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Vacuolar H^+-Translocating Pyrophosphatase Is Induced by Anoxia or Chilling in Seedlings of Rice[1]

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The present study was undertaken to determine whether vacuolar H^+-pyrophosphatase (V-PPase) might replace vacuolar H^+-ATPase under energy stress due to anoxia or chilling in anoxia-tolerant species such as rice (*Oryza sativa* L.) and corn (*Zea mays* L.). The relative transcript level of V-PPase in rice seedlings, like that of alcohol dehydrogenase 1, increased greatly under anoxia and declined again when the seedlings were returned to air. However, the distribution of transcripts in root, shoot, and seed differed somewhat from that of alcohol dehydrogenase 1. Immunoreactive V-PPase protein and V-PPase enzyme specific activity in a tonoplast fraction from rice seedlings increased progressively with time of anoxia or chilling at 10°C, showing a 75-fold increase after 6 d of anoxia, compared with a 2-fold increase of vacuolar H^+-ATPase activity. When the seedlings were returned to air, the specific activity returned to its initial level within 2 d. After 6 d of chilling at 10°C, V-PPase specific activity reached a level 20-fold of that at 25°C. In microsomes of corn roots, V-PPase specific activity did not respond to anoxia but was constitutively high. It is proposed that V-PPase can be an important element in the survival strategies of plants under hypoxic or chilling stress.

Many plants are subjected to temporary anoxia or hypoxia as a result of flooding, waterlogging, or ice encasement. Among crop plants, rice (*Oryza sativa* L.) is the most tolerant of anoxic conditions (Mocquot et al., 1981), and corn (*Zea mays* L.) is also moderately tolerant (Johnson et al., 1989). In seedlings of these species, a number of metabolic changes have been observed in response to hypoxia, and evidence has been presented that at least some of these responses are adaptive, i.e. they increase the ability of the plant to survive the stress (Johnson et al., 1989). In anoxic conditions, cessation of oxidative phosphorylation typically results in a marked decrease in ATP levels (Raymond et al., 1985). This can be alleviated in tolerant species by an increase in alcoholic, lactic, and other fermentation pathways (Kennedy et al., 1992; Menegus et al., 1993), and many of the proteins induced by hypoxia are enzymes of these glycolytic pathways (Bailey-Serres et al., 1988). Chilling likewise creates energy stress (Stewart and Guinn, 1969) due to mitochondrial dysfunction (Lyons and Raison, 1970), and like hypoxia chilling induces alcohol dehydrogenase in corn and rice seedlings (Christie et al., 1991).

As an additional adaptation to energy stress, glycolytic enzyme reactions consuming ATP can be at least partially replaced by reactions utilizing PPi as an energy source. In rice, anoxia induces an increase in Suc synthase (Ricard et al., 1991) as well as an increase in PPi:Fru-6-P 1-phosphotransferase but not phosphofructokinase (Mertens et al., 1990). Since anoxia also induces an increase in Fru-2,6-bisphosphate (Mertens et al., 1990), which activates PPi:Fru-6-P 1-phosphotransferase in the glycolytic rather than the reverse direction (Enomoto et al., 1992), these effects of anoxia favor glycolytic pathways utilizing PPi over those consuming ATP. In corn, it has been noted that, although many glycolytic enzymes are induced by hypoxia, none of these are kinases (Bailey-Serres et al., 1988). Cytosolic PPi levels seem to be independent of those of ATP (Dancer et al., 1990) and unaffected by anoxic stress (Dancer and ap Rees, 1989). Moreover, the cytoplasmic acidification that commonly results from anaerobic metabolism tends to increase the free energy of hydrolysis of PPi but has the opposite effect on the free energy of ATP hydrolysis (Davies et al., 1993). It appears therefore that PPi metabolism may play an important role in plant adaptation to anoxia.

The V-PPase (Rea and Poole, 1993) represents another enzyme that might advantageously replace an ATP-consuming one (the V-ATPase) in anoxia or chilling stress. These parallel proton pumps are ubiquitous in the plant kingdom, although present in differing proportions in various tissues and species. The reaction stoichiometries of the two enzymes (Davies et al., 1992; Schmidt and Briskin, 1993) permit them to generate approximately equal proton gradients (Hedrich et al., 1989) across the vacuolar membrane, and calculations of free energy changes (Davies et al., 1993) indicate that the V-PPase acts in the direction of PPi hydrolysis. (The V-PPase also appears to play an additional role in the transport of K^+ [Davies et al., 1992].) The question thus arises whether the V-PPase, like the PPi-dependent pathways of glycolysis, is preferentially induced in hypoxic conditions. We report here that in rice seedlings anoxia evokes a marked increase in V-PPase transcript abundance, V-PPase immunoreactive protein, and V-PPase enzyme specific activity. This enzyme is also shown to be induced in rice seedlings by chilling stress and to be present in corn roots at a constitutively high level.

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Abbreviations: ADH1, product of the alcohol dehydrogenase gene *Adh1*; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; V-ATPase, vacuolar H^+-translocating ATPase; V-PPase, vacuolar H^+-translocating pyrophosphatase.

MATERIALS AND METHODS

Plant Material

Seeds of rice (*Oryza sativa* L., var IR-36; International Rice Research Institute, Manila, Philippines) were surface sterilized by washing in 60% ethanol for 5 min, twice in distilled water, once in 0.1% $HgCl_2$ for 6 min, and four times in distilled water. The seeds were then placed on wet, sterile Miracloth (Calbiochem) in plastic containers (separate containers for each analysis time). The seeds were allowed to germinate aerobically at 25°C for 4 d in the dark. Humidified air or nitrogen was passed continuously through the containers. At specific times, individual containers were opened and seedlings were immediately used for membrane protein or RNA isolation. Corn seeds (*Zea mays* L., hybrid Earlivee; W.H. Perron, Laval, Quebec, Canada) were grown in a similar manner.

RNA Extraction

For whole plant RNA, total RNA was extracted from fresh seedlings. For tissue RNA, seedlings were frozen and maintained at −70°C throughout tissue dissection. Extraction of total RNA was carried out using the phenol extraction method described by Monroy et al. (1993). The RNA was subsequently used for both reverse transcription-PCR and northern blotting. Total RNA was used for most of the present work. When mRNA was required for comparison, it was isolated from total RNA by the use of Hybond-mAP. (Amersham) following the manufacturer's protocol.

RNA Reverse Transcription and PCR

PCR primers were designed based on known conserved sequences of V-PPase cDNAs from Arabidopsis (Sarafian et al., 1992) and barley (Tanaka et al., 1993). The upstream primer chosen was 5′-GGNGGNATCTACACTAAGGCTGC-3′, corresponding to the 745- to 767-bp region of the barley cDNA sequence. The downstream primer selected was 5′-ATTGTCACT(G/A/T)ATGGGACCATAAGC-3′, complementary to the 1513- to 1536-bp region of the barley cDNA sequence. To amplify the region within the two primers, total RNA extracted from rice seedlings as described above was used for reverse transcription, followed by PCR on the DNA produced. The protocol followed was as described by Sambrook et al. (1989) with a few changes. To synthesize cDNA from RNA, 20 μL of reverse transcription mix was made, which contained 1× PCR buffer (Bio/Can, Mississauga, Canada; 10× contains 500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton X-100), 0.5 mM each of the four deoxyribonucleoside triphosphates, 4 mM MgCl, 2.5 μM downstream primer, 20 units of reverse transcriptase (Moloney murine leukemia virus, Pharmacia), and 1 μg of total RNA (predenatured by heating at 90°C for 5 min). The reaction was allowed to proceed at 25°C for 10 min, then at 37°C for 30 min, and was then stopped by heating to 95°C for 10 min. To amplify the band by PCR, 80 μL of PCR mix was made, which contained 1× PCR buffer, 0.125 mM each of deoxyribonucleoside triphosphates, 1.25 μM upstream primer, 0.625 μM downstream primer, 0.875 mM MgCl, and 2.5 units of *Taq* polymerase (Bio/Can). The 80-μL PCR mix was added to the 20-μL reverse transcription mix, and the resulting solution was overlaid with 100 μL of mineral oil. The temperature sequence was 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C, for 30 cycles. The resulting amplified sequence (792 bp) was separated by electrophoresis in a 1% agarose gel and isolated from the gel by the phenol-freeze-fracture method as described by Huff (1991). The DNA fragment was sequenced using a T7 sequencing kit (Pharmacia) and the upstream primer, and it was found to be 89% identical with the cDNA sequence of barley V-PPase. Finally, the amplified fragment was cloned into KS^+ Bluescript vector and transformed into XL1-Blue cells by ligation of the PCR fragment to a T-tailed vector and transformation of the resulting plasmid into competent cells, as described by Finney (1993).

Analysis of Transcript Levels

RNA samples (10 μg of each) were denatured in 95% formamide at 90°C for 10 min and size fractionated by electrophoresis in 1.2% agarose-formaldehyde gels (Sambrook et al., 1989). The rRNA bands were visualized by ethidium bromide staining. The RNA was subsequently transferred to a 0.2-μm-pore nylon membrane (ICN) by capillary elution (Sambrook et al., 1989) for northern blot hybridization. Filters were incubated with ^{32}P-labeled clones of the V-PPase partial sequence (792 bp) or the full cDNA clone of rice ADH1 (Rice Genome Research Program, Tsukuba, Japan). A rRNA probe, in addition to the ethidium bromide-stained rRNA bands, was used to confirm that equal amounts of RNA were loaded on each lane. Membrane hybridization and high-stringency washes were done according to the method of Sambrook et al. (1989). The relative intensities of the autoradiogram bands were estimated by scanning with an Hewlett-Packard ScanJet IIcx and analyzed with Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Isolation of Tonoplast Membranes

Tonoplast membranes were isolated as described by Sarafian and Poole (1989) with some modifications. Rice seedlings were homogenized for 2 min in a blender along with 100 mL of homogenization medium containing 1 mM PMSF, 70 mM bis-Tris propane, 250 mM Suc, 250 mM KI, 3 mM EDTA, 3 mM EGTA, 0.2% (w/v) BSA, 10% (w/v) glycerol, 25 mM K-metabisulfite, 5 mM DTT, and 1% PVP, adjusted to pH 8.0 with Mes. The homogenates were filtered through four layers of cheesecloth and centrifuged at 3,000*g* for 10 min in a Sorvall centrifuge. Supernatants were then centrifuged at 80,000*g* for 35 min in a Beckman Ti45 rotor. The resulting microsomal pellets were resuspended in 4 mL of suspension medium consisting of 10% (w/v) glycerol, 3 mM EDTA, 3 mM EGTA, 5 mM bis-Tris propane-Mes, pH 7.0, 0.1% (w/v) BSA, and 1 mg/mL DTT. The mixture was homogenized in a glass homogenizer and layered over a four-step gradient, each layer consisting of 7.5 mL of suspension medium containing Suc at 10, 25, 32, and 40% (w/w). After centrifugation at 80,000*g* for 2 h in a Beckman SW28 rotor, the 10/25% interfaces containing tonoplast membranes were collected, diluted in 20 mL of

membrane storage medium containing 10% (w/v) glycerol, 200 mM Suc, 10 mM bis-Tris propane-Mes, pH 7.0, 1 mM DTT, and 1 mM EDTA, and centrifuged at 80,000*g* for 30 min in a Beckman Ti60 rotor. The resulting pellets were resuspended in 0.5 mL of membrane storage medium, instantly frozen in liquid nitrogen, and stored at −70°C.

Assay for V-PPase and V-ATPase Activities

V-PPase and V-ATPase activities were calculated as the rate of PPi or ATP hydrolysis, respectively, by measuring the amount of released Pi according to the Ames (1966) method. The reaction was initiated by the addition of 10 μL of 100 mM $MgSO_4$ in 0.5 mL of reaction mixture at 35°C containing up to 15 μg of protein in 50 mM KCl, 30 mM bis-Tris propane-Mes, pH 8.0, 150 μM gramicidin D, 0.1 mM $NaMoO_4$, 1 mM NaN_3, 0.1 mM EDTA with bis-Tris propane to pH 8.0, and 1 mM either ATP or PPi. The ATPase assay also contained 0.1 mM Na_3VO_4. The reaction was stopped after 20 min by the addition of 1.5 mL of stopping mixture consisting of 6 parts of 0.42% ammonium molybdate in 1 N H_2SO_4 and 1 part of 10% ascorbic acid. The A_{800} of the samples was measured after 30 min and was compared with that of standards of known Pi concentration. The V-PPase activity reported is the difference between activities measured in the presence and absence of $MgSO_4$. V-ATPase activity was taken as the difference in ATPase activities in the absence and presence of 140 nM bafilomycin-A_1 (Bowman et al., 1988). To determine protein content, membranes were solubilized in 1% Triton X-100 for 10 min. Protein assays were then performed according to the method of Bradford (1976). BSA (fraction V) was used as the protein standard.

Preliminary studies were done on tonoplast membrane fractions from aerobic and 6-d anoxia-treated seedlings to determine whether the increased V-PPase activity was coupled to increased proton pumping in the tonoplast. The method followed was based on fluorescence quenching of acridine orange as described by Rea and Poole (1985).

SDS-PAGE and Western Blotting

One-dimensional SDS-PAGE and western blotting were performed according to the method of Sarafian and Poole (1989). Ten percent acrylamide gels were run in the Bio-Rad Mini-Protean II apparatus. Detection of total protein was performed by Coomassie blue staining. The proteins were transferred to a 0.45-μm-pore nitrocellulose membrane (Millipore) in the Bio-Rad Mini Trans-blot apparatus. Immunoblotting was performed according to the method of Sarafian and Poole (1989) using rabbit polyclonal antibody against the 67-kD subunit of mung bean V-PPase kindly provided by M. Maeshima (Maeshima and Yoshida, 1989). The antibody was visualized with goat anti-rabbit IgG-conjugated horseradish peroxidase.

RESULTS

PPi Transcript Induction during Anaerobiosis

Reverse transcriptase-PCR amplification from rice RNA resulted in a unique 792-bp DNA fragment, which was 89% identical with the published barley V-PPase sequence (Tanaka et al., 1993). This fragment hybridized to two bands, approximately 3.1 and 2 kb long, of the total rice RNA blot under high-stringency conditions (Fig. 1). Northern blots using mRNA instead of total RNA still resulted in hybridization of the probe to the two bands (results not shown), which suggested that both bands were poly(A) tailed. Since the sequence of the 792-bp probe lies entirely within the open reading frame, it might hybridize to the products of more than one V-PPase gene. On the other hand, since the sizes of the published sequences of the V-PPase cDNA open reading frame in barley (Tanaka et al., 1993) and Arabidopsis (Sarafian et al., 1992) along with the surrounding regions are both 2.8 kb, and the open reading frame itself is 2.3 kb, it is also possible that the 3.1-kb band from rice corresponds to the full-length transcript, whereas the 2-kb band may be a degradation product. In any case, as shown below, similar hybridization patterns were observed for the two bands.

V-PPase transcript levels were estimated before and after exposure of 4-d-old seedlings to anoxia and compared with those of the ADH1 transcript, which has been shown to be induced during anaerobiosis in rice (Xie and Wu, 1989). Northern blots of total RNA after different times of anoxic treatment, hybridized with V-PPase- or ADH1-specific probes, are shown in Figure 1 and quantitated in Figure 2. A similar induction pattern was seen for both bands hybridizing to the V-PPase probe. The relative V-PPase transcript level was very low during aerobic conditions (0 h in anoxia). When the seedlings were subjected to anoxia, the transcript level rapidly increased, reaching a peak after 14 h, and subsequently remained at a high level for at least 48 h. When seedlings were returned to air after 24 h under anoxia, the transcript level decreased. ADH1 showed a similar pattern. It was induced under anoxia, reaching a peak after 14 h, and then stabilized to a constant level lower than its peak value. When the seedlings were returned to

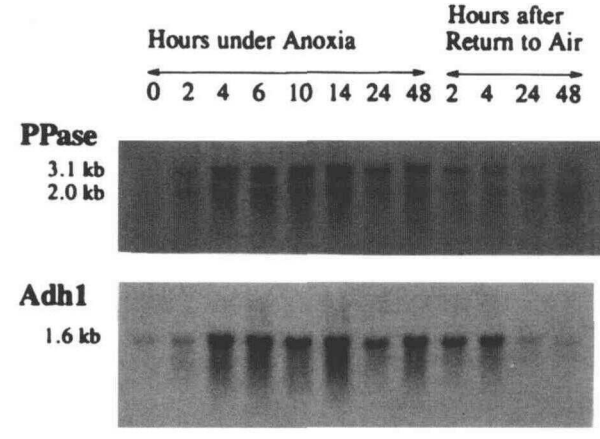

Figure 1. Northern blots of total RNA of rice seedlings after various periods of anoxia and after return to air. Total RNA (10 μg from each time) was size fractionated in formaldehyde-agarose gels and blotted onto nylon membranes. The blots were hybridized at 42°C in 50% formamide with the rice V-PPase partial clone or ADH1 full cDNA and washed at high stringency. The anoxia treatment was applied after 4 d of aerobic germination. The return to air followed 1 d of anoxic treatment.

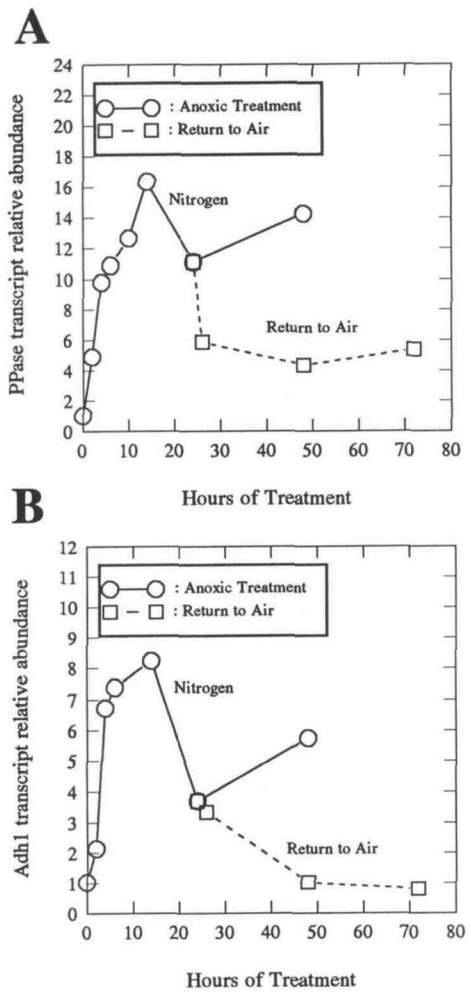

Figure 2. Relative transcript levels of V-PPase and ADH1 in rice seedlings during anoxic treatments. Data are from the experiment of Figure 1. A, Relative transcript abundance of V-PPase during anoxia. Both V-PPase bands from the autoradiograms in Figure 1 were quantified with a densitometer (Hewlett-Packard ScanJet IIcx). Since the patterns were similar, the sum of the two bands is plotted here. The relative abundance of the V-PPase was plotted during (○) and after (□) anoxia. The anoxia treatment was applied after 4 d of aerobic germination. The return to air followed 1 d of anoxic treatment. B, Relative transcript abundance of ADH1 during anoxia. Hybridized bands of the autoradiograms in Figure 1 (for ADH1) were quantified with a densitometer (Hewlett-Packard ScanJet IIcx). The relative abundance of ADH1 was plotted during (○) and after (□) anoxia. The anoxia treatment was applied after 4 d of aerobic germination. The return to air followed 1 d of anoxic treatment.

air, the ADH1 transcript decreased back to the initial aerobic level. The V-PPase showed a 17-fold transcript increase at its peak, whereas ADH1 was increased about 8.5-fold.

The transcript distribution in different tissues is shown in Figure 3. The young seedlings were separated into three parts: the root, shoot, and seed. It appears that in air the V-PPase transcript was primarily concentrated in the seed, and then the root, whereas the shoot had a relatively low V-PPase level. When the seedlings were subjected to anoxia, there was a transcript increase in all tissues. The highest increase was seen in the root and the seed, whereas the shoot showed a smaller induction. The tissue distribution for ADH1 was different from that of the V-PPase. Aerobic conditions resulted in ADH1 transcript accumulation predominantly in the seed, whereas the root and shoot levels were very low. During anaerobiosis, there was an increase in transcript levels primarily in the shoot and to a lesser extent in the root, whereas the seed level did not change dramatically. This is in agreement with previous studies by Cobb and Kennedy (1987), who showed that the ADH1 transcript is primarily localized in the shoot.

It was of interest to determine whether the V-PPase transcript was also induced in corn. Anaerobic induction of transcripts in corn is shown in Figure 3. For both V-PPase and ADH1, which is known to be induced in corn (Freeling and Bennet, 1985), there was an increase in the level of transcript during anoxia. However, the relative increase in V-PPase transcript in corn (using a rice probe) appeared to be less pronounced than in rice.

Effect of Anoxia on V-PPase Enzyme Abundance

Microsomal membranes isolated from whole rice seedlings were subjected to Suc gradient centrifugation. The 10 to 25% Suc interface was taken as a tonoplast fraction. Membrane proteins solubilized from this fraction by SDS were separated by PAGE, blotted to nitrocellulose, and probed with a V-PPase-specific polyclonal antibody to estimate the relative amounts of the V-PPase enzyme. As shown in Figure 4A, the V-PPase protein was hardly detectable in air. When seedlings were subjected to anoxia, the amount of V-PPase increased with time under stress.

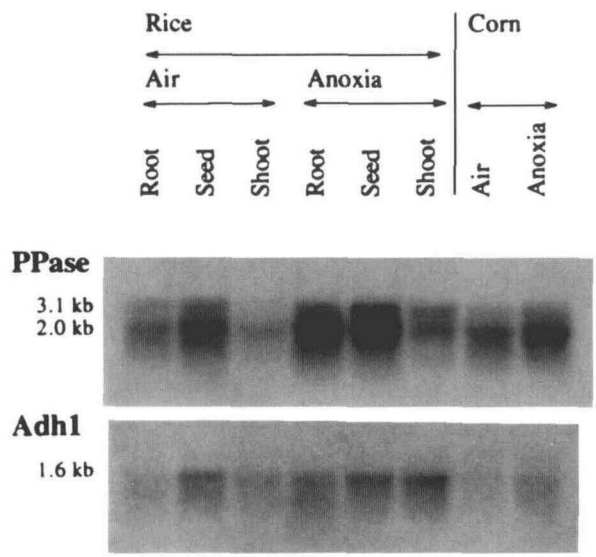

Figure 3. Organ-specific expression of V-PPase and ADH1 in rice and V-PPase and ADH1 transcript induction in corn. Total RNA was isolated from the root, seed, or shoot of rice seedlings or whole seedlings of corn after growth in air or anoxia for 1 d following 4 d of aerobic germination. All RNAs were size fractionated in formaldehyde-agarose gels (10 μg/lane) and blotted onto nylon membranes. The blots were hybridized at 42°C in 50% formamide with the rice V-PPase partial clone or rice ADH1 cDNA and washed at high stringency.

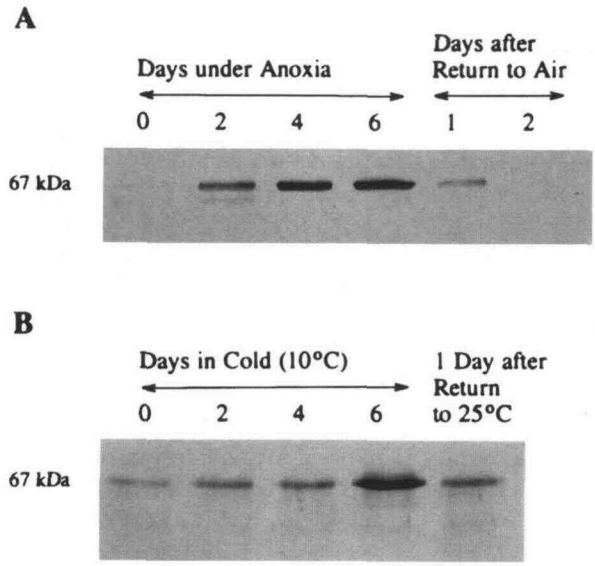

Figure 4. Immunoblots of the 67-kD catalytic subunit of V-PPase from tonoplast membranes of rice seedlings grown under anoxic or cold conditions. Protein from the 10/25% Suc interface following step density-gradient centrifugation was subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and probed with a rabbit anti-V-PPase polyclonal antibody, followed by a goat anti-rabbit IgG-conjugated horseradish peroxidase. A, Induction of V-PPase during anaerobic treatment. Seedlings were subjected to anoxia for the indicated number of days, following an initial 4-d aerobic germination period. After 4 d of anaerobic treatment the seedlings were returned to air for the indicated number of days. Two micrograms of total protein was loaded per lane. B, Induction of V-PPase enzyme during chilling. Seedlings were subjected to 10°C aerobic chilling for the indicated number of days after 4 d of aerobic germination at 25°C. After 4 d of cold treatment the seedlings were returned to 25°C for 1 d. Ten micrograms of total protein was loaded per lane.

Upon return to air, the relative amount of the enzyme decreased back to the original level within 2 d. An attempt at quantitation was made by running proteins from each treatment at a range of concentrations. These experiments suggested that during 6 d of anoxia the amount of V-PPase increased by almost 2 orders of magnitude (results not shown). This estimate is supported by estimates of enzyme specific activity shown below.

Induction of V-PPase and V-ATPase Enzyme Activities during Anoxia

Enzyme specific activities were measured in a "tonoplast" fraction obtained by step density-gradient centrifugation of a microsomal fraction from whole seedlings of rice. V-PPase enzyme specific activities during aerobic and anoxic treatments are shown in Figure 5A. The V-PPase specific activity was relatively high in young seedlings (3 d after germination) and decreased to a constant low level (0.8 μmol PPi mg^{-1} h^{-1}) after 4 d of growth in air at 25°C. When seedlings were placed under nitrogen at 25°C, the V-PPase specific activity increased linearly with time for at least 6 d, by which time it reached a value of 60 μmol PPi mg^{-1} h^{-1}. Upon return of the seedlings to air after 4 d of anoxia, the activity decreased, reaching the original aerobic levels after 2 d. Thus, during anoxia, the V-PPase specific activity levels are induced up to 75-fold, at constant temperature (25°C) in the dark. For comparison, V-ATPase specific activities are shown in Figure 5B. Anaerobic treatment caused a 2-fold increase in specific activity within the first 2 d of the treatment, when a plateau was reached (5 μmol mg^{-1} h^{-1}). Return to air resulted in a decrease in V-ATPase specific activity to the starting levels within 2 d. Therefore, although anaerobic treatment resulted in a major increase in the V-PPase specific activity, it only slightly affected the V-ATPase activity.

Since some doubt remained as to whether the tonoplast fraction used in the rice experiments represented an accurate sampling of V-PPase activity in the tissue, some measurements of V-PPase activity in the total microsomal membrane fraction were also made. For seedlings germinated for 4 d in air, the microsomal V-PPase activity was undetectable (less than 0.7 μmol PPi mg^{-1} h^{-1}), whereas after 6 d under anoxia, the microsomal V-PPase activity was approximately comparable to that in the tonoplast fraction (results not shown). Thus, the marked increase in PPase specific activity shown in Figure 5 is representative of the total vacuolar PPase activities in the tissue.

A question also arises as to what extent an increase in enzyme specific activity in anoxia may reflect a decrease in the total protein recovered in the tonoplast fraction. In other words, do the changes in total enzyme activity per gram fresh weight of tissue correspond to the observed changes in enzyme specific activity? Although the seedlings showed less growth during anoxia, the fresh weights after growth in air or nitrogen were approximately equal, i.e. any difference in fresh weights of shoots plus roots in air compared with nitrogen was presumably balanced by the decrease in fresh weight of the seed. However, after 4 to 6 d of growth under nitrogen, the amount of protein recovered in the tonoplast fraction from whole seedlings was about 40% of that from corresponding seedlings grown in air. Thus, on a fresh weight basis, the V-PPase activity increased up to 30-fold over 6 d, whereas the V-ATPase activity showed no significant change.

To determine whether the induction of V-PPase enzyme activity was accompanied by an increased capacity for PPi-dependent proton pumping, an attempt was made to assay proton pumping in the tonoplast fraction by following the fluorescence quenching of acridine orange. The results indicated that membrane vesicles isolated from rice seedlings under these conditions were not well sealed. Nevertheless, preliminary measurements suggested that, as expected, PPi-dependent proton pumping in tonoplast vesicles was greatly increased in plants subjected to anoxia (results not shown).

Regulation of V-PPase Level and Activity during Chilling Stress in Rice

Alcohol dehydrogenase has been shown to be induced by chilling (Christie et al., 1991) in both corn and rice seedlings. It was therefore of interest to determine the effect of chilling on V-PPase protein level and specific activity in rice. The response of the V-PPase protein to

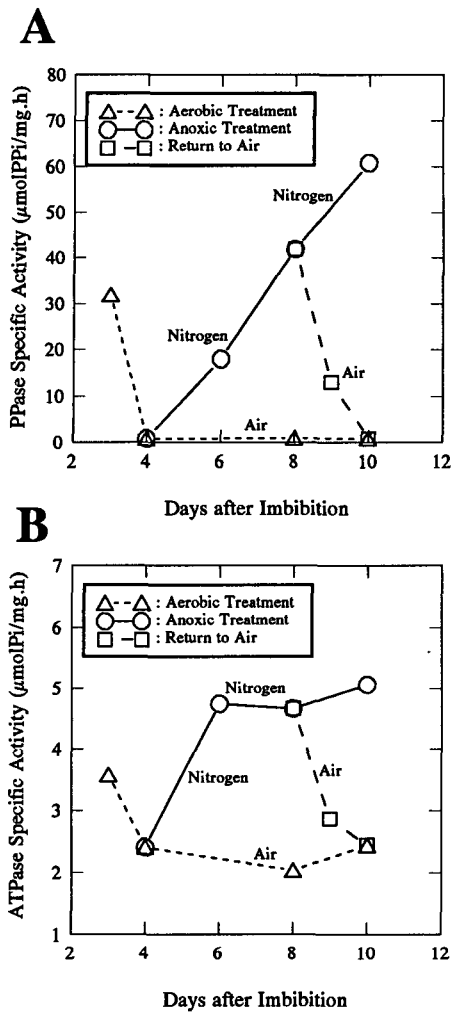

Figure 5. Effect of anoxia on the specific activities of V-PPase and V-ATPase in the tonoplast fraction from whole rice seedlings. Specific activities were assayed in 10/25% Suc interface fractions isolated at various times before (△), during (○), and after (□) anoxia. A, Specific activity of V-PPase. B, Specific activity of V-ATPase.

chilling (10°C) is shown in Figure 4B. The level of immunoreactive enzyme increased progressively with time during cold but to a somewhat lower extent than in anoxia. (Note that more protein was loaded on the gel in Fig. 4B.) When seedlings were transferred back to 25°C, the enzyme level slowly declined. Chilling also had a marked effect on the V-PPase enzyme specific activity levels as shown in Figure 6. When seedlings were subjected to an aerobic cold treatment, the V-PPase activity progressively increased to 16 μmol PPi mg^{-1} h^{-1} within 6 d. Upon return to 25°C, the activity slowly decreased. Thus, chilling treatment resulted in a 20-fold induction of V-PPase activity in rice seedlings.

Specific Activities of V-PPase and V-ATPase in Corn Seedlings

Since some increase had been observed in the relative abundance of V-PPase transcripts in corn after anoxic treatment (Fig. 3), V-PPase and V-ATPase specific activities were also measured in microsomal membranes of corn roots. The specific activity of V-PPase during growth in air was high 3 d after germination and decreased with time as in rice but stabilized at 5 to 6 d after germination at much higher levels, in the range 20 to 25 μmol PPi mg^{-1} h^{-1} in the first 5 mm of the root tip and 7 to 8 μmol PPi mg^{-1} h^{-1} in the remainder of the root (Table I). The effect of hypoxic stress was tested by acclimating the seedlings in 4% O_2 for 18 h before transferring them to strict anoxia for 2 d. These conditions have been shown to give optimal induction of alcohol dehydrogenase in corn seedlings (Johnson et al., 1989). This treatment caused a decline in both V-PPase and V-ATPase specific activities in the root tips, perhaps because of tissue damage by the anoxic treatment, whereas no change in either activity was observed in the remainder of the roots (Table I). Similar results were observed after 1 d of anoxia (results not shown). Thus, in corn, the vacuolar V-PPase is constitutively high in activity, and the slight increase in transcript abundance in anoxia shown in Figure 3 either occurs in a different tissue or may be balanced by less-efficient translation, lower stability, or lower activity of the enzyme under anoxic conditions.

DISCUSSION

Transcript Levels

Changes in V-PPase transcript level in rice seedlings following application of anoxia and on return to air paralleled changes in the level of the ADH1 transcript (Figs. 1 and 2), which showed a pattern similar to that described previously by Xie and Wu (1989). The V-PPase transcript was rapidly induced, and from 6 h to at least 48 h of anoxia, it maintained a level more than 10-fold its initial level in air. The distribution in different tissues (Fig. 3) was somewhat different for the two transcripts. Both the V-PPase and the ADH1 transcripts were initially most abundant in the seed, perhaps because the seed may be hypoxic even in air. However the level of V-PPase transcript in the root was relatively higher in air than in the shoot, and it increased markedly in anoxia, whereas ADH1 showed higher levels

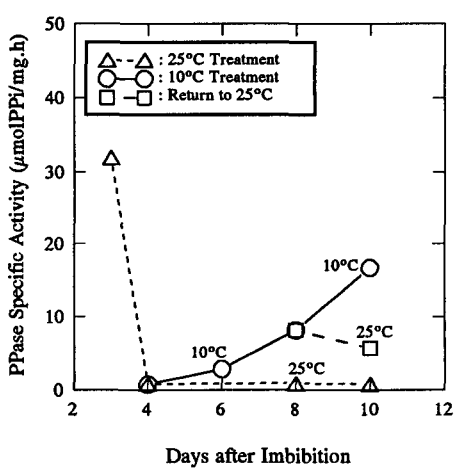

Figure 6. Effect of chilling (10°C) on V-PPase specific activity in tonoplast membranes from rice seedlings. Specific activities were assayed in 10/25% Suc interface fractions isolated at various times before (△), during (○), and after (□) chilling.

Table I. *Specific activities of vacuolar H^+-phosphohydrolases in corn root microsomes*

Enzyme specific activities were determined in microsomes from root tips 5 mm in length or from the remainder of the roots. Indicated times represent days after imbibition. Growth was under normal atmospheric levels of O_2 except for the hypoxia treatment (last column), which consisted of 3 d of growth in air, followed by 18 h of acclimation in 4% (v/v) O_2 and then 2 d in nitrogen. Data are means ± SE of two to four independent experiments.

Material	Enzyme	3 d	4.75 d	5.75 d	5.75 d/N_2
			μmol substrate mg^{-1} protein h^{-1}		
Root tips	V-PPase	39.7 ± 0.2	25.1 ± 5.9	21.4 ± 4.3	11.9 ± 0.4
	V-ATPase	4.2 ± 0.7	2.0 ± 0.3	3.5 ± 1.2	1.6 ± 0.3
Roots minus tips	V-PPase	18.0 ± 1.1	7.1 ± 1.3	7.5 ± 0.7	7.0 ± 0.9
	V-ATPase	3.4 ± 0.3	2.0 ± 0.3	2.9 ± 0.6	2.2 ± 0.5

in the shoot, as shown previously by Cobb and Kennedy (1987). We have no information about the mechanisms controlling transcript levels for V-PPase. In the case of alcohol dehydrogenase, although the two genes *Adh1* and *Adh2* in corn are both regulated at the transcriptional level by anoxia, different mechanisms appear to be involved for each gene (Paul and Ferl, 1991).

Enzyme Specific Activities in Anoxia and Chilling

As in the case of alcohol dehydrogenase (Xie and Wu, 1989), V-PPase enzyme specific activity was high during early germination in air. This is in line with the high level of V-PPase transcript observed in the seed (Fig. 3) and may result either from a degree of hypoxia during early germination or from a constitutive synthesis of the enzyme at this stage. In any case, the V-PPase enzyme specific activity decreases to a low level by 4 d after imbibition and remains low during further growth in air but increases progressively with time under anoxia (Fig. 5A). Preliminary data (not shown) indicated that the increase in V-PPase activity in anoxia was accompanied by an increased capacity for proton transport. Since the increase in immunoreactive protein paralleled the enzyme specific activity within the limits of experimental measurement (Fig. 4 and data not shown), the induction of enzyme activity under anoxia appears to be largely or entirely attributable to new enzyme synthesis. Although it has been shown that anoxia affects the relative rates of synthesis of different proteins at both the transcriptional and translational levels (Bailey-Serres and Freeling, 1990), the marked increase in V-PPase transcript level (Figs. 1 and 2) may be sufficient to account for the increased synthesis of this enzyme under anoxia.

The V-PPase, like alcohol dehydrogenase (Christie et al., 1991), was also induced by chilling, as indicated by progressive increases of enzyme specific activity (Fig. 6) and immunoreactive protein (Fig. 4B) with time at 10°C. Although the rate of increase was not quite as high as in anoxia, there was a 20-fold increase in specific activity over 6 d of chilling. Although the signal transduction pathways are not known for either anoxia or chilling responses and are not likely to be identical, it is interesting to note that cytoplasmic acidosis (Yoshida, 1994) may be an element in both cases.

V-ATPase specific activity in the tonoplast fraction from whole rice seedlings grown in air 4 to 10 d after imbibition was 2 to 2.5 μmol h^{-1} mg^{-1} protein. It is not known whether this somewhat low value reflects a relatively ineffective membrane fractionation, high protein content, or other factors (such as lack of provision of any external solutes to be accumulated by the seedlings). In any case, the specific activity of V-ATPase in air-grown seedlings was about 3-fold higher than that of the V-PPase. The V-ATPase specific activity approximately doubled during anoxia, and like the V-PPase, it returned to its original level upon re-exposure to air. However, these changes were relatively insignificant compared with the increase in V-PPase of 75-fold during the period studied. Moreover, when activities are expressed on the basis of tissue fresh weight instead of tonoplast protein (see "Results"), there was no significant change in V-ATPase activity in anoxia, whereas the V-PPase increased by 30-fold during the 6-d period studied.

V-PPase Turnover

When the seedlings were re-exposed to air after anoxia, there was a rapid decrease in the level of V-PPase immunoreactive protein (Fig. 4A) and enzyme activity (Fig. 5A), indicating a half-life of less than 24 h. If the V-PPase had a significant turnover rate during anoxia, one would expect the amount of enzyme degraded per unit time to increase in proportion to the amount of enzyme present, leading to a decreasing rate of net synthesis with time and eventually a steady-state level of enzyme. This pattern was not observed, but rather the V-PPase activity increased approximately linearly over 6 d of anoxia, whereas the mRNA maintained a more or less steady level up to at least 48 h. Thus, enzyme turnover under anoxia appears to be negligible, and the data suggest a great increase in the rate of V-PPase degradation when the tissue is returned to air. Other examples have been described in which the rapid degradation of specific proteins is initiated under specific circumstances. In certain cases (e.g. phytochrome and cyclins), degradation has been ascribed to the ubiquitin pathway (Shanklin et al., 1987; Glotzer et al., 1991), although the signal pathways leading to recognition of these proteins by the ubiquitin-conjugating system are unknown.

Physiological Significance of V-PPase Induction

In the introduction we stated that it would be advantageous to plants under energy stress due to anoxia to conserve ATP by switching to a PPi-based metabolism where possible, and examples of such metabolic changes have

been reported for glycolysis. Chilling treatments likewise create energy stress (Stewart and Guinn, 1969) due to mitochondrial dysfunction (Lyons and Raison, 1970). The induction of V-PPase by anoxia or chilling is in accordance with these ideas.

Davies et al. (1993) have shown that for probable cytosolic conditions the V-PPase acts in the direction of PPi hydrolysis and H^+ pumping into the vacuole. In corn root tips, the estimated level of PPi (Roberts, 1990) is probably lower than that assumed by Davies et al. (1993), considering the relatively high cytosolic volume in this material, but this is offset by a lower level of Pi, as well as a lower cytoplasmic pH under anoxia and a relatively small pH differential between cytoplasm and vacuole (Roberts et al., 1984); therefore, in this material also, the available evidence indicates that the V-PPase acts to energize vacuolar transport rather than to synthesize PPi.

However, if the advantage of V-PPase induction were only to conserve ATP, it is hard to understand why this enzyme increases to levels 1 order of magnitude higher than the V-ATPase. It has been demonstrated that alcohol dehydrogenase also accumulates in hypoxic corn root tips to levels much greater than those required for survival, at least under the restricted conditions of the laboratory (Roberts et al., 1989). It remains unclear whether this represents an inability of plants to regulate the enzyme to maximum advantage (although not all anaerobic proteins are induced to this extent) or whether such an apparently "excessive" response has survival value in other circumstances.

It has been proposed (Roberts et al., 1984) that survival of root tips under hypoxia is largely determined by the ability to control cytoplasmic acidosis. Although such control is no doubt a factor in survival, a role for the V-PPase in the disposal of protons produced during hypoxic metabolism can be ruled out, since in rice the vacuole actually becomes more alkaline in anoxia (Menegus et al., 1991). Moreover, rice seedlings have a variety of metabolic H^+-consuming reactions, such as glutamate decarboxylation and Arg breakdown to putrescine and NH_4^+, which can more than compensate for anoxic acidification (Menegus et al., 1993).

If the V-PPase does not appear to play a major role in the disposal of protons produced during anoxia, it is nevertheless likely to be important in maintaining the pH difference between cytoplasm and vacuole. It has been suggested (Roberts et al., 1984) that under anoxia cell death is eventually initiated by the leakage of vacuolar contents into the cytoplasm. If this is the case, the maintenance of vacuolar energization by V-PPase should have a high priority in the survival strategy of plants under anoxic stress. The V-PPase may also provide the energy to sequester the organic end products of anoxic metabolism, which accumulate in large quantities because of the Pasteur effect and other factors. Ala and putrescine are products of anoxic metabolism in rice (Menegus et al., 1993) that are likely to be accumulated in cell vacuoles by transport mechanisms indirectly energized by the V-PPase. Induction of V-PPase would thus aid in maintaining these metabolic pathways and in conserving important nutrients while preventing possible cytotoxic effects. Another function of V-PPase is likely to be the generation of the necessary turgor pressure for growth, by accumulation of Ala and K^+ (Menegus et al., 1993).

V-PPase Strategies in Corn and Rice

If, as speculated above, the anoxic induction of high levels of V-PPase may have survival value for rice seedlings, it is relevant to ask whether this enzyme is induced in other anoxia-tolerant species. Although a slight increase in the relative transcript abundance was seen in corn seedlings (Fig. 3), this was not reflected in a detectable increase in enzyme activity (Table I), at least in the roots. A similar observation has also been reported for Suc synthase: in rice (Ricard et al., 1991), both transcript and enzyme are induced by anoxia, whereas in corn (McElfresh and Chourey, 1988), an increase in transcript translatable in vitro does not seem to lead to a significant increase in enzyme levels in vivo. It may be that for these enzymes the increase in transcript may only suffice to offset other factors such as less-efficient translation. This does not mean that these enzymes are not relevant to survival of anoxic stress. Indeed, as shown in Table I, the V-PPase is constitutively maintained at high levels in corn roots, and especially in the root tips, which in both rice and corn are the tissues most sensitive to hypoxia, presumably because of their high energy requirements.

In summary, the marked induction of V-PPase by anoxia or chilling in rice and its maintenance at high levels in the hypoxia-tolerant corn, together with the theoretical advantages of PPi-based metabolism under energy stress and the apparent need to maintain tonoplast energization, lead us to the hypothesis that the V-PPase is an important element in the survival strategies of plants under hypoxic or chilling stress. The testing of this hypothesis is a subject for future research.

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LITERATURE CITED

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol **8:** 115–118

Bailey-Serres J, Freeling M (1990) Hypoxic stress-induced changes in ribosomes of maize seedling roots. Plant Physiol **94:** 1237–1243

Bailey-Serres J, Kloeckener-Gruissem B, Freeling M (1988) Genetic and molecular approaches to the study of the anaerobic response and tissue specific gene expression in maize. Plant Cell Environ **11:** 351–357

Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci USA **85:** 7972–7976

Bradford MM (1976) 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

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

Cobb BG, Kennedy RA (1987) Distribution of alcohol dehydrogenase in roots and shoots of rice (*Oryza sativa*) and *Echinochloa* seedlings. Plant Cell Environ **10:** 633–638

Dancer JE, ap Rees T (1989) Effects of 2,4-dinitrophenol and anoxia on the inorganic pyrophosphate context of the spadix of *Arum maculatum* and the root apices of *Pisum sativum*. Planta **178:** 421–424

Dancer JE, Veith R, Feil R, Komor E, Stitt M (1990) Independent changes of inorganic pyrophosphate and the ATP/ADP or UTP/UDP ratios in plant cell suspension cultures. Plant Sci **66:** 59–63

Davies JM, Poole RJ, Rea PA, Sanders D (1992) Potassium transport into plant vacuoles energized directly by a proton-pumping inorganic pyrophosphatase. Proc Natl Acad Sci USA **89:** 11701–11705

Davies JM, Poole RJ, Sanders D (1993) The computed free energy change of hydrolysis of inorganic pyrophosphate and ATP: apparent significance for inorganic-pyrophosphate-driven reactions of intermediary metabolism. Biochim Biophys Acta **1141:** 29–36

Enomoto T, Ohyama H, Kodama M (1992) Purification and characterization of pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase from rice seedlings. Biosci Biotech Biochem **56:** 251–255

Finney M (1993) Molecular cloning of PCR products. *In* FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl, eds, Current Protocols in Molecular Biology. Greene Associates and Wiley-Interscience, New York, pp 15.7.1–15.7.6

Freeling M, Bennet DC (1985) Maize *Adh1*. Annu Rev Genet **19:** 297–323

Glotzer M, Murray AW, Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway. Nature **349:** 132–138

Hedrich R, Kurkdjian A, Guern J, Fluegge UI (1989) Comparative studies on the electrical properties of the H^+ translocating ATPase and pyrophosphatase of the vacuolar-lysosomal compartment. EMBO J **8:** 2835–2841

Huff JP (1991) Rapid isolation and purification of DNA from agarose gels: the phenol-freeze-fracture method. Biotechniques **10:** 724

Johnson J, Cobb BG, Drew MC (1989) Hypoxic induction of anoxia tolerance in root tips of *Zea mays*. Plant Physiol **91:** 837–841

Kennedy RA, Rumpho ME, Fox TC (1992) Anaerobic metabolism in plants. Plant Physiol **100:** 1–6

Lyons JM, Raison JK (1970) Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol **45:** 386–389

Maeshima M, Yoshida S (1989) Purification and properties of vacuolar membrane proton-translocating inorganic pyrophosphatase from mung bean. J Biol Chem **264:** 20068–20073

McElfresh KC, Chourey PS (1988) Anaerobiosis induces transcription but not translation of sucrose synthase in maize. Plant Physiol **87:** 542–546

Menegus F, Cattaruzza L, Mattana M, Beffagna N, Ragg E (1991) Response to anoxia in rice and wheat seedlings. Changes in the pH of intracellular compartments, glucose-6-phosphate level, and metabolic rate. Plant Physiol **95:** 760–767

Menegus F, Cattaruzza L, Molinari H, Ragg E (1993) Rice and wheat seedlings as plant models of high and low tolerance to anoxia. *In* PW Hochachka, PL Lutz, T Sick, M Rosenthal, G van den Thillart, eds, Surviving Hypoxia. CRC Press, Boca Raton, FL, pp 53–64

Mertens E, Larondelle Y, Hers H-G (1990) Induction of pyrophosphate:fructose 6-phosphate 1-phosphotransferase by anoxia in rice seedlings. Plant Physiol **93:** 584–587

Mocquot B, Prat C, Mouches C, Pradet A (1981) Effect of anoxia on energy charge and protein synthesis in rice embryo. Plant Physiol **68:** 636–640

Monroy AF, Sarhan F, Dhindsa RS (1993) Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression. Evidence for a role of calcium. Plant Physiol **102:** 1227–1235

Paul AL, Ferl RJ (1991) In vivo footprinting reveals unique *cis*-elements and different modes of hypoxic induction in maize *Adh1* and *Adh2*. Plant Cell **3:** 159–168

Raymond P, Al-Ani A, Pradet A (1985) ATP production by respiration and fermentation, and energy charge during aerobiosis and anaerobiosis in twelve fatty and starchy germinating seeds. Plant Physiol **79:** 879–884

Rea P, Poole RJ (1985) Proton-translocating inorganic pyrophosphatase in red beet (*Beta vulgaris* L.) tonoplast vesicles. Plant Physiol **77:** 46–52

Rea PA, Poole RJ (1993) Vacuolar H^+-translocating pyrophosphatase. Annu Rev Plant Physiol Plant Mol Biol **44:** 157–180

Ricard B, Rivoal J, Spiter A, Pradet A (1991) Anaerobic stress induces the transcription and translation of sucrose synthase in rice. Plant Physiol **95:** 669–674

Roberts JKM (1990) Observation of uridine triphosphate:glucose-1-phosphate uridylyltransferase activity in maize root tips by saturation transfer ^{31}P-NMR. Estimation of cytoplasmic PP_i. Biochim Biophys Acta **1051:** 29–36

Roberts JKM, Callis J, Jardetzky O, Walbot V, Freeling M (1984) Cytoplasmic acidosis as a determinant of flooding intolerance in plants. Proc Natl Acad Sci USA **81:** 6029–6033

Roberts JKM, Chang K, Webster C, Callis J, Walbot V (1989) Dependence of ethanolic fermentation, cytoplasmic pH regulation, and viability on the activity of alcohol dehydrogenase in hypoxic maize root tips. Plant Physiol **89:** 1275–1278

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sarafian V, Kim Y, Poole RJ, Rea PA (1992) Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **89:** 1775–1779

Sarafian V, Poole RJ (1989) Purification of a H^+-translocating inorganic pyrophosphatase from vacuolar membranes of red beet. Plant Physiol **91:** 34–38

Schmidt AL, Briskin DP (1993) Energy transduction in tonoplast vesicles from red beet (*Beta vulgaris* L.) storage tissue: H^+/substrate stoichiometries for the H^+-ATPase and H^+-PPase. Arch Biochem Biophys **301:** 165–173

Shanklin J, Jabben M, Vierstra RD (1987) Red light-induced formation of ubiquitin-phytochrome conjugates: identification of possible intermediates of phytochrome degradation. Proc Natl Acad Sci USA **84:** 359–363

Stewart JM, Guinn G (1969) Chilling injury and changes in adenosine triphosphate of cotton seedlings. Plant Physiol **44:** 605–608

Tanaka Y, Chiba K, Maeda M, Maeshima M (1993) Molecular cloning of cDNA for vacuolar membrane proton-translocating inorganic pyrophosphatase in *Hordeum vulgare*. Biochem Biophys Res Commun **190:** 1110–1114

Xie Y, Wu R (1989) Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. Plant Mol Biol **13:** 53–68

Yoshida S (1994) Low temperature-induced cytoplasmic acidosis in cultured mung bean (*Vigna radiata* [L.] Wilczek) cells. Plant Physiol **104:** 1131–1138