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RESEARCH PAPER

Genetic and biochemical analysis of anaerobically-induced enzymes during seed germination of *Echinochloa crus-galli* varieties tolerant and intolerant of anoxia

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Abstract

To compare the regulation of anaerobic metabolism during germination in anoxia-tolerant and intolerant plants, enzymes associated with anaerobic metabolism such as sucrose synthase, aldolase, enolase, pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (ALDH) were assayed in two varieties of *Echinochloa crus-galli*, formosensis (tolerant) and praticola (intolerant). The initial and intervening enzymes of the pathway (sucrose synthase and aldolase) and enzymes in the last part of the pathway (PDC, ADH and ALDH) revealed similar changing patterns in activities during germination. This implies that each group of enzymes may be controlled by an identical regulatory mechanism. During anoxia, activities of all enzymes increased 1.5–30-fold in both varieties compared to their activities under aerobic conditions. Activities of sucrose synthase, enolase and ADH exhibited the same induction patterns under anoxia in formosensis and praticola. However, the activities of aldolase, ALDH and PDC were more strongly induced in formosensis under anoxia (1.2–2-fold) than in praticola. These enzymes were also assayed in F_3 families which varied in their anaerobic germinability. For PDC, activities under anoxia in anoxia-tolerant families were similar to those of an anoxia-intolerant family during the whole period although the family did not exhibit anaerobic germinability. This suggests that there is no correlation between PDC activity and anaerobic germinability. For ALDH, activities were more strongly induced under anoxia in anoxia-tolerant families than in anoxia-intolerant families, a trend also exhibited by the parents. This indicates that ALDH may play a role in detoxifying acetaldehyde formed through alcoholic fermentation during anaerobic germination.

Key words: Aldehyde dehydrogenase, anaerobic germination, anoxia, *Echinochloa crus-galli* var. *formosensis*, *E. crus-galli* var. *praticola*, flooding tolerance.

Introduction

Seeds of most higher plants have an absolute oxygen requirement for germination. However, germination and seedling development under anoxia have been reported in rice (Alpi and Beevers, 1983), *Echinochloa* species (Kennedy *et al*., 1992), the African legume *Erythrina caffra* (Small *et al*., 1993), and the water plant *Trapa natans* (Menegus *et al*., 1992). This laboratory has been investigating the adaptive mechanisms imparting the unusual trait of anaerobic germination in *Echinochloa* species. In the anoxia-tolerant *Echinochloa* species exam-

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Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ASP, anaerobic stress protein; TPCK, *N*-tosyl-L-phenylalaninechloromethylketone; PDC, pyruvate decarboxylase; QTL, quantitative trait loci.

ined, glycolysis and alcoholic fermentation were found to operate under anoxia (Rumpho and Kennedy, 1983*a*, *b*), but a Pasteur effect was not found (Kennedy *et al*., 1992). In addition, these *Echinochloa* species exhibit an ability to vent most of the ethanol produced during anaerobic germination to the external media and the seedlings are very tolerant of ethanol (Rumpho and Kennedy, 1983*b*; Kennedy *et al*., 1987*b*). Although mitochondria in other species including rice degenerate or exhibit abnormalities under anoxia exposure, anaerobic-tolerant *E. phyllopogon* (Stev.) Koss. (synonymous with *E. crus-galli* var. *oryzicola* Ohwi, and *E. oryzicola* Vasing.) developed intact mitochondria even under anaerobic conditions (Kennedy *et al*., 1990). Indeed, ATP was generated anoxically by the continued functioning of part of the TCA cycle (Kennedy *et al*., 1987*a*; Fox and Kennedy, 1991). The pentose phosphate pathway (Rumpho and Kennedy, 1983*a*) and lipid biosynthesis (Knowles and Kennedy, 1984) were also operative under anoxia.

By contrast to abundant biochemical data, genetic experiments have not been carried out to investigate anaerobic germination and flooding tolerance because the *Echinochloa* species used previously (e.g. *E. phyllopogon* –flooding tolerant and *E. crus-pavonis* –flooding intolerant) are incompatible for crossing. However, in the present study, two other varieties of *Echinochloa* (*E. crus-galli* var. *formosensis* Ohwi and *E. crus-galli* var. *praticola* Ohwi; hereafter, formosensis and praticola, respectively) were crossed and several generations of progeny produced. *E. crus-galli* (L.) Beauv is one of the world's worst weeds with a worldwide distribution and consists of several varieties. Formosensis and praticola exhibit physiological and morphological traits that are clearly diverse. Formosensis is one of the paddy weeds found in flooded rice fields and exhibits an ability to germinate and grow under anaerobic conditions; whereas, praticola inhabits roadsides and fields and requires oxygen for germination (Yamasue *et al*., 1990). Although praticola also inhabits a ridge between flooded rice fields (dry area), it is not found within the rice field (flooded area). This indicates that anaerobic germinability and flooding tolerance differences between formosensis and praticola influence the habitat occupied by these two weeds (Yamasue *et al*., 1990).

In this study, the specific activities of enzymes associated with glycolysis and alcoholic fermentation were assayed during germination in formosensis and praticola seeds under aerobic and anaerobic conditions. In addition, the F_2 and F_3 inbred progenies of praticola× formosensis were produced and their anaerobic germinability and flooding tolerance evaluated. Finally, F_3 families with and without anaerobic germinability were subjected to enzyme assays to investigate the regulation of anaerobic metabolism among flooding-tolerant and -intolerant F_3 families.

Materials and methods

Plant materials

Both formosensis and praticola inbred lines were obtained by self-pollination through about 10 generations. Formosensis seeds originated from a plant collected at a fallowed paddy in Uji City, Japan and praticola was a strain from a seed of praticola originally collected by Dr T Yabuno, Osaka Prefectural University. Formosensis and praticola seeds were sterilized in a 2.5% (v/v) sodium hypochlorite solution for 15 min and rinsed thoroughly with deionized water.

Germination tests

To test germination, 200 seeds were placed on wet filter paper at 30 °C in the light for 5 d in an aerobic incubator or anaerobic chamber (Forma Scientific) that was flushed continuously with a 90% nitrogen–10% hydrogen gas mixture. Germination was scored based on emergence of greater than 1 mm shoot or radicle.

F_1 seeds of praticola×formosensis were produced by conventional breeding methods and the F_1 plant was grown and selfed to collect F_2 seeds. Two-hundred F_2 seeds were subjected to a germination test in an anaerobic chamber as described above. Following the test, both germinating and non-germinating seeds were incubated in an aerobic chamber for 5 d and 198 seedlings obtained were transplanted into pots. Three panicles on each F_2 plant were covered by paraffin bags to collect self-fertilized F_3 seeds. Seeds in the three bags from each plant were combined and recognized as one family. Twenty-seven and 43 families were selected from F_3 families germinating and not germinating under anaerobic conditions at the F_2 generation, respectively, and their anaerobic germinability was subsequently analysed. Twenty seeds from each F_3 family were utilized for germination tests under anoxia and the families that exhibited high and low germination percentages (approximately 100% and 0%, respectively) were selected. One-hundred seeds of the F_3 families selected were examined again for anaerobic germinability and the families were further subjected to the enzyme assays reported here.

Enzyme assays

Seeds (3.2 g fresh weight) were incubated at 30 °C in the light for up to 7 d in an aerobic incubator or anaerobic chamber. The entire sample (seeds and/or seedlings as found for each sample) at each time point was harvested on days 1, 3, 5 and 7, frozen in liquid nitrogen, and stored at –80 °C until used. Frozen samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Frozen powder (300 mg) was transferred to a chilled microcentrifuge tube and immediately ground in cold extraction buffer (900 µl) using a small pestle, on ice. The extract was centrifuged at 16 000 *g* for 25 min at 4 °C and the supernatant used for enzyme assays. Components of the extraction buffer and assay methods are described below. For all enzyme assays, protein was quantified by the method of Bradford (1976), with BSA as the standard.

ADH (EC 1.1.1.1): Enzyme activity was measured by the method of Rumpho and Kennedy (1981). The extraction buffer contained 100 mM TRIS-HCl (pH 9.0), 20 mM $MgCl_2$, 0.1% (v/v) β-mercaptoethanol, 100 µM leupeptin, 1 mM PMSF, and 100 µM TPCK (*N*-tosyl-L-phenylalaninechloromethylketone). For the activity assay, 20 µl extract was added to a reaction mixture containing 50 mM TRIS-HCl (pH 9.0) and 1 mM NAD in a 1 ml cuvette. Ethanol (50 µl) was added last to initiate the reaction. ADH activity was assayed in the acetaldehyde synthesis direction and NAD reduction was monitored at 340 nm at 30 °C for 2.5 min.

Table 1. *Germination percentage of parents and the F_2 progeny under aerobic and anaerobic conditions*

Two-hundred seeds were placed on wet filter paper at 30 °C in the light for 5 d in either an aerobic or anaerobic incubator. Germination was scored based on emergence of greater than 1 mm shoot (anaerobic germination) or root (aerobic germination).

	Aerobic germination	Anaerobic germination
Formosensis	100%	97%
Praticola	89%	6%
F_2	96%	78%

ALDH (EC 1.2.1.3): The enzyme assay was performed as described by Liu *et al*. (2001). The extraction buffer contained 100 mM HEPES (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol, and 0.1% (v/v) Triton X-100. For the activity assay, 100 µl extract was added to a reaction mixture containing 100 mM sodium pyrophosphate (pH 9.5) and 1.5 mM NAD. Acetaldehyde (99.5%, 5.6 µl) was added to start the reaction. NAD reduction was monitored at 340 nm at 30 °C for 2.5 min.

Aldolase (EC 4.1.2.13): Enzyme activity was measured by the methods of Swamy and Sandyarani (1986). The extraction buffer contained 50 mM TRIS-HCl (pH 8.0), 0.1% (v/v) β-mercaptoethanol, 100 µM leupeptin, 1 mM PMSF, and 100 mM TPCK. For the enzyme assay, 100 µl extract was added to a reaction mixture containing 50 mM TRIS-HCl (pH 8.0), 50 mM $MgCl_2$, 5 mM dithiothreitol, and 3.3 mM phenylhydrazine-HCl. Ten microlitres of 500 mM fructose-1,6-diphosphate was added to start the reaction. The production of triose phenylhydrazine was monitored at 324 nm at 30 °C for 2.5 min.

Enolase (EC 4.2.1.11): Enzyme activity was measured by the method of Fox *et al*. (1995). Extraction buffer contained 50 mM TRIS-HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 500 µM EDTA, 0.1% (v/v) β-mercaptoethanol, 100 µM leupeptin, 1 mM PMSF, and 100 mM TPCK. For the activity assay, 100 µl extract was added to a reaction mixture containing 50 mM MOPS-KOH (pH 7.0) and 10 mM KCl. The reaction was started by the addition of 10 µl of 100 mM 2-phosphoglyceric acid. Phosphoenolpyruvate production was monitored at 240 nm at 30 °C for 2.5 min.

PDC (EC 4.1.1.1): Enzyme activity was assayed as described by Rivoal *et al*. (1990). Enzyme buffer contained 50 mM MEPS-NaOH (pH 6.2), 1 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM thiamine pyrophosphate chloride. For the assay, 100 µl extract was added to a reaction mixture containing 50 mM MEPS-NaOH (pH 6.2), 0.5 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate chloride, 0.12 mM NADH, and 660 nkat yeast ADH. In this reaction, acetaldehyde produced by PDC was reduced by ADH and the coupled NADH oxidation was monitored at 340 nm at 30 °C for 2.5 min.

Sucrose synthase (EC 2.4.1.13): Enzyme activity was measured by the method of Zeng *et al*. (1998). Extraction buffer contained 200 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, 20 mM sodium ascorbate, 1 mM PMSF, and 10% (w/v) polyvinylpolypyrrolidone. The extract was dialysed in a dialysis cassette (Pierce, 10 000 M_r cutoff) at 4 °C for 24 h against extraction buffer diluted 1:40. The buffer was changed three times during dialysis. For the assay, 20 µl extract was added to a reaction mixture (50 µl) containing 50 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 10 mM fructose, 5 mM UDP-glucose, and the mixture was incubated at 30 °C for 30 min. At the end of the reaction, the sucrose produced was measured by the anthrone method (Zeng *et al*., 1998).

ADH isozyme analysis

Crude protein extract from the ADH assay was used for ADH isozyme analysis. Twenty micrograms of protein were run in each lane in a 6.5% polyacrylamide gel. Electrophoresis and activity staining were carried out as described by Yamasue *et al*. (1990).

Results

Anaerobic germinability and flooding tolerance in formosensis, praticola and the F_2 and F_3 progenies

Germination tests were performed on seeds of formosensis, praticola and their F_2 progeny (Table 1). Under aerobic conditions, the radicle emerged first from the seeds, but root and shoot emergence occurred nearly simultaneously. In fact, germination in air was observable on day 3 in both formosensis and praticola seeds and all germinating seeds exhibited both shoot and root emergence. Germination was also initiated at day 3 under anoxia with 97% of the formosensis seeds germinated after 5 d. Only 6% of the praticola seeds germinated under anoxia. In contrast to aerobic germination where the radicle emerged first and both root and shoot growth were observed, only the shoot emerged and elongated during the entire anaerobic incubation period. Of the F_2 seeds, 78% germinated under anaerobic conditions compared to 96% in air. Assuming that anaerobic germinability is considered a discrete trait, it can be analysed by a χ^2 contingency test. The segregation ratio of anaerobic germinability in the F_2 progeny was fit to the expected ratio, 3:1 (P=0.37) (Table 2). This suggests that anaerobic germinability in *E. crus-galli* is controlled by a single gene.

The F_2 seedlings germinating under anoxia exhibited various shoot lengths (Fig. 1). Germination under anoxia was initiated from day 3 to day 5 in F_2 seeds. Some F_2 seedlings did not grow after germination, indicating that shoot length was not related to time of germination. Shoot growth rate under anaerobic conditions is one of the important factors affecting flooding tolerance because of the snorkel effect (Drew, 1997). It is proposed here that this continuous trait represents the degree of flooding tolerance. Under anaerobic conditions, only shoot emergence was observed. Of the seeds that did not germinate under anoxia, only two did not germinate even when returned to aerobic conditions. These seeds were scored as dead or dormant and eliminated from the population. The average shoot length for formosensis germinating under anoxia was 17.2 mm and for the few praticola that germinated, the average shoot length was 0.3 mm (data not shown). The F_2 progeny revealed intermediate values between praticola and formosensis (Fig. 1). If non-germinating seeds (dead or dormant) were removed from the population, the distribution was approximately normal.

Table 2. *A χ^2 test for germinating and non-germinating seeds of the F_2 progeny under anoxia*

Two-hundred seeds were subjected to a germination test under anoxia. Two of the 200 seeds were scored as dead because they did not germinate upon return to aerobic conditions after the 5 d anaerobic germination test.

	No. of germinated seeds	No. of non-germinated seeds	Expected ratio	χ^2 test	
F_2 seeds	154	44	3:1	χ^2=0.81	P=0.37

The frequency distribution for anaerobic germinability in the F_3 families originating from seed that either germinated or did not germinate under anoxia in the F_2 generation, is shown in Fig. 2. Germination frequency in the F_3 families not germinating anaerobically in the F_2 generation (grey bars in Fig. 2) was normally distributed (from 0% to 95%). In these families, 15% of the F_3 families exhibited greater than 80% germination although parents of these families did not germinate under anoxia. On the other hand, in the F_3 families whose parents germinated under anoxia (black bars in Fig. 2), all exhibited greater than 25% anaerobic germination.

Activities of anaerobically-induced enzymes during germination in formosensis and praticola

The specific activities of enzymes associated with glycolysis and alcoholic fermentation, sucrose synthase, aldolase, enolase, ADH, ALDH, and PDC, were assayed during germination of formosensis and praticola under aerobic and anaerobic conditions (Fig. 3). Under aerobic conditions, the activity of each enzyme changed over time of imbibition with an approximately similar pattern between formosensis and praticola. At day 0, a significant difference was measured for enolase activity between formosensis and praticola, but this was not observed for the other enzymes. For both formosensis and praticola, sucrose synthase and aldolase activities decreased in air and remained fairly level until the seeds germinated at day 3 and then the activities increased. At day 1, PDC, ADH and ALDH activities increased under aerobic conditions in both seed types, similar to their respective induction under anoxia. For enolase, PDC, ADH, and ALDH, aerobic activities remained low after germination at day 3.

During anoxia, the activities of all six enzymes increased 1.5–30-fold in both praticola and formosensis compared to their activities over time of imbibition in air. Activities of PDC, ADH and ALDH were strongly induced (10–30-fold), whereas sucrose synthase and aldolase were weakly induced (1.5–2-fold). The activities of sucrose synthase, enolase and ADH exhibited the same induction patterns under anoxia in formosensis and praticola in spite of the fact that praticola seeds did not germinate under anoxia. By contrast, activities of aldolase, PDC and ALDH were more strongly induced in formosensis than praticola under anoxia (1.2–2-fold). In both varieties, activities of PDC, ADH and ALDH strongly increased until germination at day 3, after which their activity slopes became level.

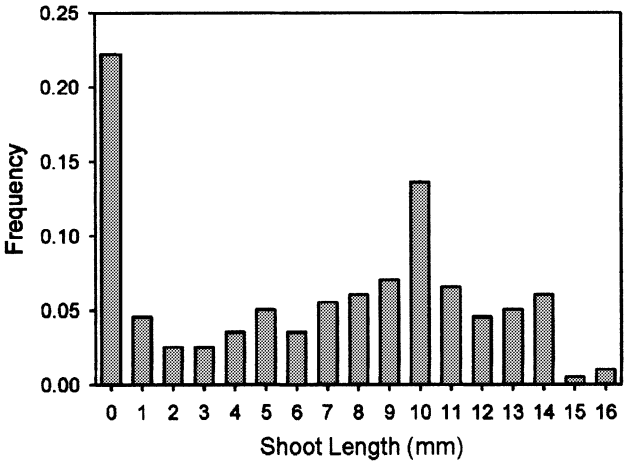

Fig. 1. Frequency distribution for shoot length of F_2 progeny. Two-hundred F_2 seeds were placed on wet filter paper at 30 °C in the light for 5 d under anaerobic conditions. The average shoot lengths for formosensis and praticola after 5 d anoxia were 17.2 mm and 0.3 mm, respectively.

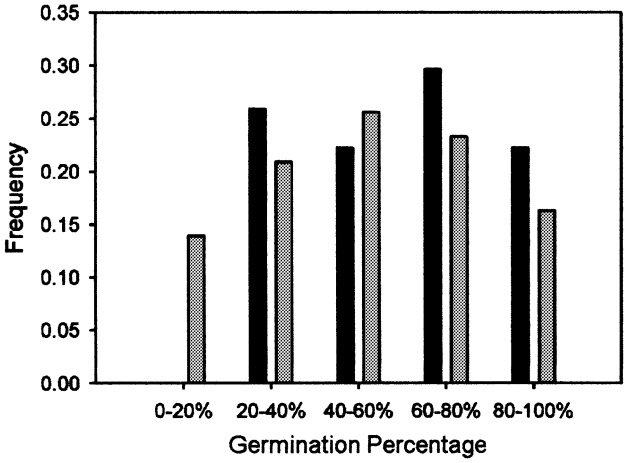

Fig. 2. Frequency distribution for germination percentage under anoxia in F_3 families. Black and grey bars indicate F_3 families whose parent did or did not germinate under anoxia in the F_2 generation, respectively. Twenty seeds from each family were placed on wet filter paper at 30 °C in the light for 5 d under anoxia.

ADH isozymes during germination under aerobic and anaerobic conditions

To understand the correlation between anaerobic germinability and the number of ADH isozymes in *E. crus-galli*, ADH isozymes were analysed in seeds of formosensis and praticola during aerobic and anaerobic germination

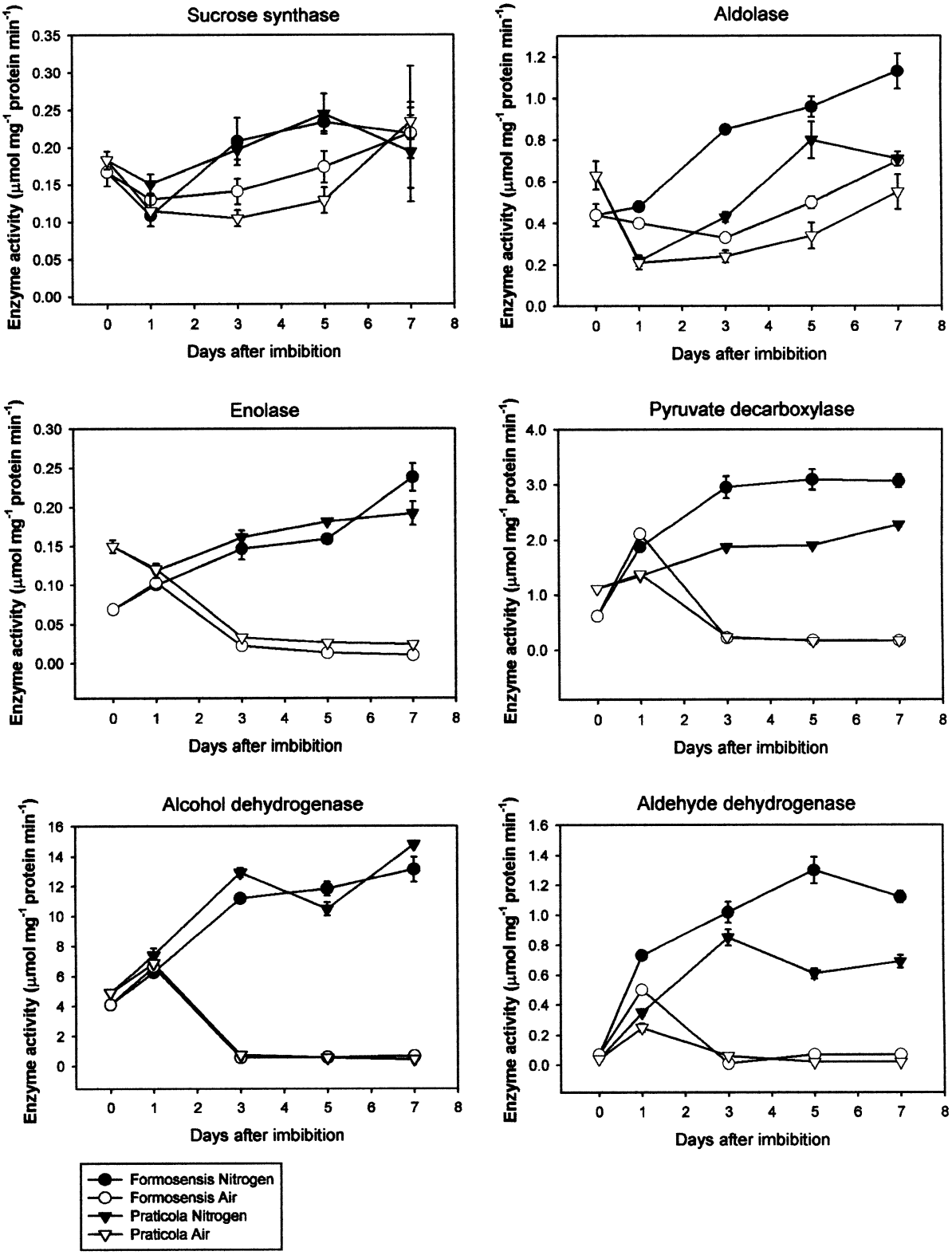

Fig. 3. Enzyme activities during germination under aerobic and anaerobic conditions in formosensis and praticola. Shoot (anaerobic) or root (aerobic) emergence was initiated on day 3 in each species, except for praticola under nitrogen in which germination did not occur. Each activity value represents an average of three measurements of the same sample. Standard deviation values are indicated when larger than the symbol.

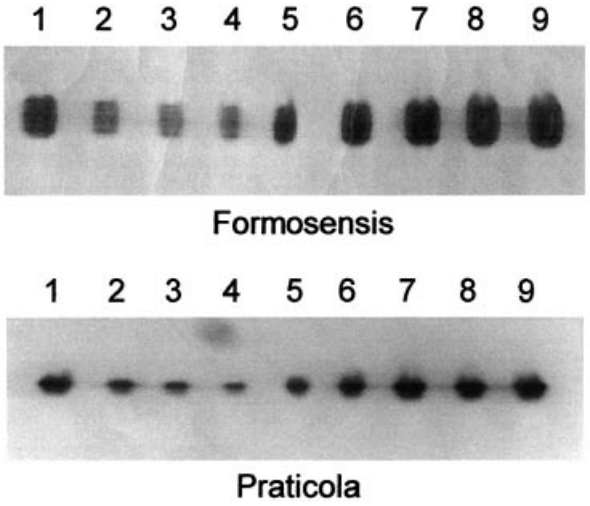

Fig. 4. ADH isozymes in formosensis and praticola seeds under aerobic and anaerobic conditions. Twenty µg of crude protein were loaded in each lane. Lanes 1–4: air 1 d, 3 d, 5 d, 7 d; lane 5: 0 d; Lanes 6–9: nitrogen 1 d, 3 d, 5 d, 7 d.

Table 3. *Germination percentage in F_3 families under aerobic and anaerobic conditions*

One hundred-seeds were placed on wet filter paper at 30 °C in the light for 5 d in either an aerobic or anaerobic incubator.

Family	Anaerobic germinability of parent seeds (F_2 seeds)	F_3 families germinability	
		Aerobic	Anaerobic
T38	Yes	100%	98%
T124	Yes	99%	97%
T179	No	99%	99%
T64	No	99%	9%
T195	No	99%	0%

(Fig. 4). ADH isozymes in formosensis and praticola consisted of five bands and a single band, respectively, during normal aerobic germination. Under anoxic stress, newly synthesized bands were not found in either formosensis or praticola although the density of each isozyme band changed correspondingly as the enzyme activities were induced during anaerobic germination.

Enzyme activities during germination in F_3 families

The differences between formosensis and praticola seeds in activities of ALDH, aldolase and PDC during anaerobic germination were measured. For enzyme assays in F_3 families, two out of the above three enzymes were selected since some F_3 sample sizes were limiting. Hence, enzymes associated with alcoholic fermentation were selected and the specific activities of ALDH and PDC were assayed in F_3 families. Nearly 100% of the seeds in the F_3 families T38, T124 and T179 showed anaerobic germinability,

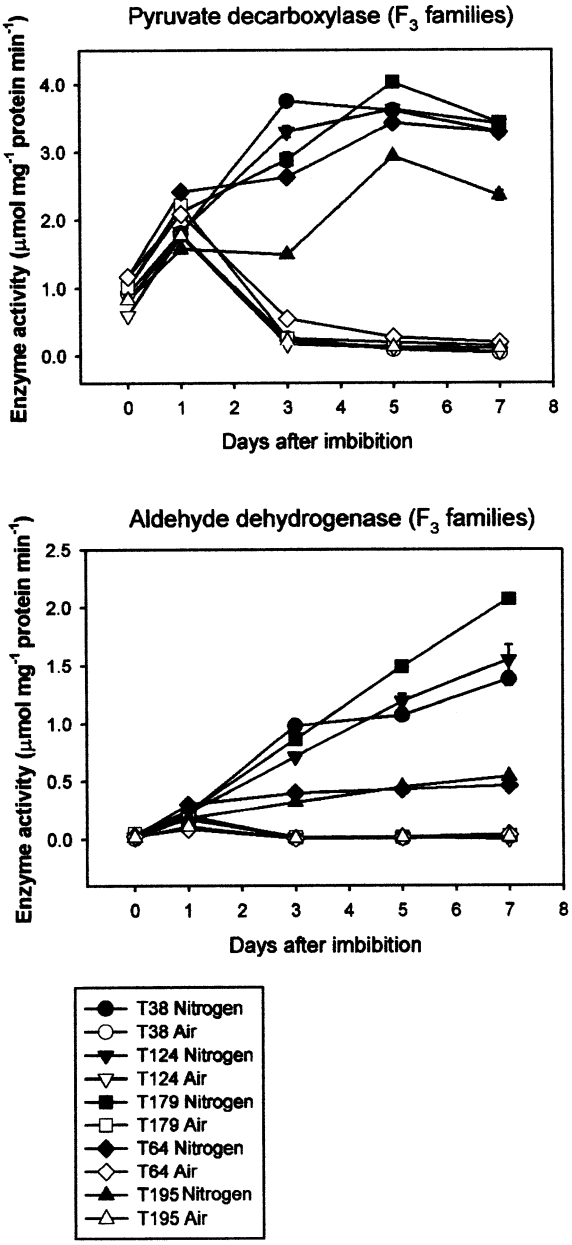

Fig. 5. Enzyme activities during germination under aerobic and anaerobic conditions in F_3 families. Shoot or root emergence was initiated on day 3 in each family. Each activity value represents the average of three measurements. Standard deviation is indicated when larger than the symbol.

while families T64 and T195 did not exhibit this ability (Table 3). Although the parent (F_2 seed) of T179 did not germinate under anoxia, the F_3 seeds revealed vigorous anaerobic germinability. The specific activities of PDC and ALDH were assayed during germination under aerobic and anaerobic conditions in five different F_3 families (Fig. 5). Each crude enzyme extract was prepared from bulked seeds of each F_3 family. For PDC, activities in all five families exhibited the same changing pattern during

germination under aerobic conditions as seen in the parents, formosensis and praticola (compare with Fig. 3). During anaerobic germination, anoxia-intolerant praticola revealed lower PDC activity than anoxia-tolerant formosensis (Fig. 3). However, the induction patterns of activity in anoxia-tolerant families (T38, T124 and T179) were similar to those in the anoxia-intolerant T64 family during the whole period under anoxia although T64 did not exhibit anaerobic germinability. For ALDH, no difference was noted among all F_3 families in activity during aerobic germination. Under anoxic stress, enzyme activities were more strongly induced in anoxia-tolerant families (T38, T124 and T179) than in anoxia-intolerant families (T64 and T195) as also measured in the tolerant and intolerant parents.

Discussion

Anaerobic germination and flooding tolerance have been well studied using flooding-tolerant and -intolerant species and genotypes. However, hybrids between tolerant and intolerant genotypes and near inbred lines of flooding-tolerance have not been utilized to investigate this unusual physiological trait which is significant for most agricultural plants.

Inheritance studies and breeding experiments of flooding tolerance have been carried out in flooding-tolerant and -intolerant rice. Mohanty and Chaudhary (1985) demonstrated that heritability of flooding tolerance in rice was relatively high and that the genetic mode for inheritance of tolerance was partially or completely dominant. Mackill *et al*. (1993) indicated that the trait was controlled by one or a few loci with a major effect. Although the gene(s) associated with anaerobic germinability in *Echinochloa* could be different from those of flooding tolerance in rice, these genetic data of F_2 segregation suggest anaerobic germinability is regulated by one dominant gene (Table 2). However, in the F_3 families, 15% of the families exhibited more than 80% anaerobic germination even though the parent of these families did not germinate under anoxia (Fig. 2). In addition, phenotypes of F_3 families whose parents germinated under anoxia were segregated from 25% to 100% (data not shown). This indicates that anaerobic germination is a complicated trait regulated by several genes, although the trait itself is discrete and requires investigation by more advanced methods such as diallel analysis and quantitative trait loci (QTL) analysis.

Under anoxia, continuous glycolytic flux requires regeneration of NAD from NADH by alcoholic fermentation (Drew, 1997). In fact, an ADH null mutant in maize demonstrated that ADH is required for extended survival under flooding conditions (Schwartz, 1969). In addition, the number of ADH isozymes in seedlings increases under flooding stress in several species (Sachs and Freeling, 1978; Small *et al*., 1993). Kennedy *et al*. (1987*b*) reported that a faster-migrating ADH band appeared under anaerobic conditions in seeds of *E. phyllopogon*, which exhibits anaerobic germinability. Allotetraploid *E. phyllopogon* is a progenitor of allohexaploid *E. crus-galli* (Yabuno, 1966). Despite this, no additional ADH bands were synthesized under anoxia in seeds of *E. crus-galli* (either formosensis or praticola) (Fig. 4). Kennedy *et al*. (1987*b*) investigated the banding patterns of ADH isozymes in seeds of several other *Echinochloa* species with and without anaerobic germinability and concluded that the number of ADH isozymes was not correlated with anaerobic germinability. Fukao *et al*. (1998) produced several ADH mutants of formosensis by γ-ray irradiation. These mutants, with various combinations of ADH bands, were subjected to anaerobic germination tests, but no correlations between ADH banding patterns and anaerobic germinability were observed (unpublished data). These data suggest that the number of ADH isozymes is not related to anaerobic germinability in *E. crus-galli*.

Under anoxic stress, plants shift metabolism from aerobic respiration to anaerobic fermentation. Most of the anaerobic stress proteins (ASPs) identified are enzymes associated with glycolysis and fermentation (Sachs *et al*., 1996; Drew, 1997). Indeed, continuous glycolytic flux and production of NAD by alcoholic fermentation are essential to maintain formation of ATP under anaerobic conditions. Here, the activities of enzymes associated with glycolysis and alcoholic fermentation were assayed during anaerobic germination of formosensis and praticola. Interestingly, an initial and also an intervening enzyme of the pathway, sucrose synthase and aldolase, respectively (hereafter, type A enzymes), exhibited similar patterns in their timing and magnitude of change under both aerobic and anaerobic conditions (Fig. 3). Likewise, the last part of the pathway, PDC, ADH and ALDH (hereafter, type B enzymes) also exhibited similar patterns (Fig. 3). Enolase exhibited an intermediate response between type A and type B enzymes in the degree of induction by anoxic stress and the different pattern on day 1 under aerobic conditions (formosensis, induced; praticola, not induced). In maize seedlings, no common patterns of change in the transcriptional level of glycolytic and fermentative enzymes were observed under hypoxia or anoxia (Andrews *et al*., 1994). On the contrary, in rice seedlings, two types of mRNA accumulation were observed in genes associated with glycolytic and fermentative pathway under submergence stress (Umeda and Uchimiya, 1994). Although in *E. crus-galli* (including formosensis and praticola), induction patterns of the enzymes at the transcriptional level have not been investigated yet, two types of induction patterns were recognized at the enzymatic level. Each type of enzyme (A and B) may be regulated by an identical mechanism such as a common transcription factor and/or signal transduction pathway.

Yamasue and Ueki (1987) pointed out that alcoholic fermentation predominates rather than conventional aerobic respiration in the early stages of aerobic germination in *E. oryzicola* seeds. Additionally, in pea and maize, fermentation was estimated to be a major pathway to regenerate ATP during the 48 h period prior to shoot or root emergence from seeds even under aerobic conditions (Raymond *et al*., 1983). The data presented here for the type B enzymes (Fig. 3) suggest that anaerobic alcoholic fermentation drives the first period of germination even under aerobic conditions and initiation of germination may require the operation of alcoholic fermentation.

In a previous report, a significant difference in enolase activities between flooding tolerant *E. phyllopogon* and intolerant *E. crus-pavonis* under anoxia was reported (Fox *et al*., 1995). However, no significant difference in enolase activity was observed between formosensis and praticola during germination under anaerobic conditions (Fig. 3). This indicates that although both *E. crus-galli* var. *praticola* and *E. crus-pavonis* are flooding-intolerant and do not exhibit anaerobic germinability, enolase activities in the two species are differentially regulated in response to anaerobic stress.

Significant differences in activity were measured in formosensis and praticola for aldolase, PDC and ALDH during anaerobic germination. PDC could act as a regulatory site for anaerobic metabolism because the enzyme is at a branch point leading to either the aerobic TCA cycle or anaerobic lactate and alcoholic fermentation. Quimio *et al*. (2000) reported that submergence tolerance was increased in transgenic rice when the *pdc1* gene was overexpressed by an actin I promoter, although the degree of tolerance was lower than for the tolerant cultivar, FR13A. However, these results for one F_3 family without anaerobic germinability (T64) and three other families with anaerobic germinability (T38, T124 and T179), revealed similar induction patterns (both timing and degree) for PDC activity during anaerobic germination (Fig. 5). Although anaerobic germination and submergence tolerance in seedlings are different physiological traits and their metabolic regulatory mechanisms may not be common, the data suggest that there is no correlation between PDC activity and anaerobic germinability in *E. crus-galli*. In contrast to PDC, ALDH activities were more strongly induced under anoxia in all anoxia-tolerant F_3 families compared to the anoxia-intolerant families (Fig. 5).

This is the same pattern observed for ALDH in tolerant formosensis versus intolerant praticola (Fig. 3). ALDH catalyses the conversion of aldehydes to their corresponding acids and detoxifies acetaldehyde produced by alcoholic fermentation. Kennedy *et al*. (1987*b*) reported that excess ethanol accumulation was found within the seed (42 μmol g^{-1} dry weight) and in the imbibition solution (562 μmol g^{-1}) after 5 d anaerobic germination of *E. oryzicola* and rice. In addition, seeds of anoxia-intolerant rice cultivars IR22 and IR42 produced much more aldehyde (48–88-fold) than those of tolerant cultivars FR13A, IR54, IR36, and Calrose under anoxia (Setter *et al*., 1994). These data imply that ALDH may play a role in the detoxification of acetaldehyde produced through ethanol metabolism during anaerobic germination and the ability to detoxify aldehydes may allow formosensis to germinate under anaerobic conditions.

Anaerobic germinability and flooding tolerance are complex traits and it seems that they are regulated by several genes. Until now, QTL of flooding tolerance has been examined only in rice. Xu and Mackill (1996) reported that a major locus (*Sub1*) for flooding tolerance was located approximately 4 cM from the RFLP marker C1232 on rice chromosome 9. Sripongpangkul *et al*. (2000) identified several QTL, including *Sub1*, that control plant elongation and submergence tolerance in rice. an RFLP linkage map of *E. crus-galli* is now being constructed for praticola and formosensis and QTL are being analysed for anaerobic germination/flooding tolerance. These studies will provide more informative results to help explain these complex physiological traits.

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