Analysis of Expression of Genes for Mitochondrial Aldehyde Dehydrogenase in Maize during Submergence and Following Re-aeration

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Acetaldehyde is oxidized from anaerobically accumulated ethanol during re-aeration in plants and may cause post-anoxic (post-hypoxic) injury. Aldehyde dehydrogenase (ALDH) can metabolize acetaldehyde to acetate, which is much less toxic. To understand the role of ALDH in alleviating post-anoxic injury of plants, we analyzed the expression of the mitochondrial ALDH genes, rf2a and rf2b, and determined the ethanol and acetaldehyde contents under submergence and subsequent re-aeration in a submergence-intolerant plant, maize (Zea mays L., inbred line B73). The level of rf2a mRNA decreased, whereas that of rf2b mRNA increased under submergence conditions. When the submerged plants were re-aerated, the transcript levels returned to the original levels. The level of mitochondrial ALDH proteins decreased under submergence and remained unchanged under re-aeration. The ALDH activity decreased under submergence and, during re-aeration, recovered to the original level much more slowly than in the case of rice (Oryza sativa L.). The content of acetaldehyde, which was produced under submergence, further increased following re-aeration. These results suggest that the lower submergence tolerance of maize compared to that of rice is partly due to a weaker ALDH activity during re-aeration.

Key Words: aldehyde dehydrogenase, acetaldehyde, post-anoxic (post-hypoxic) injury, abiotic stress, rf2, maize.

Introduction

Glycolysis and ethanolic fermentation are essential for the survival of plants under low-oxygen conditions (Perata and Alpi 1993, Sachs et al. 1996, Vartapetian and Jackson 1997). However, under low-oxygen conditions, ATP production occurs mainly by glycolysis, which is less efficient than ATP production under aerobic conditions. Under extended anaerobiosis, a plant can be injured by lower ATP production and by the production of phytotoxic acetaldehyde. This type of injury is referred to as anoxic injury or hypoxic injury (Perata and Alpi 1991, Crawford and Brändle 1996).

In addition, cells may be damaged during re-aeration following anaerobic conditions. This is known as post-anoxic injury or post-hypoxic injury (Monk et al. 1987, VanToai and Bolles 1991, Crawford et al. 1994). This type of injury can be caused by two kinds of molecules: acetaldehyde and reactive oxygen species (ROS). The latter include superoxide radicals (O₂⁻), hydroxyl radicals (OH) and hydrogen peroxide (H₂O₂). The production of ROS is induced immediately after exposure of anaerobic plant tissues to normal oxygen tension and, as a consequence, proteins, nucleic acids and membranes may undergo severe peroxidation (Wollenweber-Ratzer and Crawford 1994, Biemelt et al. 1998, Blokhina et al. 2001). Re-aeration also induces the production of acetaldehyde, as a result of the oxidation of ethanol, which is produced and accumulated by ethanolic fermentation under low-oxygen conditions (see Fig. 6; Pfister-Sieber and Brändle 1995, Zuckermann et al. 1997). Ethanol is assumed to be rapidly oxidized to acetaldehyde by alcohol dehydrogenase (ADH) and/or catalase (CAT) (Monk et al. 1987, Pavelic et al. 2000, Kreuzwieser et al. 2001). CAT probably oxidizes ethanol through the reduction of H₂O₂ that is produced during re-aeration (Monk et al. 1987, Pavelic et al. 2000).

Acetaldehyde is harmful to cells because of its tendency to form acetaldehyde-protein and acetaldehyde-DNA adducts (Perata et al. 1992, Zhang et al. 1997). Cells possess mechanisms to metabolize acetaldehyde. Aldehyde dehydrogenase [ALDH (aldehyde: NAD(P)⁺ oxidoreductase, EC 1.2.1.3)] catalyzes the conversion of aldehydes to the corresponding acids (reviewed by Sophos et al. 2001). Humans...
have at least two ALDH isozymes (cytosolic ALDH1 and mitochondrial ALDH2) that are involved in the oxidation of acetaldehyde, which is produced during the metabolism of dietary ethanol. ALDH2 is the main enzyme in the detoxification of acetaldehyde (Yoshida et al. 1998). In plants, mitochondrial ALDH genes have been identified in several species, including rice (Nakazono et al. 2000, Tsuji et al. 2003b), barley (Meguro et al. 2001), tobacco (op den Camp and Kuhlemeier 1997), Arabidopsis (Skibbe et al. 2002, Kirch et al. 2004) and maize (Cui et al. 1996, Skibbe et al. 2002). Two maize mitochondrial ALDHs have been identified, RF2A and RF2B (Cui et al. 1996, Skibbe et al. 2002), both of which can efficiently oxidize acetaldehyde (Liu et al. 2001, Liu and Schnable 2002). Cytosolic ALDH protein has also been reported in plants (Skibbe et al. 2002, Kirch et al. 2004). Nair et al. (2004) have recently observed that the cytosolic ALDH gene (ALDH1a) of Arabidopsis was responsible for the mutation of the reduced epidermal fluorescence 1 (ref1) gene. This finding suggests that cytosolic ALDH is mainly involved in the oxidation of sinapaldehyde and coniferaldehyde.

We previously reported that the transcript level of a mitochondrial ALDH gene (ALDH2a) from rice, a species that is tolerant to low oxygen conditions, increased under anaerobic conditions (Nakazono et al. 2000). The transcript level of a second rice mitochondrial ALDH gene, ALDH2b, decreased under submergence and recovered to the initial level after re-aeration (Tsuji et al. 2003a). Re-aerated rice plants were also characterized by a strong induction of mitochondrial ALDH2a protein, increased ALDH activity, and decrease in the amounts of ethanol and acetaldehyde that were produced under submerged conditions (Tsuji et al. 2003a). These results suggest that, in rice, mitochondrial ALDH (mainly ALDH2a) is involved in the alleviation of post-anoxic injury induced by acetaldehyde.

If this is the case in a submergence-tolerant plant, could the submergence intolerance of another species (maize; Fig. 1) be due to a lower ALDH activity during re-aeration? To address this question, we analyzed the expression patterns of the maize mitochondrial ALDH genes, rf2a and rf2b, which are orthologs of rice ALDH2b and ALDH2a, respectively (Skibbe et al. 2002, Tsuji et al. 2003b). We also examined the ALDH activity and the amount of acetaldehyde in maize under submerged and re-aerated conditions.

Materials and Methods

Plant materials, growth conditions and treatments

Maize (Zea mays L., inbred line B73) was germinated on a wet filter paper moisturized with tap water in a wide-mouth glass jar with a lid and was grown in the light at 25°C for 8 days. For the submergence treatment, the jars were completely filled with tap water. The submerged seedlings were kept in the dark at 25°C for up to 24h. For re-aeration, water was removed from the jars and the seedlings were kept under aerobic conditions in the dark at 25°C and examined for periods up to 48h. Unless mentioned otherwise, the analyses were performed on leaves of the seedlings that were quick-frozen in liquid nitrogen and stored at −80°C until analysis.

RNA extraction and northern hybridization

Total RNA was extracted from frozen leaves by the standard guanidine thiocyanate/CsCl method (Kingston 1991). Electrophoresis of RNA and northern hybridization were performed by the method of Saisho et al. (1997). Fragments of two maize mitochondrial ALDH genes, rf2a and rf2b, and one maize ADH gene, Adh1, were used for probes. The fragments were amplified by PCR from rf2a, rf2b and Adh1 cDNAs using the primer sets rf2a-fwd/rf2a-rev, rf2b-fwd/rf2b-rev and Adh1-fwd/Adh1-rev, respectively (see below), and labeled with a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany). Each blot was hybridized only with the specific probe and equal loading of RNA was confirmed by ethidium bromide staining of each electrophoresis gel.

The primers were as follows:

rf2a-fwd: 5′-GTACAGATCTTGTGACCGCAAT-3′
rf2a-rev: 5′-AAAGATCTTGTTGAATTCGTCTGCG-3′
rf2b-fwd: 5′-CATTGCTTAAATCACACTCCCGG-3′
rf2b-rev: 5′-TGACCATTTCTCCTGCTTGTC-3′
Adh1-fwd: 5′-CTGACGTAGCTCCGGGACCATGT-3′
Adh1-rev: 5′-TGGTCATTCAGCAAGTACCTCTTCG-3′.

Protein extraction and immunoblotting

Total proteins were extracted from leaves by the method of Xie and Wu (1989). The materials were frozen in liquid nitrogen and were ground at 4°C in extraction buffer (150 mM Tris-HCl, pH 8.0, 25% (v/v) glycerol, 2% (w/v) polyvinylpyrrolidone, 0.8% (v/v) β-mercaptoethanol and 5 mM dithiothreitol). The extracts were centrifuged and the supernatants were used for SDS-PAGE. SDS-PAGE was performed according to the method of Tsuji et al. (2003b).

A polyclonal antibody against mitochondrial ALDH was produced by injecting a rabbit with a mixture of 2 oligopeptides corresponding to internal sequences of rice ALDH2a and Arabidopsis ALDH2a (Nakazono et al. 2000). As sequences of the oligopeptides are highly conserved among the plant mitochondrial ALDH protein families, the antibody used in the present study recognized both types of mitochondrial ALDH proteins (ALDH2a and ALDH2b) (Tsuji et al. 2003b). A polyclonal antibody against rice ADH (Kadowaki et al. 1988) was kindly provided by Dr. K. Kadowaki (National Institute of Agrobiological Sciences, Tsukuba).

Immunoblotting was performed according to the method of Nakazono et al. (2000). The signal was visualized using Lumi-Light (Roche Diagnostics) and a Lumino-image analyzer LAS-1000 (FUJIFILM, Tokyo, Japan). Intensity of signal was measured with LAS-1000.

Crude enzyme extraction and ALDH assay

Crude enzyme was extracted from fresh (unfrozen)
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leaves by the method of Tsuji et al. (2003a). ALDH activity was assayed as described by Tsuji et al. (2003b).

Determination of acetaldehyde and ethanol contents

The acetaldehyde and ethanol contents in the headspace vapor of leaves were determined with a gas chromatograph-mass spectrometer (GC-MS, Hewlett-Packard, Cheadle Heath, UK) by the method of Tsuji et al. (2003a).

Results and Discussion

Maize was found to be less tolerant to submergence than rice (Fig. 1). Under our experimental conditions, maize seedlings showed symptoms of injury during re-aeration for 24 h following submergence for 24 h (Fig. 1). We determined how the expression of the mitochondrial ALDH genes, rf2a and rf2b, changed under such conditions (i.e., submergence and subsequent re-aeration). As shown in Figure 2, the transcript level of the mitochondrial ALDH gene rf2a (an ortholog of rice ALDH2b) was high under aerated conditions and decreased after submergence. In contrast, the mRNA level of rf2b (an ortholog of rice ALDH2a) was low in aerobically grown plants and increased after submergence (Fig. 2). After the seedlings were transferred to aerobic conditions, the mRNA level decreased to the pre-submergence level (Fig. 2). In rice, the mRNA level of ALDH2b decreased under submergence and recovered to the initial level, while the mRNA level of ALDH2a increased under submergence and decreased after re-aeration (Tsuji et al. 2003a). Thus, the expression patterns of the maize rf2a and rf2b genes were similar to those of rice ALDH2b and ALDH2a, respectively.

To determine the protein levels of maize mitochondrial ALDH proteins under submergence and subsequent re-aeration, an immunoblot analysis was performed using a mitochondrial ALDH-specific antibody. The amount of mitochondrial ALDH protein decreased to almost one-third of the initial amount under submerged conditions (Fig. 3).

Fig. 1. Effects of submergence and re-aeration on the growth of maize and rice seedlings. Eight-day-old seedlings were submerged for 24 h and then were re-aerated for 24 h. The growth of the rice seedlings (left) resumed after re-aeration, whereas the maize seedlings (right) were severely injured during re-aeration following submergence.

Fig. 2. Northern blot analysis of transcripts of rf2a, rf2b and Adh1 genes. Eight-day-old aerobically grown maize seedlings (0 h) were submerged for 12 and 24 h in the dark (Sub.), and then the 24 h-submerged seedlings were transferred to aerobic conditions where they were kept for 6, 12, 24 and 48 h in the dark (Re-aeration). Each lane was loaded with 10 µg total RNA. Equal loadings of total RNA were checked by ethidium bromide staining (EtBr-staining).

Fig. 3. Immunoblotting of mitochondrial ALDH (mtALDH; RF2A and RF2B) and ADH. Eight-day-old aerobically grown maize seedlings (0 h) were submerged for 12 and 24 h in the dark (Sub.), and then the 24 h-submerged seedlings were transferred to aerobic conditions where they were kept for 2, 6, 12, 24 and 48 h in the dark (Re-aeration). Each lane was loaded with 35 µg total protein.
When the submerged seedlings were returned to the aerated condition, the amount of the protein did not recover to its initial level (Fig. 3). In rice, re-aerated plants showed a strong ALDH2a induction, despite a decrease in the level of ALDH2a mRNA (Tsuji et al. 2003a). Thus, the induction of the ALDH protein after re-aeration was different between maize and rice, although we were not able to distinguish between the levels of RF2A and RF2B under our experimental conditions of immunoblotting. It is possible that rf2b mRNA was not efficiently translated either under submerged conditions or following re-aeration. Alternatively, the increase in the intensity of the band of RF2B protein might have overlapped the band of RF2A protein, since the sizes of RF2A (54.2 kDa) and RF2B (54.0 kDa) are almost the same (Liu and Schnable 2002). In other words, the level of the RF2B protein might not increase to a level that compensates for the decrease of the RF2A protein level. Thus, the total amounts of RF2A and RF2B might appear to decrease during submergence.

Maize ALDH activity fell to one-half of that in the aerobic plants under submergence and slowly recovered to the original activity level (at 0 h) after re-aeration (Fig. 4). The changes in the acetaldehyde-oxidizing ALDH activity under submergence and following re-aeration can be attributed to the total amounts of RF2A and RF2B, since cytosolic ALDH might not be involved in the oxidation of acetaldehyde (Nair et al. 2004). However, we cannot rule out the possibility that cytosolic ALDH proteins are involved in the acetaldehyde oxidation in response to submergence and following re-aeration. Further investigations should be carried out.

Although the ethanol and acetaldehyde levels of maize seedlings were low under aerobic conditions, they significantly increased after submergence for 24 h (Fig. 5A and 5B) as a result of ethanolic fermentation. The ethanol content in the re-aerated plants (at least 4 h after re-aeration) was almost the same as that in the submerged plants (Fig. 5A), and the acetaldehyde content increased 1.5–1.8 fold after re-aeration (Fig. 5B). These results suggest that, in maize, the low activity of ALDH was unable to prevent the accumulation of acetaldehyde soon after re-aeration. In rice, however, the ALDH activity quickly increased during re-aeration, reaching 3 times the activity in the aerobic plants after 4 h. The increase in the ALDH activity resulted in efficient detoxification of acetaldehyde in rice (Tsuji et al. 2003a). This difference in the metabolism of acetaldehyde between rice and maize may be one of the reasons why rice is more tolerant of submergence than maize.

As illustrated in Figure 6, we suggest that in submergence-tolerant plants such as rice, ALDH protein synthesis can increase soon after re-aeration and that acetaldehyde is quickly detoxified, thereby enabling to avoid or alleviate the injury caused by acetaldehyde under re-aerated conditions. On the other hand, in submergence-intolerant plants such as maize, it appears that acetaldehyde produced during re-aeration is not efficiently detoxified, which results in serious damage to plants after submergence. Indeed, in a submergence-tolerant aquatic plant, Potamogeton pectinatus, post-anoxic acetaldehyde accumulation was effectively suppressed (Summers et al. 2000). In contrast, in submergence-intolerant species such as Glyceria maxima and potato (Solanum tuberosum), the level of acetaldehyde increased after transfer of the plants from anoxic to aerobic conditions (Monk et al. 1987, Pfister-Sieber and Brändle 1994). We previously reported that barley, another submergence-intolerant species,
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appeared to lack submergence-inducible mitochondrial ALDH2 genes, or to express them at very low levels (Meguro et al. 2001). This evidence supports the hypothesis that submergence-intolerant species such as maize and barley are less able than submergence-tolerant species such as rice to detoxify acetaldehyde, which is produced from ethanol immediately after re-aeration, by ALDH2. During evolution, submergence-tolerant plants such as rice might acquire a mechanism, by which ALDH is induced during re-aeration, in order to avoid possible self-poisoning by post-hypoxic production of acetaldehyde.

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Literature Cited


