Plant Physiol. (1993) 101: 217–226

# Constitutive and Inducible Aerobic and Anaerobic Stress Proteins in the *Echinochloa* Complex and Rice[1]

**Cesar V. Mujer[2], Mary E. Rumpho*[3], Jih-Jing Lin[4], and Robert A. Kennedy[5]**

Department of Botany and Maryland Agricultural Experiment Station, University of Maryland, College Park, Maryland 20742

**Anaerobic stress resulted in a change in the protein accumulation patterns in shoots of several *Echinochloa* (barnyard grass) species and *Oryza sativa* (L.) (rice) as resolved by two-dimensional gel electrophoresis. Of the six *Echinochloa* species investigated, *E. phyllopogon* (Stev.) Koss, *E. muricata* (Beauv.) Fern, *E. oryzoides* (Ard.) Fritsch Clayton, and *E. crus-galli* (L.) Beauv. are tolerant of anaerobiosis and germinate in the absence of oxygen, as does rice. In contrast, *E. crus-pavonis* (H.B.K.) Schult and *E. colonum* (L.) Link are intolerant and do not germinate without oxygen. Computer analysis of the protein patterns from the four tolerant species and rice indicated that the anaerobic response is of five classes: class 1 proteins, enhanced under anaerobiosis (9 to 13 polypeptides ranging from 16–68 kD); class 2 proteins, unique to anaerobiosis (1 to 5 polypeptides ranging from 17–69 kD); class 3 proteins, remained constant under aerobiosis and anaerobiosis; class 4 proteins, prominent only in air and repressed under anoxia (3 to 7 polypeptides ranging from 19–45 kD); and class 5 proteins, unique to aerobiosis (1 to 4 polypeptides ranging from 18–63 kD). In the intolerant species, *E. colonum* and *E. crus-pavonis*, no polypeptides were enhanced or repressed under anoxia (class 1 and class 4, respectively), whereas in the tolerant *Echinochloa* species and rice, a total of at least 9 to 13 anaerobic stress proteins and 4 to 7 "aerobic" proteins were noted. Immunoblotting identified two of the major anaerobic stress proteins as fructose-1,6-bisphosphate aldolase and pyruvate decarboxylase. Based on the differential response of the intolerant species to anaerobiosis, we suggest that another set of genes, whose products may not necessarily be among the major anaerobic stress polypeptides, might confer tolerance in *Echinochloa* under prolonged anaerobic stress.**

Anaerobic stress induces a rapid change in gene expression in plants. In maize, for example, a set of 20 polypeptides designated as ASPs (originally designated anaerobic proteins but here referred to as ASPs according to Nover's convention [Nover, 1989]) is selectively synthesized in the primary root (Sachs et al., 1980). At the same time, synthesis of "aerobic" proteins is generally repressed. Anoxia induces a similar pattern of altered gene expression in barley (Hoffman et al., 1986), cottonwood (Kimmerer, 1987), tomato (Tanksley and Jones, 1981), pea (Llewellyn et al., 1987), and soybean (Tihanyi et al., 1989). In contrast, many polypeptides, including a number of aerobic proteins, are stimulated under anoxia in rice (Mocquot et al., 1981).

Several ASPs have been characterized; all are glycolytic enzymes such as ADH (Rumpho and Kennedy, 1981; Tanksley et al., 1981; Llewellyn et al., 1987; Xie and Wu, 1989), PDC (Lazslo and St. Lawrence, 1983; Kelley, 1989), fructose-1,6-bisP aldolase (Rumpho and Kennedy, 1983a; Kelley and Tolan, 1986), glucose-6-P isomerase (Kelley and Freeling, 1984), GAPDH (Russell and Sachs, 1989), and lactate dehydrogenase (Hoffman et al., 1986; Rivoal et al., 1991), or are involved in glucose-P metabolism, as is sucrose synthase (Springer et al., 1986). The identity and function of the remaining ASPs are unknown.

The effects of anaerobic stress are often studied by growing plants in air prior to an anaerobic treatment. This approach is used because most plants are unable to germinate and cannot grow under anoxia. Maize seedlings die after 72 h anaerobiosis (Sachs et al., 1980). As a consequence, only short-term effects have been measured in most studies on anaerobiosis. In contrast, some *Echinochloa* (barnyard grass) species and *Oryza sativa* L. (rice) have the capacity to germinate and grow for extended periods, 3 weeks or more, totally without oxygen. Thus, *Echinochloa* and rice are useful systems to investigate short- and long-term metabolic responses to anaerobiosis. An important distinction between these systems and other plant models for studying anoxic stress is that the former can be used without exposing the seeds to air during or after germination.

For several years now, studies in this laboratory have focused on determining the adaptive mechanisms that confer to *Echinochloa* its ability to germinate and grow anaerobically. These studies have shown that, at least in *E. phyllopogon*, enzymes controlling several metabolic processes such as alcoholic fermentation (Rumpho and Kennedy, 1981, 1983a,

[1] This work was supported by U.S. Department of Agriculture Competitive Research Grant 87-CRCR-1-2595 (R.A.K. and M.E.R.), a Herman Frasch Foundation Grant in Agricultural Chemistry (R.A.K.), and The Maryland Agricultural Experiment Station (Scientific Paper No. A6314, Contribution No. 8489).

[2] Present address: Department of Biochemistry, North Carolina State University, Raleigh, NC 27965.

[3] Present address: Department of Horticultural Sciences, Texas A&M University, College Station, TX 77843.

[4] Present address: Laboratory of Biochemical Physiology, Biological Response Modifier Program, National Cancer Institute, FCRDC, Frederick, MD 20702.

[5] Present address: Department of Biology, Texas A&M University, College Station, TX 77843.

* Corresponding author; fax 1–409–845–0627.

Abbreviations: ADH, alcohol dehydrogenase; ASP, anaerobic stress protein; GAPDH, glyceraldehyde-3-P dehydrogenase; NEpHGE, nonequilibrium pH gradient gel electrophoresis; PDC, pyruvate decarboxylase; TBST, Tris-buffered saline with Tween-20; TCA cycle, tricarboxylic acid cycle.

1983b), glycolysis (Rumpho and Kennedy, 1983a; Kennedy et al., 1987b), the oxidative pentose phosphate pathway (Kennedy et al., 1983; Rumpho and Kennedy, 1983a), lipid metabolism (VanderZee and Kennedy, 1982; Knowles and Kennedy, 1984; Everard and Kennedy, 1985), and mitochondrial activity (Kennedy et al., 1987a; Fox and Kennedy, 1991) operate under anaerobic conditions. Recently, we (Fox and Kennedy, 1991) reported that a complete TCA cycle was present in *E. phyllopogon* under anoxia and that most TCA cycle enzymes in nitrogen-grown seedlings exhibited in vitro activities comparable to those grown in air.

In this study, we investigated changes in the pattern of gene expression at the protein level in several species of *Echinochloa* grown under anaerobiosis. Because this genus represents a wide spectrum of flood tolerance, it is an interesting system for comparing species-specific responses to anaerobiosis. Of the six species studied, *E. phyllopogon* and *E. oryzoides* are flood tolerant and confined to aquatic environments. *E. muricata* is semi-tolerant and found along streambanks, whereas *E. crus-galli*, *E. colonum*, and *E. crus-pavonis* are flood intolerant and found only in drier sites (Barrett and Wilson, 1981). In the laboratory, rice and all of the *Echinochloa* species except *E. colonum* and *E. crus-pavonis* were able to germinate and grow under a strict nitrogen atmosphere (Kennedy et al., 1990). Here we demonstrated that the tolerant species exhibited a common response to anoxia: 3 to 7 proteins were prominent in air, 9 to 13 proteins were enhanced in nitrogen, 1 to 4 proteins were uniquely expressed in air, and 1 to 5 proteins were unique to anaerobic growth. In contrast, the intolerant species exhibited similar protein patterns after 5 d of aerobic or anaerobic growth.

## MATERIALS AND METHODS

### Plant Materials

Seeds of six *Echinochloa* species [*E. phyllopogon* (Stev.) Koss, *E. muricata* (Beauv.) Fern, *E. oryzoides* (Ard.) Fritsch Clayton, *E. crus-galli* (L.) Beauv., *E. crus-pavonis* (H.B.K.) Schult, and *E. colonum* (L.) Link] and *Oryza sativa* (L.) were surface sterilized with 50% Clorox for 10 min, rinsed four times with deionized water, and germinated in Petri dishes lined with two layers of filter paper wetted with 5 mL of sterile distilled water. Aerobically germinated seedlings were grown in the dark in an incubator at 28°C, whereas anaerobically germinated seedlings were grown in an anaerobic chamber at 28°C (Forma Scientific, Inc.) and flushed continuously with a 90% nitrogen–10% hydrogen gas mixture. Five days after shoot emergence, seedlings were harvested and frozen with liquid nitrogen, and the shoots were separated from seeds and roots. All tissues were stored at −70°C until they were used. For *E. crus-pavonis* and *E. colonum*, the seeds were first grown in air for 5 d prior to varying periods of anaerobic treatment (1, 2, or 3 d).

### Protein Extraction

Total proteins were extracted according to Hurkman and Tanaka (1986) with the following modifications: 2 g fresh weight of shoot tissue were ground to a fine powder in a mortar containing liquid nitrogen. When the temperature reached approximately 0°C, 10 mL of cold, pH 9.2, extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% [v/v] 2-mercaptoethanol, 1 mM PMSF, 0.1 mM leupeptin, and 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone) was added. The homogenate was clarified by centrifugation at 2000*g* for 5 min at 4°C. Proteins were extracted from the supernatant with an equal volume of water-saturated phenol containing 0.1% hydroxyquinoline. After incubation with shaking for 10 min at room temperature, the phases were separated by centrifugation at 10,000*g* for 10 min at 4°C. Proteins were precipitated from the phenol phase by addition of 5 volumes of 0.1 M ammonium acetate in methanol and incubated overnight at −20°C. The precipitate was washed three times with cold 0.1 M ammonium acetate in methanol. The pellet was dried using a stream of nitrogen gas and redissolved in 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, and 2% ampholytes (0.4% pH 3–10 Pharmalyte [Sigma] and 1.6% pH 5–8 Bio-Lyte [Bio-Rad]). The insoluble material was removed by centrifugation for 5 min in a microcentrifuge. Total protein was measured using a modified Bio-Rad protein assay reagent with BSA as a standard and 1 μL of extract buffer included with each standard.

### Two-Dimensional Gel Electrophoresis

Two-dimensional PAGE was performed with modifications according to O'Farrell et al. (1977). Samples containing 100 μg of protein were loaded onto the acidic end for NEpHGE. The anode (upper) buffer was 8 mM $H_3PO_4$ and the cathode (lower) buffer was 0.02 N NaOH. The gels were electrophoresed for 0.5 h at 200 V and 4 h at 500 V for a total of 2100 V × h. After electrophoresis, the gels were equilibrated for 30 min in 2.3% SDS, 10% glycerol, and 5% 2-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) and stored at −70°C.

The second dimension SDS-PAGE was performed as described by Laemmli (1970). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 then destained and silver-stained using a Bio-Rad silver stain kit, or were transferred to Immobilon P membrane by electroblotting for 2 h at 100 mA constant current at 4°C using the methods of Towbin et al. (1979).

### Computer Analysis

All silver-stained gels were subjected to computer analysis for comparison of protein patterns between air- and nitrogen-grown seedlings as described by Hruschka et al. (1983) and Hruschka (1984). Black and white slides of the gels were digitized with a Charge Coupled Device Camera and Datacube image processor hosted by a Sun 3 workstation. The images were smoothed with a Gaussian filter and the background removed with a La Placian filter, then brought into registration with a two-dimensional quadrator least square polynomial equation and viewed with a Grinnel 270 image processor hosted by a Hewlett-Packard 1000F computer. In the resulting image, common proteins appeared yellow, aerobic proteins were red, and anaerobic proteins were green. The intensity of each protein spot was manually adjusted, enabling the identification of unique aerobic and anaerobic

proteins. This reduced the chances of error arising from larger, more intense spots on one gel masking out smaller, less intense spots on a second gel. In the color reproductions, this was not always apparent and at times only the more intense color was obvious. This was especially true when there were extreme differences in the intensities of the two corresponding spots. However, all proteins that were found to be enhanced or unique following these procedures are indicated by arrows in the figures and discussed in the text and figure legends.

### Immunostaining

After electroblotting of two-dimensional gels, the Immobilon P membranes were immunostained using the protocol described in the Promega Technical Manual for the Horseradish Peroxidase System. Briefly, the Immobilon P membranes were subjected sequentially to: (a) incubation with TBST and 1% BSA for 30 min; (b) incubation with 50 mL of diluted antisera in TBST for 1 h (1:500 dilution for sucrose synthase; 1:1000 dilution for fructose-1,6-bisP aldolase, pyruvate decarboxylase, peroxidase, glucose-6-P dehydrogenase, ADH, and GAPDH); (c) three 10-min washes in TBST; (d) incubation with 50 mL of a 1:2500 dilution of anti-immunoglobulin G horseradish peroxidase conjugate in TBST for 30 min; (e) three 10-min washes in TBST before finally staining with 4-chloro-1-napthol.

## RESULTS

In the following experiments, we have investigated the induction of the anaerobic response in several *Echinochloa* species and rice using two-dimensional gel electrophoresis. We have focused these studies on the protein patterns of 5-d-old shoots of seedlings exhibiting widely varying tolerance to flooding and anaerobiosis.

### Anaerobic Response of Tolerant Species

Computer analysis of the two-dimensional gel electrophoresis protein patterns indicated that, at least among the four tolerant *Echinochloa* species (*E. phyllopogon, E. oryzoides, E. muricata,* and *E. crus-galli*) (Fig. 1, A–H and Fig. 2A) and in rice (Fig. 3, A and B), the response to anoxia was one of five types: class 1, proteins enhanced under anaerobiosis; class 2, proteins unique to anaerobiosis; class 3, proteins that remained constant during both aerobiosis and anaerobiosis; class 4, proteins that remained prominent in air but were repressed under anoxia; and class 5, proteins unique to aerobiosis. Class 3 proteins were constitutive, whereas class 1, 2, 4, and 5 proteins were inducible.

In all tolerant species, a major class 1 protein with a molecular mass ranging from 52.5 to 57.5 kD was noted (52.5 kD in *E. phyllopogon,* 56.2 kD in *E. muricata,* 54.9 kD in *E. oryzoides,* and 57.5 kD in *E. crus-galli*). *E. muricata* had two polypeptides of 56.2 kD, whereas in rice this major polypeptide had a molecular mass of 55.6 kD. In contrast, no class 1 proteins were noted in the intolerant species *E. colonum* and *E. crus-pavonis* (Fig. 1, I–L).

Based on molecular mass, class 1 proteins (i.e. those enhanced under nitrogen) were grouped into three major clusters (boxed in Fig. 1, D and H). The most prominent group, designated as cluster "a", contained polypeptides that ranged in mass from 35.5 kD in *E. crus-galli* to about 46.2 kD in *E. phyllopogon* (Table I). The number of polypeptides in this cluster varied in each species: nine in *E. phyllopogon;* five in *E. muricata, E. oryzoides,* and rice; and four in *E. crus-galli.* Cluster "b" contained polypeptides that ranged in mass from 23.7 to 26.3 kD. In *E. muricata,* there were five different polypeptides in cluster "b", whereas in *E. phyllopogon, E. oryzoides, E. crus-galli,* and rice there were 3, 1, 1, and 1, respectively. A group of low molecular mass ASPs (16–20 kD), designated as cluster "c," was found only in *E. oryzoides* and *E. crus-galli* (Fig. 1, E and H). The former had two polypeptides and the latter had four.

Class 2 proteins are unique to anaerobiosis. In general, this group of proteins was not as prominent as those in class 1. Computer analysis greatly facilitated the identification of this group (Fig. 2). In contrast with class 1 proteins, this group of proteins did not cluster in a specific location of the gel and their number and intensity were much lower. Except for *E. phyllopogon,* which had one unique anaerobic polypeptide of 63 kD, and *E. muricata,* which had five ASPs (16.6–69.2 kD), all other *Echinochloa* species exhibited two low molecular mass polypeptides (16.8–24.5 kD) (Table II; also see open arrows in Fig. 1, B, D, F, H, J, and L). On the other hand, rice had three unique ASPs (Table II and Fig. 3B).

In both air and nitrogen, class 3 proteins remained constant and constituted the majority of the background spots. Most of these polypeptides are possibly products of housekeeping genes.

Class 4 proteins were prominent in air but were repressed under anoxia. As in class 1, this group clustered in the tolerant species (see closed arrowheads in Fig. 1, A, C, E, and G). Their masses ranged from 19.3 kD in *E. phyllopogon* to about 45.2 kD in *E. crus-galli.* There were five polypeptides in this class in *E. phyllopogon,* four in *E. muricata,* three in *E. oryzoides,* and seven in *E. crus-galli* (Table III). Rice had five polypeptides in this group, ranging in mass from 21.6 to 27.5 kD.

The last class of proteins (class 5) was unique to aerobiosis. As in class 2, the number and intensity of proteins in this class were also low. Among the tolerant *Echinochloa* species, the number of unique aerobic proteins varied from one in *E. oryzoides* to four in *E. muricata* (see open arrows in Fig. 1, A, C, E, and G). Their molecular masses ranged from 17.8 kD in *E. crus-galli* to 57.5 kD in *E. muricata* (Table II). Rice had one unique aerobic protein at 63.1 kD (Fig. 3A).

Our estimation of the number of major ASPs and aerobic proteins was conservative. Other polypeptides that may be slightly enhanced under both aerobic and anaerobic conditions may not have been included in our classification.

### Anaerobic Response of Intolerant Species

When seeds of the two intolerant species, *E. crus-pavonis* and *E. colonum,* were kept under anaerobic conditions they failed to germinate. However, the seeds remained viable even after 14 d of anaerobic treatment, as indicated by their germination upon exposure to air. In addition, if the intoler-

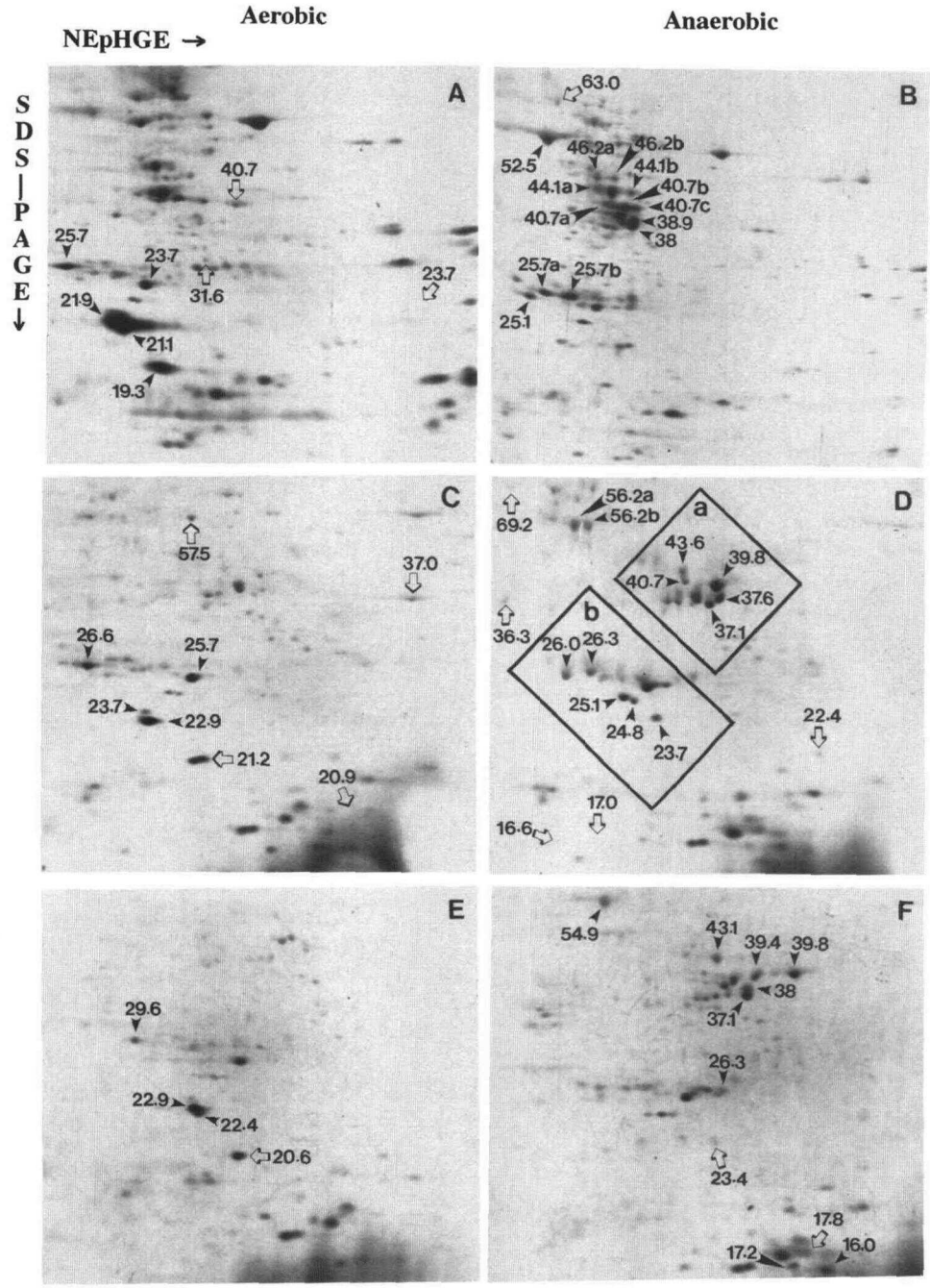


**Figure 1.** Constitutive and inducible protein patterns of *E. phyllopogon* (A and B), *E. muricata* (C and D), *E. oryzoides* (E and F), *E. crus-galli* (G and H), *E. colonum* (I and J), and *E. crus-pavonis* (K and L) on two-dimensional polyacrylamide gels double-stained with Coomassie brilliant blue and silver. Total proteins (100 μg) isolated from shoots of 5-d-old seedlings grown either in air (A, C, E, G, I, and K) or nitrogen (B, D, F, H, J, and L) were electrophoresed in the first dimension by NEpHGE (pH 3–10) and in the second dimension by SDS-PAGE (12.5%). In panels J and L, the seeds were first germinated and grown for 5 d in air prior to anaerobic treatment for 24 h. In each panel, class 1 (enhanced under nitrogen) and class 4 (repressed under anoxia) proteins are labeled by closed arrowheads, and class 2 (unique to anaerobiosis) and class 5 (unique to aerobiosis) proteins are indicated by open arrows. Numbers specify molecular masses in kD of the indicated proteins. The three major class 1 protein clusters, designated as a, b, and c, are boxed in panels D and H.

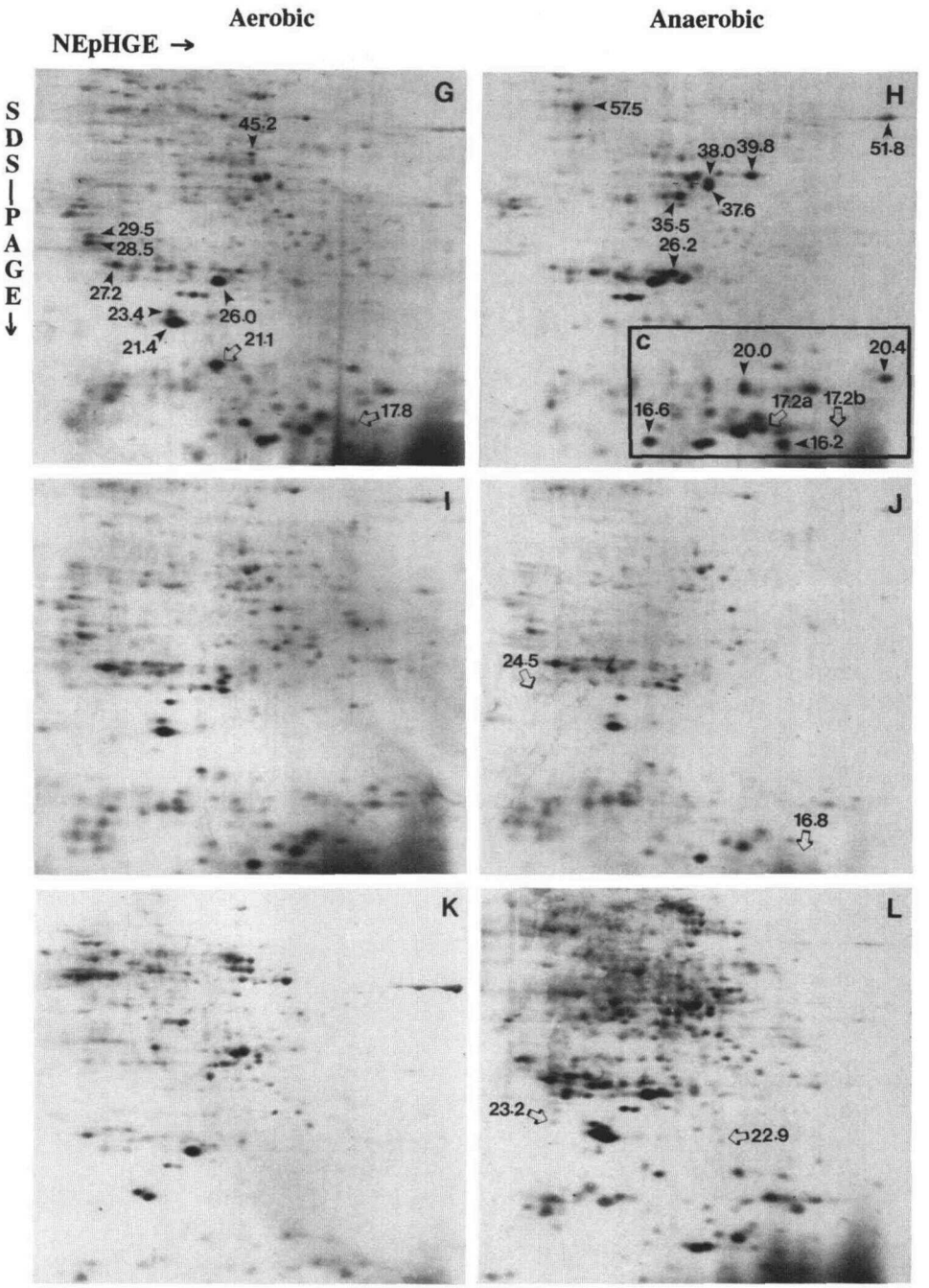


**Figure 1.** (continued).

ant seeds were given a short aerobic exposure (1–12 h) before anaerobic incubation, they subsequently germinated upon return to anaerobic conditions and exhibited both root and shoot growth (unpublished observations). Thus, the effect of varying periods of anoxia on the intolerant seeds was studied by germinating them in air prior to subjecting them to anoxia.

In *E. crus-pavonis* seedlings, grown for 5 d in air and transferred to nitrogen for 24 h, many minor proteins were enhanced (Figs. 1L and 2B). This enhancement was transient because after 44 and 88 h of anaerobiosis, the minor proteins were no longer enhanced and the protein pattern was similar to that of seedlings grown in air (data not shown). Likewise, a similar protein pattern was obtained from seeds that were kept under nitrogen for 5 d then grown in air for 7 d. In *E. colonum*, the protein patterns were similar between air-grown seedlings and those grown in air for 5 d and subjected to anaerobiosis for 24 h (Fig. 1, I and J). However, unlike in *E. crus-pavonis*, no other minor proteins were enhanced. In neither species were the five classes of response to anoxia, typical among the tolerant species, observed.

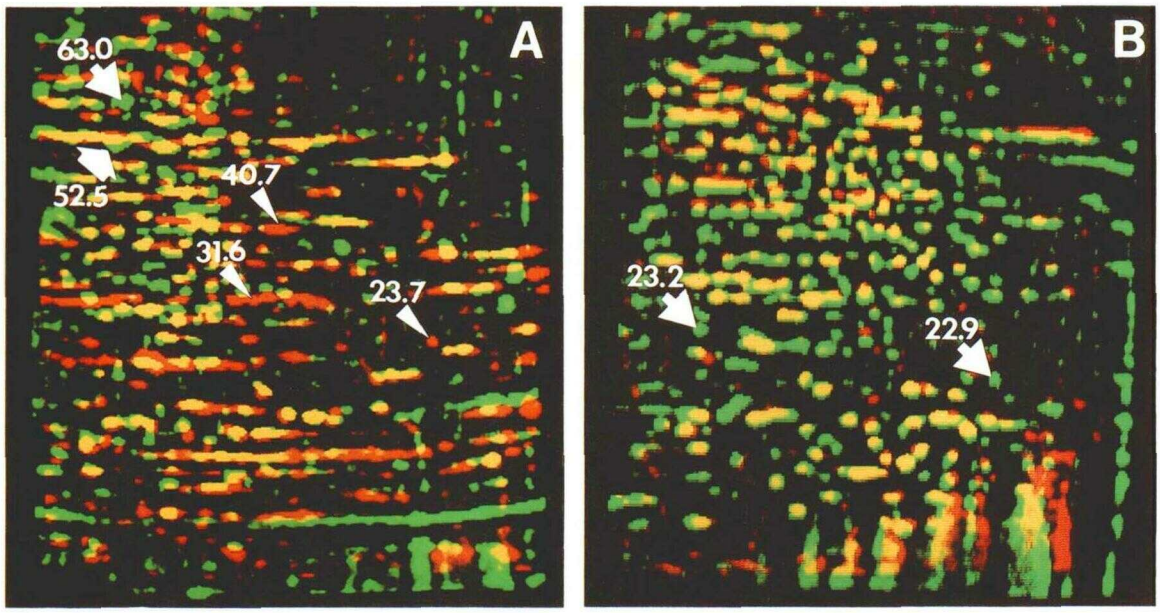


**Figure 2.** Representative computer-enhanced images of Figure 1A (*E. phyllopogon*, air) superimposed on Figure 1B (*E. phyllopogon*, nitrogen) (A) and Figure 1K (*E. crus-pavonis*, air) superimposed on Figure 1L (*E. crus-pavonis*, nitrogen) (B). Proteins of the aerobically grown shoots appear red and those of the anaerobically grown shoots appear green. Proteins common to both aerobically and anaerobically grown shoots appear yellow. Three unique aerobic proteins (class 5 [white arrowheads]) were detected in *E. phyllopogon* (tolerant) with molecular masses of 23.7, 31.6, and 40.7 kD (A). One unique anaerobic polypeptide (class 2 [thin white arrows]) of 63.0 kD was detected in *E. phyllopogon* (A) and two in *E. crus-pavonis* (B) with molecular masses of 22.9 and 23.2 kD. In addition, a major class 1 protein (enhanced under anaerobiosis [thick white arrow]) with a molecular mass of 52.5 kD was noted in *E. phyllopogon* (A).

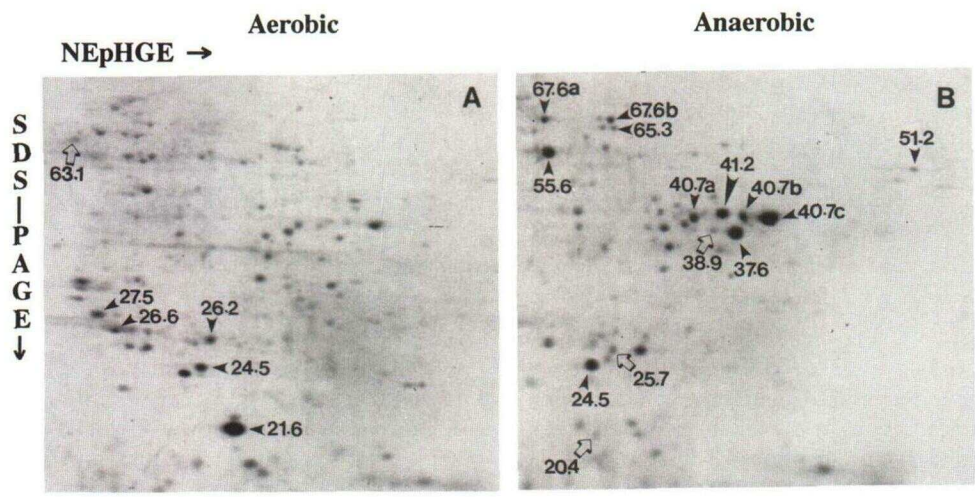


**Figure 3.** Constitutive and inducible protein patterns of rice on two-dimensional polyacrylamide gels double stained with Coomassie brilliant blue and silver. Total proteins (100 μg) isolated from shoots of 5-d-old rice seedlings grown either in air (A) or nitrogen (B) were electrophoresed in the first dimension by NEpHGE (pH 3–10) and in the second dimension by SDS-PAGE (12.5%). Class 1 and class 4 proteins are labeled by closed arrowheads, and class 2 and class 5 proteins by open arrows. Numbers specify molecular masses in kD of the indicated proteins.

**Table I.** *Major anaerobic proteins (class 1) of Echinochloa and rice*

Numbers are molecular masses of the individual proteins, and letters in parentheses indicate polypeptides having the same mass.

| Cluster | Species | | | | |
|---|---|---|---|---|---|
| | *E. phyllopogon* | *E. muricata* | *E. oryzoides* | *E. crus-galli* | *O. sativa* |
| | | | *kD* | | |
| | 52.5 | 56.2 (a,b) | 54.9 | 57.5 | 67.6 (a,b) |
| | | | | 51.8 | 65.3 |
| | | | | | 55.6 |
| | | | | | 51.2 |
| A | 46.2 (a,b) | 43.6 | 43.1 | 39.8 | 40.7 (a,b,c) |
| | 44.1 (a,b) | 40.7 | 39.8 | 38.0 | 41.2 |
| | 40.7 (a,b,c) | 39.8 | 39.4 | 37.6 | 37.6 |
| | 38.9 | 37.6 | 38.0 | 35.5 | |
| | 38.0 | 37.1 | 37.1 | | |
| B | 25.7 (a,b) | 26.3 | 26.3 | 26.2 | 24.5 |
| | 25.1 | 26.0 | | | |
| | | 25.1 | | | |
| | | 24.8 | | | |
| | | 23.7 | | | |
| C | | | 17.2 | 20.4 | |
| | | | 16.0 | 20.0 | |
| | | | | 16.6 | |
| | | | | 16.2 | |

### Identity of Class 1 and Class 4 Proteins

In an attempt to begin to identify the major aerobic and ASPs, we have employed antisera raised against known ASPs from other sources (e.g. maize). We have used several different antisera raised against fructose-1,6-bisP aldolase, PDC, GAPDH, sucrose synthase, glucose-6-P dehydrogenase, peroxidase, and ADH to identify the polypeptides. Two isozymes of fructose-1,6-bisP aldolase (38 and 41 kD) were noted on the immunoblots of the four tolerant species and in *E. crus-pavonis* (Fig. 4a). Anaerobiosis enhanced the expression of the 38-kD isozyme in the tolerant species but not in *E. crus-pavonis*. *E. colonum* had only one form of aldolase (41 kD), which seemed to be unaffected by anaerobiosis. Two-dimensional gel electrophoresis of the same extracts from *E. phyllopogon* further separated each isozyme into three distinct polypeptides (Fig. 4, b and c). All six polypeptides were enhanced under anaerobiosis (Fig. 4c) and corresponded to six ASPs on our two-dimensional protein gels.

Except for *E. phyllopogon*, which has only one form of PDC, the other five *Echinochloa* species and rice all had three isozymes (60, 65, and 75 kD) (Fig. 5). The isozymes were enhanced under anoxia in all species except *E. crus-galli* and *E. crus-pavonis*.

One form of peroxidase (58 kD) that was enhanced in air and repressed under anoxia was detected in the four tolerant *Echinochloa* species (Fig. 6). In the intolerant species, peroxidase was equally expressed in air and under nitrogen.

The immunoblots of the enzymes GAPDH, sucrose synthase, glucose-6-P dehydrogenase, and ADH reproduced less clearly due to weaker antisera reactions and are not shown here. However, two GAPDH polypeptides (28 and 29 kD) of similar intensity were noted in all *Echinochloa* species studied.

None of the major aerobic or anaerobic polypeptides corresponded to sucrose synthase. However, an 87-kD polypeptide appeared on the immunoblots of two-dimensional gels of both air-grown and anaerobically grown seedlings, with similar intensity under air and nitrogen. Two isozymes (28 and 29 kD) of glucose-6-P dehydrogenase were detected in

**Table II.** *Unique aerobic (class 5) and anaerobic (class 2) proteins of Echinochloa complex and rice*

| Species | Aerobic | Anaerobic |
|---|---|---|
| | *kD* | |
| *E. phyllopogon* | 23.7 | 63.0 |
| | 31.6 | |
| | 40.7 | |
| *E. muricata* | 20.9 | 16.6 |
| | 21.2 | 17.0 |
| | 37.0 | 22.4 |
| | 57.5 | 36.3 |
| | | 69.2 |
| *E. oryzoides* | 20.6 | 17.8 |
| | | 23.4 |
| *E. crus-galli* | 17.8 | 17.2a |
| | 21.1 | 17.2b |
| *E. colonum* | —[a] | 16.8 |
| | | 24.5 |
| *E. crus-pavonis* | — | 22.9 |
| | | 23.2 |
| *O. sativa* | 63.1 | 20.4 |
| | | 25.7 |
| | | 38.9 |

[a] None was detected.

**Table III.** *Major aerobic proteins (class 4) of Echinochloa and rice*

| Species | | | | |
|---|---|---|---|---|
| *E. phyllopogon* | *E. muricata* | *E. oryzoides* | *E. crus-galli* | *O. sativa* |
| | | *kD* | | |
| 25.7 | 26.6 | 29.6 | 45.2 | 27.5 |
| 23.7 | 25.7 | 22.9 | 29.5 | 26.6 |
| 21.9 | 23.7 | 22.4 | 28.5 | 26.2 |
| 21.1 | 22.9 | | 27.2 | 24.5 |
| 19.3 | | | 26.0 | 21.6 |
| | | | 23.4 | |
| | | | 21.4 | |

all *Echinochloa* species studied. The response to anaerobiosis varied for each species, i.e. hardly expressed in *E. phyllopogon*; enhanced under nitrogen in *E. oryzoides*, *E. muricata*, *E. crus-galli*, and rice; repressed under nitrogen but enhanced in air in *E. crus-pavonis*; and totally repressed in air in *E. colonum*. The ADH antisera reacted nonspecifically and generated a pattern where most of the proteins stained positively. Because of the nonspecific reaction, it is difficult to assign the precise location of the enzyme on the immunoblot.

We are also currently in the process of microsequencing some of the unique and enhanced anaerobic proteins.

## DISCUSSION

In plants such as maize and soybean, anaerobiosis induces the synthesis of a few select ASPs concomitant with a general repression of aerobic genes. This repression seems to be fixed because aerobic proteins are never synthesized under anoxia and the plant dies within 3 d (Sachs et al., 1980). However, in *Echinochloa* and rice the response to anoxia is different, because after the initial response to anaerobiosis, where ASPs are selectively synthesized, aerobic protein synthesis resumes within 24 h. In the present study, we looked at the changes in the protein patterns of different *Echinochloa* species exposed to anaerobiosis from the onset of imbibition through 5 d of growth.

At the protein level, the anaerobic response was remarkably similar among the tolerant *Echinochloa* and rice species (i.e. all exhibited five classes of response). This similarity suggests a common mechanism for the induction of anaerobic proteins. Except for a few class 5 proteins, all of the constitutive and inducible proteins present in air were also expressed under anaerobiosis. Thus, it appears that for extended survival under anaerobic stress, most of the aerobic proteins must be synthesized as well. Such a strategy is consistent with the continued operation of all of the major respiratory and biosynthetic pathways that we have documented in *E. phyllopogon* during anoxia (Kennedy et al., 1992). Failure to maintain at least basal levels of such aerobic proteins probably results in a relatively quick death, as in maize (Sachs et al., 1980). Among the tolerant *Echinochloa* and rice species, the induction of major ASPs seemed to coincide temporally with the repression of the major aerobic proteins. Whether the occurrence of these two events is coordinated by a common factor similar to the aerobic respiration control and fumarate and nitrate reduction systems found in bacteria (Spiro and Guest, 1991) needs to be investigated.

On the other hand, there were some species-specific differences in the number of polypeptides within each class of proteins and in the number of class 1 protein clusters among the species. These differences may result partly from differences in ploidy levels among the different species (Yabuno, 1964) by altering the number of alleles specifying particular polypeptides. It is interesting to note that among the tolerant *Echinochloa* and rice species, a 52.5- to 57.5-kD polypeptide was induced during anoxia, with the specific molecular mass depending on the species. In *E. phyllopogon* grown in air and labeled in vivo for 4 h under anoxia, the 52.5-kD polypeptide was also a major protein (unpublished data). However, this protein was never induced in the *Echinochloa* species that failed to germinate under anaerobic conditions. It is possible that this protein aids in germination under anoxia among the tolerant *Echinochloa* species. By internal amino acid sequence analysis, we have preliminarily identified this polypeptide as

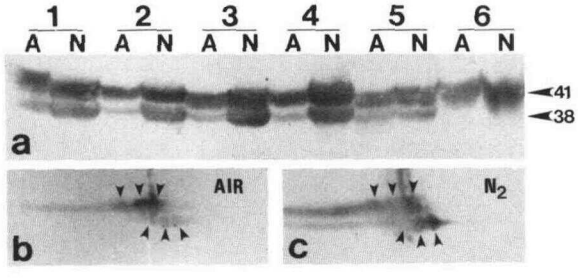


**Figure 4.** a, Immunoblots of fructose-1,6-bisP aldolase from *E. phyllopogon* (lane 1), *E. oryzoides* (lane 2), *E. muricata* (lane 3), *E. crus-galli* (lane 4), *E. crus-pavonis* (lane 5), and *E. colonum* (lane 6). Total proteins (50 μg) isolated from shoots of 5-d-old seedlings grown either in air (A) or nitrogen (N) were subjected to SDS-PAGE (12.5%), electroblotted to Immobilon P membrane, and immunostained as discussed in "Materials and Methods." Masses of the indicated proteins are in kD. b and c, *E. phyllopogon* protein extracts (200 μg) from aerobically (b) and anaerobically (c) grown seedlings were electrophoresed in the first dimension by NEpHGE (pH 3–10) and in the second dimension by SDS-PAGE (12.5%), electroblotted to Immobilon-P membrane, and immunostained as described in "Materials and Methods." Closed arrowheads indicate a number of aldolase polypeptides.

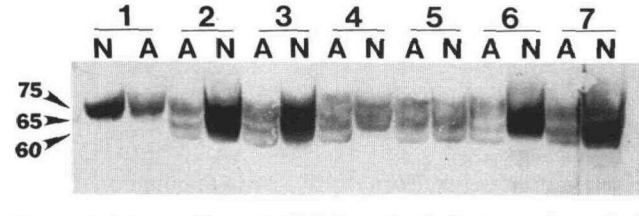


**Figure 5.** Immunoblots of PDC from *E. phyllopogon* (lane 1), *E. oryzoides* (lane 2), *E. muricata* (lane 3), *E. crus-galli* (lane 4), *E. crus-pavonis* (lane 5), *E. colonum* (lane 6), and rice (lane 7). Total proteins (50 μg) isolated from shoots of 5-d-old seedlings grown either in air (A) or nitrogen (N) were subjected to SDS-PAGE (12.5%), electroblotted to Immobilon-P membrane, and immunostained as described in "Materials and Methods." Molecular masses of the indicated proteins are in kD.

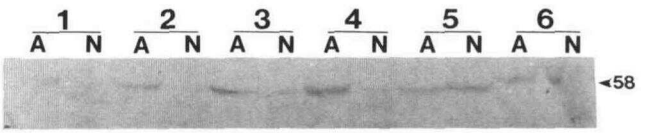


**Figure 6.** Immunoblots of peroxidase from *E. phyllopogon* (lane 1), *E. oryzoides* (lane 2), *E. muricata* (lane 3), *E. crus-galli* (lane 4), *E. crus-pavonis* (lane 5), and *E. colonum* (lane 6). Total proteins (50 μg) isolated from shoots of 5-d-old seedlings grown either in air (A) or nitrogen (N) were subjected to SDS-PAGE (12.5%), electroblotted to Immobilon-P membrane, and immunostained as described in "Materials and Methods." Molecular masses of the indicated proteins are in kD.

enolase (EC 4.2.1.11) (unpublished data). The significance of this finding relative to our previous biochemical studies on the anaerobic metabolism of *E. phyllopogon* (Rumpho and Kennedy, 1981, 1983a, 1983b) is being pursued.

In soybean roots, Tihanyi et al. (1989) reported three classes of response to anaerobiosis based on enzyme activity: those induced severalfold, including ADH, fructose 1,6-bisP aldolase, PDC, phosphoglucomutase, and lactate dehydrogenase; those having constant activity in both air and under anaerobiosis, exemplified by glucose-6-P isomerase, sucrose synthase, and malate dehydrogenase; and those whose activity decreased 90% after 1 d of anaerobiosis. This type of response is shown by peroxidase. Similarly, in this study immunoblotting identified fructose-1,6-bisP aldolase and PDC as class 1 proteins, whereas peroxidase was designated a class 4 protein. We (Rumpho and Kennedy, 1983a) have previously reported that fructose-1,6-bisP aldolase activity in *E. phyllopogon* grown for 7 d after imbibition under nitrogen was enhanced 2.7-fold compared with that in air-grown plants. In addition, the activities of other enzymes controlling glycolysis and the oxidative pentose phosphate pathway in *E. phyllopogon* have been measured (Rumpho and Kennedy, 1981, 1983a). Based on specific activity, phospho*enol*pyruvate carboxylase, malic enzyme, malate dehydrogenase, and glucose-6-P dehydrogenase are class 4 proteins (prominent only in air and repressed under anoxia), whereas phosphofructokinase is a class 3 protein (constant under air or anoxia).

More recently, Fox and Kennedy (1991) reported the activities of several TCA cycle enzymes of *E. phyllopogon* and rice grown under aerobic and anaerobic conditions. Except for succinate dehydrogenase, which is a class 4 protein, the activities of the remaining enzymes changed at different stages of growth. Hence, putting them into classes may be less meaningful. Surprisingly, sucrose synthase was not a major ASP in our two-dimensional gels despite the fact that immunoblots of one-dimensional PAGE indicated that more sucrose synthase was synthesized under anoxia and fluorograms of one-dimensional SDS-PAGE exhibited an enhanced 87-kD polypeptide (Kennedy et al., 1990). Sucrose synthase was a major ASP in maize (Sachs et al., 1980; Springer et al., 1986).

The two intolerant species (*E. crus-pavonis* and *E. colonum*) did not exhibit the anaerobic response typical of the four tolerant species. In *E. crus-pavonis* in particular, no proteins were induced or repressed continuously during anaerobiosis. Instead, several minor polypeptides were transiently enhanced for up to 24 h. Thereafter, the protein patterns in air and anoxia were similar, indicating that synthesis of aerobic proteins is required for extended growth. These results suggest that the intolerant species may utilize a different mechanism to survive and grow during anaerobic stress. Because the intolerant species failed to induce ASPs, yet exhibited the ability to grow under anaerobic conditions (if first aerobically primed), another set of genes may confer tolerance, possibly by producing some other factor imparting tolerance. Then, as in the tolerant species, the usual set of aerobic proteins are synthesized.

In contrast, among the tolerant *Echinochloa* species, two sets of genes may confer tolerance to anaerobic stress. The first set is rapidly induced in response to stress, resulting in the synthesis of major ASPs, as we have shown in this study. The second set, whose identity is unknown, may be responsible for metabolic maintenance under prolonged anaerobic stress. The presence of this second set of genes was inferred from the differences in the response of the tolerant and intolerant species to anoxia. The concept of two sets of genes conferring stress tolerance has recently been suggested for rice cells adapting to water stress (Borkird et al., 1991).

The present study suggests that the abilities to germinate and to grow under anaerobiosis are two distinct physiological events. *E. crus-pavonis* and *E. colonum* normally do not germinate under anoxia. However, when germination was first initiated in these species in air, transfer to a nitrogen atmosphere did not prevent growth and development of the seedlings. Because anaerobic growth can occur in these species under these conditions, the initiation of germination appears to be a separate process. On the other hand, the inability of these two intolerant species to germinate without the oxygen pretreatment may be due to the absence or repression of an essential factor. Lack of induction of the major ASPs in the intolerant species is consistent with this hypothesis.

Alternatively, a germination inhibitor may be activated by an oxygen-sensitive enzyme present only in the intolerant species. In this scenario, the inhibitor is inactivated in the presence of oxygen but is activated under anoxia and germination is prevented.

Based on controlled breeding experiments and inheritance studies, Yamasue et al. (1989) have reported that two epistatic genes were responsible for anaerobic germination in *Echinochloa*. Reciprocal crosses between *E. crus-galli* var *formosensis* (tolerant) and *E. crus-galli* var *praticola* (intolerant) yielded $F_1$ seeds that germinated under nitrogen. $F_2$ seeds derived by selfing $F_1$ seeds segregated with a 13:3 ratio: 13 seeds capable of and 3 seeds incapable of anaerobic germination and growth under flooding. The authors suggested that two major genes control the production of an inhibitor to germination under flooded conditions but concluded that neither of these genes was *ADH*.

Other explanations for anaerobic survival in *Echinochloa* were considered. (a) The ability to germinate and grow under anaerobiosis may depend upon sequential processes regulated at the germination level. Once germination was initiated, growth under anoxia could continue. This hypothesis implies that similar postgermination anaerobic metabolism operates in both tolerant and intolerant species. However,

our results are inconsistent with this hypothesis because ASPs induced in the tolerant species were not detected in the intolerant species. (b) A factor (perhaps a *trans*-acting factor) may regulate both processes, analogous to the aerobic respiration control and fumarate and nitrate reduction systems in *Escherichia coli* (Spiro and Guest, 1991). This factor might be expressed only in the tolerant species under anoxia. Currently, we are trying to discern which regulatory mechanism(s) controls anaerobic germination and growth in *Echinochloa*.

## ACKNOWLEDGMENTS

We thank Dr. William R. Hruschka for the computer analysis and use of the facilities at the Instrumentation and Sensing Laboratory, USDA, Beltsville, MD; Dr. Theodore C. Fox for the technical support in computer analysis; Dr. Philip Kelley for the fructose-1,6-bisP aldolase, PDC, and sucrose synthase antisera; Dr. Karen Koch for sucrose synthase antiserum; Dr. Mark Lagrimini for the peroxidase antiserum; Dr. Martin M. Sachs for the GAPDH antiserum; and Dr. Drew Schwartz for the ADH antiserum.

Received May 4, 1992; accepted September 24, 1992.


## LITERATURE CITED

**Barrett SCH, Wilson BF** (1981) Colonizing ability in the *Echinochloa crus-galli* complex (barnyard grass). I. Variation in life history. Can J Bot **59:** 1844–1860

**Borkird C, Simoens C, Villarroel R, Van Montagu M** (1991) Gene expression associated with water-stress adaptation of rice cells and identification of two genes as hsp 70 and ubiquitin. Physiol Plant **82:** 449–457

**Everard JD, Kennedy RA** (1985) Physiology of lipid metabolism during anaerobic germination of *Echinochloa crus-galli* var *oryzicola* under anoxic germination conditions (abstract No. 534). Plant Physiol **77:** S-98

**Fox TC, Kennedy RA** (1991) Mitochondrial enzymes in aerobically and anaerobically germinated seedlings of *Echinochloa* and rice. Planta **184:** 510–514

**Hoffman NE, Bent AF, Hanson AD** (1986) Induction of lactate dehydrogenase isozymes by oxygen deficit in barley root tissue. Plant Physiol **82:** 658–663

**Hruschka WR** (1984) Computerized comparison of two autoradiographs. Clin Chem **30:** 2037–2039

**Hruschka WR, Massie DR, Anderson JD** (1983) Computerized comparison of two-dimensional electrophoretograms. Anal Chem **55:** 2345–2348

**Hurkman WJ, Tanaka CK** (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol **81:** 802–806

**Kelley PM** (1989) Maize pyruvate decarboxylase mRNA is induced anaerobically. Plant Mol Biol **13:** 213–222

**Kelley PM, Freeling M** (1984) Anaerobic expression of maize glucose phosphate isomerase. I. J Biol Chem **259:** 673–677

**Kelley PM, Tolan DR** (1986) The complete amino acid sequence for the anaerobically induced aldolase. Plant Physiol **82:** 1076–1080

**Kennedy RA, Fox TC, Dybiec LD, Rumpho ME** (1990) Biochemical adaptations to anoxia in rice and *Echinochloa* seeds. *In* RB Taylorson, ed, Recent Advances in the Development and Germination of Seeds. Plenum Press, New York, pp 151–163

**Kennedy RA, Fox TC, Siedow JN** (1987a) Activities of isolated mitochondria and mitochondrial enzymes from aerobically and anaerobically germinated barnyard grass (*Echinochloa*) seedlings. Plant Physiol **85:** 474–480

**Kennedy RA, Rumpho ME, Fox TC** (1987b) Germination physiology of rice and rice weeds: metabolic adaptations to anoxia. *In* RMM Crawford, ed, Plant Life in Aquatic and Amphibious Habitats. Blackwell Scientific, Oxford, UK, pp 193–203

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

**Kennedy RA, Rumpho ME, VanderZee D** (1983) Germination of *Echinochloa crus-galli* (barnyard grass) seeds under anaerobic conditions. Respiration and response to metabolic inhibitors. Plant Physiol **72:** 787–794

**Kimmerer T** (1987) Alcohol dehydrogenase and pyruvate decarboxylase activity in leaves and roots of Eastern cottonwood (*Populus deltoides* Bartr.) and soybeans (*Glycine max* L.). Plant Physiol **84:** 1210–1213

**Knowles LO, Kennedy RA** (1984) Lipid biochemistry of *Echinochloa crus-galli* during anaerobic germination. Phytochemistry **23:** 529–532

**Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

**Lazslo A, St Lawrence P** (1983) Parallel induction and synthesis of PDC and ADH in anoxic maize roots. Mol Gen Genet **192:** 110–117

**Llewellyn DJ, Finnegan EJ, Ellis JG, Dennis ES, Peacock WJ** (1987) Structure and expression of an alcohol dehydrogenase 1 gene from *Pisum sativum* (cv. "Greenfast"). J Mol Biol **195:** 115–123

**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

**Nover L** (1989) Anaerobic stress. *In* L Nover, D Neumann, K-D Scharf, eds, Heat Shock and Other Stress Response Systems of Plants. Springer-Verlag, New York, pp 89–90

**O'Farrell PH, Goodman HM, O'Farrell PO** (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12:** 1133–1142

**Rivoal J, Ricard B, Pradet A** (1991) Lactate dehydrogenase in *Oryza sativa* L. seedlings and roots. Identification and partial purification. Plant Physiol **95:** 682–686

**Rumpho ME, Kennedy RA** (1981) Anaerobic metabolism in germinating seeds of *Echinochloa crus-galli* (barnyard grass). Metabolite and enzyme studies. Plant Physiol **68:** 165–168

**Rumpho ME, Kennedy RA** (1983a) Activity of the pentose phosphate and glycolytic pathways during anaerobic germination of *Echinochloa crus-galli* (barnyard grass) seeds. J Exp Bot **155:** 1–7

**Rumpho ME, Kennedy RA** (1983b) Anaerobiosis in *Echinochloa crus-galli* (barnyard grass) seedlings. Intermediary metabolism and ethanol tolerance. Plant Physiol **72:** 44–49

**Russell DA, Sachs MM** (1989) Differential expression and sequence analysis of the maize glyceraldehyde-3-phosphate dehydrogenase gene family. Plant Cell **1:** 793–803

**Sachs MM, Freeling M, Okimoto R** (1980) The anaerobic proteins of maize. Cell **20:** 761–767

**Spiro S, Guest JR** (1991) Adaptive responses to oxygen limitation in *Escherichia coli*. Trends Biochem Sci **16:** 310–314

**Springer B, Werr W, Starlinger P, Bennett DC, Zokolica M, Freeling M** (1986) The shrunken gene on chromosome 9 of *Zea mays* L. is expressed in various plant tissues and encode an anaerobic protein. Mol Gen Genet **205:** 461–468

**Tanksley SD, Jones RA** (1981) Effects of $O_2$ stress on tomato alcohol dehydrogenase activity: description of a second ADH coding gene. Biochem Genet **19:** 397–409

**Tihanyi K, Fontanell A, Thirion J-P** (1989) Gene regulation during anaerobiosis in soya roots. Biochem Genet **27:** 719–730

**Towbin H, Staehelin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

**VanderZee D, Kennedy RA** (1982) Plastid development in seedlings of *Echinochloa crus-galli* var *oryzicola* under anoxic germination conditions. Planta **155:** 1–7

**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

**Yabuno T** (1964) Cytotaxonomic study of the genus *Echinochloa*. PhD thesis. Kyoto University, Kyoto, Japan

**Yamasue Y, Hamada M, Kusanagi T** (1989) Anaerobic seed germination of *Echinochloa* weeds: the inherent mode and relationship with isozymes of alcohol dehydrogenase. Mem Coll Agric Kyoto Univ **135:** 43–52