition, ADH-deficient mutant of maize seedlings, which can not produce ethanol, was more sensitive to low temperature than the wild-type seedlings (Peters and Frenkel, 2004). Therefore, ethanol accumulation could be responsible for submergence-induced low-temperature tolerance. This hypothesis is supported by the increased low-temperature tolerance induced by exogenously applied ethanol (Fig. 4).

ADH gene expression in rice seedlings was induced by low-temperature stress conditions (Christie et al., 1991; Minhas and Grover, 1999). Low-temperature stress also increased the induction of ADH and accumulation of ethanol in rice roots (Figs. 2 and 3). Thus, the induction of ADH and accumulation of ethanol in low-temperature stress conditions may lead to adaptation to the low temperature to some extent in the roots by altering the physical properties of membrane lipids.

Chilling injury occurs in rice seedlings at the early developmental stage if temperature drops to below 10-15°C (Levitt, 1980). The findings in the present research suggest that submergence pretreatment may increase tolerance of rice to low-temperature stress conditions due to ethanol accumulation.

References

Christie, P.J., Hahn, M. and Walbot, V. 1991. Low-temperature accumulation of alcohol dehydrogenase-1 mRNA and protein activity in maize and rice seedlings. Plant Physiol. 95 : 699-706.

Crawford, R.M.M. and Braendle, R. 1996. Oxygen deprivation stress in a changing environment. J. Exp. Bot. 47 : 145-159.

Dolferus, R., Ellis, M., de Bruxelles, G., Trevaskis, B., Hoeren, F., Dennis, E.S. and Peacock, W.J. 1997. Strategies of gene action in Arabidopsis during hypoxia. Ann. Bot. 79 (Suppl.) : 21-31.

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

Feng, A.L., Tang, X. and Wang, X. 2000. Changes of microsomal membrane properties in spring wheat leaves (Triticum aestivum L.) exposed to enhanced and ultraviolet-B radiation. J. Photochem. Photobiol. 57 : 60-65.

Frenkel, C. and Erez, A. 1996. Induction of chilling tolerance in cucumber (Cucumis sativus) seedlings by endogenous and applied ethanol. Physiol. Plant. 96 : 593-600.

Graham, D. and Patterson, B.D. 1982. Response of plants to low, non-freezing temperatures : proteins, metabolism and acclimation. Annu. Rev. Plant Physiol. 33 : 347-372.

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance. Role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41 : 187-223.

Hahn, M. and Walbot, V. 1989. Effects of cold-treatment on protein synthesis and mRNA levels in rice leaves. Plant Physiol. 91 : 930-938.

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

Jarillo, J.A., Leyva, A., Salinas, J. and Martínez-Zapater, J.M. 1993. Low temperature induces the accumulation of alcohol dehydrogenase mRNA in Arabidopsis thaliana, a chilling-tolerant plant. Plant Physiol. 101 : 833-837.

Kato-Noguchi, H. 2000. Evaluation of the importance of lactate for the activation of ethanolic fermentation in lettuce roots in anoxia. Physiol. Plant. 109 : 28-33.

Kato-Noguchi, H. 2001. Submergence tolerance and ethanolic fermentation in rice coleoptiles. Plant Prod. Sci. 4 : 62-65.

Kato-Noguchi, H. 2002. Ethanol sensitivity of rice and oat coleoptiles. Physiol. Plant. 115 : 119-124.

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

Levitt, J. 1980. Response of plants to environmental stresses : chilling, freezing, and high temperature stresses. In T.T. Kozlowsky ed., Physiological Ecology : A Series of Monographs, Texts, and Treatises, Ed. 2, Vol 1. Academic Press, New York. 23-64.

Minhas, D. and Grover, A. 1999. Transcript levels of genes encoding various glycolytic and fermentation enzymes change.
in response to abiotic stresses. Plant Sci. 146 : 41-51.

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

Perata, P. and Alpi, A. 1991. Ethanol-induced injuries to carrot cells. The role of acetaldehyde. Plant Physiol. 95 : 748-752.

Peters, J.S. and Frenkel, C. 2004. Relationship between alcohol dehydrogenase activity and low-temperature in two maize genotypes, Silverado F, and Adh1Adh2 doubly null. Plant Physiol. Biochem. 42 : 841-846.

Perata, P. and Alpi, A. 1991. Ethanol-induced injuries to carrot cells. The role of acetaldehyde. Plant Physiol. 95 : 748-752.

Peters, J.S. and Frenkel, C. 2004. Relationship between alcohol dehydrogenase activity and low-temperature in two maize genotypes, Silverado F, and Adh1Adh2 doubly null. Plant Physiol. Biochem. 42 : 841-846.

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

Saltveit, M.E. and Morris, L.L. 1990. Overview of chilling injury in horticultural crops. In : C.Y. Wang ed., Chilling Injury of Horticultural Crops. CRC Press, Boca Raton, FL. 3-15.

Saltveit, M.E. 1994. Exposure to alcohol vapours reduces chilling-induced injury of excised cucumber cotyledons, but not of seedlings or excised hypocotyl segments. J. Exp. Bot. 45 : 813-821.

Saltveit, M.E., Peiser, G. and Rab, A. 2004. Effect of acetaldehyde, arsenite, ethanol, and heat shock on protein synthesis and chilling sensitivity of cucumber radicles. Physiol. Plant. 120 : 556-562.

Setter, T.L., Ellis, M., Laureles, E.V., Ella, E.S., Senadhira, D., Mishra, S.B., Sarkarung, S. and Datta, S. 1997. Physiology and genetics of submergence tolerance in rice. Ann. Bot. 79 (Suppl.) : 67-77.

Sung, D.Y., Kaplan, F., Lee, K.J. and Guy, C.L. 2003. Acquired tolerance to temperature extremes. Trend Plant Sci. 8 : 179-187.

Tadege, M., Dupuis, I. and Kuhlemeier, C. 1999. Ethanolic fermentation, new functions for an old pathway. Trend Plant Sci. 4 : 320-325.

Thomasow, M.F. 1999. Plant cold acclimation : freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 : 571-579.

Uemura, M. and Yoshida, S. 1986. Studies on freezing injury in plant cells. II. Protein and lipid changes in the plasma membranes of Jerusalem artichoke tubers during a lethal freezing in vivo. Plant Physiol. 80 : 187-195.

Vartapetian, B.B. and Jackson, M.B. 1997. Plant adaptations to anaerobic stress. Ann. Bot. 79 (Suppl.) : 5-20.

Yoshida, S. 1994. Low temperature-induced cytoplasmic acidosis in cultured mung bean (Vigna radiate (L.) Wilczek) cells. Plant Physiol. 104 : 1131-1138.