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University of Alberta

Characterization of hypoxically inducible lactate dehydrogenase in maize

by

Mary Elizabeth Christopher

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Molecular Biology and Genetics

Department of Biological Science

Edmonton, Alberta

Fall 1997

University of Alberta

Library Release Form

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Title of Thesis: Characterization of hypoxically inducible lactate dehydrogenase in maize

Degree: Doctor of Philosophy

Year this Degree Granted: 1997

12, 9932 – 88 Avenue
Edmonton, Alberta, Canada
T6G 2E9

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University of Alberta

Faculty of Graduate Studies and Research

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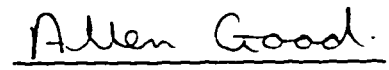

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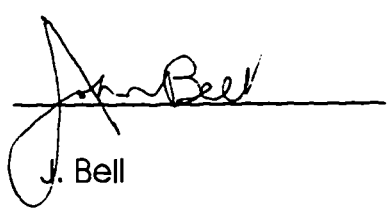

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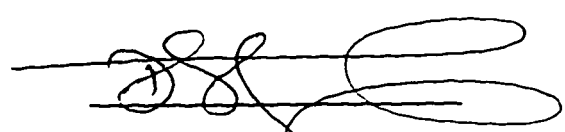

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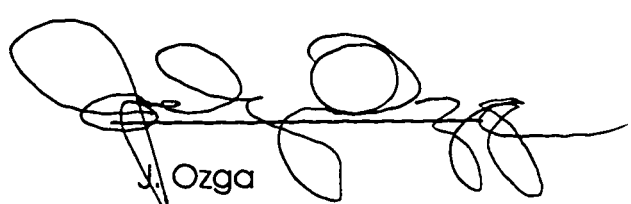

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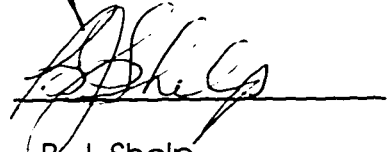

B. J. Shelp

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Abstract

Lactate dehydrogenase (LDH; EC 1.1.1.27) activity increased 3.5 fold in maize roots exposed to six days of hypoxic treatment. The increase in activity during the first 24 hours was concomitant with a rise in LDH protein concentration and increased activity of all five LDH isozymes. LDH protein concentrations decreased after 24 hours of hypoxic treatment, at which time LDH activity was still increasing, suggesting a change in the specific activity of the enzyme. Prolonged hypoxia significantly affected the *in vitro* stability of the enzyme, such that in hypoxic conditions LDH had a half-life 60% of that of the enzyme present in aerobic conditions. Hypoxic transcript profiles indicated that two *ldh* genes were induced, one being expressed at higher levels and with an earlier induction profile than the other.

Concomitant cold, heat or desiccation stresses inhibited the hypoxic response. Salt stress increased *ldh* transcription in both aerobic and hypoxic conditions without mediating an increase in LDH enzyme activity. Treatment with protein synthesis inhibitors demonstrated that hypoxic *ldh* expression occurred in the presence of cytosolic protein synthesis inhibitors (cycloheximide, CHX), but not with organellar protein synthesis inhibitors (chloramphenicol). These results suggest that the transcription factors required for hypoxic induction are present in aerobic

root tissue and that mitochondrial protein synthesis is important in either the detection or the transduction of the hypoxic signal. CHX treatment of aerobic plants resulted in a dramatic increase in *ldh* expression and ethanol treatment increased both aerobic and hypoxic *ldh* expression. Studies with calcium and Ruthenium Red also demonstrated roles for organellar and extracellular calcium in the *ldh* signal transduction pathway. Together these results demonstrated that maize *ldh* was regulated at the transcriptional, post-transcriptional or translational and post-translational levels. A comparitive analysis was performed with alanine aminotransferase (*alaat*), a hypoxically induced gene involved with amino acid metabolism.

An 8.2 kilobase region of the maize genome, spanning a region with homology to maize *ldh1* was sequenced in an attempt to isolate the second *ldh* gene. Although this region contained a *ldh* pseudogene rather than a functional gene, it yielded information regarding maize genome organization.

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2,4-D	2,4-dichlorophenoxyacetic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ADC	Arginine decarboxylase
ADH	Alcohol dehydrogenase
AdoMet	S-adenosyl-L-methionine
ADP	Adenosine diphosphate
AEC	Adenylate energy charge
AlaAT	Alanine aminotransferase
AMP	Adenosine monophosphate
ANP	Anaerobic polypeptide
AOX	Alternative oxidase
ARE	Anaerobic response element
ARF-B2	ARE binding factor – B2
ATP	Adenosine triphosphate
B73	Maize inbred line
BMS	Black Mexican Sweet maize cell culture line
bp	Base pair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus 35S promoter
CAP	Chloramphenicol
CFU	Colony forming unit
CHX	Cycloheximide
Ci	Curie
cpm	Counts per minute
Crusader	Maize inbred line
CTAB	Hexadecyltri-methylammonium bromide
cv.	Cultivar
d	Day
DR	Direct repeat
DTT	Dithiothreitol
E	Einstein
EC	Enzyme Commission of the International Union of Biochemistry
EDTA	Ethylene diamine tetraacetic acid
EF-1α	Translation elongation factor 1α
EGTA	Ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetra-acetic acid

Eno	Enolase
FK	Fructokinase
FPLC	Fast performance liquid chromatography
FW	Fresh weight
g	Gram
GABA	4-aminobutyrate
GABA-T	GABA transaminase
gapC	Gene encoding GAPDH
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBF1	G-box binding factor 1
G-box	CCACGTGG
GC motif	GCCC / GCCGCG / CCGCG
GCBP-1	GC-binding protein
GDC	Glutamate decarboxylase
GDH	Glutamate dehydrogenase
GDP	Guanosine diphosphate
Gene 1005	Xyloglucan endotransglycosylase homolog
Gene 1032	Maize ANP of unknown function
GF14ϖ	Arabidopsis thaliana G-box binding protein
GK	Glucokinase
GT motif	GGTTT
GTP	Guanosine triphosphate
GUS	β-glucuronidase
h	Hour
H^+-PPase	H^+-translocating pyrophosphatase
i.e.	For example
I.U.	International unit
IP_3	Inositol 1,4,5-triphosphate
kb	Kilobase
kD	Kilodalton
KLH	Keyhole limpet hemocyanin
kPa	KiloPascals
L	Litre
LDH	Lactate dehydrogenase
LP-like element	Retrotransposon-like element containing sequences with homology to ldh and pdc
LTR	Long terminal repeat

m	Meter
M	Molar
min	Minute
MOPS	3-(N-morpholino)propanesulphonic acid
M_r	Molecular weight
mRNA	Messenger RNA
MS	Murashige and Skoog
MTA	5'-methylthioadenosine
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NDPK	Nucleoside diphosphate kinase
NiR	Nitrite reductase
NMR	Nuclear magnetic resonance
NR	Nitrate reductase
NTP	Nucleotide triphosphate
ORF	Open reading frame
P3377	Maize cell suspension culture line
Pa	Pascal
PAGE	Polyacrylamide electrophoresis
pAtC4B/E1.8	Plasmid containing *Arabidopsis thaliana* actin coding region
pBI221	Plasmid containing CaMV 35S promoter-GUS reporter gene-NOS terminator
PBS	Primer binding site
pCG22	Plasmid containing *Arabidopsis thaliana* cyclophilin coding region
PDC	Pyruvate decarboxylase
PEG	Polyethylene glycol
PFK	Phosphofructokinase
PG1	Glucose-6-phosphate isomerase
pH_c	Cytoplasmic pH
pH_e	External pH
Pi	Inorganic phosphate
PK	Pyruvate kinase
pIdh1Sal1.0	Plasmid containing maize Idh1 coding region
pIdh1XSB	Plasmid containing maize Idh1 3' UTR
PMSF	Phenyl-methyl-sulfonyl fluoride
PPi	Inorganic pyrophosphate
PPT	Polypurine tract
PVP	Polyvinylpyrrolidone

rpm	Revolutions per minute
rpt	Repeat
RR	Ruthenium red
rRNA	Ribosomal RNA
RWC	Relative water content
s	Second
SDS	Sodium dodecyl sulphate
sh1	Gene encoding SS1
SM	Sodium chloride, magnesium sulfate buffer
sp.	Species
SS	Sucrose synthase
SSA	Succinic semialdehyde
SSA-D	SSA dehydrogenase
SSC	Socium chloride, sodium citrate
SSPE	Sodium chloride, sodium phosphate
$T_{1/2}$	Half-life
TCA	Trichloroacetic acid
TE	Tris-EDTA buffer
TGA1	G-box-like binding factor
tRNA	Transfer RNA
U	Units of enzyme activity
UTR	Untranslated region
v/v	Volume per volume
V-ATPase	Vacuolar H^+-translocating ATPase
V-PPase	Vacuolar H^+-translocating pyrophosphatase
w/v	Weight per volume
W22	Maize inbred line

1. Introduction

1.1 *The hypoxic root environment*

Non-waterlogged soils possess a large number of air filled pores which facilitate diffusion of oxygen through the soil into the root. This inward radial diffusion of oxygen through the root surface is the dominant mechanism of oxygen entry into roots in well-drained soils or in aerated culture medium (Armstrong et al., 1994). As these pores become water filled oxygen diffusion to the roots decreases in comparison to that of well drained soils (Laanbroek, 1990). Root oxygen consumption together with soil microorganism oxygen consumption consumes soil oxygen within one day (d) of flooding (Ernst, 1990). Oxygen concentrations in well-drained soils are similar to those of ambient air (21% (v/v)) decreasing to 1.9% (v/v) after two days of waterlogging and remaining at that level for 15 d (Drew and Sisworo, 1979). Oxygen distribution in waterlogged soil is not uniform. Waterlogging produces a steep oxygen gradient, with only the first few millimeters of soil being oxidized (Laanbroek, 1990).

In waterlogged soils, microbial activity is determined primarily by the availability of electron acceptors for the oxidation of both organic and inorganic compounds. Reduction processes by soil microorganisms occur according to a fixed sequence: oxygen (O_2), nitrate (NO_3^-), manganese (IV) oxide (MnO_2), ferric oxide ($Fe(OH)_3$), sulphate (SO_4^{2-}) and carbon dioxide (CO_2) to produce water, ammonium (NH_4^+), N_2O, N_2, Mn^{2+}, Fe^{2+}, H_2S, S^-, S^0 and methane (CH_4) (Table 1-1) (Ernst, 1990; Laanbroek, 1990). Elevated concentrations of Mn^{2+}, Fe^{2+} and H_2S are toxic to various cellular enzymes (Drew, 1990; Ernst, 1990). Additionally, some organic acids produced by anaerobic microorganisms affect membrane integrity (Drew, 1990).

Table 1-1: Sequence of reduction processes in waterlogged soil

Event	Substrates	Products
Disappearance of oxygen	$O_2 + 2\ H_2$	$2\ H_2O$
Disappearance of nitrate	$2\ NO_3^- + 5\ H_2 + 2\ H^+$	$N_2 + 6\ H_2O$
	$NO_3^- + 4\ H_2 + 2\ H^+$	$NH_4^+ + 3\ H_2O$
Appearance of manganous ions	$MnO_2 + H_2 + 4\ H^+$	$Mn^{2+} + 2\ H_2O$
Appearance of ferrous ions	$2\ Fe(OH)_3 + H_2 + 4\ H^+$	$2\ Fe^{2+} + 6\ H_2O$
Disappearance of sulphate	$(SO_4)^{2-} + 4\ H_2$	$S^{2-} + 4\ H_2O$
Appearance of methane	$CO_2 + 4\ H_2$	$CH_4 + 2\ H_2O$

Adapted from Laanbroek, 1990

Nitrate absorption decreases during hypoxia (Drew and Sisworo, 1979; Konings and Verschuren, 1980; Ernst, 1990; Laanbroek, 1990), as does uptake of phosphate and potassium (Drew and Sisworo, 1979; Morard and Silvestre, 1996). With prolonged hypoxia, Ca^{2+} and Mg^{2+} uptake also decreases (Morard and Silvestre, 1996). Some of the effects of waterlogging on barley, a relatively flood sensitive plant, can be alleviated by calcium nitrate treatment (Drew et al., 1979). This suggests that some of the adverse effects of waterlogging are secondary to nutrient deprivation.

Roots of grasses possess a rhizosheath, an aggregation of soil particles, rhizobacteria and mucilage (released from both the root cap and rhizobacteria). This is located over root regions containing living root hairs and epidermal cells and is absent from the elongation region of the root tip and region of root hair development (McCully, 1995). Plants which develop aerenchyma (gas filled spaces in the root) either constitutively or inducibly (Section 1.3.1) can have an effective radial oxygen loss, thus oxygenating the rhizosphere and reducing the toxic effects of soil anoxia (Kramer, 1951; Ernst, 1990; Laanbroek, 1990). In flooded soils, apical oxygen concentrations eventually decline to a point where either growth can no longer be sustained or where rhizosphere protection against soil toxins is no longer possible. Together these determine the rooting depth of the flooded plant (Justin and Armstrong, 1987).

Maize, the object of study of this thesis, is moderately waterlogging tolerant. It can grow in wet soils that are saturated with water for most of the year, in damp soils that are occasionally wet and in soils that are normally moist (i.e. typical field soils) (Justin and Armstrong, 1987). Although maize can grow in waterlogged soils, growth in these soils renders plants more susceptible to *Pythium arrhenomanes*, the causative agent of root rot and a common pathogen of wet soils (Yanar et al., 1997).

1.2 *Hypoxia mediated effects*

1.2.1 Structural and anatomical

The adverse effects of flooding or waterlogging are visible as (a) chlorosis, (b) epinastic curvature and change in the angle of the petiole, (c) formation of callus tissue along the stem but primarily at the water line, (d) formation of adventitious roots, (e) hypertrophy of the stem at the water line, and (f) death of the primary root (Kramer, 1951). Chlorosis results from dehydration due to a reduced capacity of waterlogged roots

to absorb and conduct water (Kramer, 1951). The other effects are presumably due to disturbances in either the translocation of carbohydrates, or hormone status (Kramer, 1951).

1.2.2 Cellular

Hypoxia (reduced oxidative phosphorylation) and anoxia (absence of oxidative phosphorylation) differentially affect mitochondrial integrity. In hypoxic cells, mitochondria are maintained within the cell until the protoplasts disintegrate. In contrast, mitochondria in anoxic cells develop irregular shapes and dilated cristae prior to their disintegration within 24 to 48 hours (h) (Campbell and Drew, 1983; Couée et al., 1992). Mitochondria isolated from anoxic rice have decreased synthesis of mitochondrial proteins, although *in organello* studies demonstrate that most polypeptides synthesized by aerobic mitochondria can be produced if sufficient ATP is present. Anoxic rice mitochondria can carry out protein synthesis without a functional respiratory chain, a feature not observed in aerobic mitochondria (Couée et al., 1992).

Hypoxia also alters the ultrastructure of endoplasmic reticulum, Golgi and chromatin, and increases the length of the cell cycle (Drew, 1990).

1.2.3 Cytoplasmic pH

Regulation of cytoplasmic pH (pH_c) is important in co-ordinating the activities of diverse enzyme catalyzed pathways, membrane transport and regulatory agents (i.e. Ca^{2+}, inositol 1,4,5-triphosphate (IP_3)) in a manner appropriate to fulfill the required task (Felle, 1989). Protons may act as intracellular messengers by modulating pH_c, cell wall acidification, intramolecular or local proton shifts within a membrane or pH shifts localized to certain cytoplasmic regions (Felle, 1989). Two models of pH_c regulation have been proposed. The biophysical pH stat model proposes that ATPases pump H^+ ions out of the cytosol into the extracellular medium. The metabolic pH stat model proposes that lower pH values activate enzymes that catalyze proton-consuming decarboxylation reactions (Snedden et al., 1992).

Studies of pH_c have utilized a variety of methods, including microelectrodes, ^{31}P-NMR (nuclear magnetic resonance) and pH sensitive dyes. Studies of pH_c in maize have used both excised root tips and intact roots. Results differed slightly, depending on the root source and the method, but the common feature of all studies on maize is that pH_c decreases within the first few minutes (min) of anoxia (Roberts et al.,

1984a and b; Saint-Ges et al., 1991; Xia and Roberts, 1996). This is associated with an increase in NADH, indicating a rapid inhibition of oxidative phosphorylation (Roberts et al., 1984b). Later studies confirmed the rapid decrease in pH_c, from 7.5 to 6.9 (Saint-Ges et al., 1991; Xia and Roberts, 1996) but, in contrast to earlier studies, demonstrated a gradual increase in pH_c following the initial decline. A steady state pH of 7.1 to 7.2 was reached after 25 min of anoxia (Saint-Ges et al., 1991). These results were corroborated by Roberts et al., 1992. The discrepancy between the earlier and later experimental results was attributed to a more rapid transition from aerobic to anoxic conditions in the later experiments, resulting in greater metabolic synchrony within the root tips.

In maize *adh1*$^+$ (alcohol dehydrogenase) lines exposed to hypoxia pH_c decreases from 7.3 to 6.8 during the first h gradually decreasing to pH 6.5 after 30 h. Maize *adh1*$^-$ lines have a greater pH_c decrease, to 6.6 within the first 2.5 h of hypoxia, gradually decreasing to 6.2 after 22 h (Roberts et al., 1984a).

Rice, a hypoxia tolerant plant, also undergoes cytoplasmic acidification upon exposure to anoxia (Menegus et al., 1991). During the first 10 h of anoxia, pH_c decreases from 7.4 to 7.0, remaining stable for ~4 h, followed by cytoplasmic alkalization. During this time, although metabolic rate, glucose–6-phosphate concentration and ethanolic fermentation increase, lactate production is very limited. This differs from wheat and maize, which produce significantly more lactate (Menegus et al., 1991). It is suggested that lactate is the major contributor to pH_c acidification (Davies et al., 1974; Vayda et al., 1995), although some studies show that the time course of the decrease in pH_c does not strictly correlate with that of lactate accumulation (Saint-Ges et al., 1991). The production of lactate early in the hypoxic response is the basis for the pH stat hypothesis (Davies et al., 1974). This hypothesis proposes that lactate dehydrogenase (LDH), which has an alkaline pH optimum, is active at the onset of anoxia. Lactate accumulation decreases the pH_c to inhibit LDH and activate pyruvate decarboxylase (PDC), which has an acidic pH optimum. This results in competition between LDH and PDC for pyruvate (Davies et al., 1974).

Analysis of metabolites, by NMR, during anoxia demonstrates that pH_c regulation is triphasic. In the first 15 min of anoxia (phase 1), the 0.5 to 0.6 pH unit drop is ascribed to the production of lactate and alanine. In the second phase (10 to 75 min), pH_c increases 0.2 pH units as malate is decarboxylated to alanine and anaerobic glycolysis yields lactate and ethanol. The production of ethanol permits rapid cessation of cytoplasmic acidosis caused by fermentation of glucose to lactate and alanine. In the third phase (30 to 360 min) pH_c remains stable as 4-aminobutyrate (GABA) synthesis from glucose consumed protons (Menegus et al., 1989; Roberts et al., 1992; Crawford et al., 1994).

Studies on mung bean (*Vigna radiata*) suspension cultures demonstrate a correlation between chilling sensitivity and decreases in pH_c. Cells in the early stage of exponential growth (actively growing cells) are more sensitive to chilling stress than cells in the late stages of exponential growth (Yoshida, 1994). This could resolve the observation that maize root tips are the most sensitive to anoxic stress, dying before the remainder of the root (Roberts et al., 1984 a and b), since cells in the root tip are the most metabolically active and are growing.

Death of maize root tips is correlated with decreases in pH_c and acidification of the external solution (pH_e) (Roberts et al., 1984a; Xia and Roberts, 1996). Studies on sycamore (*Acer pseudoplatanus*) cell suspensions demonstrate that when pH_e declines below 6.5, respiratory CO_2 released into the media remains as a gas and therefore does not affect pH_e (Gout et al., 1992). This study also demonstrates that pH_c is independent of pH_e when pH_e is between 4.5 and 7.5. In contrast, pH_e affects the pH_c of the green algae *Chlorella fusca* and *C. vulgaris* when cells are maintained anaerobically but not when cells are maintained aerobically (Küsel et al., 1990). The pH of most flooded soils is between 6.7 to 7.2 regardless of the pH of the well drained soil, with soil organic content having a critical effect on pH_e (Laanbroek, 1990).

The studies on sycamore suspension cultures also demonstrates that pH_c is poorly regulated when NTP concentrations are low. This suggests that plasma membrane ATPases can have an important role in the control of pH_c by maintaining the required pH gradient across the plasma membrane (Gout et al., 1992). Recently it was observed that plasma membrane H^+-ATPases operate under anoxia in intact maize roots (Xia and Roberts, 1996). However, comparisons of pH_c regulation in root tips hypoxically-pretreated with those that had not been pretreated, demonstrated that mechanisms other than plasma membrane H^+ transport are involved in maintaining the pH gradient across the plasma membrane (Xia et al., 1995; Xia and Roberts, 1996).

In rice, the vacuolar H^+-translocating pyrophosphatase (V-PPase) is induced during anoxia (Carystinos et al., 1995). V-PPase is one of the vacuolar proton pumps and its activity during times of energetic stress may serve to conserve ATP by switching to a PPi-based metabolism. In maize, exposure of hypoxically-pretreated roots to anoxia results in a decrease in V-PPase protein and enzyme activity in the root tips but not in the remainder of the root. Its activity during anoxia could function to maintain the pH differential between the cytosol and the vacuole. The resulting accumulation of the products of anoxic metabolism (alanine and putrescine) in the vacuole could preserve nutrients, coincidentally preventing their possible cytotoxic effects (Carystinos et al., 1995). Experimental evidence suggests that H^+-PPases maintain vacuolar acidity

under cellular conditions that favour accumulation of ADP (Brauer et al., 1997), such as occurs during prolonged hypoxia.

1.2.4 Molecular

Anoxia decreases ATP concentrations within the first 2 min. This decrease is sufficient to bring aminoacyl tRNA synthetase reactions almost to equilibrium thus slowing the overall rate of protein synthesis. Within 15 min, dissociation of polyribosomes to monosomes occurs (Bailey-Serres and Freeling, 1990). Studies on potatoes demonstrate that translation elongation factor EF-1α becomes stably associated with polysomes at the onset of hypoxia (Vayda et al., 1995). EF-1α is involved in the presentation of aminoacyl tRNAs to the A site of the ribosome. Its association with ribosomes is usually transient as cleavage of ribosome bound GTP to GDP and Pi by a ribosome associated hydrolase results in EF-1α release. As a result, EF-1α is not usually associated with polysomes. When the pH of aerobic tuber extracts decreases from 6.8 to 6.0 or lower, EF-1α becomes associated with polysomes. This suggests that ribosomal release of EF-1α is inhibited by a lower pH_c, possibly by eliciting conformational changes either in EF-1α or the ribosomal proteins with which it interacts (Vayda et al., 1995).

Monitoring the incorporation of ^{3}H-Leucine into protein by two-dimensional polyacrylamide electrophoresis (PAGE) determined that progressive changes in protein synthesis occur during anoxia (Sachs et al., 1980). In maize, within the first h of strict anoxia, there is a decrease in the synthesis of aerobic proteins and a synthesis of a class of proteins of ~33 kilodaltons (kD) termed the transition peptides. The synthesis of the transition peptides decreases within 3 to 5 h of anoxia being replaced by ~20 anaerobic polypeptides (ANPs), whose synthesis is detected within 1.5 h of anoxia. ANPs remain stable for 5 to 70 h, depending on the particular ANP (Sachs et al., 1980). Soybean, pea and cotton, all of which are flood intolerant, synthesize fewer ANPs than maize (Millar and Dennis, 1996a).

In vitro translation studies demonstrate that mRNAs encoding aerobic proteins are present within the cell during the first 5 h of anoxia. This presumably allows rapid synthesis of aerobic proteins should the O_2 concentration return to normal (Sachs et al., 1980). Many of the genes induced by anoxia have an increased transcription rate as well as an increase in transcript stability (Drew, 1990).

1.3 *Strategies for survival*

There are three main theories regarding resistance to oxygen deficit: (1) oxygen transport theory, (2) ion toxicity theory, and (3) metabolic adaptation theory (reviewed in Ernst, 1990). Briefly, the oxygen transport theory suggests that flood resistant plants avoid anoxia by supplying oxygen to the roots from the shoots via aerenchyma formation with subsequent radial oxygen loss through the roots to oxidize the root environment (Armstrong, 1967). The ion toxicity theory suggests that the toxicity of high concentrations of iron, manganese and sulphide are diminished in resistant plants (Jones and Etherington, 1970). The metabolic adaptation theory suggests that the production of organic acids and the avoidance of ethanol accumulation are important for flood tolerance (Crawford, 1966).

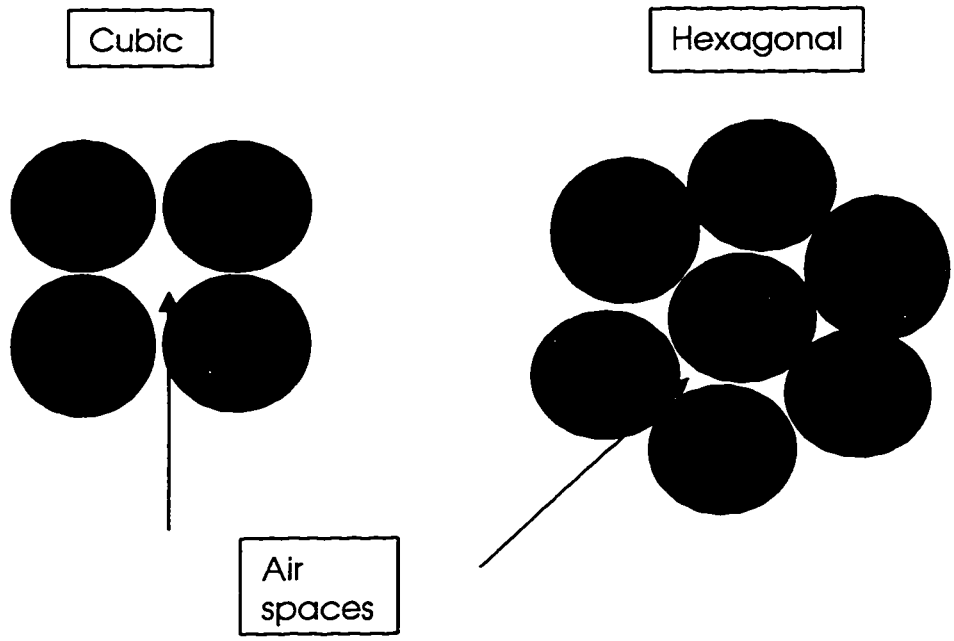
1.3.1 Strategies to increase oxygen supply to the roots

The most notable feature of roots of flood tolerant plants is the presence of aerenchyma. Aerenchyma are gas filled spaces, or lacunae, in the root. Aerenchyma are formed either schizogenously or lysigenously. Schizogenous aerenchyma are produced through differential division and separation of cells in the root cortex, as occurs in *Rumex* sp. (Justin and Armstrong, 1987). Lysigenous aerenchyma are produced through lysis of cortical cells, as occurs in maize (Campbell and Drew, 1983) and rice (Jackson, 1990). The net result of aerenchyma formation is the diffusion of oxygen from aerial parts of the plants to the roots resulting in a relatively aerobic cortex and a relatively hypoxic stele (Campbell and Drew, 1983; Jackson, 1990; Armstrong et al., 1994). Experiments using microelectrodes demonstrate that the difference in oxygen concentration between the cortex and the stele was more substantial toward the base of the root than nearer the apex (Armstrong et al., 1994).

The root cortex contains cells arranged in two major configurations, cubic or hexagonal (Figure 1-1). Cells arranged hexagonally are common in the medullas of most plants, in the outer cortex of some aerenchymatous plants and in the cortex of most non-aerenchymatous plants. Hexagonal arrangement is associated with mechanical strength whereas cubic arrangement is more porous and allows oxygen transport. Cubic arrangement is the predominant cortical configuration in wetland and flood tolerant plants that develop aerenchyma. Cubic cellular arrangement minimizes cell wall contact between adjacent cells leading to easier cell separation. In plants containing cells in both arrangements

(i.e. maize), aerenchyma formation originates in cells located within the cubic zone (Justin and Armstrong, 1987).

Figure 1-1: Cortical cell configurations

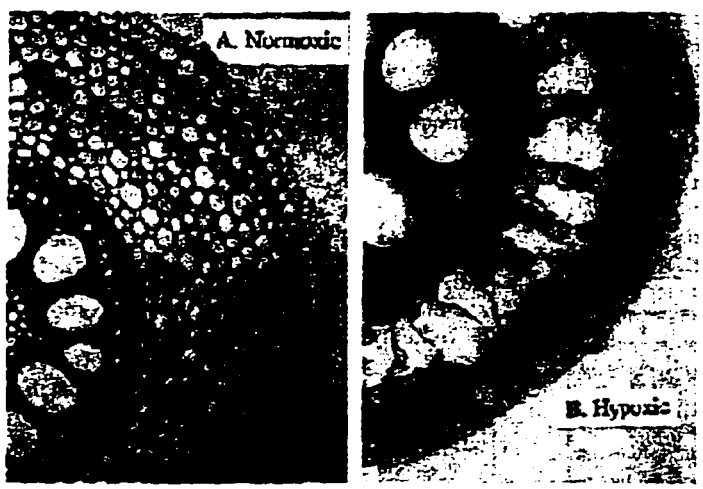


Adapted from Justin and Armstrong, 1987

In maize, aerenchyma formation occurs through lysis of cells in the midcortex of adventitious roots (Campbell and Drew, 1983; Drew et al., 1994). Aerenchyma development occurs about 5 to 15 mm from the root tip. Initially, there is a loss of vacuolar solutes and cell turgor in a contiguous group of cells. The cell walls collapse inwardly (because of damage to the plasma membrane), leaving a volume of ion-poor liquid behind. The cells contain few recognizable organelles at this point. The ion-poor liquid is gradually lost, leaving an air-filled cavity surrounded by a membrane derived from the tonoplast and plasma membranes, and a cell wall derived from the collapsed walls of the affected cells (Campbell

and Drew, 1983; Drew et al., 1994; van der Weele et al., 1996). The resulting cortex contains a spoke-like radial file of cells which remain alive (although possessing fewer than normal organelles) separated by large, gas-filled spaces (Figure 1-2) (Campbell and Drew, 1983).

Figure 1-2: Maize root tip sections from aerobic and hypoxic roots



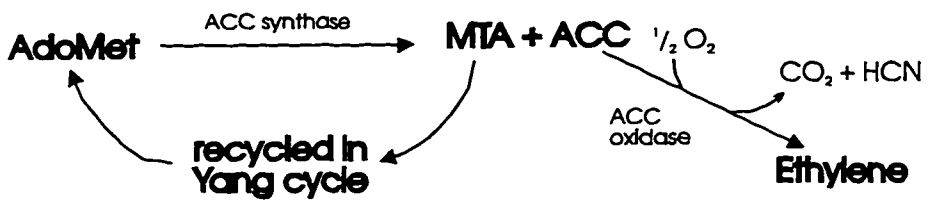
Transverse sections of maize nodal roots following 96 hours of (A) aerobic or (B) hypoxic treatment. Sections were stained with toluidine blue and are magnified X56. The hypoxic root section demonstrates aerenchyma formation. Cells in the midcortex have disappeared being replaced with air-filled spaces with the persistence of some radial files of intact cells.
Adapted from He et al., 1996

Aerenchyma formation impairs nutrient uptake. Some mature aerenchymatous spaces contain liquid, probably exuded from the remaining living cortical cells during time of high root turgor pressure. Dye tracer experiments indicate that the symplastic pathway (transport through the cytosol) is important for ion movement across the cortex into this fluid in the aerenchymatous space. Ions in the aerenchymatous fluid rapidly diffuse to other parts of the plant (van der Weele et al., 1996).

Aerenchyma production is stimulated by ethylene (Drew et al., 1994). Ethylene biosynthesis requires molecular oxygen to convert 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Figure 1-3)

(reviewed in Theologis, 1992). ACC is produced in anoxic root tip cells and is converted to ethylene in the adjacent subapical zone (Drew et al., 1994). Hypoxia increases the activity of several enzymes involved in ethylene biosynthesis including ACC synthase (Wang and Arteca, 1992; He et al., 1994; Scott et al., 1996) and ACC oxidase (Scott et al., 1996). Ethylene is speculated to be the signaling molecule which triggers the induction of several cell wall degrading enzymes including cellulase (He et al., 1994; Scott et al., 1996) and a xyloglucan endo-transglycosylase homologue (gene 1005) (Saab and Sachs, 1996). Aminooxyacetic acid stimulates ACC synthase activity in both aerobic and hypoxic tomato plants (Wang and Arteca, 1992). Because aminooxyacetic acid can cross the mitochondrial membrane, and hypoxia affects mitochondrial integrity, it is speculated that hypoxia and aminooxyacetic acid induce ACC synthase by the same mechanism (Wang and Arteca, 1992).

Figure 1-3: Final steps of the ethylene biosynthetic pathway



Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid, AdoMet = S-adenosyl methionine, MTA = 5'-methylthioadenosine.
Adapted from Theologis, 1992

In addition to hypoxia, aerenchyma in maize is also induced in response to a number of other environmental conditions including nitrate (Konings and Verschuren, 1980) or phosphate deficiency (Drew et al., 1994), desiccation (Campbell and Drew, 1983) or mechanical

impedance (Scott et al., 1996). Hypoxia increases ethylene biosynthesis and entrapment within the roots, and nitrate or phosphate deficiencies increase the sensitivity of the cells to ethylene (Drew et al., 1994). In rice however, aerenchyma formation is constitutive and is not subject to close regulation by environmental conditions (Jackson, 1990).

The process of aerenchyma development is similar to apoptosis (He et al., 1996). Apoptosis occurs in plants in several situations in response to environmental or developmental signals including the maturation and death of xylem cells (Mittler and Lam, 1995). It is a gene-regulated process in which the dying cell actively participates and involves DNA fragmentation stimulated by Ca^{2+} activation of endonucleases (reviewed in: Vaux, 1993; He et al., 1996). Ca^{2+} has a role in aerenchyma formation as experiments using Ca^{2+}-calmodulin inhibitors and EGTA inhibit aerenchyma formation. Chemicals which increase cellular Ca^{2+} concentrations increase aerenchyma formation during hypoxia as well as stimulating its production in aerobic conditions (Jackson, 1994; He et al., 1996). Aerenchyma development has been experimentally shown to involve G-proteins, phosphoinositides and protein phosphorylation / dephosphorylation (He et al., 1996) although the signal transduction pathway has yet to be determined.

Other features of flood-tolerant plants that increase the supply of oxygen to the roots include petiole elongation and negative gravitropism. Petiole elongation results in enhanced shoot growth below the water surface (depth accommodation response) thus restoring leaf-air contact. This feature is observed in a wide variety of amphibious plants including *Racunculus sceleratus, Oryza sativa, Rumex palustris* (Jackson, 1990; Banga et al., 1996). These effects are mediated by accelerated cell division, cell wall elongation and extensibility in the growing internode, increased photosynthesis in the foliage above the water surface and preferential transport of the photosynthate from the leaves to the growing internode (Jackson, 1990). Negative gravitropism which induces roots to grow upwards occurs in *Myrica gale, Viminaria juncea, Melaleuca quinquenervia* and sunflower (Jackson, 1990).

Shallow root systems (*Zostera marina*) which maintain roots in the narrow area of oxidized soil are important for some wetland species (Justin and Armstrong, 1987; Drew, 1990). Some wetland plants (*Rumex palustris* and *Rumex thyrsiflorus*) exhibit a large increase in the number of auxin induced aerenchymatous adventitious roots upon flooding (Visser et al., 1995; Visser et al., 1996).

1.3.2 Strategies to reduce root oxygen consumption

Strategies to reduce oxygen consumption are limited to root mortality in the deeper soil layers (Ernst, 1990) or conservation of oxygen within the roots. Strategies to conserve root oxygen supply include a high root radius (*Aster* sp., *Rumex* sp.) or a thickened hypodermis (*Carex* sp., *Oryza* sp.) (Justin and Armstrong, 1987).

1.3.3 Modification of carbon flow

Complete submergence of a plant during flooding results in a wide variety of metabolic changes resulting from restricted access of the shoot to CO_2 and O_2. Because terrestrial plants are limited to CO_2 as their sole carbon source, the major effect of flooding is, therefore, the inhibition of photosynthesis. This results in the roots having an inadequate carbohydrate supply and consequently lacking energy for normal cellular metabolism. Submerged macrophytes, however, can maintain their carbohydrate supply by using bicarbonate (Ernst, 1990).

In aerobic conditions, root respiration involves the catabolism of glucose to pyruvic acid via glycolysis (Figure 1-4). Pyruvic acid is then oxidized in the Krebs cycle to CO_2, with the electrons released being transferred to O_2 by the electron transport chain. This process results in 36 moles of ATP being produced per mole of glucose consumed. The net effect is described in Equation 1-1 (Salisbury and Ross, 1985).

Equation 1-1: Summary of aerobic glycolysis

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O + 688\ kcal$$

Root respiration rates vary widely between plants, so that under non-limiting oxygen conditions, roots of some plants (oilseed rape, corn) require more oxygen than others (wheat, cucumber, tomato, violet, strawberry) (Morard and Silvestre, 1996). Root weight, temperature, transfer of photosynthate from aerial parts (diurnal and meteorological) and CO_2 concentration also affect root respiration rates (Morard and Silvestre, 1996).

Changes in the rate of ATP-generating processes (mitochondrial electron transport) are usually accommodated by adjustments in ATP-consuming processes (growth). This maintains a relatively constant energy state within the cell over a wide range of metabolic conditions (Plaxton, 1992). The adenylate energy charge (AEC, Equation 1-2) is used to define the energy status of the cell.

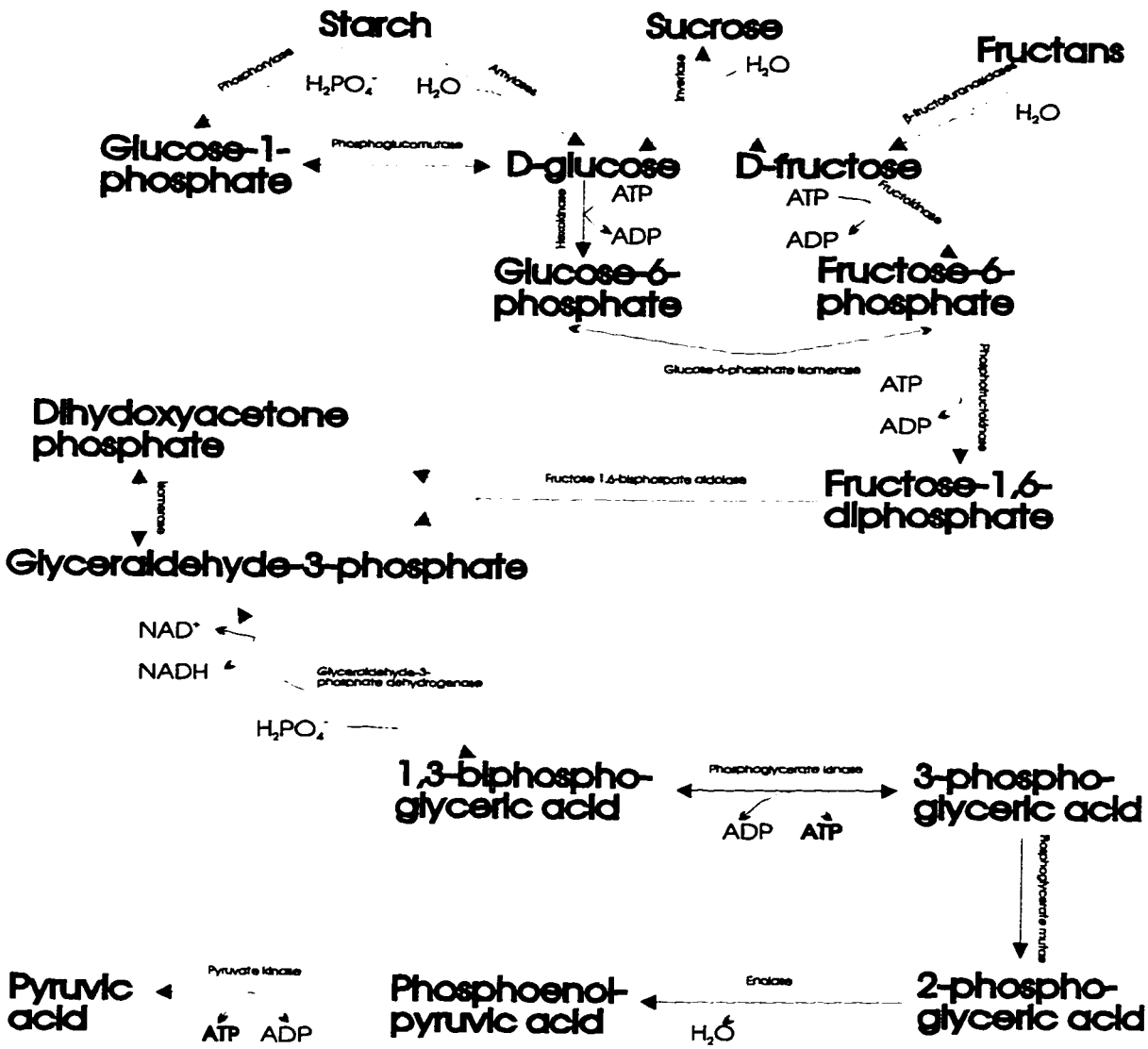
Equation 1-2: Adenylate energy charge

$$AEC = \frac{(ATP) + \frac{1}{2}(ADP)}{(ATP) + (ADP) + (AMP)}$$

Adenylate energy charge is usually maintained between 0.7 and 0.9. Decreases in adenylate energy charge are typically prevented by an increased metabolic flux through ATP-regenerating pathways and a decreased flux through ATP-consuming pathways (Plaxton, 1992). During anoxic treatment the adenylate energy charge progressively decreases to ~0.6 in both hypoxically pretreated and non-hypoxically pretreated maize root tips. However hypoxically pretreated root tips have significantly more ATP and adenine nucleotides relative to non-hypoxically pretreated root tips (Johnson et al., 1989).

Root respiration includes both the cytochrome and the alternative oxidase (AOX) pathway. Electron transfer through the cytochrome pathway is coupled at three sites to the generation of an electrochemical gradient and ATP production. AOX electron transfer has a single site therefore significantly decreasing ATP yield (Vanlerberghe et al., 1997). Cytochrome oxidase has a very high affinity for O_2; therefore in isolated mitochondria a very low concentrations of O_2 is required to drive respiration. At the tissue level, the limiting concentration of O_2 for respiration (critical oxygen pressure) is much greater than in isolated mitochondria. In maize (at 25°C), critical oxygen pressure is 30 and 10 kPa in root tips and the older mature zones, respectively (Drew, 1990).

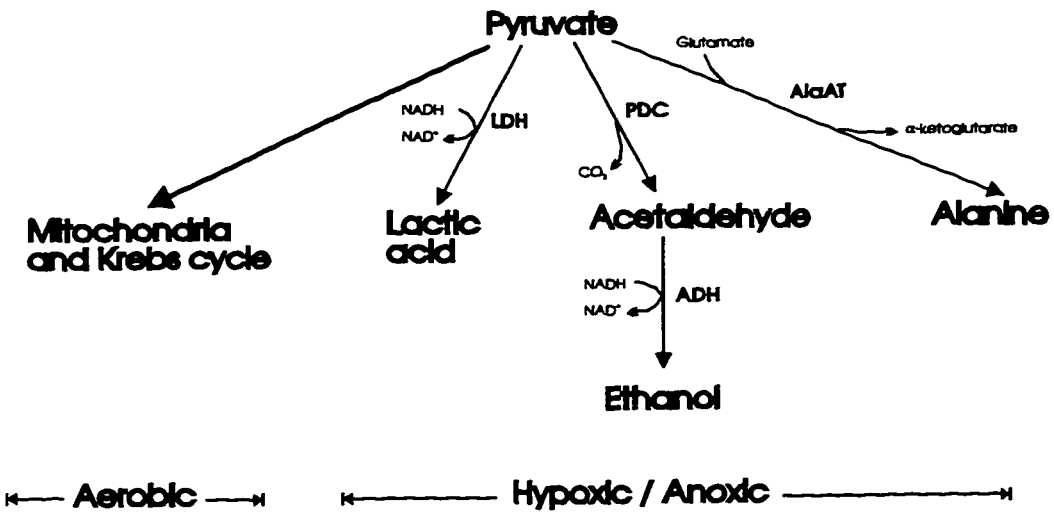
Figure 1-4: The glycolytic pathway



---- Degradative reactions not usually considered to be part of glycolysis

From Salisbury and Ross, 1985

Figure 1-5: Fates of pyruvate



Abbreviations: ADH = alcohol dehydrogenase, AlaAT = alanine aminotransferase, LDH = lactate dehydrogenase, PDC = pyruvate decarboxylase.
From Salisbury and Ross, 1985

Pyruvate, a strong activator of AOX (Vanlerberghe et al., 1995), accumulates when there is an imbalance between respiratory carbon metabolism and electron transport (Figure 1-5). Elevated pyruvate concentrations result in fermentation, even in aerobic conditions (Vanlerberghe et al., 1997). Antimycin A inhibits cytochrome C reductase and therefore the cytochrome pathway. Plants exposed to antimycin A must have high AOX activity in order to survive. Wild type or transgenic tobacco plants expressing high levels of AOX have no adverse morphological effects or ethanol production following 24 h of antimycin A treatment. In contrast, transgenics with decreased AOX activity (*aox* antisense or co-suppressed) have visible injury to the leaves as well as an increase in ethanol production. These results imply that activation of AOX by upstream respiratory metabolism can prevent aerobic fermentation when there is an imbalance between respiratory carbon metabolism and the capacity of mitochondrial electron transport (Vanlerberghe et al., 1995).

Plants modify their carbon flow in response to flooding by increasing their glycolytic rate (Pasteur effect). Sensitive plants have increased ethanol production whereas tolerant plants produce products such as malate and fructosan in addition to ethanol (Heimovaara-Dijkstra et al., 1994). Increases in cellular concentrations of malate raise pH_c

(Heimovaara-Dijkstra et al., 1994), thus minimizing the adverse effects of hypoxia on pH_c. However, the time course of increases in malate and citrate differ from the increase in pH_c. Some flood tolerant plants are also able to generate reducing power for glycolysis by using nitrate as a final electron acceptor instead of O_2 (Vanlerberghe and Turpin, 1990; Morard and Silvestre, 1996).

Ethanolic fermentation results in a significantly decreased ATP production per mole of glucose consumed, 2 moles of ATP versus 36 moles generated during aerobic conditions. In the mitochondria of rice grass (*Echinochloa*), additional ATP is generated by substrate-linked phosphorylation in the TCA cycle, which appears to function anaerobically (Drew, 1990).

In maize root tip cells, at 25°C, anoxia inhibits oxidative phosphorylation within 2 min, resulting in an increase in NADH to a new steady state level (Drew, 1990). Lactate, synthesized initially, may be responsible for the decrease in the cytoplasmic pH from 7.3 to 6.8 during the first 20 min. Lactate formation is replaced by ethanol as the enzymes, PDC and ADH, sequentially convert pyruvate to acetaldehyde and ethanol. Cytoplasmic acidification could be the triggering signal for ethanol production in maize root tips. Evidence for this arises from the observation that CO_2 present in the medium at the onset of hypoxia results in a more rapid ethanol production, although the relative accumulation of ethanol produced over time is not affected. Increasing concentrations of CO_2 in the media negatively correlate with pH_c (Roberts et al., 1984a). Cytoplasmic pH is maintained at 6.8 for ~10 h until leakage of protons from the vacuole decreases it further, resulting in cell death within 20 to 24 h. The vacuole, the major proton storage compartment, requires continuous activity of tonoplast H^+-translocating ATPases to maintain the steep proton gradient across the tonoplast membrane. Fermentation, however, does not provide sufficient energy for long term activity of these ATPases, resulting in the observed decrease in pH_c after 10 h of anoxia (Drew, 1990).

LDH interconverts pyruvate and lactate using NAD^+ / NADH as cofactors. In addition, LDH can utilize the substrates 2-hydroxybutyrate, glyoxylate and oxalate (Bais et al., 1989). The glyoxylate pathway is important in fatty acid metabolism, specifically in the conversion of the acetate units of Acetyl-CoA to succinic and malic acids in the glyoxysome (Salisbury and Ross, 1985). Activation of the glyoxylate cycle in senescent tissue occurs in a variety of species, possibly because it acts as a salvage pathway for the conversion of membrane lipids into sucrose which can be transported from the senescing tissue (Graham et al., 1994).

Experiments with barley using ^{14}C-glucose, determined that in hypoxic conditions, glucose is converted to ethanol (46%), alanine (47%)

and lactate (3%) with the lactate being secreted into the surrounding medium (Hoffman et al., 1986). There is no change in the flux of ^{14}C from (U-^{14}C)glucose to lactate and ethanol during anoxic treatment of transgenic tomato plants expressing a barley *ldh* cDNA under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter. In both transgenic and control plants, hypoxic pretreatment increases the flux to lactate and promotes lactate efflux to the medium. These results suggest that there is a very low flux control coefficient for lactate fermentation and that lactate secretion exerts a major control over long-term lactate glycolysis *in vivo* (Rivoal and Hanson, 1994).

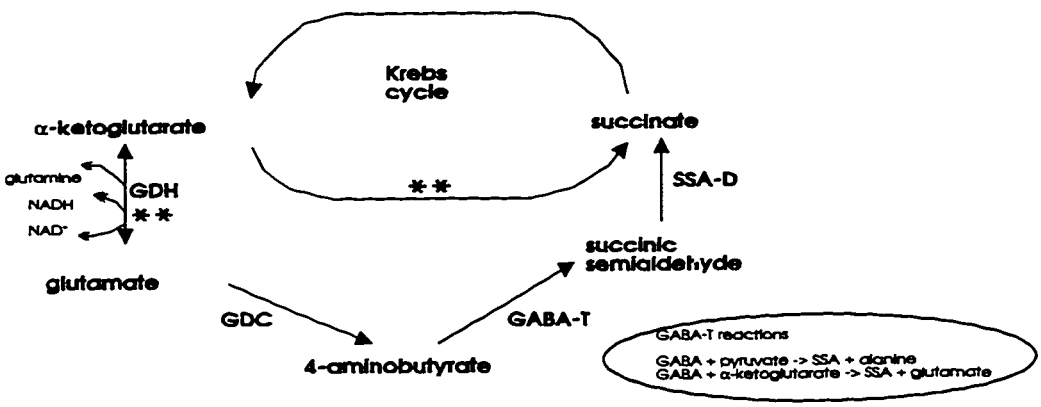
Species of the halophyte *Limonium* produce high (>1), medium (0.5 to 0.8) or low (<0.1) ratios of lactate to ethanol (Rivoal and Hanson, 1993). Most species excrete the lactate into the medium, with a direct correlation between lactate production and excretion. Alanine is the major amino acid produced and the proportion of labeled alanine excreted in the media is very different from that of lactate, suggesting that lactate efflux is specific and not just the result of a generalized increase in membrane permeability (Rivoal and Hanson, 1993).

In red bell pepper (*Capsicum annuum*) fruit exposed to anoxia, respiration rate decreases by 60% (within 30 min) and acetaldehyde and ethanol concentrations increase (within 2 to 3 h). Within 5 min of re-exposure to oxygen, acetaldehyde production increases 2 to 10 fold. The increase is transient if the prior anoxic period is short, whereas repeated or long anoxic exposures result in acetaldehyde remaining elevated for several h (Zuckermann et al., 1997). Ethanol can be converted to acetaldehyde by ADH, in a NAD^+ dependent conversion, or via a H_2O_2-dependent catalase-controlled peroxidation (ethanol + H_2O_2 → acetaldehyde + $2H_20$). It is unlikely that a significant surplus of NAD^+ is available to ADH during the first few min of oxygen re-exposure and thus ethanol peroxidation is the most likely source of the increased acetaldehyde. Conversion of superoxide radical (O_2^-) within the mitochondrion by superoxide dismutase is the most likely source of H_2O_2. The high concentration of reducing equivalents (NADH) within mitochondria of anoxic tissue may favour a rapid generation of active oxygen species (i.e. O_2^-, H_2O_2, OH^-) during the first few min of post-anoxia (Zuckermann et al., 1997). Senescence of plant organs and tissues is associated with free radical formation (Acevedo and Scandalios, 1996). The post-anoxic ethanol peroxidation rate is dependent on the rate of H_2O_2 production, reaching a peak value and then decreasing as mitochondria resume normal functioning (Zuckermann et al., 1997). It has been suggested that the reduction products of oxygen may initiate events leading to heat shock protein synthesis. In heat-shocked anoxic rice embryos, heat shock proteins are synthesized in addition to ANPs,

although the heat shock protein profile differs from that of aerobic heat shocked embryo's (Mocquot et al., 1987).

GABA is increased during hypoxia, cold, and insect and mechanical damage (Shelp et al., 1995). The GABA shunt (Figure 1-6) permits glutamate carbon to enter the Krebs cycle via an alternative pathway. Cytoplasmic acidification stimulates glutamate decarboxylase (GDC) activity to produce GABA which, in the presence of pyruvate and GABA transaminase, is converted to alanine and succinic semialdehyde (SSA), which enters the Krebs cycle (Shelp et al., 1995).

Figure 1-6: GABA shunt



** Steps bypassed by GABA shunt to allow glutamate carbons to enter the Krebs cycle

Abbreviations: GABA = 4-aminobutyrate, GABA-T = GABA transaminase, GDC = glutamate decarboxylase, GDH = glutamate dehydrogenase, SSA = succinic semialdehyde, SSA-D = SSA dehydrogenase.
Adapted from Shelp et al., 1995

1.3.4 Synthesis of anaerobic proteins

A number of proteins whose expression is enhanced during hypoxia and anoxia have been identified. Maize synthesizes 20 major ANPs within 1.5 h of anaerobic treatment (Sachs et al., 1980). These proteins include those involved in ethylene biosynthesis, aerenchyma formation, glycolysis, fermentation, amino acid synthesis and transcription regulation (Table 1-2). Additional proteins, with a variety of functions, identified in other species are listed in Table 1-3.

Table 1-2: Hypoxically / anoxically induced proteins identified in *Zea mays*

Protein	Comments	ARE-like element	Reference
I. Ethylene biosynthesis			
ACC oxidase	a, c		Scott et al., 1996
ACC synthase	a, c		He et al., 1994; Scott et al., 1996
II. Aerenchyma formation			
Cellulase	a, c		He et al., 1994; Scott et al., 1996
Gene 1005 (xyloglucan endo-transglycosylase homolog)	a, b		Peschke and Sachs, 1994; Saab and Sachs, 1996
III. Transcription regulation			
G-box binding factor (GBF1)	b		de Vetten and Ferl, 1995
IV. Amino acid biosynthesis			
Alanine aminotransferase (AlaAT)	a		Good and Crosby, 1989a
V. Starch / sucrose mobilization			
Sucrose synthase (SS)	b, d	yes	McElfresh and Chourey, 1988; Olive et al., 1990
Glucokinase (GK)	a		Bouny and Saglio, 1996
VI. Stem glycolytic enzymes			
Phosphoglucomutase	b		Bailey-Serres et al., 1988
Fructokinase (FK)	a		Bouny and Saglio, 1996
Glucose-6-phosphate isomerase (PG1)	b	yes	Kelley and Freeling, 1984b
Phosphofructokinase (PFK)	a		Bouny and Saglio, 1996
Fructose 1,6-bisphosphate aldolase	b, d	yes	Kelley and Freeling, 1984a; Olive et al., 1990; Christie et al., 1991
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	a, e (*gapC3*), f and g (*gapC4*)	yes	Russell and Sachs, 1992; Köhler et al., 1995; Köhler et al., 1996
Phosphoglycerate mutase	b		Bailey-Serres et al., 1988
Enolase	a		Bailey-Serres et al., 1988; Bouny and Saglio, 1996
Pyruvate kinase (PK)	a		Bouny and Saglio, 1996
VII. Ethanol fermentation			
Pyruvate decarboxylase (PDC)	b, d	yes	Christie et al., 1991; Bouny and Saglio, 1996; Hossain et al., 1996
Alcohol dehydrogenase (ADH)	a, b, d, h	yes	Dennis et al., 1985; Christie et al., 1991; Peschke and Sachs, 1994
Lactate dehydrogenase (LDH)	a	yes	Hoffman et al., 1986; Good and Paetkau, 1992; Xia and Saglio, 1992
VIII. Unknown function			
Gene 1032	b		Peschke and Sachs, 1994

Comments: Induced by (a) hypoxia, (b) anoxia, (c) mechanical impedance, (d) chilling (10°C), (e) heat shock, (f) UV light, (g) wounding and (h) salt. ARE-like element: the presence of an anaerobic response element (Section 1.5.1) within the promoter region of the gene encoding the particular protein.

Table 1-3: Additional hypoxic / anoxic induced proteins identified in other species

Protein	Plant Species	Comments	Reference
I. Amino acid biosynthesis			
Arginine decarboxylase (ADC)	*Oryza sativa*	b, d	Reggiani, 1994
II. Sucrose / starch mobilization			
α-amylase	*Oryza sativa*	b,	Guglielminetti et al., 1995
III. GABA shunt			
Glutamate decarboxylase (GDC)	*Glycine max*	a, c	Shelp et al., 1995
IV. Proton pumps			
Vacuolar H^+-translocating ATPase (V-ATPase)	*Oryza sativa*	b,	Carystinos et al., 1995
Vacuolar H^+-translocating pyrophosphatase (V-PPase)	*Oryza sativa*	b,	Carystinos et al., 1995
V. Kinases			
Nucleoside diphosphate kinase (NDPK)	*Oryza sativa*	b,	Guglielminetti et al., 1995
VI. Oxygen sensor (?)			
Hemoglobin	*Hordeum vulgare*	a,	Taylor et al., 1994
VII. Oxygen radical removal			
Superoxide dismutase (SOD)	*Iris pseudacorus*	b	Monk et al., 1987

Comments: Induced by (a) hypoxia, (b) anoxia, (c) cold and (d) salt.

ADH is essential for long-term survival during both hypoxia and anoxia (Schwartz, 1969). Maize *adh* null mutants do not survive anoxia longer than 24 h (Schwartz, 1969). Studies on maize lines differing in ADH activity, demonstrates that pH_c regulation and the ability of root tips to survive prolonged hypoxia is largely independent of ADH (except when ADH activity is approximately that of *adh1* null mutants). This suggests that ADH alone cannot determine hypoxic tolerance. *In vivo*, the control of flux along metabolic pathways is generally distributed among several enzymes. No single enzyme is "rate-limiting" until its activity decreases to 10 to 20% of wild type, at which point that enzyme can significantly limit flux (Roberts et al., 1989). PDC has been suggested to be the rate limiting enzyme in the fermentation pathway (Bucher et al., 1994) as transgenic tobacco plants over-expressing PDC increase ethanolic fermentation when treated with respiratory inhibitors under aerobic conditions without increasing *adh* transcription. This suggests that anaerobic transcription is triggered directly through an oxygen sensing mechanism and not by the metabolic consequences of oxygen limitation. In barley roots hemoglobin transcripts parallel, rather than anticipate, the expression of *adh* and *ldh*

suggesting that hemoglobin does not function as the oxygen sensor (Taylor et al., 1994).

1.3.5 Miscellaneous

Species capable of anaerobic germination, including *Oryza sativa* and *Erythrina caffa* can reduce and assimilate nitrate (Mattana et al., 1994), an important feature since nitrate absorption is reduced during hypoxia (Drew and Sisworo, 1979; Konings and Verschuren, 1980; Ernst, 1990; Laanbroek, 1990).

Plants growing in areas that are frequently flooded also have a shorter life cycle and produce seeds that can survive flooding (Blom et al., 1994). Negative gravitropism in shoots (leaf epinasty) decreases light intensity on the leaf thereby decreasing water loss and wilting (Jackson, 1990). Stomatal closure also serves to decrease transpiration (Jackson, 1990). Leaf reorientation to a more vertical position occurs in a few species including *Rumex palustris* (Banga et al., 1996).

Root shedding, a feature of the waterfern *Azolla filiculoides*, is speculated to allow movement and / or dispersion to better conditions (Uheda and Kitoh, 1994). This shedding response has many similarities with aerenchyma formation and is stimulated by respiratory inhibitors, including sodium azide and sodium cyanide. Root maturity is important in the response to respiratory inhibitors. Roots <5 mm possess an abscission layer but are not shed, whereas roots >10 mm are shed completely. The authors hypothesize that, in response to inhibitors of respiration, large cells at the base of the root expand and round up because of rapid absorption of water, thus increasing the distance between cells and facilitating their separation (Uheda and Kitoh, 1994). Similarly, aerenchyma occurs in the more mature zones of the root (>5 mm from the tip) and not in the immature regions (<5 mm from the tip) (Campbell and Drew, 1983; Drew et al., 1994).

Eelgrass (*Zostera marina*), a shallow-water submerged angiosperm is unique in that it fluctuates diurnally between aerobic respiration and anaerobic fermentation. During the day it produces sufficient photosynthetically derived O_2 to maintain aerobic respiration. During the night, metabolism slows by 30% and is anaerobic, producing ethanol (95%) and lactate and alanine (5%) (Drew, 1990).

1.4 *Hypoxia versus anoxia*

Hypoxia is the term used to describe situations in which oxidative phosphorylation in cells or tissues is slowed by external oxygen concentrations. Anoxia is used to describe situations in which the oxygen concentration is so low that oxidative phosphorylation is negligible, relative to ATP production by glycolysis and fermentation (He et al., 1996; Morard and Silvestre, 1996). These terms can apply to all or part of the root system (Morard and Silvestre, 1996).

1.4.1 Testing

Studies of plants responses to hypoxia have been performed in soil, agar or, more commonly, in hydroponic cultures. In these conditions, localized hypoxia within the root is more common than hypoxia throughout the entire root system (Morard and Silvestre, 1996). Root asphyxiation, in which plants are submerged in sealed containers previously sparged with nitrogen gas, have been used to study anoxia.

The survival of rice grown in agar mixed with a 1% solution of soil extract is identical to those grown in soil, but lower than those grown in 1% agar alone. This suggests that soil microorganisms, microelements and organic compounds are important in determining hypoxic survival (Setter and Ella, 1994). However, soil conditions are very difficult to reproduce. Flooded soils invoke nutrient deficiency and microelement toxicity in addition to anoxic / hypoxic stress; thus, growth in a soil-less media is used so that the effects of hypoxia or anoxia alone can be studied.

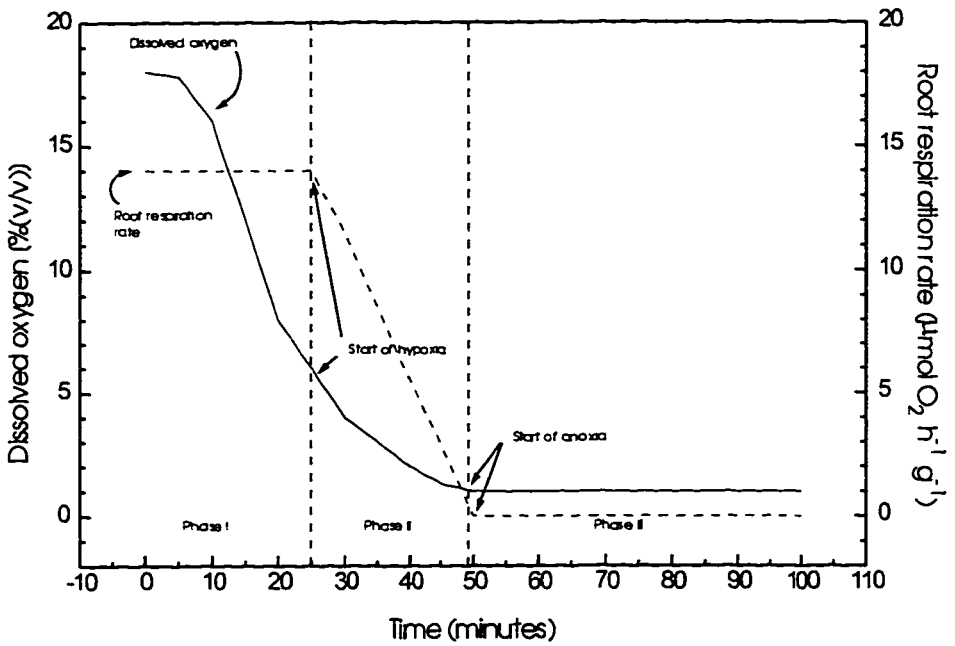
Hydroponic culture is the medium of choice for most studies as plant responses mimic those of soil waterlogging without the compounding problem of anaerobic soil microorganisms which produce fermentation products toxic to the plants (Morard and Silvestre, 1996). Hypoxia in hydroponic cultures is mediated either by bubbling a gas (usually nitrogen) through the culture, or by placing the roots in an airtight container so that oxygen is depleted by root oxygen consumption. Both methods result in a gradual depletion of oxygen similar to that in natural conditions (Figure 1-7).

Studies with wheat and cucumber examining oxygen concentrations in hydroponic tanks indicate that oxygen is usually depleted within one h of removing the oxygen supply. Oxygen depletion is phasic, corresponding to three different respiration rates and profiles (Figure 1-7). In phase I oxygen depletion is linear down to oxygen concentrations of 4 to 6% partial pressure, corresponding to a constant respiration rate. During this phase root cell oxygen requirement is satisfied

and oxygen concentration is not limiting. In phase II, oxygen concentrations in the media become limiting. This is characterized by a progressive change in the rate of oxygen depletion and a decrease in respiration rate, as cells become hypoxic. Phase III oxygen concentrations correspond to 1% partial pressure, the limit capacity for dissolved oxygen use by root cells. At this time, root respiration rate tends towards zero (anoxic) (Morard and Silvestre, 1996).

ADH activity in maize roots decreases after 24 hours of growth in hydroponic tanks bubbled with 40% O_2. This suggests that plants in hydroponic tanks bubbled with air are slightly hypoxic and that pretreatment with 40% O_2 may be necessary to avoid hypoxic induction (Andrews et al., 1993). However, since root respiration is stable at dissolved oxygen concentrations greater than 4 to 6% (v/v) (Figure 1-7), the experimental control plants described in this thesis were maintained in hydroponic tanks bubbled with air.

Figure 1-7: Dissolved oxygen concentration and root respiration in a hydroponic medium



Depletion of dissolved oxygen by wheat roots (solid line) and calculated respiration rates of root systems (dotted line) in a hydroponic medium.
Adapted from Morard and Silvestre, 1996

Reports on the severity of flooding stress suggest that the developmental stage of the plant is important (Andrews et al., 1994; Van Toai et al., 1995). Most cultivated seeds do not germinate under anoxia. Starchy seeds (i.e. maize) can germinate at decreased oxygen pressures, but the seedlings are very sensitive to anoxic stress. Most established plants do not survive anoxia well (Van Toai et al., 1995). Studies on maize demonstrates a significant survival difference between 2 and 3 d old seedlings subjected to anoxic stress, with 2 d old seedlings having a much higher survival rate and ATP content after 48 h of anoxia than 3 d old seedlings. Abscisic acid and hypoxic pretreatment improves the survival of the older but not the younger seedlings (Van Toai et al., 1995).

Maize seedlings possess an inhibitor of ADH activity. The activity of this inhibitor progressive increases in the scutellum, shoot, leaf and roots of the seedling (Ho and Scandalios, 1975). The activity of this inhibitor is very low before 40 h post-germination and its increased activity is concomitant with a decrease in ADH activity. The presence of this inhibitor may partially contribute to the lower survival of older seedlings exposed to anoxic stress. Comparisons of ADH activity in 3- and 5-d old maize roots demonstrate that ADH activity is higher in the younger plants (Andrews et al., 1994). Consistent with the observation of higher ADH activity in younger plants is the improved survival of 2-d-old maize plants, conditioned with 1% glucose in the flooding buffer (Van Toai et al., 1995). No protective effect is seen in 3-d-old plants. The younger plants, with higher ADH activity, have enhanced ethanolic fermentation relative to 3-d-old plants and thus synthesize more NAD^+, which is required for functioning of the stem glycolytic pathway. Studies on pollen also demonstrate that the availability of carbohydrate (and not oxygen supply) has a major influence on the rate of ethanolic fermentation (Bucher et al., 1995).

Roots of intact seedlings survive longer than isolated maize root tips (Van Toai et al., 1995). Evaluation of the ATP content of roots indicates that intact roots maintain higher concentrations of ATP during anoxic stress relative to isolated root tips (Van Toai et al., 1995).

1.4.2 Benefits of hypoxic pretreatment before anoxic exposure

Studies of the benefits of hypoxic pretreatment prior to anoxic shock have been done primarily on maize. Hypoxic pretreatment improves viability of maize root tips from <24 to 96 h, increases ATP and total nucleotide concentrations and adenylate energy charge. A noticeable Pasteur effect is observed in hypoxically pretreated root tips upon subsequent anoxic treatment (Johnson et al., 1989; Drew, 1990; Bouny

and Saglio, 1996). This Pasteur effect is sustained for longer periods of time than in non-hypoxically pretreated root tips (Hole et al., 1992). Hypoxically pretreated root tips incubated in the presence of exogenous glucose produce more ethanol than non-hypoxically pretreated root tips incubated in the same medium (Hole et al., 1992). In intact roots, anoxia impedes phloem unloading of sugars suggesting that intact hypoxically pretreated roots can tolerate anoxia if the energy status of the cell is adequate for phloem unloading and symplastic transport of endogenous sugars into the apical meristem (Hole et al., 1992).

Hypoxic pretreatment results in increased activity of many of the glycolytic enzymes including GK, FK, PFK, GAPDH, PK, PDC, ADH, LDH and enolase (Eno) (Bouny and Saglio, 1996). Hypoxic pretreatment improves pH_c regulation on exposure to anoxia, relative to non-hypoxically pretreated root tips (Xia and Roberts, 1996). Hypoxic pretreatment also results in increased lactic acid production relative to non-hypoxically pretreated root tips; however in hypoxically pretreated root tips, lactic acid is primarily extracellular whereas in non-hypoxically pretreated root tips it is primarily intracellular (Bouny and Saglio, 1996).

1.5 *Regulation of genes encoding anaerobic proteins*

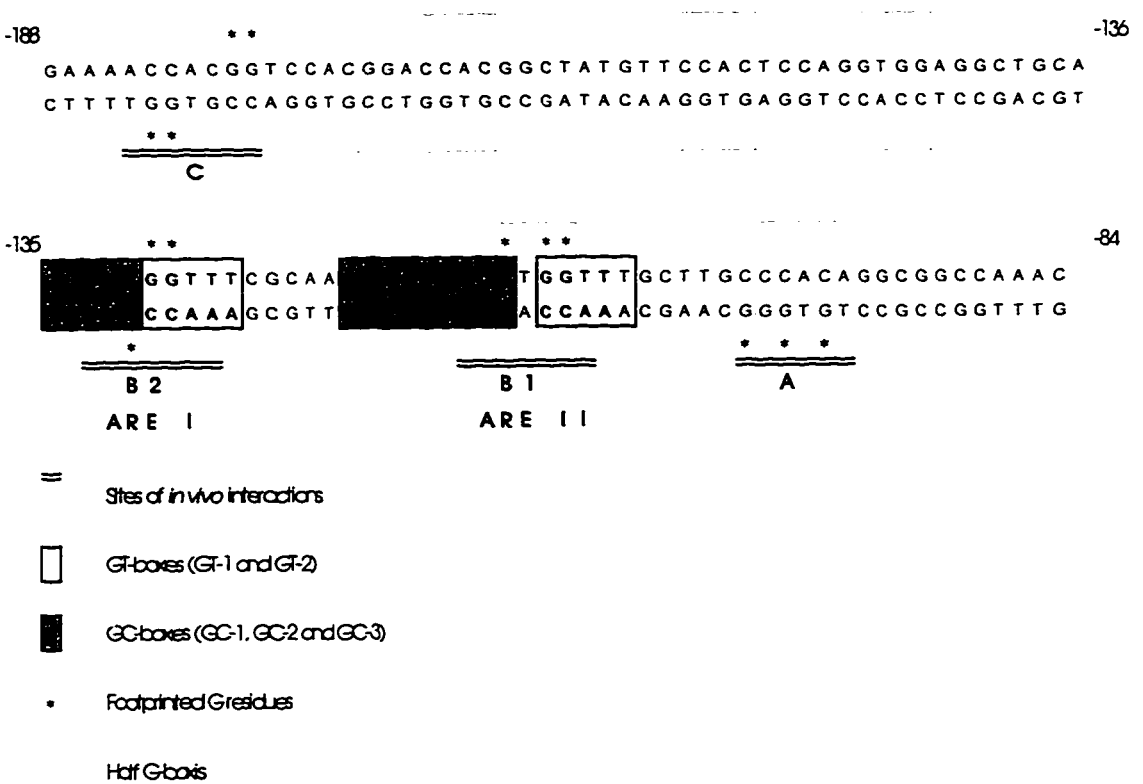
1.5.1 Transcriptional control

Most investigations on the transcriptional control of anaerobically induced genes have focused on *adh* in both maize and *Arabidopsis thaliana*. An element in the maize promoter, essential for anaerobic induction of *adh1*, is termed the anaerobic response element (ARE). This element, located from −135 to −106 relative to the transcription start site, is composed of three GC-rich and two GT-rich domains (Figure 1-8) (Walker et al., 1987; Olive et al., 1991). The ARE is essential and sufficient for anaerobic induction in maize protoplasts (Walker et al., 1987; Olive et al., 1991) and in transgenic rice (Kyozuka et al., 1994). The ARE is composed of two subregions, ARE I (-135 to -126) and ARE II (-115 to -106), both with core consensus sequences of GGTTT. ARE II dimers are more effective than ARE I dimers in evoking anoxic induction in electroporated maize protoplasts. Spacing between the two subregions is important, suggesting that the subregions act cooperatively (Olive et al., 1990). Mutations in the GGTTT sequence eliminate hypoxic activation of gene expression (Olive et al., 1991).

Several protein binding sites have been identified in the maize *adh1* promoter: C, B2, B1 and A (Figure 1-8). Sites A and C have proteins bound only in anaerobic conditions, whereas B1 and B2 (components of the

ARE) have proteins bound constitutively. However, the footprint of the protein bound to region B2 changes upon hypoxic induction (Ferl and Nick, 1987; Paul and Ferl, 1991). Proteins bound to region B2 may physically mark the *adh1* promoter to assist in recruitment of additional components of the transcription apparatus or directly participate in the recognition of induction conditions with a change in conformation at the onset of anaerobiosis. Alternatively, they may facilitate binding of transcription factors at sites A and C via protein-protein contacts or by generating the appropriate chromatin structure for recognition (Ferl and Nick, 1987).

Figure 1-8: Maize *adh1* promoter elements

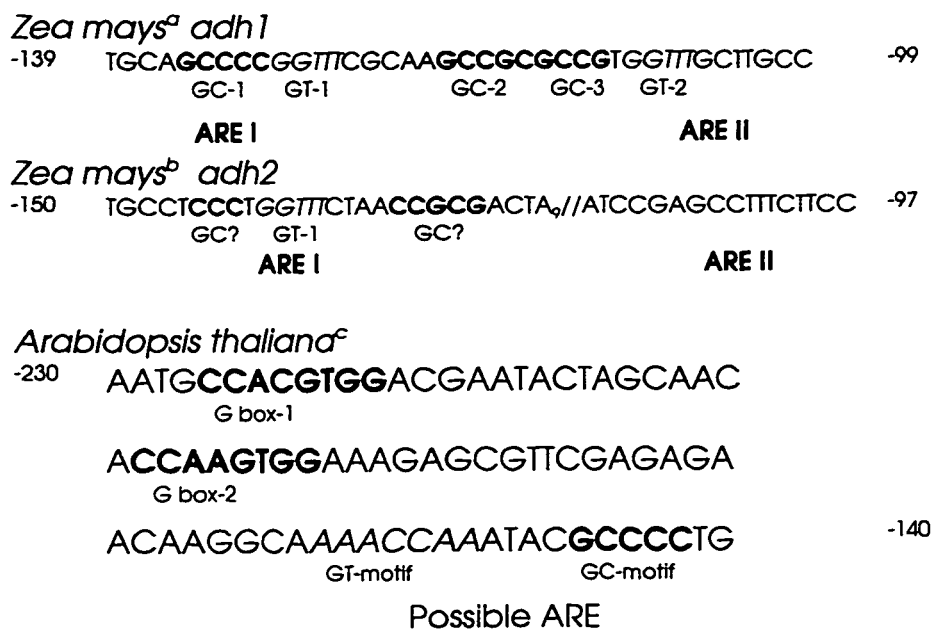


Adapted from de Vetten and Ferl, 1995

ARE binding factor B2 (ARF-B2) which binds region B2, is a multi-component complex. ARF-B2α binds DNA with low specificity. When complexed with ARF-B2χ, a negatively charged polypeptide, ARF-B2α binds the B2 region with increased specificity (Ferl, 1990). The GC binding protein (GCBP-1) binds GC-boxes within the ARE (Olive et al., 1991). Mutations that inhibit GCBP-1 binding prevent hypoxic *adh1* activation (Oliver et al., 1991). G-box binding factor 1(GBF1), a basic leucine zipper protein, binds a maize *adh1* promoter fragment containing the region -140 to +20. The maize *adh1* promoter lacks a complete G-box, but contains many half G-boxes (GTGG). However these sequences do not appear to be critical for hypoxic induction. The mobility of GBF1 in nuclear extracts differs from that of recombinant GBF1. Additionally, GBF1 activity in aerobic nuclear extracts is approximately the same as in hypoxic nuclear extracts. These results suggest that GBF1 likely interacts with other proteins (de Vetten and Ferl, 1995). Mutations in the region -124 to -116 result in high aerobic expression of *adh1* in maize protoplasts possibly because of the removal of a repressor binding site or the generation of a weak activator binding site (Olive et al., 1991).

Maize *adh* is induced by anaerobiosis, chilling (10°C) (Christie et al., 1991) and salt (Peschke and Sachs, 1994). *Arabidopsis thaliana adh* is additionally induced by dehydration and cold (4°C) (Jarillo et al., 1993; Dolferus et al., 1994). The *Arabidopsis thaliana* promoter appears to be more complex than the maize promoter with important sequences being located from -230 to -140, relative to the transcription start site (Figure 1-9). Within this region are two G-boxes and an ARE-like element. The G box-1 element, a perfect palindrome bound by GBF1 (Schindler et al., 1992) and TGA1 (G-box-like binding factor), is important for the cold and dehydration responses and is responsive to Abscisic acid. The ARE-like element contains both GT- and GC- motifs which appear to be part of a general stress response element. Mutations within these motifs decrease expression during cold, dehydration and anoxic stress as well as in the unstressed control plants (Dolferus et al., 1994). The transcriptional activator, GF14ϖ, binds the *Arabidopsis thaliana* GBF1 / G-box element complex (Lu et al., 1992; Lu et al., 1994).

Figure 1-9: *adh* promoter elements

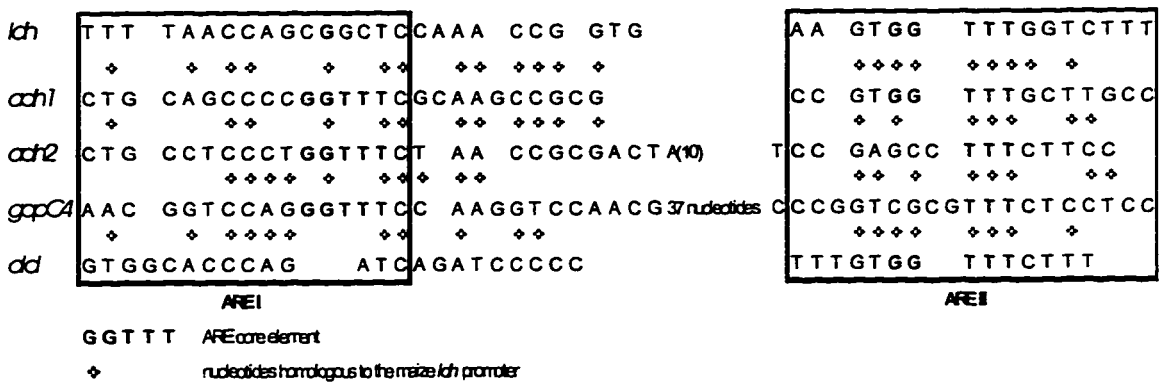


a: de Vetten and Ferl, 1995; b: Walker et al., 1987; c: Dolferus et al., 1994

Cotton (*Gossypium hirsutum*), an allotetraploid, contains at least four *adh* genes. One genomic clone, *adh2b*, has been examined for homology to the maize *adh1* promoter. The promoter region contains a G-box like element and a GT-1 core sequence in addition to six GT motifs, four of which are in the 5' untranslated region (UTR) with the remainder in the first intron (Millar and Dennis, 1996b). The location of these GT-motifs is interesting as GT-motifs have been determined to be important elements in the maize *adh1* ARE (Walker et al., 1987; Olive et al., 1991).

Maize *ldh1* possesses a sequence with homology to the ARE (Figure 1-10) with the homology being greater in ARE subregion II than in subregion I (Table 1-4).

Figure 1-10: Comparison of the maize *Idh* ARE with AREs from other maize genes encoding ANPs



Reference: *Idh* – Good and Paetkau, 1992; *adh1* and *adh2* – Walker et al., 1987; *gapC4* – Köhler et al., 1995; *ald* – Dennis et al., 1988

Table 1-4: Homology of the ARE of maize genes encoding ANPs to the maize *Idh* ARE

	% homology to *Idh* ARE	
	ARE I	ARE II
adh1	44%	56%
adh2	38%	47%
gapC4	44%	42%
ald	57%	57%

Reference: *Idh* – Good and Paetkau, 1992; *adh1* and *adh2* – Walker et al., 1987; *gapC4* – Köhler et al., 1995; *ald* – Dennis et al., 1988

1.5.2 Post-transcriptional control

Introns of several hypoxically induced genes enhance gene expression in transient assays (Vain et al., 1996). The enhancement in gene expression is speculated to be due to effects on splicing.

It is suggested that transiently expressed genes have unstable transcripts. This would enable them to respond rapidly to changes in the environment; conversely, genes whose expression must be buffered from rapid changes have stable transcripts (Green, 1993; Sullivan and Green, 1993). Regulation by altered mRNA stability is suggested by discrepancies between the transcription rate, as measured by *in vitro* nuclear run-on assays, and transcript accumulation (measured by RNA blotting techniques) (Sullivan and Green, 1993). Genes within a gene family can have similar transcription rates but different mRNA half-lives ($T_{1/2}$), resulting in their respective mRNAs accumulating to different levels (Thompson et al., 1989).

Numerous observations indicate that degradation of transcripts may be coupled to translation. For example: (1) association of components involved in mRNA decay with polyribosomes, (2) cycloheximide induced accumulation of some unstable transcripts (mRNA decay requires either the action of labile cellular factors or active translation facilitates degradation), (3) decreased mRNA $T_{1/2}$ resulting from frameshift mutations which induce premature translation termination (activation of ribosome-associated RNAses or increased nuclease sensitivity of mRNA regions devoid of ribosomes), (4) translation initiation and mRNA stability mediated by poly(A) binding protein (Green, 1993).

1.5.3 Translational control

Hypoxic conditions affect the ratio of monoribosomes to polyribosomes (Bailey-Serres and Freeling, 1990; Bailey-Serres and Dawe, 1996). Within one h of hypoxia, there is an increase in the number of monoribosomes and ribosomal subunits. Prolonged hypoxia results in an accumulation of nontranslating ribosomes and an increase in the numbers of small polyribosomes (<5 ribosomes per transcript). Return to aerobic conditions results in recovery of a normal ribosome profile (Bailey-Serres and Freeling, 1990; Bailey-Serres and Dawe, 1996). In maize root tips the ratio of monoribosomes to polyribosomes changes from 0.3, during aerobic conditions, to 3 following 45 min of hypoxia (Webster et al., 1991). The rate of protein synthesis strongly correlates with the number, size and activity of polyribosomes. *In vivo* labeling experiments demonstrate both

physical and chemical modifications to protein components of hypoxic ribosomes (Bailey-Serres and Freeling, 1990).

Changes in pH_c can participate in translation regulation at the level of elongation and termination (Webster et al., 1991). The elongation reaction of isolated maize root tip polysomes has a pH optimum of 7.5. Decreasing the pH to 6.5 results in a 50% decrease in the elongation rate. Stalled ribosomes remain attached to the mRNA, due to inhibition of either elongation or termination reactions (Webster et al., 1991). EF-1α becomes stably associated with polysomes in hypoxic potato tubers resulting in translation elongation arrest (Vayda et al., 1995). Another study suggested that the accumulation of monoribosomes during hypoxia is due to impaired translation initiation (Fennoy and Bailey-Serres, 1995). This study also demonstrated that oxygen concentration has a discriminatory effect on the loading of ribosomes onto specific mRNAs. Under both aerobic and anoxic conditions, *adh* mRNA is associated with large polyribosomes (>5 ribosomes), whereas *ant* (mitochondrial adenine translocator) mRNA is associated with large polyribosomes during aerobic conditions, but is less abundant and associated with small polyribosomes during anoxia. It is possible that sequences in the *adh1* mRNA confer a ribosome loading advantage. Maize *adh1* and *adh2* 5′ UTRs have diverged sequences except for an 18 nucleotide region of homology (Figure 1-11) (Dennis et al., 1985). It is speculated that this conserved sequence may have a role in anaerobic specific translation.

Figure 1-11: Maize *adh1* and *adh2* 5'UTR homology region

adh1	+46	G	G	T	C	T	C	G	G	A	G	T	G	G				A	T	C	G	A	+63
		◆		◆	◆	◆		◆	◆	◆	◆	◆	◆	◆				◆	◆	◆	◆	◆	
adh2	+70	G	T	T	C	T	T	G	G	A	G	T	G	G	T	C	C	A	T	C	G	A	+90

From Dennis et al., 1985

Translation initiation is affected by the 5′ cap (5′-7mGppp), the length and secondary structure of the 5′ UTR, nucleotide sequences around the start codon and the 3′ UTR via interactions with the cap (Bailey-Serres and Dawe, 1996). Studies on the maize cell line P3377, in which radiolabeled *in vitro* transcribed RNAs were electroporated into protoplasts, demonstrated a sequence between +82 and +161 of *adh1*

mRNA which specifically enhances translation of the reporter gene under hypoxic conditions (Bailey-Serres and Dawe, 1996). Constructs A_{161}GA and A_{161}G both contain 107 nucleotides of the 5' UTR and the first 54 nucleotides of the *adh1* coding region fused to the GUS reporter gene, followed by a 68 nucleotide poly(A) tract. Construct A_{161}GA also contains 186 nucleotides of the *adh1* 3' UTR. The construct lacking the *adh1* 3' UTR has a higher aerobic expression of GUS compared to when it is present, but a lower hypoxic expression. This suggests that sequences in the 3' UTR interact with those in the 5' UTR to facilitate translation during hypoxic conditions (Bailey-Serres and Dawe, 1996).

In rice, the pattern of *in vitro* translation products obtained from mRNA isolated from anoxic roots is more complex than the pattern of the corresponding *in vivo* labeled proteins (Breviario et al., 1994). This suggests that, in rice, the control of gene expression during the first hours of anoxia is primarily at the translational level. Additionally, more and greater varieties of proteins are synthesized in the coleoptile relative to the root. This is consistent with observations that rice coleoptiles, but not roots, grow rapidly in flood conditions. Some proteins are detectable by *in vitro* translation but not by *in vivo* labeling suggesting the existence of a negative translational mechanism (Breviario et al., 1994). Similar studies in maize suggest that *gapC3/4* are selectively translated (Russel and Sachs, 1992).

In maize, although sucrose synthase (SS) protein concentrations and isozyme profiles are unaltered by anoxia, SS enzyme activity decreases and *sh* transcripts (encoding SS1 isozyme) increase. This suggests that regulation of *sh* is at the post-transcriptional level (McElfresh and Chourey, 1988). Since SS is involved in starch mobilization and maize lacks starch reserves, accumulation of SS would be unnecessary. Rice seeds, however, contain starchy reserves and during anoxia SS activity and protein and mRNA levels increase (Ricard et al., 1991).

1.5.4 Components of the signal transduction pathway

Pharmacological agents have been used to study the components of the hypoxic / anoxic signal transduction pathway. These agents are very useful when mutants are not available; however, uptake across the root can be a significant problem and must be considered in interpretation of results.

Calcium has a role in the induction of *adh* and *sh1*. Caffeine treatment of aerobic cells increases intracellular calcium concentrations and ADH activity (Subbaiah et al., 1994a; Subbaiah et al., 1994b) but not ethylene synthesis (He et al., 1996). Ruthenium red (RR), an inhibitor of

organellar calcium release, decreases *adh1* and *sh1* transcripts, ADH activity and root survival. RR affects the anoxia-induced increase in intracellular calcium in maize suspension cultures more so than the caffeine induced increase. This suggests that different intracellular calcium stores are affected by anoxia and caffeine treatment (Subbaiah et al., 1994b). Chelators, present in the external media, have no effect on anoxic survival or transcript levels suggesting that calcium uptake across the plasma membrane is not necessary for the anoxic response (Subbaiah et al., 1994b).

Studies on maize suspension cultures using a fluorometric dye to monitor changes in intracellular calcium demonstrate that there is an increase in intracellular calcium on anoxic exposure, which decreases on reoxygenation. The increase in the calcium signal starts as a hot spot around the nucleus and subsequently spreads through out the cell. Cells pretreated with RR prior to anoxic exposure lose the anoxic increase in intracellular calcium (Subbaiah et al., 1994b).

In *Arabidopsis* two calcium peaks occur in cotyledons and leaves following anoxic exposure but there is no detectable increase in calcium in roots and hypocotyls (Sedbrook et al., 1996). Maize roots, in contrast, exhibit a monophasic calcium increase (Subbaiah et al., 1994a). Roots of *Arabidopsis thaliana* have increased *adh* transcripts even though no calcium peaks are evident (Sedbrook et al., 1996). This emphasizes the need to study the hypoxic / anoxic response in both monocot and dicot species.

Ca^{2+} effects are mediated by binding of Ca^{2+} to specific target proteins in a reversible manner (Bush, 1993). These target proteins include calmodulin, protein kinases, protein phosphatases, ion transporters, cytoskeletal proteins and lipases. The target proteins then alter the activity of a variety of effector proteins responsible for the final cellular response (Bush, 1993). Ca^{2+} / calmodulin can directly stimulate enzyme activity thus modulating the activity of specific metabolic pathways (Snedden et al., 1995). GDC activity, which catalyzes the first step in the GABA shunt, is stimulated by Ca^{2+} / calmodulin binding *in vitro* (Snedden et al., 1995)

Phosphorylation / dephosphorylation of proteins is a major component of signal transduction pathways. *Arabidopsis thaliana* GBF1 has significantly enhanced binding activity to a G-box oligonucleotide upon phosphorylation and decreased, but still detectable, binding when dephosphorylated (Klimczak et al., 1992). GBF1 interacts with GF14ϖ (Lu et al., 1992), a component of a multigene family (Rooney and Ferl, 1995). GF14ϖ binds Ca^{2+} and is phosphorylated at serine residues. The phosphorylated form has a higher affinity for Ca^{2+} than the nonphosphorylated form. Proteolytic studies suggest that Ca^{2+} binding,

presumably in the EF hand motif, causes a conformational change in the protein (Lu et al., 1994).

The study of the hypoxic / anoxic signal transduction pathway is still in its infancy. Although several proteins have been identified which interact with sequence elements shown to be important in the response, a lot of work is still required to understand the signaling pathway and even to determine the exact signal that is recognized by the cell.

1.6 *Plant lactate dehydrogenases*

LDH has not been extensively studied in plants and most of the work has focussed on barley and rice. In barley, LDH activity in aerobic roots decreases with the age of the plant. However, this decrease does not affect the inducibility of LDH under hypoxic conditions (Good and Crosby, 1989b).

Barley possesses two tightly linked *ldh* loci that are 92% identical (Hondred and Hanson, 1990). This small amount of divergence is consistent with a relatively recent gene duplication event. Hypoxic treatment increases the intensity of a single 1.5 kilobase (kb) transcript. Transcripts remain at elevated levels for 2 to 4 d of hypoxic treatment before decreasing (Hondred and Hanson, 1990). LDH enzyme activity remains high for the duration of hypoxic treatment and its catalytic activity is in significant excess relative to the activity required to generate lactate. Enzyme activity is induced through out the whole root system, but is highest in the tip region (Hoffman et al., 1986). LDH activity is also induced by hypoxic treatment of barley aleurone layers (Hanson and Jacobsen, 1984).

In rice, LDH activity is barely detectable in seedlings and is very low in roots. Hypoxic treatment does not result in increases in LDH activity in seedlings, but does result in a <2 fold increase in LDH activity in roots of 30 d old plants (Rivoal et al., 1991). This is consistent with observations that rice produces relatively low amounts of lactate compared to other species (Menegus et al., 1991).

In white spruce suspension cell cultures (*Picea glauca*), ADH and AlaAT are hypoxically, but not anoxically induced. LDH, however, is not induced in either condition (Good et al., 1990).

1.7 *Goals of this thesis*

Maize was selected as the object of study as it is the monocot model organism chosen for most studies of the hypoxic / anoxic response. LDH was studied since LDH activity has been speculated to play a pivotal role in the early hypoxic response (Davies-Roberts pH stat hypothesis).

The first item to be addressed was the determination of the hypoxic induction profile of maize LDH. The maize *ldh1* gene possesses an ARE within its promoter region and thus would be expected to respond to hypoxic conditions in a manner similar to other ANPs. Therefore the first hypothesis being tested was that *ldh* induction is similar to that of other ANPs. This hypothesis was examined by:

- Time course studies of LDH expression from aerobic to several days of hypoxia, examining LDH activity, LDH protein and *ldh* transcript profiles during that period.
- Time course studies of LDH expression in maize cell cultures. This would also provide useful information as to whether maize cell cultures could be used for analysis of the *ldh1* promoter in transient assays.
- Examination of factors affecting LDH activity *in vitro*, specifically to determine LDH *in vitro* stability under both aerobic and hypoxic conditions and to attempt to understand the reasons for the differences between the enzyme stability in both conditions.

The second item to be addressed was the isolation of the second *ldh* gene. Maize possesses two genes encoding ADH (Dennis et al., 1985) and SS (Peschke and Sachs, 1994), three genes encoding PDC (Peschke and Sachs, 1993) and four genes encoding GAPDH (Köhler et al., 1995). The genes encoding all of these proteins are regulated differently in response to oxygen deprivation. Thus the hypothesis being tested was that maize *ldh1* and *ldh2* respond differently to hypoxic stress. This hypothesis was examined by:

- The isolation, sequencing and analysis of the sequence of a second *ldh* gene.
- Probing northern blots with probes specific to the cloned *ldh* genes.

The third item to be addressed was whether *ldh* induction was specific to the hypoxic response in order to determine its usefulness in the study of the hypoxic signal transduction pathway. Mitochondrial respiration can be affected by stresses other than hypoxia, thus it is possible that *ldh* could also be induced in these conditions. The hypothesis being tested was that *ldh* induction is specific to the hypoxic response in that oxygen deprivation and not mitochondrial respiration is the trigger for the hypoxic signal transduction pathway. This hypothesis was examined by:

- Examination of the effect of abiotic stresses that affect mitochondrial respiration on *ldh* expression.
- Examination of the effects of inhibiting mitochondrial protein synthesis on *ldh* expression.

The fourth item to be addressed was the determination of some of the components of the *ldh* hypoxic signal transduction pathway. The hypotheses being tested were that new transcription factor synthesis is required during hypoxia to facilitate hypoxic *ldh* expression and that Ca^{2+} is a component of the *ldh* signaling pathway. These hypotheses were examined by:

- Examination of the effects of protein synthesis inhibitors on *ldh* expression during aerobic and hypoxic conditions.
- Examination of the effects of Ca^{2+} and Ca^{2+} inhibitors on the hypoxic expression of *ldh*.

The fifth item to be addressed was the examination of another hypoxically inducible gene, *alaat*. The hypothesis being tested was that *alaat* is regulated by nitrogen deficiency and not hypoxia *per se*, as nitrogen deficiency occurs very early in the hypoxic response and is speculated to be the cause of a number of adverse effects. This hypothesis was examined by:

- Characterization of the response of *alaat* to hypoxia
- Characterization of the response of *alaat* to abiotic stresses which affect mitochondrial respiration.
- Characterization of the response of *alaat* to nitrogen deficiency and relief from nitrogen deficiency.

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2. Characterization of hypoxically inducible lactate dehydrogenase from maize roots[1]

2.1 *Introduction*

Hypoxic stress, mediated by water excess, results in a variety of molecular, cellular and physiological changes. An organism's survival during low oxygen conditions is determined by its ability to maintain sufficient energy for metabolism and is associated with production of ethanol, lactate, malate, succinate and alanine (reviewed in Ricard et al., 1994). Several hypoxically induced enzymes involved in generating some of these products have been identified, including ADH (Freeling, 1973; Good and Crosby, 1989) and LDH (Hanson and Jacobsen, 1984; Hoffman et al., 1986; Good and Crosby, 1989) (Tables 1-2 and 1-3).

LDH activity is thought to be involved in the short term (<8 h) hypoxic response. The pH stat hypothesis (Davies et al., 1974; Roberts et al., 1984a) proposes that lactate is initially produced from pyruvate, resulting in a lowered pH_c. This results in inhibition of LDH activity and the activation of PDC, triggering ethanolic fermentation. Support for this hypothesis is based on comparisons of pH_c with ethanol production, on the reduced survival of *adh1* null mutants which are dependent on lactic fermentation for their energetic needs (Roberts et al., 1984b) and on transgenic tomato plants overexpressing barley *ldh* (Rivoal and Hanson, 1994).

Several observations have been made which question the role of LDH in mediating the switch from lactate to ethanol production. Menegus et al. (1989) observed that pH_c alkalization is detectable after 8 h of hypoxia in intact maize seedlings. Saint-Ges et al. (1991) observed that, in excised maize root tips, pH_c decreases in parallel with decreasing nucleotide triphosphate (NTP) levels contrary to the pH-stat hypothesis. NMR studies have also shown that three different acid producing fermentations occur within the first 15 min of hypoxia (Table 2-1) (Roberts et al., 1992), thus the activity of enzymes other than LDH may have a role in pH_c acidification.

Table 2-1: Early acid-producing fermentations

Glucose	→	Lactate
Glucose + Glutamine	→	Alanine + Glutamate
Glucose + Aspartate	→	Alanine + Malate + Succinate

From Roberts et al., 1992

[1] A version of this chapter has been published. Christopher ME and Good AG (1996) Plant Physiol 112: 1015-1022

In addition to the proposed role of LDH in the short term response, a role for LDH during long term (>8 h) hypoxia is suggested by the observation that LDH activity remains elevated for at least 6 d in barley, wheat, maize and rye roots (Hoffman et al., 1986; Good and Crosby, 1989). In barley, *ldh* transcript levels increase after 2 d of hypoxia and start to decrease after 4 d of hypoxia (Hondred and Hanson, 1990) whereas LDH activity continues to increase for at least 14 d (Hoffman et al., 1986).

In nature, the transition from normoxia to hypoxia is slow (24 to 48 h) depending on the soil temperature and the respiratory activities of roots and microorganisms (Drew, 1990). This allows the plants to adapt physiologically to the declining oxygen levels. The majority of experiments on maize have been performed under anoxic conditions during which LDH activity is suppressed (Hanson and Jacobsen, 1984; Taylor et. al., 1994) and aerenchyma formation is inhibited (He et al., 1994). Consequently, the experimental protocols used throughout this thesis utilized young maize plants in which the roots were subjected to hypoxic stress with the shoots and leaves remaining in ambient air. This simulates conditions similar to those encountered by maize plants grown in the field, insofar as only the roots are submerged. Field grown plants experience a number of simultaneous stresses and thus results cannot be directly compared to plants grown hydroponically or in soil under controlled conditions (Robertson et al., 1994).

It has been shown that *ldh* transcript levels in barley increase during hypoxia (Hondred and Hanson, 1990; Taylor et al., 1994). The ARE required for hypoxic induction of maize *adh1* (Walker et al., 1987) has also been found in the maize *ldh* promoter (Good and Paetkau, 1992). This suggests that *ldh* and *adh* may show similar induction profiles. The hypothesis being tested in this chapter is that maize *ldh* induction is similar to that of other ANPs.

To date, most of the analysis of *ldh* in monocots has been done using barley (Hanson and Jacobsen, 1984; Hoffman et al., 1986; Good and Crosby, 1989; Hondred and Hanson, 1990). The characterization of the hypoxic response of maize *ldh* is important for several reasons.

- since most of the *adh* work has been done on maize (Freeling, 1973; Walker et al., 1987; Paul and Ferl, 1991), the comparison between maize *adh* and maize *ldh* is the most relevant; and
- in contrast to maize *adh* which is highly expressed in developing seeds (Good and Crosby, 1989; Peschke and Sachs, 1994) and in anoxic and hypoxic roots, *ldh* has only been shown to be inducible by hypoxia in roots.

2.2 *Materials and methods*

2.2.1 Plant materials and conditions

Zea mays inbred lines, Crusader (Stokes Seeds, St. Catharines, Ontario) and B73 (U.S. Department of Agriculture, Ames, IA), were germinated in vermiculite. Ten-d-old plants were transferred to hydroponic tanks filled with 0.5X Hoagland's solution (Table 2-2) and grown at 20°C for 2 weeks (12-/12-h light/dark cycle, 200 $\mu E\ s^{-1}\ cm^{-1}$). Barley (*Hordeum vulgare* cv Leduc) was grown in vermiculite for 5 d prior to transfer to hydroponic conditions. Maize line B73 was selected as it had been used previously in studies of hypoxia / anoxia (Russell and Sachs, 1992; Fennoy and Bailey-Serres, 1995; Xia et al., 1995), but due to difficulty in obtaining sufficient seed for analysis, the line Crusader was used instead. Both lines had similar responses to hypoxia. The barley cultivar Leduc was used as it had been used previously in the study of hypoxia (Muench and Good, 1994).

Hydroponic tanks were 20 L fish tanks, painted black to maintain dark conditions for root growth. The tanks were covered with a plexiglass lid containing 18 holes. The plants were placed in holes in foam plugs, which were then placed in the plexiglass lid such that the leaves remained in ambient conditions and the roots were immersed (Figure). Tanks were sparged continuously with air until induction, at which time the plants were transferred to tanks sparged with 100% nitrogen. Oxygen concentration in the hydroponic tanks was monitored with an oxygen meter (YSI Instruments, Yellow Springs, OH). Dissolved oxygen concentrations were 9.1 and 1.0 mg L^{-1} for aerobic and hypoxic hydroponic tanks, respectively.

Figure 2-1: Hydroponic tank set up

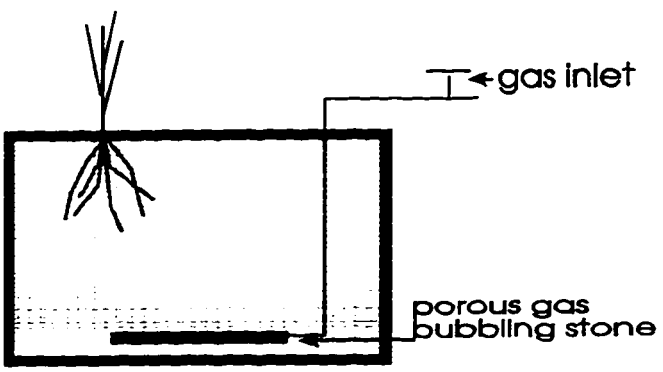


Table 2-2: Hoagland's nutrient solution for hydroponic culture

Salt	Hoagland's 20X Stock	
	Regular	Nitrogen free
$Ca(NO_3)\cdot 4H_2O$	18.8 g L^{-1}	
$CaCl_2$		11.76 g L^{-1}
$MgSO_4\cdot 7H_2O$	10.4 g L^{-1}	10.4 g L^{-1}
KNO_3	13.2 g L^{-1}	
KCl		8.95 g L^{-1}
$NH_4H_2PO_4$	2.4 g L^{-1}	
NaH_2PO_4		3.12 g L^{-1}
Fe EDTA solution	20 mL L^{-1}	20 mL L^{-1}
Na_2EDTA (33.3 g L^{-1})		
NaOH (4 g L^{-1})		
$FeCl_2\cdot 4H_2O$ (17.8 g L^{-1})		
HCl (0.15 g L^{-1})		
Micronutrient solution	2 mL L^{-1}	2 mL L^{-1}
H_3BO_3 (2.86 g L^{-1})		
$MnCl_2\cdot 4H_2O$ (1.81 g L^{-1})		
$ZnSO_4\cdot 7H_2O$ (0.222 g L^{-1})		
Na_2MoO_3 (0.018 g L^{-1})		
$CuSO_4\cdot 5H_2O$ (0.079 g L^{-1})		

2.2.2 Maize cell cultures

The maize BMS (Black Mexican Sweet) cell culture line was analyzed as it had been used previously in the analysis of the maize *adh1* promoter (Walker et al., 1987; Olive et al., 1990). BMS cell cultures were maintained in a media containing 4.3 g L^{-1} MS basal salt media (Sigma), 2 mg L^{-1} 2,4-D, 5 mg L^{-1} thiamine, 20 g L^{-1} sucrose, 0.15 g L^{-1} asparagine, pH 5.8. Four-d-old cultures were transferred to induction flasks as previously described (Good et al., 1990) and air was bubbled through for 24 h prior to exposure to either 100% N_2 or 98% N_2, 2% O_2. At various intervals 5 mL of culture was withdrawn for enzyme analysis. Cells were centrifuged for 5 min at 4,000 rpm, weighed, ground in 5 volumes of extraction buffer (Section 2.2.3), sonicated at 4°C for 1 min to lyse the cells, centrifuged for 3 min at 8,000 rpm and then assayed for enzyme activity and protein concentration.

2.2.3 Assay of LDH activity

Roots were rinsed, blotted, weighed, and ground with sand in a mortar and pestle in ice-cold extraction buffer containing 0.1 M Tris-HCl,

pH 8.5, 10 mM sodium borate, 10 mM DTT and 15% (v/v) glycerol. After centrifugation for 3 min, a small aliquot was removed for protein determination. Bovine serum albumin (BSA) was added to the remainder of the supernatant to a final concentration of 5 mg mL^{-1}. For results reported as µmol min^{-1} g^{-1} fresh weight (FW), roots were ground in an extraction buffer containing BSA.

LDH assays were performed in the pyruvate to lactate direction (Equation 2-1) (Hoffman et al., 1986). Enzyme activity was measured by following the decrease in absorbance (Ultrospec Plus, LKB) at 340 nm as NADH was oxidized to NAD^+. Activity was measured at 22°C in a 1 mL volume containing 0.13 M Tris-HCl (pH 8.0), 1 optical density unit of NADH, 3 mM 4-methyl pyrazole, 15 mM Na pyruvate and 200 µL of root extract. 4-methyl pyrazole was added to inhibit the coupled reactions of PDC and ADH (Bouny and Saglio, 1996). Nonspecific dehydrogenase activity was measured by following the decrease in absorbance at 340 nm prior to the addition of the substrate (pyruvate). LDH activity was determined by subtracting nonspecific dehydrogenase activity from the activity observed following substrate addition (LDH plus nonspecific dehydrogenase activity). All enzyme activities were linear with respect to time. All activities are reported as µmol min^{-1} and all assays were done in duplicate immediately after extraction and the means calculated.

Equation 2-1: LDH reaction assayed

$$\text{pyruvate} + \text{reduced coenzyme I (NADH)} + H^+ \xrightarrow{\text{pH 8.0}} \text{L-lactate} + \text{oxidized coenzyme I } (NAD^+)$$

2.2.4 Assay of ADH activity

ADH assays were performed in the ethanol to acetaldehyde direction (Equation 2-2) (Hanson and Jacobsen, 1984). Enzyme activity was measured by following the increase in absorbance at 340 nm as NAD^+ was reduced to NADH. Activity was measured at 22°C in a 1 mL volume containing 0.1 M Tris-HCl (pH 9.0), 0.1 M ethanol, 1 mM NAD^+ and 50 µL of root extract or 100 µL of cell culture extract. All activities are

reported as μmol min^{-1} and all assays were done in duplicate and the means calculated.

Equation 2-2: ADH reaction assayed

ethanol + oxidized coenzyme 1 (NAD^+)
$\xrightarrow{pH\ 9.0}$
acetaldehyde + reduced coenzyme 1 (NADH) + H^+

2.2.5 Protein determination

Protein concentrations, determined by the Bradford assay (Bradford, 1976), were measured spectrophotometrically at 595 nm. BSA was used as a standard. This assay is based on a shift of the absorption maximum of Coomassie Brilliant Blue G-250 (Bio-Rad) from 465 nm to 595 nm, upon binding of the dye to protein.

2.2.6 Determination of LDH stability

The stability of LDH was determined by assaying LDH activity in root extracts prepared from 0- or 72-h hypoxically-induced maize roots, immediately after preparation and after incubation of the extract for various times on ice. The $T_{1/2}$ of LDH activity was determined graphically by plotting the percentage of the original LDH activity remaining in the sample against the incubation time.

2.2.7 Effect of protease inhibitors on LDH stability

Protease inhibitors (Table 2-3) were added to the extract immediately after grinding to a final concentration of 1 mM PMSF, 1 μg mL^{-1} pepstatin A, 2 μg mL^{-1} leupeptin and 2 μg mL^{-1} aprotinin or added directly to the extraction buffer just prior to grinding the root tissue. No difference was seen in the results obtained between the two methods of sample preparation.

Table 2-3: Properties of protease inhibitors

Inhibitor	Active against	Inactive against	Effective concentration	Stock solution
Aprotinin	kallikrein trypsin chymotrypsin plasmin	papain	1-2 μg mL^{-1}	10 mg mL^{-1} in 0.01 M HEPES (pH 8.0)
Leupeptin	plasmin trypsin papain cathepsin B	chymotrypsin pepsin cathepsins A and D	1-2 μg mL^{-1}	10 mg mL^{-1} in water
Pepstatin A	pepsin cathepsin D	trypsin plasmin chymotrypsin elastase thermolysin	1 μg mL^{-1}	1 mg mL^{-1} in ethanol
PMSF (phenyl-methyl-sulfonyl fluoride)	chymotrypsin trypsin		100 μg mL^{-1}	1.74 mg mL^{-1} (10 mM) in isopropanol

From Sambrook et al., 1989

2.2.8 Inhibitors of LDH stability

LDH activity from aerobic maize root extracts was more stable over the time course tested than LDH activity from hypoxic maize roots. To determine if aerobic roots contained some factor that would increase the stability of LDH from hypoxic root extracts, mixing experiments were done. Aerobic root extracts were mixed with hypoxic root extracts in a 1:1 and 2:1 ratio, and tested for LDH activity immediately and after incubation on ice for 20, 60, 120 or 240 min, in parallel with the aerobic and hypoxic root extracts. Root samples from three separate plants were assayed in each experiment (n=3) and the experiment was replicated three times.

To determine if hypoxic maize root extracts contained a destabilizing factor, 72-h hypoxically-induced root extracts (untreated or boiled for 3 min) were incubated with either rabbit LDH (Sigma) or partially purified barley LDH (Section 2.2.9). Recovery of the LDH spike was determined after incubation of the LDH spike with root extracts for various times. The dilution control had a volume of extraction buffer, equivalent to the volume of the LDH spike, added to the root extract. The LDH activity in this

control was subtracted from that of the test to ascertain the LDH activity of the spike. The spike activity retained was compared to the spike control (LDH spike was added to a volume of extraction buffer equal to the volume of root extract in the test samples).

An attempt to purify the putative inhibitor was made. Root extracts were passed through an FPLC Superose-6 gel filtration column (Pharmacia), and proteins were eluted with a 0.1 M Tris-HCl (pH 8.0), 0.15 M NaCl and 14.5 mM β-mercaptoethanol. Fractions were incubated with rabbit LDH (Sigma) and tested for recovery of the spike immediately and after 40 min incubation on ice.

2.2.9 Purification of maize LDH

Purification of maize LDH was attempted in order to generate a maize LDH specific antibody of high titre and for use in LDH inhibitor assays. Twenty grams of 72-h hypoxically-induced maize roots were ground in extraction buffer (Table 2-4) (Hoffman and Hanson, 1986). Extracts were centrifuged for 5 min at 4°C at 12,000 rpm and filtered through Whatman #4 paper into a pre-chilled flask. The extract was loaded onto a Cibacron Blue 3GA-agarose (Sigma, C-1285) FPLC column (Pharmacia) previously equilibrated with equilibration buffer (Table 2-4). The column was washed with 200 mL of wash buffer at a flow rate of 2 mL min^{-1}. Bound dehydrogenases were eluted with elution buffer at a flow rate of 1.5 mL min^{-1}. Fractions (4.5 mL) were collected and assayed for LDH enzyme activity; however, enzyme activity was not detected in any fraction.

Barley LDH was partially purified so that a plant source of LDH could be used in the inhibitor assays. Following the Cibacron Blue 3GA column, fractions possessing LDH activity were pooled and loaded onto a mono-Q column equilibrated with mono-Q wash buffer (Table 2-4). The column was washed with 25 mL mono-Q wash buffer at a flow rate of 0.5 mL min^{-1} and bound proteins eluted with a 0 to 0.5 M KCl gradient at the same flow rate. Fractions (1 mL) were collected and assayed for LDH activity.

Table 2-4: Buffer Composition

Buffer		Composition
Extraction buffer		0.1 M Tris-acetate (pH 8.8) 10 mM Na borate 5 mM DTT 2 mM EDTA 10% (v/v) glycerol
Cibacron Blue 3GA-agarose		
	Equilibration buffer	40 mM Tris-acetate (pH 8.8) 2 mM EDTA 0.5 mM DTT
	Wash buffer	40 mM Tris-acetate (pH 7.9) 0.3 M KCl 2 mM EDTA 0.5 mM DTT
	Elution buffer	40 mM Tris-acetate (pH 7.9) 0.15 M KCl 2 mM EDTA 0.5 mM DTT 0.5 mg mL^{-1} NADH
Mono-Q		
	Wash buffer	50 mM Tris-HCl (pH 8.0) 1 mM DTT
	Elution buffers	50 mM Tris-HCl (pH 8.0) 1 mM DTT 0 and 0.5 M KCl

2.2.10 Preparation of a LDH synthetic peptide

The amino acid sequence of maize LDH1, determined by translation of the nucleic acid sequence (Good and Paetkau, 1992), was computer analyzed to determine surface regions that could be used to generate a synthetic peptide (underlined in Figure 2-2). The synthetic peptide, Val-Ser-Gly-Asp-Ser-Ser-Thr-Pro-Thr-Ser (bold in Figure 2-2), conjugated to both BSA and Keyhole Limpet Hemocyanin (KLH) was synthesized by the Alberta Peptide Institute, Edmonton, AB. This peptide was selected on advice of the Alberta Peptide Institute as it lacked the amino acids His, Cys, Trp and Met. Additionally, amino acid identity between the maize and rabbit LDHs was lower in this region. The lyophilized peptide was reconstituted in sterile phosphate buffered saline.

Figure 2-2: Maize LDH1 amino acid sequence

MET	LYS	LYS	ALA	THR	SER	LEU	SER	GLU	LEU	GLY	PHE	ASP	ALA	GLY	15
ASP	ALA	SER	SER	GLY	PHE	PHE	ARG	PRO	VAL	SER	GLY	ASP	SER	SER	30
THR	PRO	THR	SER	GLN	HIS	HIS	ARG	ARG	ARG	LEU	THR	LYS	VAL	SER	45
VAL	ILE	GLY	ALA	GLY	ASN	VAL	GLY	MET	ALA	ILE	ALA	GLN	THR	ILE	60
LEU	THR	ARG	ASP	LEU	ALA	ASP	GLU	ILE	ALA	LEU	VAL	ASP	ALA	VAL	75
PRO	ASP	LYS	LEU	ARG	GLY	GLU	MET	LEU	ASP	LEU	GLN	HIS	ALA	ALA	90
ALA	PHE	LEU	PRO	ARG	THR	ARG	LEU	VAL	SER	GLY	THR	ASP	MET	SER	105
VAL	THR	ARG	GLY	SER	ASP	LEU	VAL	ILE	VAL	THR	ALA*	GLY*	ALA*	ARG*	120
GLN*	ILE*	[redacted]	GLY*	GLU*	THR*	ARG*	LEU*	[redacted]	LEU	LEU	GLN	ARG	ASN	VAL	135
ALA	LEU	PHE	ARG	LYS	ILE	VAL	PRO	PRO	LEU	ALA	GLU	GLN	SER	HIS	150
ASP	ALA	LEU	LEU	LEU	VAL	VAL	SER	ASN	PRO	VAL	ASP	VAL	LEU	THR	165
TYR	VAL	ALA	TRP	LYS	LEU	SER	GLY	PHE	PRO	ALA	SER	ARG	VAL	ILE	180
GLY	SER	GLY	THR	ASN	LEU	[redacted]	SER	SER	ARG	PHE	ARG	PHE	LEU	LEU	195
ALA	GLU	HIS	LEU	ASP	VAL	ASN	ALA	GLN	ASP	VAL	GLN	ALA	TYR	MET	210
VAL	GLY	GLU	[redacted]	GLY	[redacted]	SER	SER	VAL	ALA	VAL	TRP	SER	SER	VAL	225
SER	VAL	ALA	GLY	MET	PRO	VAL	LEU	LYS	SER	LEU	GLN	GLU	SER	HIS	240
ARG	CYS	PHE	ASP	GLU	GLU	ALA	LEU	GLU	GLY	ILE	ARG	ARG	ALA	VAL	255
VAL	ASP	SER	ALA	TYR	GLU	VAL	ILE	SER	LEU	LYS	GLY	TYR	[redacted]	SER	270
TRP	ALA	ILE	GLY	TYR	SER	VAL	ALA	SER	LEU	ALA	ALA	SER	LEU	LEU	285
ARG	ASP	GLN	ARG	ARG	ILE	HIS	PRO	VAL	SER	VAL	LEU	ALA	ARG	GLY	300
PHE	HIS	GLY	ILE	PRO	ASP	GLY	THR	THR	SER	SER	SER	ALA	CYS	PRO	315
PRO	ARG	ARG	PRO	ARG	ARG	ARG	PRO	GLY	ARG	ARG	GLU	MET	GLU	LEU	330
THR	GLU	GLU	GLU	ALA	LYS	ARG	LEU	ARG	ARG	SER	ALA	LYS	THR	ILE	345
TRP	GLU	ASN	CYS	GLN	LEU	LEU	GLY	LEU							360

From Good and Paetkau, 1992

The amino acids in bold (25 to 34) were selected for preparation of the synthetic LDH peptide. The underlined amino acids are those predicted to be surface amino acids. Amino acids directly involved in catalysis (Hondred and Hanson, 1990) are striped. Amino acids in the active site loop are starred and those that are absolutely conserved across all species (Wigley et al., 1992) are shaded.

2.2.11 Preparation of antiserum against the LDH peptide

The KLH-peptide conjugate (500 μg mL^{-1} in phosphate buffered saline) was mixed with an equal volume of Freund's complete adjuvant and mixed to form a thick emulsion. Two rabbits were injected; one was injected with 0.5 mL of the mixture at one intramuscular site and the other was injected with 0.25 mL at four subcutaneous sites. Three and six weeks later the rabbits were similarly injected but this time the KLH-peptide conjugate was mixed with Freund's incomplete adjuvant. The rabbits were bled and tested for antibody production.

2.2.12 LDH activity gels

Nondenaturing gel electrophoresis was performed using 7% (w/v) acrylamide slab gels as previously described (Muench and Good, 1994). Thirty-five micrograms of protein was loaded, and gels were run at 15 milliamps at 4°C for 12 h and stained for LDH activity (Hanson and Jacobsen, 1984). The gel was immersed in 100 mL of 0.15 M Tris-HCl (pH 8.0) buffer containing 10 g L^{-1} lithium L(+) lactate, 1 g L^{-1} NAD^+, 0.2 g L^{-1} $MgCl_2$, 0.1 g L^{-1} nitroblue tetrazolium chloride, 25 mg L^{-1} phenazine methosulphate and 0.1 M 4-methylpyrazole and incubated at 37°C until LDH catalyzed the reduction of the tetrazolium salt to the blue coloured formazan which was visibly detectable. Phenazine methosulphate was present to act as an intermediate electron carrier between NADH and nitroblue tetrazolium chloride.

2.2.13 LDH western blotting

Fifteen micrograms of protein was loaded onto 10% (w/v) SDS-acrylamide mini-gels (Bio-Rad) and subjected to electrophoresis in 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% (w/v) SDS. Gels were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) at 4°C according to the manufacturer's specifications (Trans-Blot transfer cell, Bio-Rad). Transfer efficiency was determined by staining the membrane with 2% Ponceau S in 30% (v/v) trichloroacetic acid (TCA), 30% (v/v) sulfosalicylic acid. Antibody staining was essentially as described previously (Good and Crosby, 1989) using a 1:600 dilution of anti-barley LDH antibody, a gift of Andrew Hanson (Hondred and Hanson, 1990). This antibody has been shown to react with barley (Hondred and Hanson, 1990), rice (Rivoal et al., 1991) and maize LDH isozymes and subunits in both their native and denatured states. The antibody generated against the KLH-LDH synthetic peptide conjugate reacted with the KLH-LDH peptide conjugate and not with the BSA-LDH peptide conjugate on Western blots. Thus the antibody appeared to be recognizing the KLH protein and not the LDH synthetic peptide and therefore was not useful.

2.2.14 Isolation of mRNA

Total RNA was isolated from 1.5 to 3 g of aerobic or hypoxically-induced maize root tissue according to the protocol of Yeh et al., 1990. Roots were washed, blotted dry and ground to a fine powder in liquid N_2.

The powder was homogenized in twice the volume of guanidinium buffer (7.5 M guanidinium-HCl, 25 mM Na citrate, 0.5% (w/v) lauryl sarcosinate, 0.1 M β-mercaptoethanol), centrifuged for 10 min at 12,000 g at 4°C, filtered through miracloth and extracted sequentially with an equal volume of phenol / chloroform / isoamylalcohol (25:24:1) and chloroform / isoamylalcohol. The aqueous phase was mixed with an equal volume of cold isopropanol and 0.1 volumes of 3 M Na acetate (pH 5.2) and total RNA was precipitated by overnight incubation at -80°C. The RNA was pelleted by centrifugation at 13,000 rpm at 4°C for 45 min, and the pellet was resuspended in 500 µL of sterile water.

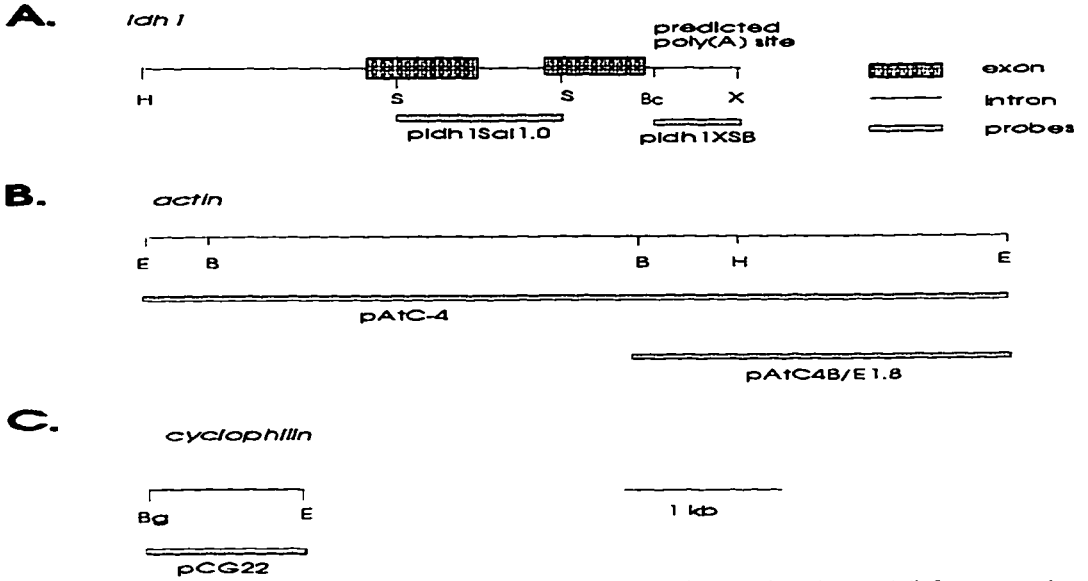
Poly(A)$^+$ RNA was subsequently isolated using an mRNA isolation kit according to the manufacturers protocol (PolyATract kit, Promega) and the concentration determined spectrophotometrically.

2.2.15 Hybridization probes

Hybridization probes (Figure 2-3) included two maize *Idh1* genomic clones (Good and Paetkau, 1992). Plasmid pIdh1Sal1.0 containing most of exon 1, intron 1 and part of exon 2 was used to analyze *Idh* expression. Plasmid pIdh1XSB containing the 3' UTR was used to confirm *Idh1* identity. Plasmids pAtC4B/E1.8, an *Arabidopsis thaliana actin* genomic clone derived from pAtC4 (Nairn et al., 1988), and pCG22, an *Arabidopsis thaliana cyclophilin* clone (Gasser et al., 1990) were used to assess RNA loading.

Double stranded DNA fragments were recovered from agarose gels by adsorption to silica matrix (Boyle and Lew, 1995) following enzymatic digestion. The DNA fragments, excised in as small a piece of agarose gel as possible, were released from the gel matrix by dissolving the gel in twice its volume of 6 M NaI and incubating at 55°C until melted. Silica suspension (100 mg mL^{-1}, Sigma S-5631) was added and the DNA adsorbed for 5 min on ice prior to washing twice with 0.5 mL of cold washing buffer (50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, and 50% (v/v) ethanol). The DNA was eluted in one pellet volume of water by incubating for 3 min at 55°C followed by centrifugation for 30 sec. The elution was repeated and both aliquots were pooled. The fragments recovered were radiolabelled using the random primer method. DNA (100 ng) in 0.2 M Hepes (pH 6.6), 0.004 OD U random primers, 0.05 mM Tris-HCl (pH 7.5), 0.05 mM EDTA, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 mM dNTPs, 50 µCi (^{32}P)dCTP (>3000 mmol µL^{-1}), 10 µg BSA, 5 U Klenow was incubated 5 h at room temperature after denaturation by boiling. Unincorporated nucleotides were removed by passage of the reaction mix through Nick columns (Pharmacia).

Figure 2-3: Hybridization probes



Partial restriction maps of northern hybridization probes. (A) Maize *Idh1* genomic clone (Good and Paetkau, 1992), (B) *Arabidopsis thaliana actin* genomic clone (Nairn et al., 1988), and (C) *Arabidopsis thaliana cyclophilin* genomic clone (Gasser et al., 1990). Restriction sites indicated: B = BamHI, Bc = BclI, Bg = BglII, E = EcoRI, H = HindIII, S = SalI, X = XbaI.

2.2.16 Northern analysis

One microgram of poly(A)$^+$ RNA was denatured in a denaturation buffer (1X MOPS, 50% (w/v) formamide, 2.2 M formaldehyde), run on a 1.2% formaldehyde agarose gel (1X MOPS, 2.2 M formaldehyde) at 90 volts for 3 to 4 h and blotted onto a nylon membrane (GeneScreen Plus, DuPont) by the capillary method. Hybridization was in 50% (w/v) formamide, 5X SSPE, 5X Denhardt solution, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 100 μg mL^{-1} sonicated salmon sperm DNA. Blots were hybridized at 42°C for 16 h and washed twice in 2X SSPE at room temperature for 15 min each, and then washed twice in 2X SSPE, 2% (w/v) SDS at 65°C for 45 min each. Membranes were exposed to autoradiographic film at -80°C for 24 to 72 h. Equality of RNA loading on poly(A)$^+$ northerns was assessed by reprobing the northerns with either an actin or cyclophilin cDNA clone (Figure 2-3). Blots probed sequentially were washed with 0.1X SSPE, 1% (w/v) SDS at 80°C for 20 min, followed by autoradiographic exposure at -80°C to confirm probe removal prior to reprobing.

2.2.17 Densitometric analysis

Western and northern blots were scanned and analyzed by densitometry (model GS 670 scanning densitometer, Bio-Rad).

2.3 *Results*

Hypoxically treated maize plants did not appear morphologically different from aerobically maintained control plants. In some experiments, the root of hypoxically treated plants developed reddish-brown deposits. These plaques can most likely be attributed to deposits of ferric hydroxide (Ernst, 1990). Aerenchyma formation and high root porosity in hypoxic plants leads to radial oxygen loss thus oxidizing ferrous iron (Fe^{2+}) to the relatively insoluble ferric form (Fe^{3+}).

2.3.1 Stability of maize LDH

Hoffman et al. (1986) determined that barley LDH was stable *in vitro* for at least 4 h in the presence of Tris-HCl, DTT, BSA and sodium borate. For this reason barley plants were induced in parallel with maize plants to control for any variance in LDH induction and assay conditions. Barley LDH activity in aerobic and hypoxically induced root extracts was stable over the 4 h time period tested, unlike maize, in which LDH activity was very unstable in hypoxically induced root extracts and, to a lesser extent, in aerobic root extracts (Table 2-5). BSA was essential for maize LDH stability *in vitro* ($T_{1/2}$ of 110 min when BSA was present compared with 30 min when BSA was absent). There was no significant difference in LDH stability when roots were ground in an extraction buffer containing BSA or when BSA was added immediately after grinding (Table 2-5 lines 3 to 6). These results suggest that phenolics alone may not mediate the decline in *in vitro* LDH stability. BSA confers protection against phenolics, compounds possessing protein cross-linking, anti-fungal and anti-bacterial activities (Salisbury and Ross, 1985). Phenolics are derived from phosphoenol pyruvic acid and erythrose-4-phosphate, components generated by glycolysis and either the pentose phosphate pathway or the Calvin cycle, respectively.

To increase the stability of LDH *in vitro*, protease inhibitors were utilized. When root extracts were treated with PMSF, aprotinin, leupeptin, or pepstatin A, minimal or no effect was seen on the stability of LDH

(Table 2-5) suggesting that protease activity was not the most likely cause of maize LDH instability.

Table 2-5: Effect of BSA and protease inhibitors on LDH activity in barley and maize roots

Plant	Relative LDH Activity at Time					$T_{1/2}$
	5 min	20 min	1 h	2 h	4 h	min
Barley						
Aerobic	100	83.3 (9.3)	85.2 (11.2)	86.2 (9.8)	101.3 (4.6)	
Hypoxic	100	99.1 (6.8)	100.2 (5.6)	110.3 (2.9)	110.4 (6.1)	
Maize						
Aerobic (a)	100	87.4 (7.1)	76.3 (4.2)	74.1 (4.3)	59.9 (1.6)	>240
Aerobic (b)	100	88.3 (4.2)	74.3 (5.9)	66.7 (4.8)	46.3 (3.8)	210 (30)
Hypoxic (a)	100	73.5 (2.8)	59.0 (2.8)	47.3 (2.2)	36.2 (2.8)	110 (10)
Hypoxic (b)	100	78.7 (0.7)	56.3 (2.3)	43.0 (2.3)	36.7 (2.2)	90 (15)
–BSA (b)	100	58.8 (9.3)	30.9 (5.6)	18.2 (8.6)	15.0 (4.6)	30 (10)
+PMSF (b)	100	81.7 (2.8)	62.7 (4.6)	48.7 (6.1)	39.7 (1.8)	100 (10)
+Leupeptin (b)	100	76.4 (4.4)	51.2 (3.8)	42.5 (1.6)	32.7 (1.1)	100 (40)
+Aprotinin (b)	100	66.4 (3.4)	44.6 (2.6)	37.7 (1.8)	28.8 (4.2)	55 (5)
+Pepstatin A (b)	100	72.7 (6.2)	59.3 (3.4)	51.7 (4.5)	39.3 (1.9)	100 (30)

Aerobic and 72-h hypoxically-induced maize and barley root extracts with the addition of PMSF, leupeptin, aprotinin, or pepstatin A (where indicated) were assayed immediately and then kept on ice for 20 min, or for 1, 2, or 4 h prior to re-assaying. BSA was present during grinding of roots in extraction buffer (a) or added immediately after grinding (b). LDH activity in hypoxic root extracts was ~3 times that of LDH activity in aerobic root extracts. Each value is the mean of at least two separate experiments (+SE). $n > 5$ for all samples tested.

In aerobic conditions, when LDH activity was low, the *in vitro* $T_{1/2}$ of LDH stability was approximately 4 h. LDH activity increased 2-fold (Section 2.3.2) in maize plants hypoxically induced for 72 h. At these elevated levels, the *in vitro* $T_{1/2}$ of LDH stability was reduced to 110 min, approximately 60% less stable than the enzyme present under aerobic conditions. The decline in *in vitro* LDH stability was rapid, with a 25% decline in stability within the first 20 min. A buffer containing sodium ascorbate, NAD^+, EDTA, DTT, and glycerol as protective agents was used for LDH measurements in *Oryza sativa* (Rivoal et al., 1991); however, maize LDH activity was extremely low in this buffer. Roots from the same plant, divided in half, were ground in this buffer and in the standard extraction buffer. LDH activity of 72 h hypoxic roots ground in the ascorbate buffer

was 22% of that ground in the standard extraction buffer. Consequently, all LDH assays were analyzed immediately after extraction in the standard extraction buffer.

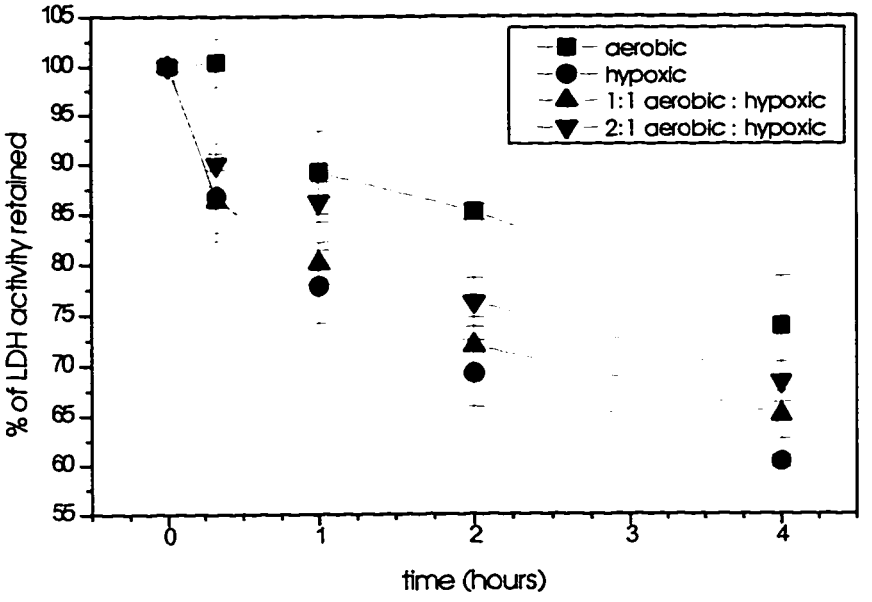
To determine if aerobic root extracts from maize possessed some factor which stabilized LDH, mixing experiments were performed. In the mixing experiments, at all time points, the observed LDH activity was 98 to 100% of that predicted from the activities of LDH in aerobic and 72 h hypoxic root extracts alone (Table 2-6). The addition of aerobic root extracts to hypoxic root extracts did not prevent the immediate decline in LDH activity observed with hypoxic root extracts alone (20 min time-point; Figure 2-4). Longer incubation of the aerobic / hypoxic extract mixtures on ice resulted in a slightly increased recovery of LDH activity relative to the hypoxic extracts alone, but the stability of the enzyme was not restored to that of the aerobic enzyme. This was similar to the results observed with barley root extracts. When aerobic and hypoxic barley root extracts were mixed 1:1, the activities observed were 89 to 100% of that predicted from each tissue alone (Hoffman et al., 1986). Furthermore, addition of aerobic root extracts to hypoxic root extracts did not result in destabilization of the induced LDH activity during 2 h of storage on ice (Hoffman et al., 1986).

Table 2-6: Percentage of expected LDH activity recovered in mixing experiments

Ratio aerobic:hypoxic	Time of incubation on ice				
	5 min	20 min	1 h	2 h	4 h
1:1	101.3 ± 0.9	97.8 ± 1.6	100.4 ± 1.8	99.1 ± 1.2	102.1 ± 1.7
2:1	101.1 ± 1.5	99.6 ± 1.7	105.9 ± 3.0	104.3 ± 1.7	105.6 ± 2.3

Root extracts from aerobic and 72-h hypoxically-induced plants were mixed, LDH activity determined and then the samples were incubated on ice for 20, 60, 120 or 240 min prior to reassaying. The activities of the aerobic and hypoxic root samples, determined at all timepoints, were used to predict the contribution that each extract had on the LDH activity of the mixed samples. Actual activity detected is reported as a percentage of that expected (n=3). The experiment was replicated three times.

Figure 2-4: Effect of mixing aerobic root extracts with hypoxic root extracts on recovery of LDH activity



Aerobic, 72-h hypoxic, 1:1 aerobic:hypoxic mixtures and 2:1 aerobic:hypoxic mixtures of maize root extracts were tested for LDH activity immediately and after incubation on ice for 20, 60, 120, and 240 min (n=3). The experiment was replicated three times and the mean of all results determined (+SE).

To investigate the *in vitro* change in LDH $T_{1/2}$ further, maize extracts were spiked with either rabbit LDH or partially purified barley LDH (Figures 2-5 to 2-8; Table 2-7). No differences in results were observed with either the plant or animal source of LDH. Attempts to purify the induced form of maize LDH were unsuccessful due to its very short *in vitro* $T_{1/2}$.

Hypoxic induction increased the recovery of the barley LDH spike relative to that in aerobic root extracts (Table 2-8) suggesting that aerobic root extracts possessed a factor which inhibited or destabilized LDH activity. This idea was supported by the observation that a higher proportion of the spike was recovered from root extracts that had been either boiled or diluted prior to spike addition. However, if a heat labile inhibitor or destabilizer of LDH activity was present in aerobic root extracts, then the mixing experiments should have yielded LDH activities less than that predicted (Table 2-6). Also, attempts to purify this factor using a gel

filtration column were unsuccessful. Subsequently, the experiments which suggested the presence of an inhibitor / destabilizer in aerobic root extracts were repeated and none yielded any conclusive evidence of an inhibitor. In summation, all of the early experiments suggested the presence of an inhibitor / destabilizer of LDH activity in aerobic root extracts while none of the later experiments did. There were no obvious differences in the experimental parameters between the two groups of experiments. Possibly seasonal fluctuations or prolonged seed storage could account for this discrepancy.

Table 2-7: Purification of barley LDH

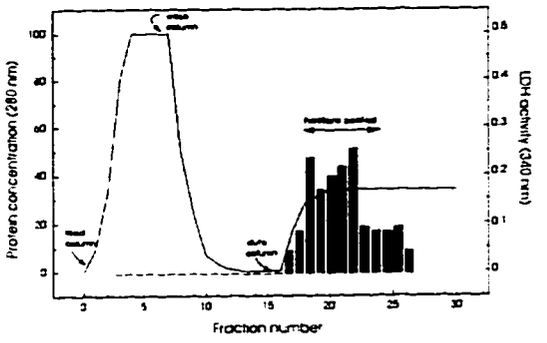
Sample	Fold purification	DH specific ctivity (I.U. g^{-1} rotein)	Total LDH activity (I.U.)	% recovery
neat	1	197.2	18170	100.0
cibacron blue	7.1	1401.5	3780	20.8
mono-Q	53.1	10462.5	2267	12.5

Table 2-8: Recovery of barley spike LDH spike from maize root extracts

	% of LDH spike activity recovered				
	Induction series			Dilution series	
h N_2	Crude	Boiled	Dilution	Crude	Boiled
0	59.3 + 3.2	96.4 + 1.1	0	37.1	95.3
24	78.9 + 4.2	90.4 + 1.8	1/2	63.7	72.4
48	74.0 + 6.9	93.7 + 5.2	1/4	86.1	87.2
			1/16	94.0	92.2
			1/64	84.8	99.5

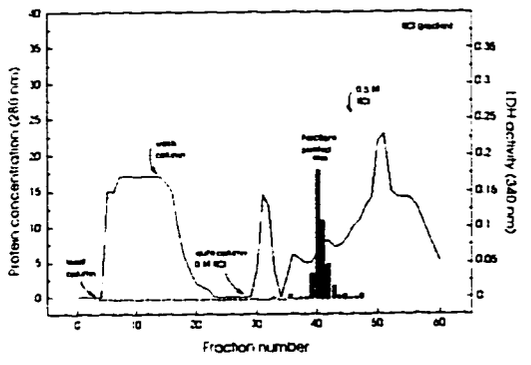
Aerobic, 24- and 48-h hypoxically-induced maize root extracts were spiked with partially purified barley LDH and tested for recovery of the spike after 20 min incubation on ice. Crude extracts were iced 3 min and boiled extracts were boiled 3 min and iced 3 min prior to spike addition. Dilution of the root extract was in extraction buffer containing 5-mg mL^{-1} BSA. Results from a representative experiment are shown (n=3; +SE). The induction series was replicated four times and the dilution series was replicated twice.

Figure 2-5: Barley LDH purification – Cibacron Blue



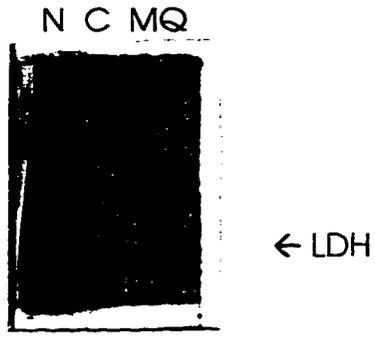
Root extracts were loaded onto a Cibacron Blue 3GA-agarose column. The column was washed with a Tris-acetate buffer, pH 7.9 and bound dehydrogenases eluted with a Tris-acetate-NADH buffer, pH 7.9. Fractions (4.5 mL) were collected and assayed for LDH activity. Fractions possessing LDH activity were pooled and loaded onto a Mono-Q column. The protein elution profile is indicated as a solid line and LDH activity as a bar graph. Fractions pooled are indicated.

Figure 2-6: Barley LDH purification – Mono-Q



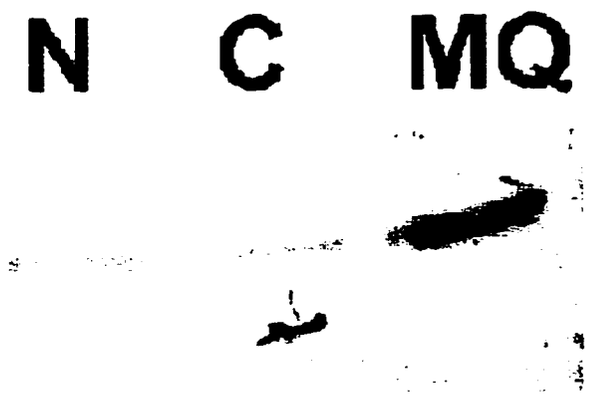
Cibacron Blue 3GA-agarose fractions possessing LDH activity were pooled and loaded onto a Mono-Q ion exchange column. Proteins were eluted with a 0 to 0.5 M KCl gradient and 1-mL fractions were assayed for LDH activity. The protein elution profile is indicated as a solid line, KCl gradient as a dotted line and LDH activity as a bar graph. Fractions pooled are indicated.

Figure 1-7: Barley LDH purification - purification profile



Silver stained SDS-PAGE of the partially purified barley LDH. 20 µg of neat (N), 0.5 µg of Cibacron Blue 3GA fraction (C), and 0.5 µg of Mono-Q fraction (MQ) were loaded onto 10% (w/v) acrylamide-SDS gels. The band of M_r ~ 40,000 predicted to be LDH is indicated.

Figure 2-8: Barley LDH purification – Western analysis



Western blot of the barley LDH purification process. Samples were loaded onto 10% (w/v) acrylamide-SDS gels as in Figure 2-7, electroblotted onto Nitrocellulose, probed sequentially with a 1:500 dilution of anti-barley LDH and 1:7500 dilution of goat anti-rabbit alkaline phosphatase conjugate followed by staining for alkaline phosphatase activity.

2.3.2 Hypoxic induction of maize LDH

In maize roots, ADH activity was assayed in parallel to LDH activity to confirm that the hydroponic treatment resulted in stimulation of the hypoxic response. ADH activity increased during hypoxic treatment (Figure 2-9) confirming that the plants were hypoxic.

LDH enzyme activity increased within 8 h of hypoxic induction (Figure 2-9). LDH activity increased 1.5 fold after 48 h of hypoxic induction. This is similar to the hypoxic induction observed in 3-d-old maize seedlings (Xia and Saglio, 1992) and rice (Rivoal et al., 1991), but less than that in barley (Hoffman et al., 1986; Good and Crosby, 1989). LDH activity continued to increase for at least 6 d, at which time roots from hypoxically treated plants had 3.5 times more LDH activity than roots from aerobically maintained plants. When plants were allowed to recover in aerobic conditions for 48 h after 48 h of exposure to hypoxia, LDH activity decreased to about 70% of the level found at 48 h. This is similar to barley, in which LDH activity (on a fresh weight basis) declined, with an apparent $T_{1/2}$ of 2 d, when aeration was restored (Hoffman et al., 1986).

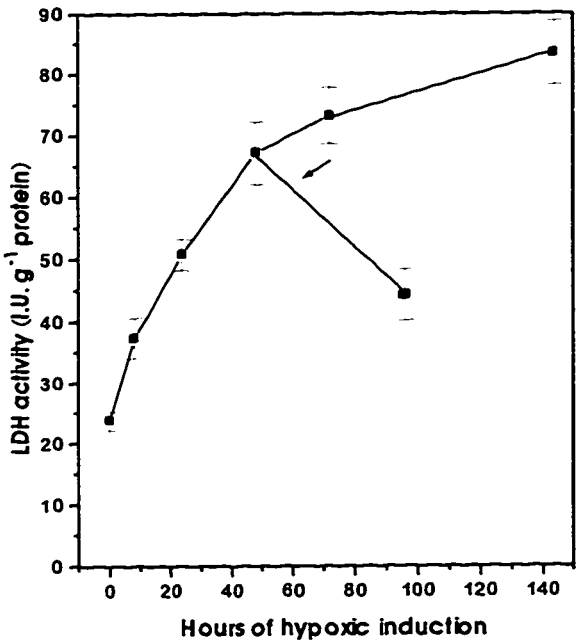
Maize cell cultures did not demonstrate an increase in LDH activity when cultures were transferred to conditions of 100% N_2 (anoxic) or 98% N_2, 2% O_2 (hypoxic) (Figure 2-10), whereas ADH activity increased in both conditions. Thus, cell culture transient assays, to analyze the maize *ldh1* promoter for sequence elements critical to the hypoxic response, would be of limited use.

2.3.3 Gel electrophoresis and immunological detection of the hypoxically inducible LDH

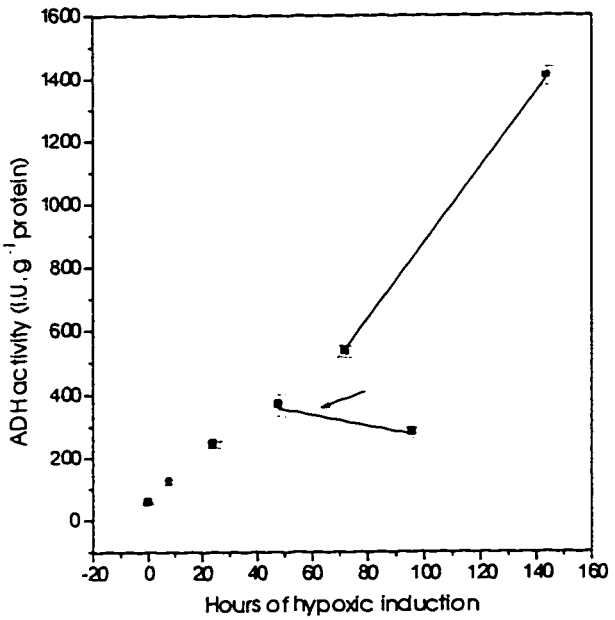
LDH activity in aerobically grown maize roots was very low. Five bands were detectable on LDH activity gels, consistent with the presence of two *ldh* genes whose protein subunits associate randomly to form a tetramer. All LDH isozymes increased during hypoxic induction (Figure 2-11). Barley LDH isozyme profiles are differentially affected by the degree of hypoxia (Hoffman et al., 1986). Exposure to 1% O_2 affected primarily the three central bands, whereas exposure to 0.3% O_2 affected the three most cathodal bands (Hoffman et al., 1986). In rice, the most basic isozymes are predominant in all conditions (Rivoal et al., 1991). It is interesting to note that, although LDH has a short *in vitro* $T_{1/2}$, it was detectable in native gels that had been subjected to electrophoresis for approximately 12 h.

Figure 2-9: Effect of hypoxia on LDH and ADH activity in maize roots

A.



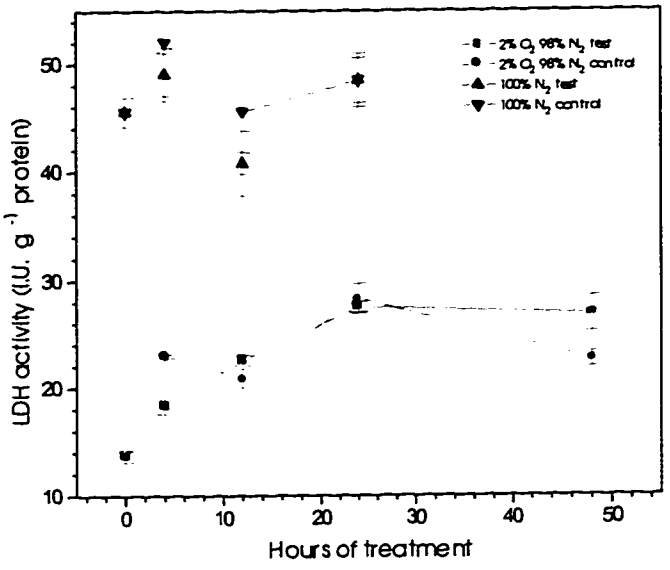
B.



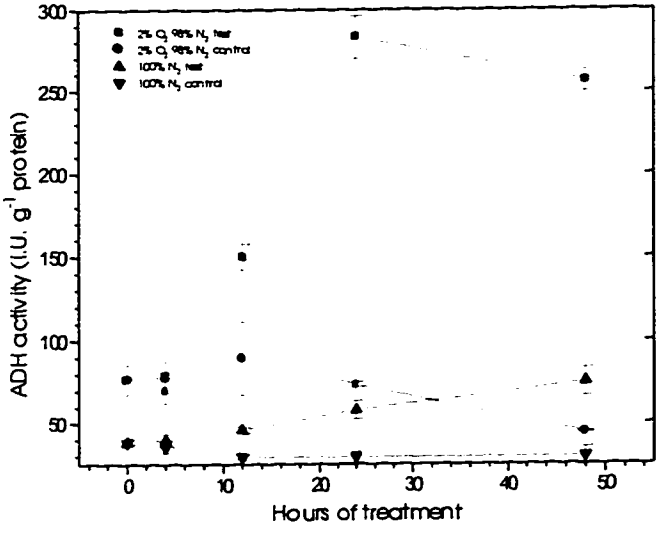
Effect of hypoxia on (A) LDH and (B) ADH activity in maize roots exposed to 0, 8, 24, 72, and 144 h of hypoxic stress, and from roots aerobically recovered for 48 h after 48 h of hypoxic treatment. Each time point is the mean of data obtained from a representative experiment (n=5; +SE). The experiment was replicated four times. LDH and ADH assays were performed immediately after extraction. The arrow indicates the return to aerobic conditions.

Figure 2-10: Effect of hypoxia and anoxia on LDH and ADH activity in maize cell cultures

A.

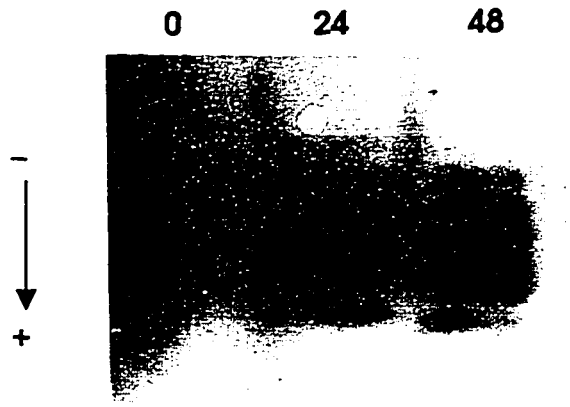


B.



Effect of hypoxia (2% O_2, 98% N_2) and anoxia (100% N_2) on (A) LDH and (B) ADH activity in maize suspension cultures. Logarithmically growing cells were bubbled with air for 2 d prior to transfer to flasks which were bubbled with air (aerobic control) or 100% N_2 (anoxic test) or 2% O_2, 98% N_2 (hypoxic test). Each time point is the mean of data obtained from a representative experiment (n=4; + SE). The experiment was replicated four times.

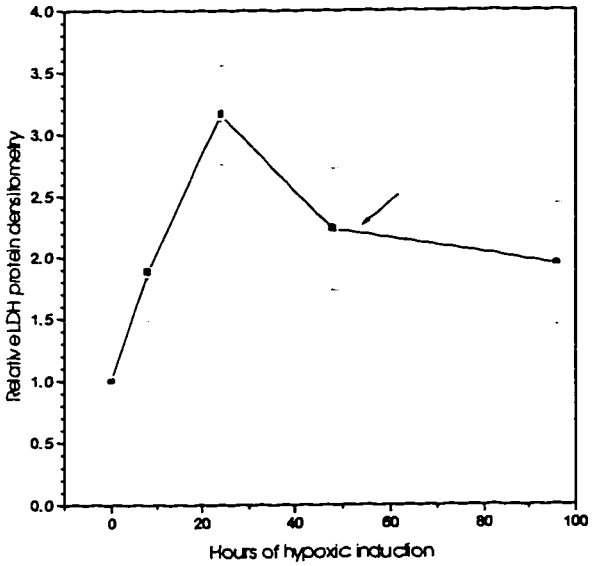
Figure 2-11: Maize LDH isozyme profile



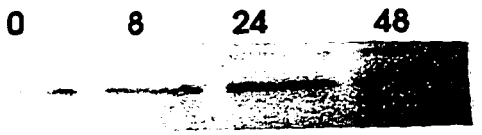
Roots were exposed to 0, 24, and 48 h of hypoxia. Root extracts run on non-denaturing PAGE were stained for LDH activity (n=3). The experiment was replicated three times.

Figure 2-12: LDH protein levels in hypoxic maize roots

A.



B.



(A) SDS immunoblot of roots exposed to 0, 8, 24, and 48 h of hypoxic stress and roots aerobically recovered for 48 h after 48 h of hypoxic treatment were analyzed by densitometry. Each time point is the mean of data obtained from four replicas (+SE). The arrow indicates the return to aerobic conditions. (B) SDS-PAGE immunoblot probed with barley anti-LDH antiserum.

Western blot analysis of denaturing PAGE demonstrated the presence of a polypeptide (M_r approximately 40,000), with LDH protein peaking at 24 h (3-fold induction; Figure 2-12). LDH enzyme activity, however, continued to increase for up to 6 d (Figure 2-9), suggesting that the specific activity of LDH increased during prolonged hypoxic induction. This is similar to barley LDH, which exhibits a higher specific activity for the induced enzyme than the aerobic enzyme (Good and Crosby, 1989).

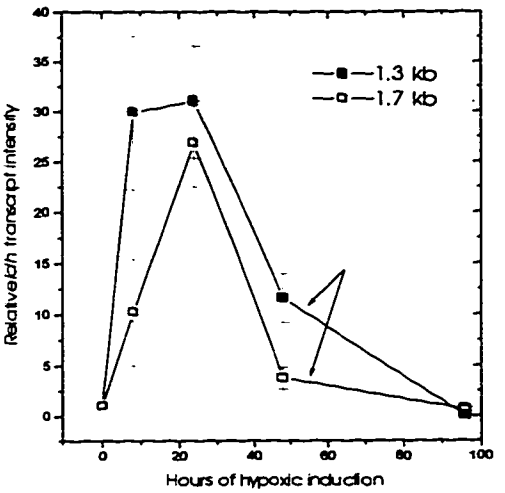
2.3.4 Hypoxically induced accumulation of *ldh* mRNA

Hybridization to a rRNA probe has standardly been used to assess equality of RNA loading between different samples. Throughout this thesis, poly(A)$^+$ RNA was used for analysis, thus necessitating the need for a different control probe. Also, run-on transcription assays have demonstrated that rRNA progressively decreases during 24 h of anoxic treatment (Fennoy and Bailey-Serres, 1995). Monitoring RNA hybridization signals during stress responses by comparison of the test signal to a control signal can lead to incorrect interpretations of results. For the results to be meaningful, the control probe must hybridize to a RNA whose concentration has been shown, unambiguously, to remain unaffected by the stress. To circumvent this problem, two control probes were used – actin and cyclophilin. Densitometric analysis of northern blots probed with actin suggested that actin mRNA increased during anoxic shock in maize seedlings (Fennoy and Bailey-Serres, 1995). However, in hypoxically treated maize seedlings, actin mRNA appears to be slightly decreased (Figure 2-13). Cyclophilin hybridization paralleled actin. Although both control probes appeared to be slightly affected by hypoxic stress, they were the best available.

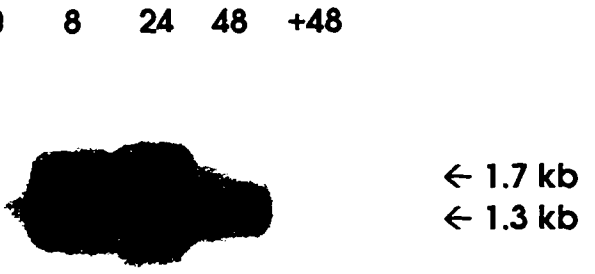
Northern analysis demonstrated that there was a very rapid induction of two *ldh* transcripts of approximately 1.3 and 1.7 kb (Figure 2-13). The transcript induction profiles differed, with the 1.3 kb transcript being generally present at higher levels than the 1.7 kb transcript. A significant increase in transcript levels can be observed as early as 4 h after hypoxic induction (the earliest time point tested; data not shown). The 1.3 kb transcript was induced approximately 30 fold at 8 h, and remained at that level for approximately 16 h before gradually decreasing (Figure 2-13). The increase in the 1.7 kb transcript was apparently delayed, with a peak at 24 h (approximately 27-fold) and a return to the aerobic level by 48 h. Return of plants to aerobic conditions for 2 d after exposure to hypoxic conditions for 2 d resulted in a decrease in transcript levels to those of the aerobic controls.

Figure 2-13: Hypoxic induction of *Idh* transcripts

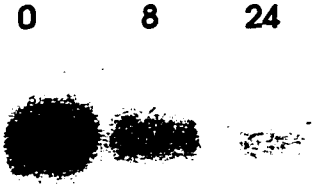
A.



B.



C.



(A) Densitometric analysis; (B) Northern blot probed with maize *Idh1* (pIdh1Sal1.0); (C) Northern blot probed with actin (pAtC4B/E1.8). One microgram of poly(A)$^+$ RNA isolated from maize roots exposed to 0, 8, 24, and 48 h of hypoxic stress and from roots aerobically recovered for 48 h after 48 h of hypoxic treatment was probed with maize *Idh1*and Arabidopsis thaliana actin. A representative northern blot is shown. Duplicate samples (n=2) from three separate experiments were analyzed (+SE). The arrows indicate return to aerobic conditions. Relative transcript intensity, determined from densitometric analysis, was the ratio of intensity of the *Idh1* hybridizing signal to the *actin* hybridizing signal.

2.4 *Discussion*

The hypoxic response, with emphasis primarily on ethanol production by ADH, has been extensively studied in maize. ADH activity is essential for hypoxic survival, since *adh* null mutants do not survive 24 h of anoxia (Lemke-Keyes and Sachs, 1989). Lactate fermentation has not as yet been demonstrated to be essential for hypoxic survival; however, LDH activity is suggested to play a role early in the hypoxic response by mediating the switch from lactate to ethanolic fermentation (Davies et al., 1974; Roberts et al., 1984a; Rivoal and Hanson, 1994). LDH activity remains elevated for several days (Hoffman et al., 1986; Good and Crosby, 1989), suggesting that LDH has a role in the long-term hypoxic response. The present study also suggests that LDH has a long-term role in the ability of young maize plants to survive hypoxic stress, as enzyme activity remained high for at least 6 d after exposure to hypoxic stress (Figure 2-8). Several other interesting features of maize *ldh* regulation were identified (a) *in vitro* stability of LDH enzyme activity changed upon hypoxic induction, and (b) induction of two *ldh* tanscripts, with different induction profiles suggested the presence of two *ldh* genes that are differentially regulated.

One significant difference between maize and barley is that, *in vitro*, maize LDH enzyme activity is unstable, with the aerobic enzyme being significantly more stable than the hypoxically induced enzyme (Table 2-5). This observation could be indicative of an *in vivo* change in LDH stability. It does require, however, that LDH assays performed on maize plants be tested immediately after extraction in order for meaningful results to be obtained.

The difference in the stability of the aerobic or induced forms of the enzyme could be the result of (1) a stabilizing factor present in aerobic roots. This could either be titrated out by the high levels of LDH activity present in hypoxically induced roots or its synthesis could be repressed by hypoxia, (2) a destabilizing factor synthesized during hypoxia (unlikely, as this would be very energy inefficient in already stressed cells), (3) changes in pH_c during hypoxia which could affect the stability of the enzyme, and (4) intrinsic instability of the enzyme.

Although the experimental results presented here did not address all the possible reasons for the decreased stability of LDH during hypoxia, the following evidence suggests that the maize enzyme was intrinsically unstable: (1) protease inhibitors had no effect on slowing the rate of decline in LDH activity (Table 2-5), (2) partially purified barley and mammalian LDHs were not destabilized by hypoxic root extracts suggesting that a destabilizing factor was not present in maize during hypoxia (Table 2-8), and (3) mixing equal volumes of aerobic and hypoxic

extracts had no effect on increasing stability relative to hypoxic extracts alone (Figure 2-4).

ADH activity during germination appears to be controlled by an inhibitor in maize (Ho and Scandalios, 1975) and *Erythrina caffa* (Small et al., 1993). This inhibitor is detected by decreased recovery of ADH activity in mixing experiments similar to those described in this chapter. The early experiments described in this chapter reproducibly demonstrated the potential existence of an inhibitor but later experiments refuted the idea. Analysis of the experimental parameters in the early and later experiments yielded no explanation for the discrepancy in the results.

In rats, experiments involving crushing of sciatic and optic nerves demonstrated that stress affects post-translational addition of amino acids onto proteins (Shyne-Athwal et al., 1986). In sciatic nerves, which can be regenerated, protein modification increases, whereas in optic nerves, which cannot be regenerated, protein modification decreases. Amino acid addition modifies protein stability by adding or removing a signal for ubiquitin-mediated protein degradation. The *in vivo* $T_{1/2}$ of a protein is affected by the identity of the amino-terminal amino acid (Bachmair et al., 1986). Some amino acids are stabilizing giving $T_{1/2}$s > 20 h, whereas others can decrease the $T_{1/2}$ to two to three min (Bachmair et al., 1986). Western analysis of denaturing acrylamide gels (Figure 2-12) did not demonstrate any modification in the size of the LDH detected in aerobic or hypoxic conditions, suggesting that this is not the cause of the decrease in *in vitro* stability of the hypoxic form of the enzyme.

Glucose starvation contributes to the decreased survival response of non-hypoxically pretreated maize root tips exposed to anoxic shock compared to hypoxically pretreated maize root tips (Hole et al., 1992). Glucose starvation in maize root tips increases protein degradation by increasing the activities of both acidic endopeptidases and carboxypeptidases. The endopeptidases present in starved and non-starved conditions differ in their sensitivity to protease inhibitors and pH optimum (James et al. 1993). In non-starved conditions, maize root meristems contain primarily cysteine, aspartic and metallo-proteases and to a lesser extent, serine proteases. Starved root tips contain primarily serine and cysteine proteases. Sensitivity of endopeptidases to cycloheximide (CHX) treatment during glucose starvation suggests that new proteases are induced and synthesized during starvation (James et al., 1993). The observation that a variety of protease inhibitors had no effect on the stability of the hypoxic form of maize LDH (Table 2-5) suggests that that there is no cause and effect relationship between protease inhibitor profiles and LDH *in vitro* stability.

An *adh* mutant (*Adh1-CN*) in *Phaseolus acutifolius* has been isolated. This mutant has both decreased activity and stability due to a Ser substitution of a highly conserved Phe located near the catalytic site

(Garvin et al., 1994). Also, *Bacillus stearothermophilus* LDH mutants containing an Asn substitution for Asp at amino acid 143 (equivalent to Asp 129 in maize LDH, Figure 2-2), an amino acid directly involved in catalysis, have decreased activity, stability and isoelectric point relative to the wild type enzyme (Nobbs et al., 1994).

Amino acid identity between the catalytic domains of maize LDH1 (Good and Paetkau, 1992) and barley LDHA and LDHB (Hondred and Hanson, 1990) is 90%, except for a 33-amino acid sequence, in which it drops to 40% (Figure 2-14). Amino acid changes within this region may be responsible for the difference in LDH stability between maize and barley.

Figure 2-14: Homology of maize and barley LDH catalytic domains

```
Maize LDH1  G I R R A V V D S A Y E V I S L K G Y T S W A I G Y S V A S L A A S L
Barley LDHA ❖ ❖ ❖ ❖ ❖ ❖ ❖ G G ❖ ❖ ❖ ❖ ❖ G ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
Barley LDHB ❖ ❖ ❖ ❖ ❖ ❖ ❖ G G ❖ ❖ ❖ ❖ ❖ G ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ T ❖ ❖

Maize LDH1  L R D Q R R I H P V S V L A R G F H G I P D G T T S S S A C P P R R P
Barley LDHA ❖ ❖ ❖ ❖ ❖ ❖ V ❖ ❖ ❖ ❖ ❖ ❖ ❖ S ❖ ❖ ❖ ❖ ❖ S ❖ ❖ H E V F L S L ❖ A ❖ L G
Barley LDHB ❖ ❖ ❖ ❖ ❖ ❖ V ❖ ❖ ❖ ❖ ❖ ❖ ❖ A ❖ ❖ ❖ ❖ ❖ S ❖ ❖ H E V F L S L ❖ A ❖ L G

Maize LDH1  R R R P ♦ G R R E M E L T E E E A K R L R R S A K T I W E N C Q L L G L
Barley LDHA ❖ A G Y L ❖ V A ❖ ❖ D ❖ ❖ ❖ A ❖ ❖ A Q ❖ ❖ ❖ ❖ ❖ ❖ ❖ L ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
Barley LDHB ❖ G G I L ❖ V A ❖ ❖ D ❖ ❖ ❖ A ❖ ❖ A Q ❖ ❖ ❖ ❖ ❖ ❖ ❖ L ❖ ❖ ❖ ❖ ❖ ❖ ❖ D ❖
```

Reference: Maize LDH1 – Good and Paetkau, 1992; Barley LDHA and LDHB – Hondred and Hanson, 1990

Amino acids identical with maize LDH1 are indicated with ❖. Amino acid missing from maize LDH1 is indicated with ♦.

Studies on electrophoretically pure human LDH-1 molecules suggest that several forms of LDH with differing stabilities exist (Xue and Yeung, 1995). Studies on a variety of *adh* low and null mutants demonstrate that their respective isozymes can be activated by immunoprecipitation with non-inactivating antisera. This suggests that variation in specific activity of isozymes is dependent on enzyme conformation rather than catalytic efficiency (Irish and Schwartz, 1987). A similar phenomenon could occur in maize roots. During hypoxia the ratio of inactive molecules could be higher than during aerobic conditions. As the holoenzyme becomes inactivated, it would result in an apparent decrease in enzyme stability. It will be interesting to determine whether LDH antisera has any effect on

stabilizing LDH activity over time. LDH is prevalent in animals and is therefore a poor immunogen. This results in antisera with low titres and high background. Because of the number of variables involved and the lack of a highly specific antibody this experiment was not performed.

Maize LDH enzyme activity increased within 8 h of hypoxic treatment, continuing to increase for up to 6 d, with the slope of increased activity being maximal during the first 2 d of treatment (Figure 2-9). This differs from barley, which has a linear increase in LDH activity for at least 5 d (Hoffman et al., 1986). Rice, a hypoxia tolerant species, has a slight induction of LDH activity upon exposure to low oxygen conditions although even the induced levels are very low (Rivoal et al., 1991).

Several of the enzymes involved in the hypoxic response have been reported to have a catalytic potential significantly higher than would be required for the glycolytic flux observed (Hoffman et al., 1986; Roberts et al., 1989). Menegus et al. (1989) observed that during anoxia, lactate levels in maize increased from <0.2 to 2.0 μmol g^{-1} fresh weight after 8 h. This study found that LDH activity increased from 51 to 100 μmol min^{-1} g^{-1} fresh weight during the same period. This indicates a significant excess of catalytic ability at both normoxia and 8 h of hypoxia. The specific activity of LDH appeared to increase during hypoxic treatment as enzyme activity increased three fold in 3-d hypoxically-induced roots (Figure 2-9), whereas LDH protein peaked at 24 h (2.1 fold increase, Figure 2-12) declining thereafter. The quintessential question is, why does LDH activity and specific activity increase during a time when LDH stability decreases? This could be a phenomenon that allows rapid inactivation of LDH once aerobic conditions are re-established; however, this study showed that enzyme activity decreased with a $T_{1/2}$ of ~2 d on return to aerobic conditions (Figure 2-9). *ldh* mutants may be beneficial in investigating this question, however, to date, none have been isolated. The absence of a visible phenotype makes screening of mutagenized maize lines extremely time consuming.

It has been suggested that increased tolerance to hypoxia correlates well with the ability to synthesize a variety of glycolytic end products (Ricard et al., 1994). Lactate production is a source of NAD^+ generation. The ability to continuously synthesize lactate and NAD^+ for the duration of hypoxic stress may explain the observation of increased LDH activity over several days. To prevent accumulation of lactate and consequently decreases in pH_c to lethal levels (Roberts et al., 1984a), the lactate must be excreted from the cell. A very high flux to lactate has been observed in barley aleurone layers, with the majority being excreted into the surrounding medium (Hanson and Jacobsen, 1984). However, during this testing, aleurone layers were bathed in a nutrient solution containing chloramphenicol. Kemp and Small (1994) observed that, in *Erythrina*, chloramphenicol inhibited ADH activity in hypoxic

conditions; therefore, the observed flux to lactate in barely aleruone layers may be artificially high. Other evidence suggesting a lactate transporter mechanism has been found in maize (Xia and Saglio, 1992), *Limonium* species (Rivoal and Hanson, 1993) and in transgenic tomato plants expressing a barley *ldh* cDNA (Rivoal and Hanson, 1994).

Bais et al. (1989) observed that LDH can catalyze the production of oxalate from glyoxylate in rat liver. This conversion produces glycolate as an intermediate (Equation 2-3) (Salisbury and Ross, 1985). Production of the intermediate glycolate generates NAD^+, which can be utilized in glycolysis. Participation in enzymatic reaction other than the reduction of pyruvate may thus necessitate high levels of enzyme activity during hypoxia.

Equation 2-3: LDH catalyzed conversion of glyoxylate to oxalate

$$\text{Glyoxylic acid} + NADH + H^+ \rightarrow \text{Glycolic acid} + NAD^+$$

$$\text{Glycolic acid} + NAD^+ \rightarrow \text{Oxalic acid} + NADH + H^+$$

Hypoxia results in a significant increase in the amounts of two different *ldh* transcripts (Figure 2-13). Maize *adh1* and *adh2* have different footprints under both aerobic and induced conditions (Paul and Ferl, 1991), suggesting regulation by different signal transduction pathways. Maize *ldh1* and *ldh2* may also be differentially regulated based on the different induction profiles of the two transcripts.

The contrasts between LDH activity and *ldh* transcript levels suggest that post-transcriptional regulation is present in addition to transcriptional regulation. This regulation could be at the translational level as several studies have demonstrated translational regulation of hypoxically induced genes (Bailey-Serres and Freeling, 1990; Fennoy and Bailey-Serres, 1995).

Induction of two different sized transcripts would be expected to result in the generation of polypeptides differing in size and / or charge. LDH is a tetrameric molecule, with the polypeptide products of two genes

randomly associating to produce five isozymes (Hanson et al., 1987). Analysis of maize LDH activity gels indicates that five isozymes are present and that the amounts of all isozymes increase upon exposure to hypoxic stress (Figure 2-11). Native gels were electrophoresed for ~ 12 h prior to staining for LDH activity. This is interesting in light of the inability to purify maize LDH. The first step in LDH purification involved affinity chromatography. It could be that some factor remained associated with LDH during chromatography changing its conformation / reducing its activity whereas this would be separated from the LDH tetramer during electrophoresis.

In summary, this chapter analyzed the induction profile of maize *ldh* in comparison to barley *ldh* and maize *adh*. It demonstrated that maize *ldh* was regulated in a fashion similar to other ANPs. The work presented in this chapter also suggested that LDH could have a role in the long-term hypoxic response, although the exact contribution of LDH to hypoxic survival has yet to be elucidated.

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3. Isolation of a *lactate dehydrogenase* pseudogene from maize

3.1 *Introduction*

The majority of plants studied to date contain at least two *ldh* genes. Crosses between barley inbred lines containing different LDH isozymes demonstrated the presence of two *ldh* loci (Hoffman et. al., 1988) and two *ldh* cDNA clones have subsequently been isolated (Hondred and Hanson, 1990). Several maize ANPs, including ADH, SS, PDC and GAPDH, are encoded by more than one gene. Of those that have been mapped, *adh1*, *pdc3* and gene *1032* map to chromosome 1, *adh2* and *gpc3* map to chromosome 4, *gpc4* and gene *1005* map to chromosome 5, *ald1*, *pdc1* and *pdc2* map to chromosome 8, and *enol* and *sh1* map to chromosome 9 (Peschke and Sachs, 1994).

Studies examining genome organization in grass species indicate extensive colinearity between maize, sorghum and rice (Dean and Schmidt, 1995). Although rice and maize diverged more than 50 million years ago, 85% of rice clones cross-hybridize to maize sequences with a high proportion of the rice sequences being duplicated in maize (Dean and Schmidt, 1995). The high frequency of duplicated loci in maize indicates the occurrence of polyploidization followed by extensive restructuring of the genome (reviewed in Lapitan, 1992). Several of these duplicated regions have been identified and include regions on chromosomes 2 and 7, chromosomes 3 and 8, and chromosomes 6 and 8 (Dean and Schmidt, 1995).

While there may be high homology between the coding region of duplicated genes, their regulatory regions often differ significantly leading to differences in regulation. The genes encoding ADH, SS, GAPDH and PDC are some examples. Two *adh* genes have been isolated in maize (Dennis et al., 1985). Studies on these two genes and their products indicate tissue specificity in expression, the preferential induction of *adh1* during hypoxia and anoxia (Dennis et al., 1985), and significant differences in the footprint profiles of the two genes (Paul and Ferl, 1991). Two genes also encode maize SS, *sh1* and *ss2*, which are differentially expressed during development and anoxic shock (Peschke and Sachs, 1994). All three maize PDC genes (*pdc1* to *pdc2*) are hypoxically induced: however, they have different induction profiles (Peschke and Sachs, 1993). Finally, only two of the four maize GAPDH genes (*gapC1* to *gapC4*) are anoxically induced (Köhler et al., 1995).

The observation of extensive gene duplication in maize suggests that maize would possess more than one *ldh* gene. Additional suggestive evidence comes from observations that native gel profiles of an inbred maize line contain five LDH isozymes, and Northern analysis demonstrating the presence of two transcripts differing in size and induction profiles (Chapter 2). A single *ldh* gene has previously been isolated and cloned from *Zea mays* (Good and Paetkau, 1992). Thus work presented in this chapter aimed to address the question of whether maize *ldh1* and *ldh2* are regulated differently. As no differences in the tissue specificity of LDH isozymes have been documented, the identification of a second *ldh* gene from maize would be useful in studying regulatory differences and similarities between the two genes.

3.2 *Materials and methods*

3.2.1 Southern blots

Genomic DNA was isolated from leaf tissue of three-week-*old Zea mays* cv. W22 plants using the protocol of Murray and Thompson (1980). Leaf tissue (0.5 g), ground to a fine powder in the presence of liquid nitrogen and a small amount of glass beads (425-600 µm, Sigma), was mixed with 5 mL of lysis buffer (1% (w/v) hexadecyltrimethyl ammonium bromide (CTAB), 5% (w/v) polyvinyl pyrrolidone (PVP), 1.4 M NaCl, 10 mM Tris-HCl (pH 8.0), 20 mM EDTA, 350 mM β-mercaptoethanol). Following incubation at 65°C for 30 min in the presence of 50 µg RNAse and SDS (final concentration 3.5%) the mixture was extracted once with 5 mL of phenol-chloroform-isoamylalcohol and twice with 5 mL chloroform-isoamylalcohol. The DNA was precipitated with 2.5 times the volume of ethanol, mixed by inversion and incubated on ice overnight. DNA was recovered by centrifuging at 9,500 rpm in a microcentrifuge for 30 min. The pellet was washed with 80% (v/v) ethanol, air dried and resuspended in 150 µL TE.

Ten µg of genomic DNA was digested with HindIII, EcoRI and BamHI (225 U, Pharmacia) according to the manufacturers recommendation, in the presence of 4 mM spermidine. The restricted genomic DNA was sequentially extracted with phenol-chloroform-isoamylalcohol, twice with chloroform-isoamylalcohol and ethanol precipitated prior to loading onto a 0.8% (w/v) agarose gel. Following electrophoresis the DNA was transferred to Gene Screen Plus (DuPont) using an alkaline transfer protocol. The membrane was wetted in water and equilibrated in 0.4 N NaOH for 15 min. The gel was treated with 0.25 N HCl for 20 min and then a capillary blot apparatus was set up using 0.4 N NaOH as the transfer

buffer. After a 10 h transfer, the membrane was rinsed with 2X SSC for 2 min and air-dried.

Pre-hybridization was performed at 42°C in 1% (w/v) SDS, 2X SSC, 10% (w/v) dextran sulfate, 50% (v/v) formamide. Radiolabeled probes (10^7 cpm, Section 2.2.15) in a 2 mL volume containing 1 mg salmon sperm DNA were boiled for 10 min, iced 15 min and then added to the pre-hybridization solution. Hybridization was at 42°C for 18 h. The membrane was washed with 2X SSC at room temperature for 10 min, twice with 2X SSC, 1% (w/v) SDS at 42°C for 20 min, and twice with 0.2X SSC, 1% (w/v) SDS at 42°C for 20 min.

3.2.2 Screening of the genomic library

Genomic DNA from *Zea mays* L. cv. W22 leaf tissue was cloned into λ-Dash® II by Stratagene Corp. *Zea mays* is a diploid organism with a genome size of 1 C equaling 3.2 X 10^9 bp (Croy et al., 1993). Thus to screen a genomic library for a single copy gene would entail screening 10^6 λ plaques containing an average insert size of 17 kilobases (kb). Approximately 500,000 plaques were plated on 15 – 150 mm plates using XL1-Blue MRA(P2) (Stratagene) as the host strain. The plaques were lifted onto nitrocellulose membranes (Nitro-plus MSI) and probed with the maize *Idh1* genomic clone pIdh1Sal1.0 (Figure 2-3) labeled as previously described (Section 2.2.15). Plaque hybridization was performed at 60°C for 14 h in 30 mL hybridization buffer (5X SSC, 1% (w/v) SDS, 5X Denhardts, 100 μg mL^{-1} salmon sperm DNA). Blots were washed twice in 2X SSC for 5 min at room temperature, and twice in 2X SSC and 0.1% (w/v) SDS for 15 min at 60°C. Putative positive plaques were eluted into 1 mL SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% (v/v) gelatin) and replated in a secondary and tertiary screen.

DNA was extracted from the positive clones by a λ miniprep protocol (Grossberger, 1987). Ten mL overnight lysates of the λ clones grown in XL1-Blue MRA(P2) were centrifuged 10 min at 2000 rpm to pellet cellular debris and the supernatant was recentrifuged for 30 min at 30,000 rpm. The pellet was dissolved in 200 μL SM buffer, digested with 200 μg proteinase K for 2 h at 37°C, phenol and chloroform extracted, ethanol precipitated and resuspended in 100 μL TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0). The DNA was digested with restriction enzymes (BRL and Pharmacia) and analyzed by Southern blotting to determine the number of different positive clones present.

Clones, which generated a different Southern profile to the *Idh1* clone previously isolated, were subjected to large scale λ DNA isolation in order to generate a restriction map and to facilitate subcloning into

plasmid vectors. Ten mL of chloroform was added to 500 mL of overnight lysates, incubated 15 min at 37°C, centrifuged at 11,000 g for 10 min followed by addition of 500 μg of both RNAseA and DNAseI and incubation at room temperature for 30 min. NaCl was added to a final concentration of 1 M. Following incubation at 4°C for 1 h the debris was pelleted by centrifugation at 11,000 g for 10 min. The phage were precipitated by the addition of 50 g of PEG 8000 and incubated and centrifuged as above. The pellet was resuspended in 10 mL of λ-diluent (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA), chloroform extracted and the aqueous layer centrifuged at 25,000 rpm for 2 h at 4°C. The phage pellet was resuspended in 1.6 mL λ-diluent and treated with SDS and EDTA to final concentrations of 2% (w/v) and 10 mM, respectively. After heating for 10 min at 65°C, proteinase K was added to a final concentration of 200 μg mL^{-1} and allowed to digest for 90 min at 55°C. Proteins were removed by phenol extraction, followed by a phenol-chloroform and two chloroform extractions. The aqueous layer was ethanol precipitated and the λ DNA was resuspended in TE.

Restriction digestions were performed according to standard protocols using a variety of enzymes (BRL and Pharmacia). Subclones were generated by cloning fragments from λ clone 39-121 into pBluescript SK^+ (Stratagene) according to standard procedures.

3.2.3 Sequence analysis

DNA isolated from pBluescript SK^+ subclones isolated from λ39-121 were treated with exo III and mung bean nuclease according to the manufacturers protocol, to generate nested deletions (ExoIII / Mung bean deletion kit, Stratagene). Deletions into the insert were made from both directions. Plasmid DNA, for exo III deletions and sequence analysis, was isolated by Wizard™ *Plus* Minipreps (Promega) according to the manufacturers protocol. Double stranded sequencing of both DNA strands using T3, T7 and specific sequencing primers (Table 3-1) was performed by the dideoxy chain termination method (Sanger et al., 1977) according to the manufacturers protocol using a sequencing kit (T7 Sequencing kit, Pharmacia). Reactions were run on a standard 6 % polyacrylamide - 8-M urea sequencing gel.

The DNA sequence was subjected to a BLAST search (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/BLAST/blast_databases.html).

Table 3-1: Sequence specific primers used for sequencing Zmldh2

Primer	Sequence (5' → 3')	mer	% G+C	T_m
MCH1	CGGATCCACTAGTTCTAGAGA	20	50%	60°C
MCH2	ACGCAGGGCCAAGAACATGTA	20	55%	62°C
MCH3	GGATCCACTAGTTCTAGAGC	20	50%	60°C
MCH4	TACAGAGAGCACATATCAGC	20	45%	58°C
MCH5	TTTTACAACGTCGTGACTGGG	21	48%	62°C
MCH6	ACTAATGACTCAACTGGACG	20	45%	58°C
MCH7	GCCAACATCTCGCTCACAAG	20	55%	62°C
MCH8	GAGCCGTCGTAGCCACCGAG	20	70%	68°C
MCH9	ATCTGGAGGAGTCGAGGTTG	20	55%	62°C
MCH10	CAGGTCGGACCCAGAGCGGC	20	75%	70°C
MCH11	CACATCGCCAGGATGCTGCC	20	65%	66°C
MCH13	ATCGACTCGCAGTGGCAGCTTG	22	59%	70°C
MCH14	AGGGTGCGAATCATAAGC	18	50%	54°C
MCH15	AACATCTCGCTCAACAAGCG	19	53%	58°C
MCH16	GTTTCACTGGAGCAGGTC	18	56%	56°C
MCH17	GTGCACTGCTTGGCTATG	18	56%	56°C
MCH18	GCTACCCATGTCAGACATG	18	50%	54°C
MCH19	CCACAGTTACGTCCCATAG	19	47%	56°C
MCH20	CCCACGAACCCTAACGTA	18	56%	56°C
MCH21	CGCCTATTTCTCAATTCTG	18	44%	52°C
MCH22	TTGAGCCGAGGTTGTAGAAC	19	53%	58°C
MCH23	GTGGCCCATTGACAAATAG	18	50%	54°C
MCH24	CTGTGGTGCTAAACAAATAGG	20	45%	58°C
MCH25	GATATGTCGAGCACACTTGG	18	50%	54°C
MCH26	TTCTGAAGGCAGGGATTGG	19	53%	58°C
MCH27	CAAGTCCAGACCCATAATC	19	47%	56°C
MCH28	TTGACTGTACCCGCCGATC	19	58%	60°C
MCH29	GAAACAGCGAGTAAGGGAG	19	53%	58°C
MCH31	TGGATCACGAGGAACATAGAAC	21	43%	60°C
MCH32	CAGGGCAATATGCAAGAC	18	50%	54°C
MCH35	CTCCTTCTAGCCGTTG	16	56%	50°C
MCH36	ATCTTGGCGTAGTGGTC	17	53%	52°C
MCH37	CAAAACAAGAAATACAAATACC	22	32%	58°C
MCH38	TTTGGGCAGTCGCGTAG	17	59%	54°C
MCH42	CCGAGTCAACGACAAGAG	17	59%	54°C
MCH43	CACAGAAAGCGAGAGG	17	59%	54°C
MCH44	CAGTCATGCTGTTTATATTG	20	35%	54°C
MCH45	CCTCGCACCTGTTCTCC	16	63%	52°C
MCH46	CCGGTTTGCTGAAGAATG	18	50%	54°C
MCH47	TCTAACAATTCCCCCACTTC	18	44%	52°C
MCH48	AGAACTCGGGGAGAACAG	17	53%	52°C
MCH49	GCATTTCAGTTGGTCTTTTC	20	40%	56°C
MCH50	AAGACCAACTGAAATGCC	18	44%	52°C
MCH51	GGCTTGATTATGGTG	18	44%	52°C
MCH52	CCATTACACACCATAGAG	18	44%	52°C
MCH53	CGTGAGAGACCCTTAGTG	18	56%	56°C
MCH54	TAGAAAAGTGCATCGAGGG	18	50%	54°C
MCH55	CAGCGATGAGGAAATACAC	18	50%	54°C
MCH56	CAGTTGTATGTCTTTTGTC	20	40%	56°C
MCH57	AGGGATCGGCAATTAGG	17	53%	52°C
MCH58	GCTGCAAAAGGGTATTCC	18	50%	54°C
MCH59	GCGAGTGACAGGGCTG	16	69%	54°C

Primers synthesized by the Molecular Biology Service Unit (MCH1-MCH3, MCH13–MCH38, MCH53-MCH59), Dr. Strobeck's laboratory (MCH4–MCH11), Research Genetics (MCH42–MCH52). $T_m = 4(G+C) + 2(A+T)$ (Clontech).

3.2.4 Primer extension

Primers (Table 3-2 and Figure 3-1), specific to different 5' regions of the *Idh1* mRNA, were kinase labeled according to the protocol of Sambrook et al., (1989) in a 30 µL volume. The reaction contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 1 µg oligonucleotide, 30 µCi(γ-^{32}P)ATP (ICN, specific activity 7000 Ci mmol^{-1}), 5 mg mL^{-1} BSA, 20 U T4 polynucleotide kinase (Pharmacia), and was incubated at 37°C for 1 h. The reaction was stopped by the addition of EDTA to a final concentration of 15 mM and heating at 65°C for 10 min.

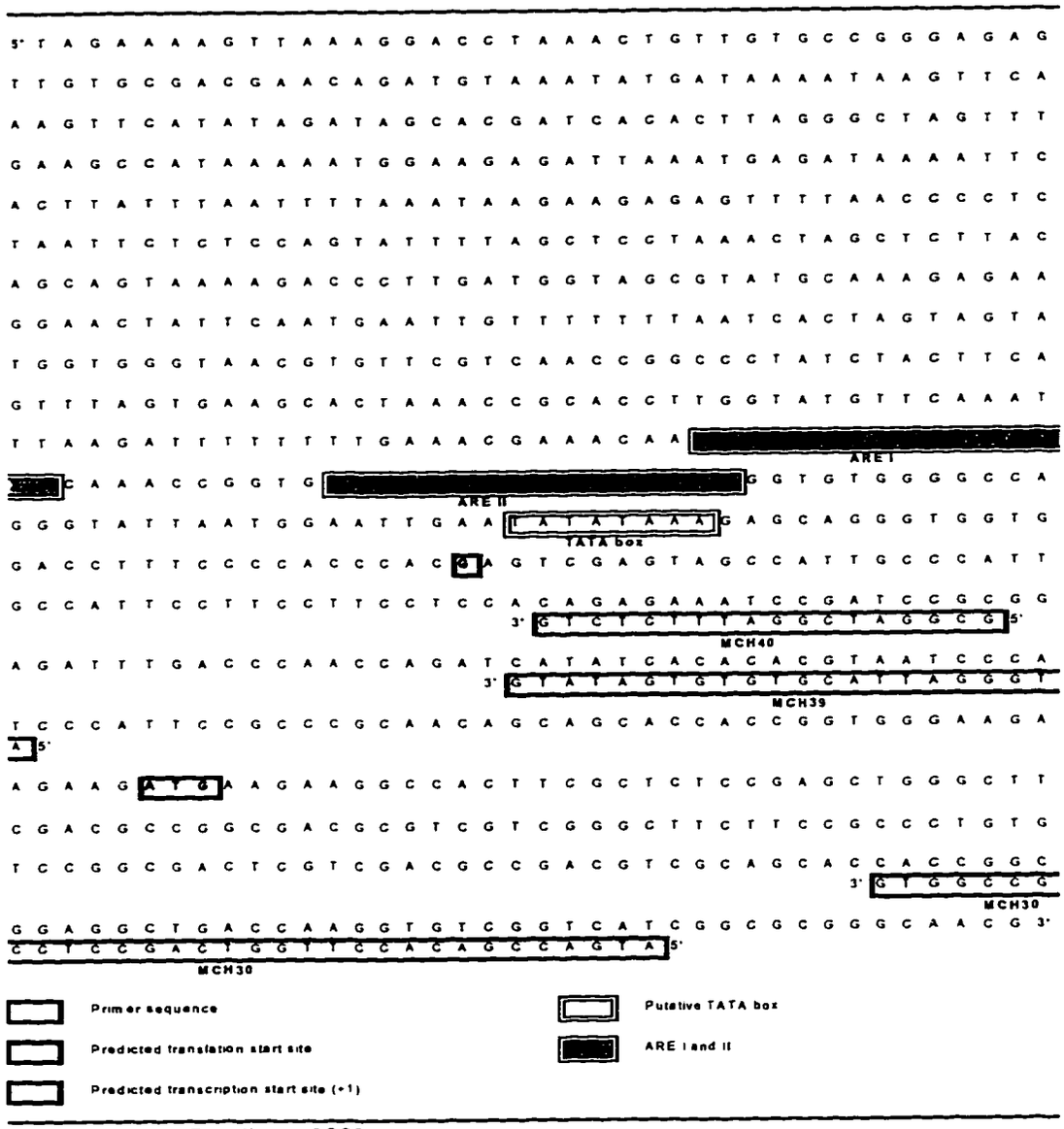
Total RNA (10 µg) isolated from 24-h hypoxically-induced roots was combined with 1 µL of the (γ-^{32}P)ATP end labeled primer in a total volume of 14 µL, heated to 70°C for 10 min, then chilled on ice. After centrifugation, buffer, dNTP's, DTT and 200 U of M-MLV-reverse transcriptase (BRL) were added. Final concentrations in a 20 µL volume were 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM each dNTP and 10 mM DTT. The reaction was incubated at room temperature for 10 min, 42°C for 50 min and terminated at 90°C for 5 min. The RNA was hydrolyzed by adding NaOH to a final concentration of 0.4 M and incubating at 65°C for 30 min. The NaOH was neutralized by an equal molar concentration of HCl and the cDNA was recovered by ethanol precipitation. After washing, the pellet was resuspended in 50 µL TE buffer and analyzed on a standard acrylamide-urea sequencing gel.

Table 3-2: Primers used for primer extension of maize *Idh1*

Primer	Sequence (5' → 3')	mer	% G+C	T_m
MCH30	A T G A C C G A C A C C T T G G T C A G C C T C C G C C G G T G	32	66%	79°C
MCH39	A T G G G A T T A C G T G T G T G A T A T G	22	41%	62°C
MCH40	G C G G A T C G G A T T T C T C T G	18	56%	56°C

Primers were synthesized by the Molecular Biology Service Unit.

Figure 3-1: Maize *Idh1* promoter



From Good and Paetkau, 1992

3.2.5 Preparation of gene specific probes

The maize *Idh1* specific probe was isolated from pIdh1Sal1.7 cloned into pT7T318U. This clone contains most of exon two plus 1.3 kb of

sequence 3' to the polyadenylation site (AATAAAAAC at position 382 in the clone). Plasmid DNA was digested with XbaI (Pharmacia) and self-ligated to generate pldh1SX0.6. This clone was digested with SalI and BclI, blunt ended with Klenow and self-ligated to generate pldh1XSB (Figure 2-3A). Sequence confirmation of pldh1XSB was performed to ensure accuracy of plasmid construction. This clone contains the 3' UTR of maize *ldh1* plus 235 nucleotides downstream of the polyadenylation site (320 nucleotides total). No coding sequences are present in this clone. DNA for *ldh1* specific probe preparation was isolated from a PvuII digest of this plasmid and therefore contained some plasmid sequences. Fragment isolation and labeling was as previously described (Section 2.2.15)

Probes to determine whether the *ldh2* clone isolated was expressed were labeled by PCR. Plasmid pZmldh2Hind4.8 (10 ng) was mixed with 25 ng of each primer in a 26 µL volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.01% (v/v) gelatin, 3 mM $MgCl_2$, 0.02 mM dATP, dGTP and dTTP, 0.002 mM dCTP, 50 µCi α^{32}P-dCTP and 0.05 U Taq polymerase. PCR was performed in GeneAmp 9600 (Perkin Elmer) using the following program: 2 min at 94°C, 35 cycles of (96°C for 30 sec, 55°C for 36 sec, 72°C for 84 sec), a final extension of 5 min at 72°C and holding at 4°C. The probe was purified by isolation through Nick columns (Pharmacia). Primer pairs used were MCH26 and MCH47 which generated a 350 base pair product from the region 1.5 kb downstream of the region of *ldh* homology.

3.3 *Results*

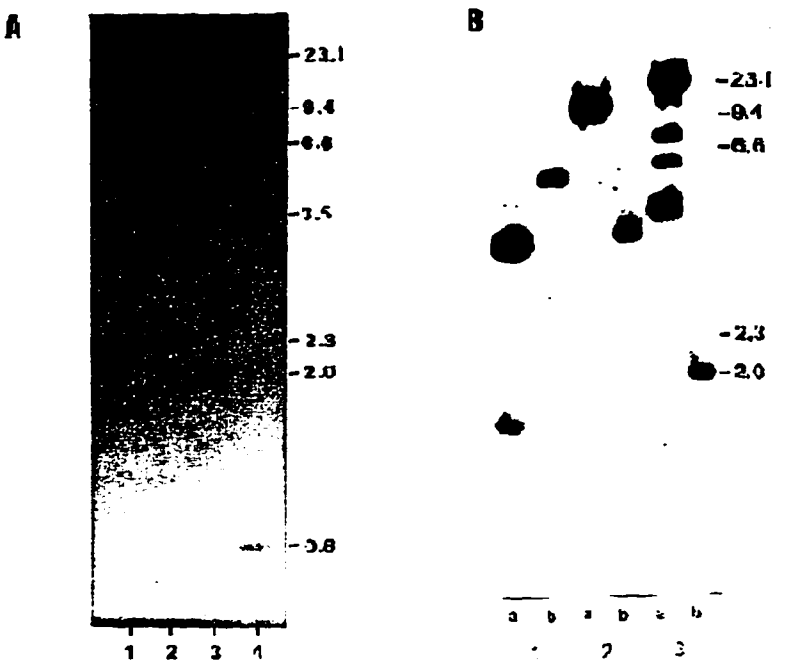
3.3.1 Comparison of maize genomic Southern with *ldh* clones isolated

Southern blots (Figure 3-2) of maize genomic DNA probed with maize *ldh1* coding region (pldh1Sal1.0) yielded a pattern of hybridizing fragments not all of which could be accounted for by the first *ldh* genomic clone isolated from maize (Good and Paetkau, 1992). Screening of a maize genomic library *ldh1* resulted in 7 positive clones of which 2 gave the same restriction digest patterns but were different from that predicted for *ldh1*. Paetkau and Good (unpublished) had previously isolated five independent λ clones, all of which were confirmed as *ldh1* based on restriction patterns and Southern analysis.

Clone λ 39-121 was selected for further analysis. Southerns of genomic DNA and the λ clones *ldh1* and 39-121 demonstrated that clone 39-121 accounts for some of the additional bands observed in the W22 genomic Southern (Figure 3-2 and Table 3-3). The differences in the hybridizing intensities in the W22 genomic Southern suggests that 39-121

contains a *ldh* gene which is not completely homologous to *ldh1*. Additional bands in the genomic Southern, unaccounted for by both the *ldh1* and 39-121 clones, indicate that additional *ldh* genes may be present.

Figure 3-2: Genomic and λ DNA Southern blots



(A) W22 genomic Southern blot; (B) W22 λ Southern blot. DNA digested with HindIII (lane 1), EcoRI (lane 2), BamHI (lane 3) was probed with pldh1Sal1.0. (A) Lane 4 is a *ldh1* marker lane. (B) Lane (a) is a *ldh1* λ clone and lane (b) is 39-121 λ clone.

Table 3-3: Identification of *Idh* hybridizing fragments in the W22 genomic Southern

Restriction enzyme	W22 genomic DNA	*Idh* λ clone containing the corresponding fragment
HindIII	~14.0 kb	
	~8.9 kb	
	~7.2 kb	*Idh1*
	~4.8 kb	39-121
	~3.3 kb	
	~1.65 kb	*Idh1*
EcoRI	~20.0 kb	*Idh1*
	~14.0 kb	*Idh1*
	~11.0 kb	
	~5.6 kb	
	~3.4 kb	39-121
BamHI	~19.0 kb*	*Idh1*
	~10.0 kb*	*Idh1*
	~6.8 kb	*Idh1*
	~5.8 kb	
	~4.0 kb	
	~3.5 kb	*Idh1*
	~1.95 kb	39-121

* indicates fragments likely generated as the result of incomplete digestion

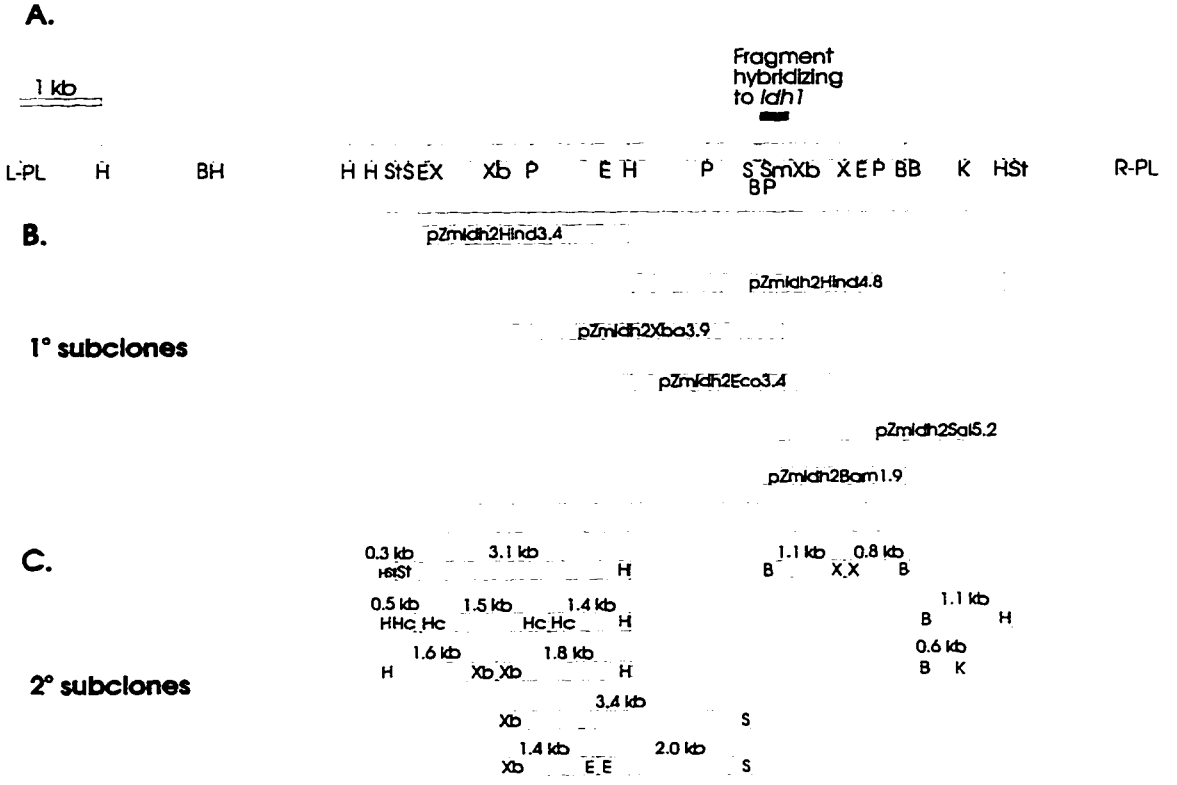
3.3.2 Isolation and sequence analysis of a second maize *Idh* genomic clone

From approximately 500,000 plaques screened, seven positive clones were isolated. Restriction digests and Southern blot analysis of the seven λ clones yielded two patterns of clones. Five clones demonstrated identical restriction patterns with the pattern being the same as λ Idh7, a clone containing the *Idh1* gene previously identified (Good and Paetkau, 1992). The final two clones had a different restriction pattern from the other five, but the restriction pattern between these two clones was identical. Clone λ 39-121 was subjected to a detailed restriction analysis. A restriction map was determined (Figure 3-3A) and a number of subclones generated to assist with sequencing (Figure 3-3B and C).

The pZmIdh2Bam1.9 clone (Figure 3-3B) was sequenced initially as it contained the region hybridizing to *Idh1*. A region of high similarity to *Idh1* was detected (84% identity over 244 nucleotides) (Figure 3-4) and 70% identical at the amino acid level (over 84 amino acids) (Figure 3-5). This

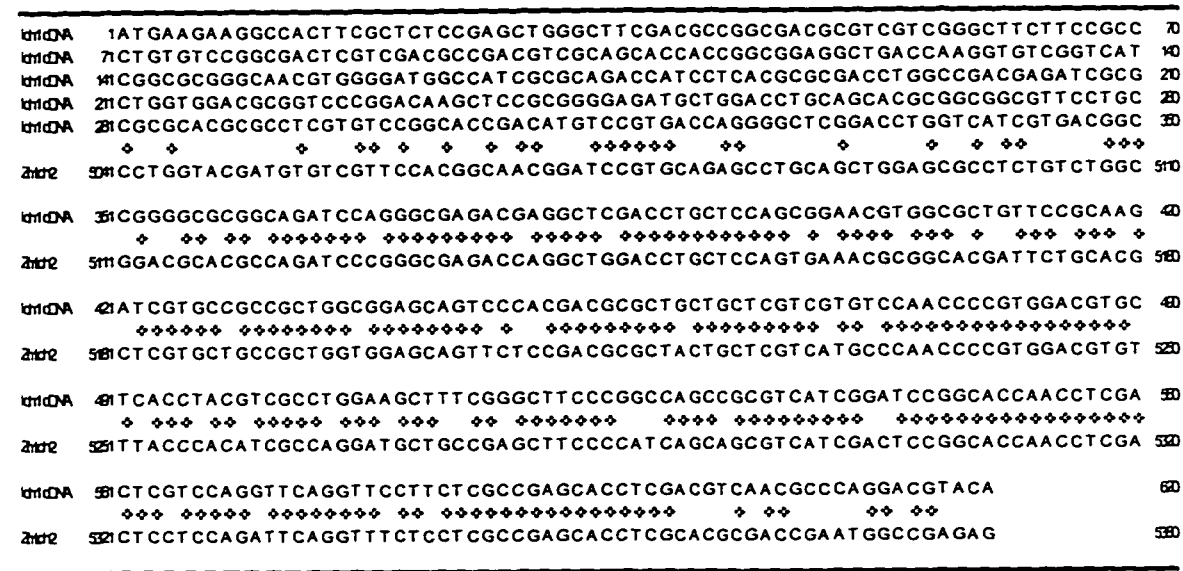
region is homologous to a wide variety of both eukaryotic and prokaryotic *Idh* genes (Table 3-4) and includes the region coding for amino acids in the active site loop, AGARQIQGETRL (Wigley et al., 1992). As the whole *Idh2* gene was not present in this clone, additional clones were sequenced. In totality, an 8227 nucleotide region was sequenced on both strands (Figure 3-6) and subjected to a BLAST search; however, additional regions of homology to *Idh1* were not discovered. Most (~66%) monocot introns are less than 120 nucleotides, ~12% are greater than 600 nucleotides with the remainder intermediate between the two (Simpson et al., 1993). Upstream of the region of homology 5107 nucleotides were sequenced with an additional 2869 nucleotides sequenced downstream. The region sequenced on either side of the *Idh1* homology region should span even a large intron, therefore, it is likely that this clone contains a pseudogene rather than a functional gene.

Figure 3-3: Restriction map of λ 39-121 and plasmid clones generated



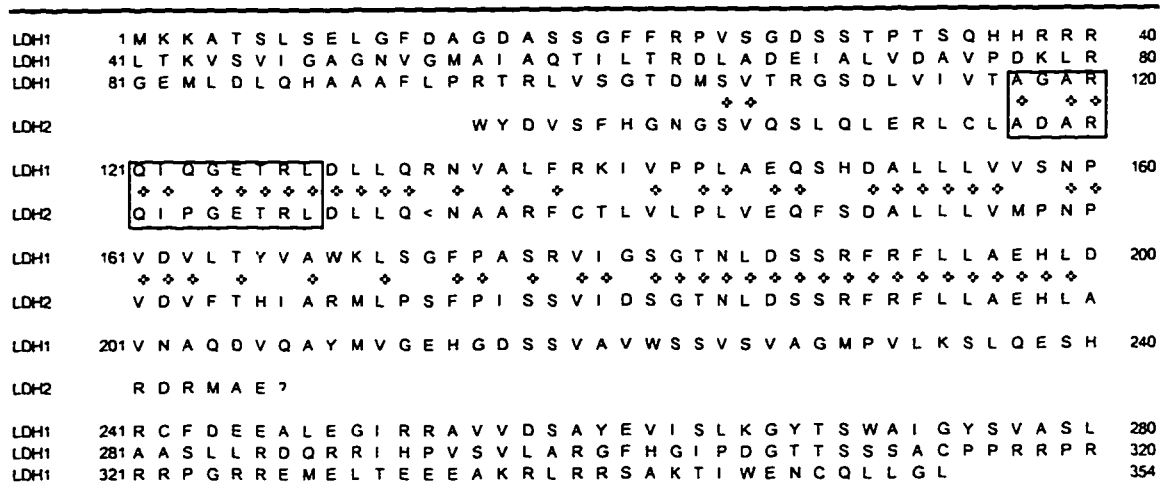
(A) Restriction map of the 14.6 kb W22 genomic insert in λ clone 39-121; (B) Primary plasmid subclones generated; (C) Secondary plasmid subclones generated from the primary subclones. Abbreviations: B = BamHI, E = EcoRI, Hc = HincII, H = HindIII, K = KpnI, L-PL = left polylinker site, P = PvuII, R-PL = right polylinker site, S = SalI, Sm = SmaI, St = SstI, Xb = XbaI, X = XhoI.

Figure 3-4: Homology of Zmldh2 with *Zea mays ldh1*



Homology of the Zmldh2 clone in the region 5041 to 5380 with *Zea mays ldh1* (Good and Paetkau, 1992) exon 1 nucleotides 281 to 620 (3' nucleotide of exon 1). Identical nucleotides are indicated with ❖.

Figure 3-5: Amino acid alignment between maize LDH1 and LDH2



Amino acid sequence predicted from the nucleic acid sequence of *ldh1* (Good and Paetkau, 1992) and *ldh2*. Boxed amino acids are those located within the active site loop (Wigley et al., 1992).

Table 3-4: Homology of Zmldh2 ORF3A with *ldh* genes

Species	Gene	Genbank number	% identity	Identical nucleotides	Region of homology	Reference
Zea mays	*ldh1*	Z11754	82%	207	251	Good and Paetkau, 1992
Hordeum vulgare	*ldhB*	M55684	80%	201	251	Hondred and Hanson, 1990
Hordeum vulgare	*ldhA*	M55685	78%	196	251	Hondred and Hanson, 1990
Oryza sativa	*ldh*	D13817	76%	192	250	Li et al., 1993
Fundulus heterodlitus	*ldhB*	L23787	60%	152	251	Bernardi et al., 1992
Fundulus parvipinnis	*ldhB*	L23780	59%	148	250	Bernardi et al., 1992
Sphyraena argentea	*ldhA*	U80000	59%	155	259	Holland et al., 1996 (unpublished)
Sphyraena idiastes	*ldhA*	U80001	59%	155	259	Holland et al., 1996 (unpublished)
Sphyraena lucasana	*ldhA*	U80002	59%	155	259	Holland et al., 1996 (unpublished)
Thermus aquaticus	*ldh*	D00585	58%	145	249	Ono et al., 1990
Caenorhabditis elegans	*ldh*	U15420	58%	143	245	Tsoi and Li, 1994
Petromyzon marinus	*ldh*	M74064	57%	143	250	Stock and Whitt, 1992
Bacillus caldotenax	*ldh*	M19395	53%	148	275	Zuelli et al., 1987
Bacillus caldolyticus	*ldh*	M19394	52%	133	251	Zuelli et al., 1987
Bacillus stearothermophilus	*ldh*	M19396	52%	133	251	Zuelli et al., 1987
Bifidobacterium	*ldh*	M33585	59%	102	172	Minowa et al., 1989
Deinococcus radiodurans	*ldh*	D63899	58%	99	168	Narumi and Watanabe, 1996
Mus musculus	*ldhX*	L10389	52%	131	250	Wu et al., 1987
Mus musculus	*ldhC*	X04752	52%	130	250	Sakai et al., 1987

Homologies with the Zmldh2 clone in the region 5041 to 5380 were identified using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/blast_databases.html) and the references were obtained by searching with the Genbank accession number (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=n).

Figure 3-6: Sequence of Zmldh2

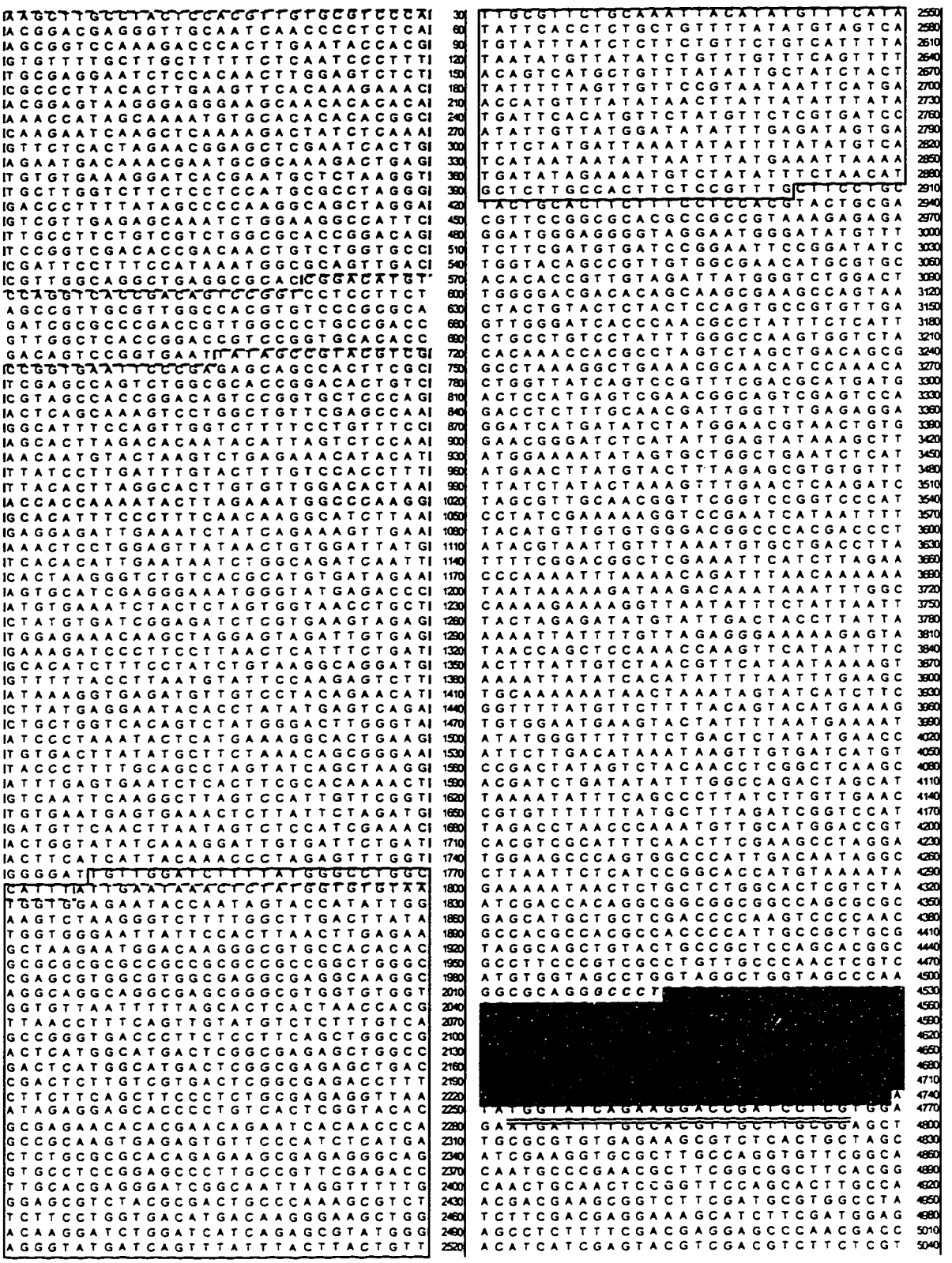
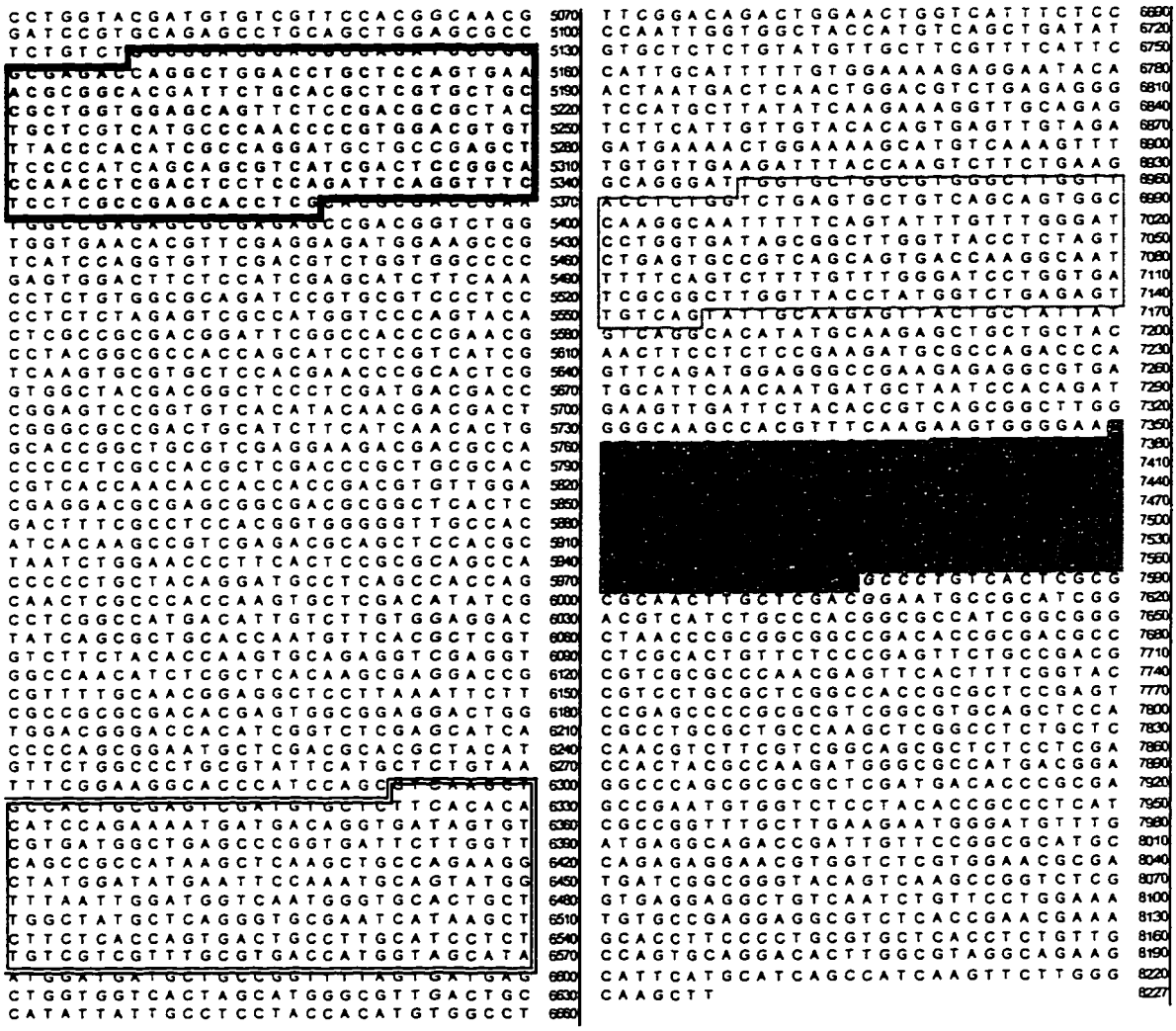
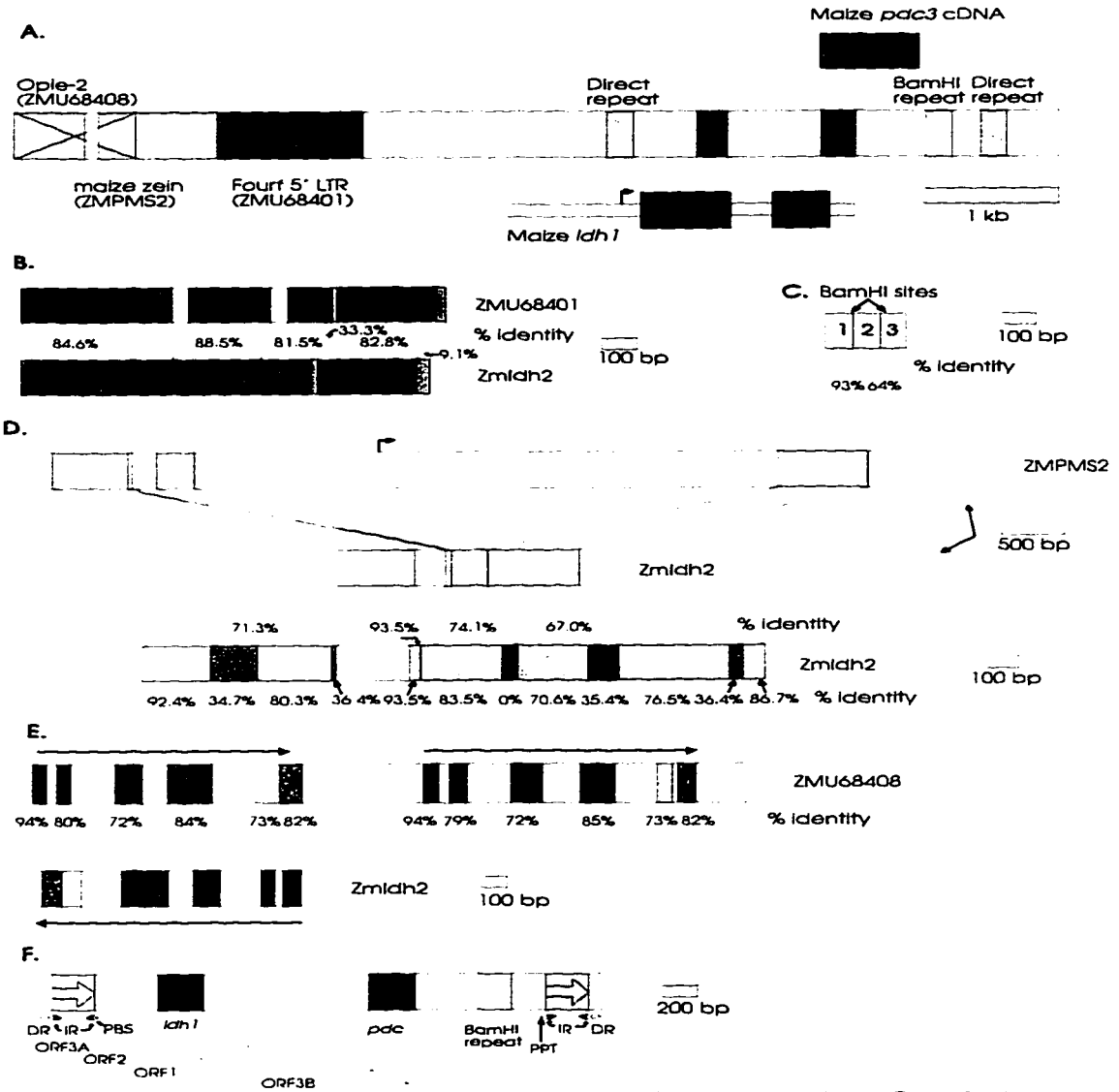


Figure 3-6 continued: Sequence of Zmldh2



Sequence of Zmldh2. Nucleotides outlined with dashes are homologous to a maize 19 kDa zein gene, ZMPMS2 (Quayle et al., 1989). Nucleotides homologous to the maize retrotransposon Opie-2, ZMU68408 (SanMiguel et al., 1996) are lightly shaded. Nucleotides outlined with a single line are homologous to the maize retrotransposon Fourf 5' LTR, ZMU68401, (SanMiguel et al., 1996). Nucleotides homologous to maize *ldh1* (Good and Paetkau, 1992) are bolded and outlined with a single heavy line, and those with homology to *pdc* (Peschke and Sachs, 1993) are outlined with a double line. The direct repeats are darkly shaded and the BamHI repeat is outlined with a dotted line. The target site duplicated nucleotides are bolded and italicized. The primer binding site (PBS) is double underlined and the polypurine tract (PPT) is singly underlined.

Figure 3-7: Features of Zmldh2 clone



Features identified through sequence analysis and BLAST searches of the Zmldh2 clone. (A) Cartoon of the entire 8227 nucleotides sequenced indicating regions homologous to maize zein (ZMPMS2), Fourf retrotransposon 5' LTR (ZMU68401), Opie-2 retrotransposon LTR (ZMU68408), maize *ldh1* and maize *pdc*, direct repeat elements, and a BamHI repeat element. (B) Blow-up of the region homologous to the Fourf 5' LTR (ZMU68401) (SanMiguel et al., 1996) indicating nucleic acid identity with the Zmldh2 plus strand. (C) Blow-up of the BamHI repeat element indicating nucleic acid identity between the three regions. (D) Blow-up of the region homologous to the maize zein gene (ZMPMS2) (Quayle et al., 1989) indicating nucleic acid identity with Zmldh2 plus strand. This is blown up further to indicate areas of high and low homology. (E) Blow-up of the region homologous to the Opie-2 retrotransposon (ZMU68408) (SanMiguel et al., 1996) indicating nucleic acid identity with the Zmldh2 minus strand. (F) Blow-up of the LP-like element indicating the location of the direct repeats in the target DNA (DR), the inverted repeats at each end of the direct repeats (IR), the primer binding site (PBS), the polypurine tract (PPT), the regions homologous to maize *ldh1* (Good and Paetkau, 1992) and *pdc* (Peschke and Sachs, 1993) and the BamHI repeat. ORFs present within this region are ORF1, ORF2 and ORFs 3A and 3B in reading frames 1,2 and 3, respectively. The *ldh* homology is in ORF3A and *pdc* homology is in ORF3B.

Several interesting features were determined from sequence analysis (Figure 3-7). First, there is a region of duplication of 226 nucleotides located at positions 4513-4739 and 7349-7575 (Figures 3-6 and 3-7). These two regions are completely identical (Figure 3-8). These direct repeats contain an inverted repeat of six nucleotides at each end (5' end of repeat: 5' TGTTAG 3', 3' end of repeat: 5' CTAACA 3'). BLAST searches using this 226-nucleotide region yielded no sequences with significant homology. At the 5' end of the upstream and at the 3' end of the downstream duplicated region is a 5 base pair (bp) direct repeat (5' GCCCT 3'). The presence of direct repeats possessing small inverted repeats at each end and a target site duplication is highly suggestive of a transposable element / retrotransposon.

Other features of retrotransposons are the presence of a polypurine tract (PPT) just upstream of the 3' long terminal repeat (LTR) and a polymerase binding site (PBS) with homology to the 3' end of a tRNA two nucleotides downstream of the 5' LTR (Arkhipova et al., 1995). These features are also found between the two direct repeats. Three nucleotides downstream of the 5' repeat is the sequence 5' TGGTATCAGAAGGACCGATCCTCG 3' which is complementary to the 3' end of maize $tRNA_i^{Met}$ (Figure 3-9). A PPT (5' AAGTGGGGAA 3') is located immediately 5' to the downstream repeat (Table 3-5). Active retrotransposons contain ORFs encoding *gag* (protease) and *pol* products (reverse transcriptase, integrase and RNAse H) (Arkhipova et al., 1995). A search for ORFs within this 2609 bp region demonstrated the presence of four ORFs which can be translated to produce polypeptide products of >100 amino acids (Figure 3-7F). None of these ORFs detected any homology to *gag* or *pol* in a BLAST search, but ORF3A contained the nucleotides possessing homology to *ldh1* (Good and Paetkau, 1992) (Figures 3-4 and 3-7). ORF3B contained nucleotides with homology to *pdc* (Peschke and Sachs, 1993) (Figure 3-7 and 3-10; Table 3-6). Homology begins at the start of the *pdc* cDNA with 85.4% identity over the first 220 nucleotides. This region also shows various amounts of homology to other *pdc* genes isolated from plants (Table 3-6). These observations suggest that the λ clone 39-121 does indeed contain a *ldh* pseudogene that is located within a defective retrotransposon. This region will be referred to as "LP-like element" for a retrotransposon-like element containing sequences with homology to *ldh* (L) and *pdc* (P).

Second, there is a region of similarity surrounding two closely situated BamHI sites (Figures 3-7 and 3-11) located within the LP-like element. This region was sequenced using primers on either side of the repeat and clone pZmldh2Hind4.8 (Figure 3-3) and therefore is not an artifact of subcloning. A BamHI repeat has been isolated from *Nicotiana tabacum* (Matyasek et al., 1989) but this possesses no similarity to the repeat found

in this clone. The repeat can be subdivided into three regions. Region 1 and 2 show 93% identity, 2 and 3 show 64% identity and 1 and 3 show 65% identity.

Two regions upstream of the repeat region were identified through a BLAST search as having high homology to a maize zein gene and two retrotransposons (Figure 3-7). The regions of homology to the maize 19 kDa zein gene (ZMPMS2) (Quayle et al., 1989) (Figures 3-12 and 3-13) are separated by a large gap (Figure 3-7D). The homology to the zein gene is in a region 1.5 kb upstream of the coding sequence and 2.3 kb downstream of the coding region. Homology between regions varies from 67% to 93.5%. Within these regions of homology are blocks of higher (mean of 83.4%) and lower (mean of 28.6%) homologies. Interestingly, the upstream region of homology between the zein and Zmldh2 sequence coincides with homology to the LTR of the retrotransposon Opie-2 (ZMU69408) (SanMiguel et al., 1996) (Figures 3-7E and 3-12) inserted in the inverse orientation. The overall identity between Opie-2 LTR and Zmldh2 is 81% and 68% between Opie-2 LTR and the zein upstream region. Opie-2 insertion generates a target site duplication of GGACC (Table 3-5) (SanMiguel et al., 1996). This sequence is not observed on the Zmldh2 minus strand immediately 5' to the start of homology with Opie-2 LTR suggesting that this region corresponds to the 3' LTR of Opie-2.

Immediately adjacent to the zein / Opie-2 LTR homology region is a region with homology to the maize retrotransposon Fourf 5' LTR and PBS (ZMU68401) (SanMiguel et al., 1996) (Figures 3-7B and 3-13). The overall identity between Fourf 5' LTR and Zmldh2 is 79.3%. There are two 30 nucleotide regions present in Fourf which are absent in Zmldh2. There are two regions with low homology (mean 20%) and when these regions are excluded, homology between the two sequences increases to 84.6%.

Figure 3-8: Identity between the two direct repeats in Zmldh2

```
rpt1   4501 G G C G C A G G G C C C T T G T T A G A T A T A T T A G G T  4530
                ❖ ❖           ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7337 A A G A A G T G G G G A A T G T T A G A T A T A T T A G G T  7366

rpt1   4531 G G G T T A T G A G C T A T G G T C A T G T G T A G T A G A  4560
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7367 G G G T T A T G A G C T A T G G T C A T G T G T A G T A G A  7396

rpt1   4561 T G A G C T A T G G G T T A T G T G G G C T T G A G G A G A  4590
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7397 T G A G C T A T G G G T T A T G T G G G C T T G A G G A G A  7426

rpt1   4591 T A T A A A T A T A G G C T G A G T C A T T T G T A A A A G  4620
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7427 T A T A A A T A T A G G C T G A G T C A T T T G T A A A A G  7456

rpt1   4621 G C A T C A A A C A A C A G A A T A C A A A T A C C A A T T  4650
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7457 G C A T C A A A C A A C A G A A T A C A A A T A C C A A T T  7486

rpt1   4651 C A C T G A G A C C C C G T C T A G C T C T C G T C T T C C  4680
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7487 C A C T G A G A C C C C G T C T A G C T C T C G T C T T C C  7516

rpt1   4681 T T C T G T C T T C C C A G C T C C A A T C T C G G A T C T  4710
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7517 T T C T G T C T T C C C A G C T C C A A T C T C G G A T C T  7546

rpt1   4711 C C T T T C T T C T A C C T T C T G T G G T G C T A A C A A  4740
            ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖ ❖
rpt2   7547 C C T T T C T T C T A C C T T C T G T G G T G C T A A C A G  7576

rpt1   4741 T A T G G T A T C A                                        4750

rpt2   7577 C C C T G T C A C T                                        7586
```

Identity of the two direct repeats (rpt) located in Zmldh2 between 4501 to 4750 and 7337 to 7586. The direct repeats in the target DNA, 5' to rpt1 and 3' to rpt2, are bolded. Identical nucleotides are indicated with ❖.

Figure 3-9: Alignment of the putative PBS in the LP-like element with $tRNA_i^{Met}$

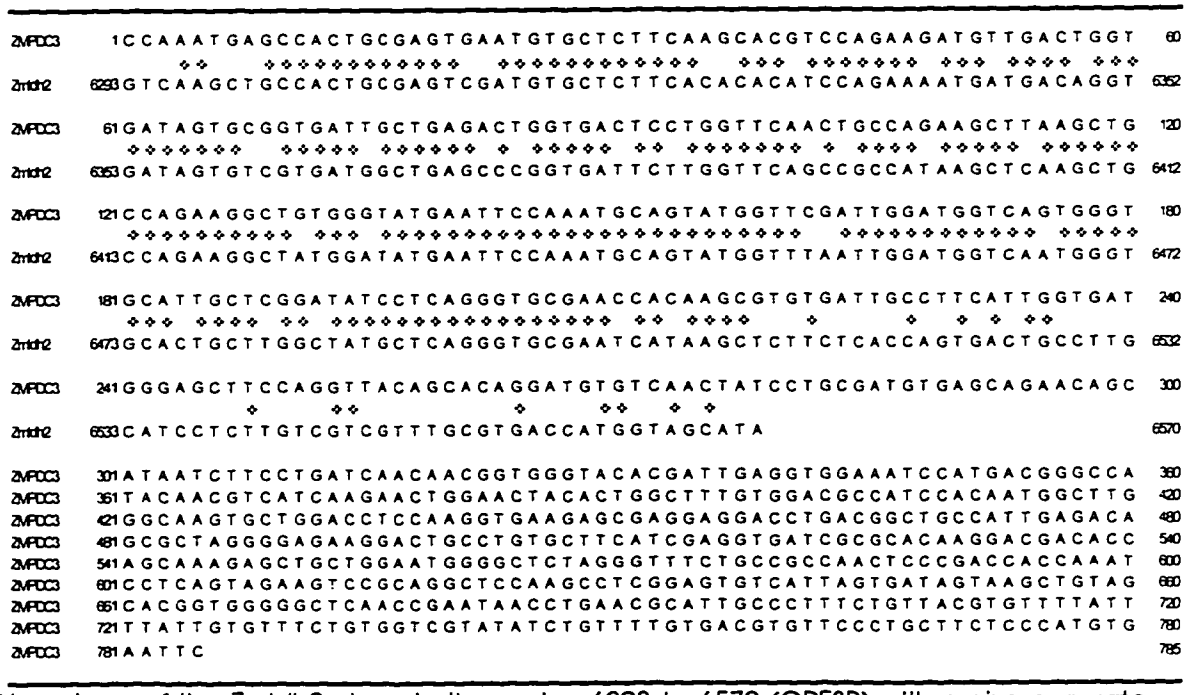
```
                                                A
                                          G     C A
                                          C G   C A                 C
tRNA_i^Met     3'  A C C A U A G U C U          U       G C U A G G A     C 5'
LP-like        5'  T G G T A T C A G A          A       C G A T C C T     G 3'
                                          A G   C                   C
                                          G
```

Table 3-5: Homology of the LP-like element with Ty1 / *copia*-like elements

Name	Size	LTR	DR	PBS	PPT	Reference
Opie-2	9.0 kb	1.3 kb	GGACC	TGGTATCGGAGCCGT	AGGGGGAG	SanMiguel et al., 1996
Ji-3	8.5 kb	1.3 kb	CGAAG	TGGTATCAGAGCCCG	AGGGGGAG	SanMiguel et al., 1996
Ji-4	10.0 kb	1.3 kb	GATTC	TGGTATCTGAGCCCG	AGGGGGAG	SanMiguel et al., 1996
Fourf	7.0 kb	1.1 kb	TAATC	CCAAAAACCTAAT	TGGTGGGG	SanMiguel et al., 1996
Victim	5.5 kb	0.1 kb	CTCAC	TGGTACCAGAGCC	GCGGGGGG	SanMiguel et al., 1996
Bs1	3.2 kb	0.3 kb	GCCAC	TGGTACAAAGGTCACC	AAGGTGGGGGG	Jin and Bennetzen, 1989
LP-like	3.0 kb	0.2 kb	GCCCT	TGGTATCAGAAGGACC	AAGTGGGGAA	This work

Abbreviations: DR – direct repeat in target DNA; PBS – primer binding site; PPT – polypurine tract.

Figure 3-10: Homology of ORF3B with *Zea mays* pyruvate decarboxylase



Homology of the Zmldh2 clone in the region 6293 to 6570 (ORF3B) with maize pyruvate decarboxylase (ZMPDC3) cDNA (Peschke and Sachs, 1993) nucleotides 1 to 217. Identical nucleotides are indicated with ❖.

Table 3-6: Homology of ORF3B with *pdc* genes

Species	Gene	Genbank number	% Identity	Identical nucleotides	Region of homology	Reference
Zea mays	*pdc3*	Z21722	85%	179	209	Peschke and Sachs, 1993
Oryza sativa	*pdc2*	U27350	79%	167	209	Huq et al., 1995
Nicotiana tabacum	*pdc1*	X81854	71%	153	214	Bucher et al., 1995
Nicotiana tabacum	*pdc2*	X81855	73%	95	130	Bucher et al., 1995
Arabidopsis thaliana	*pdc1*	U71121	72%	53	73	Dolferus et al., 1996 (unpublished)
Arabidopsis thaliana	*pdc2*	U71122	73%	139	189	Dolferus et al., 1996 (unpublished)
Pisum sativum	*pdc1*	Z66543	69%	144	208	Mucke et al., 1996

Homologies were identified using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/blast_databases.html) and the references were obtained by searching with the Genbank accession number (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=n).

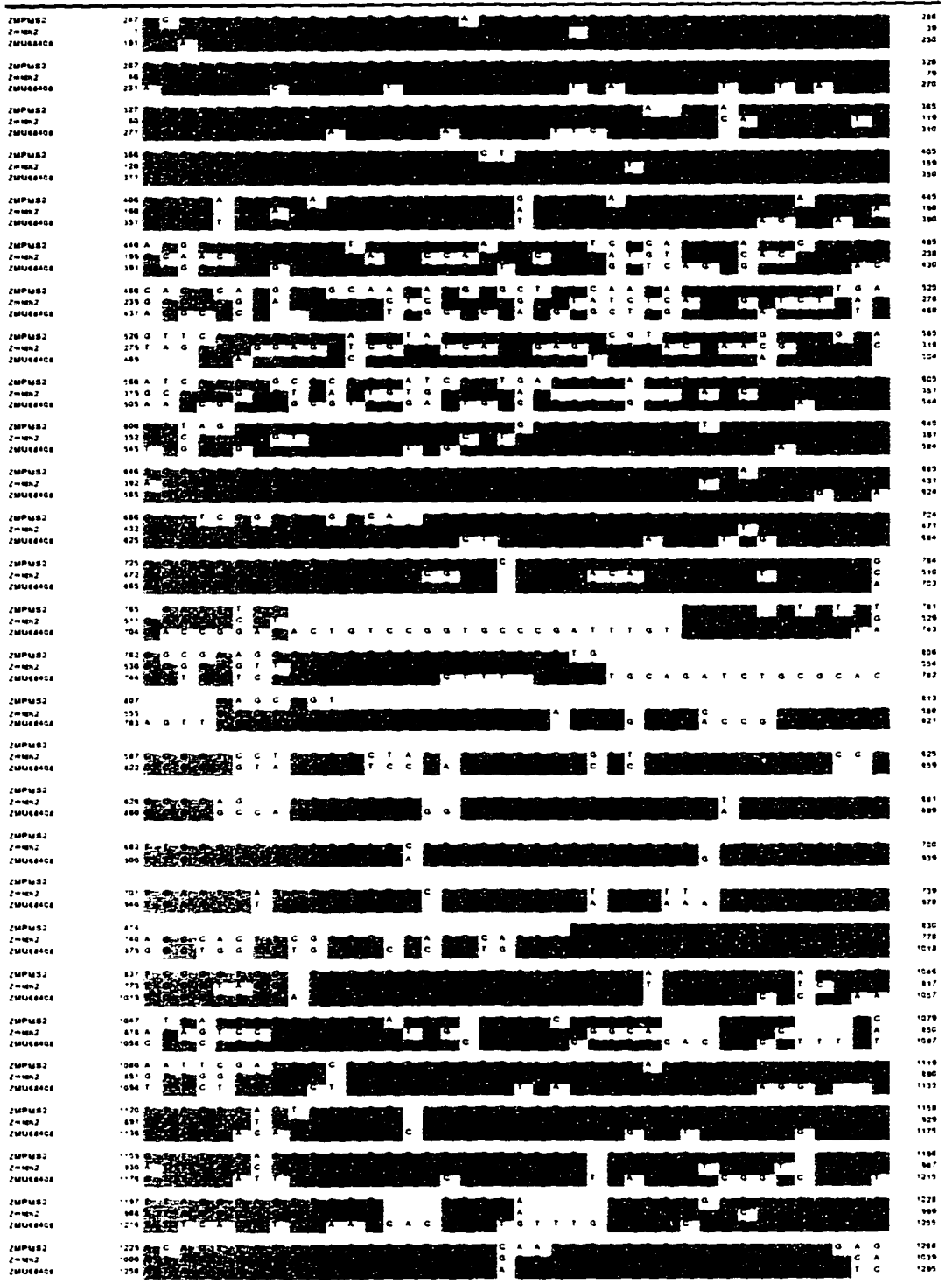
Figure 3-11: Homology between the three repeated regions in the BamHI repeat element

```
6939 T G G T G C T G G C G T G G G C T T G G T T A C C T C T G G T C T G A G T G C T G T C  6981
7023 T G G T G A T A G C G     G C T T G G T T A C C T C T A G T C T G A G T G C C G T C  7062
7105 T G G T G A T C G C G     G C T T G G T T A C C T A T G G T C T G A G A G T T G T C  7144

6982 A G C A G T G G C C A A G G C A A T T T T T C A G T A T T T  G T T T G G G A T C C  7022
7063 A G C A G T G A C C A A G G C A A T T T T T C A G T C T T T T G T T T G G G A T C C  7104
7145 A G T A T T G   C A A G A G T T A C T G C T A T T A T     G T C A G G C A C A T  7181
```

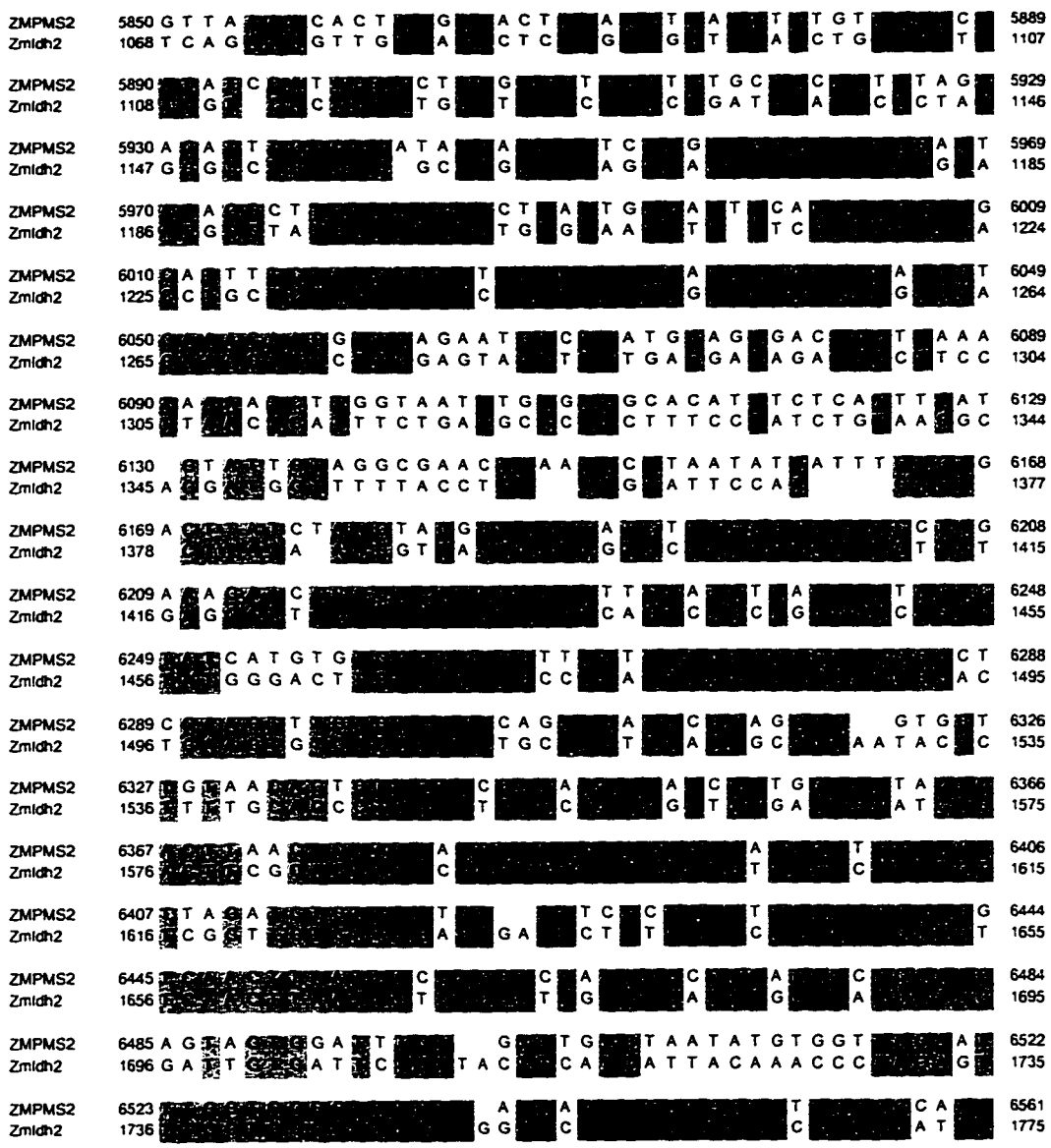
Homology in the Zmldh2 clone in the region 6939 to 7181. Nucleotides identical between at least two of the repeat regions are shaded. The BamHI site is bolded.

Figure 3-12: Homology of Zmldh2 with a *Zea mays* gene encoding a zein protein (ZMPMS2) and the retrotransposon Opie-2 LTR (ZMU68408)



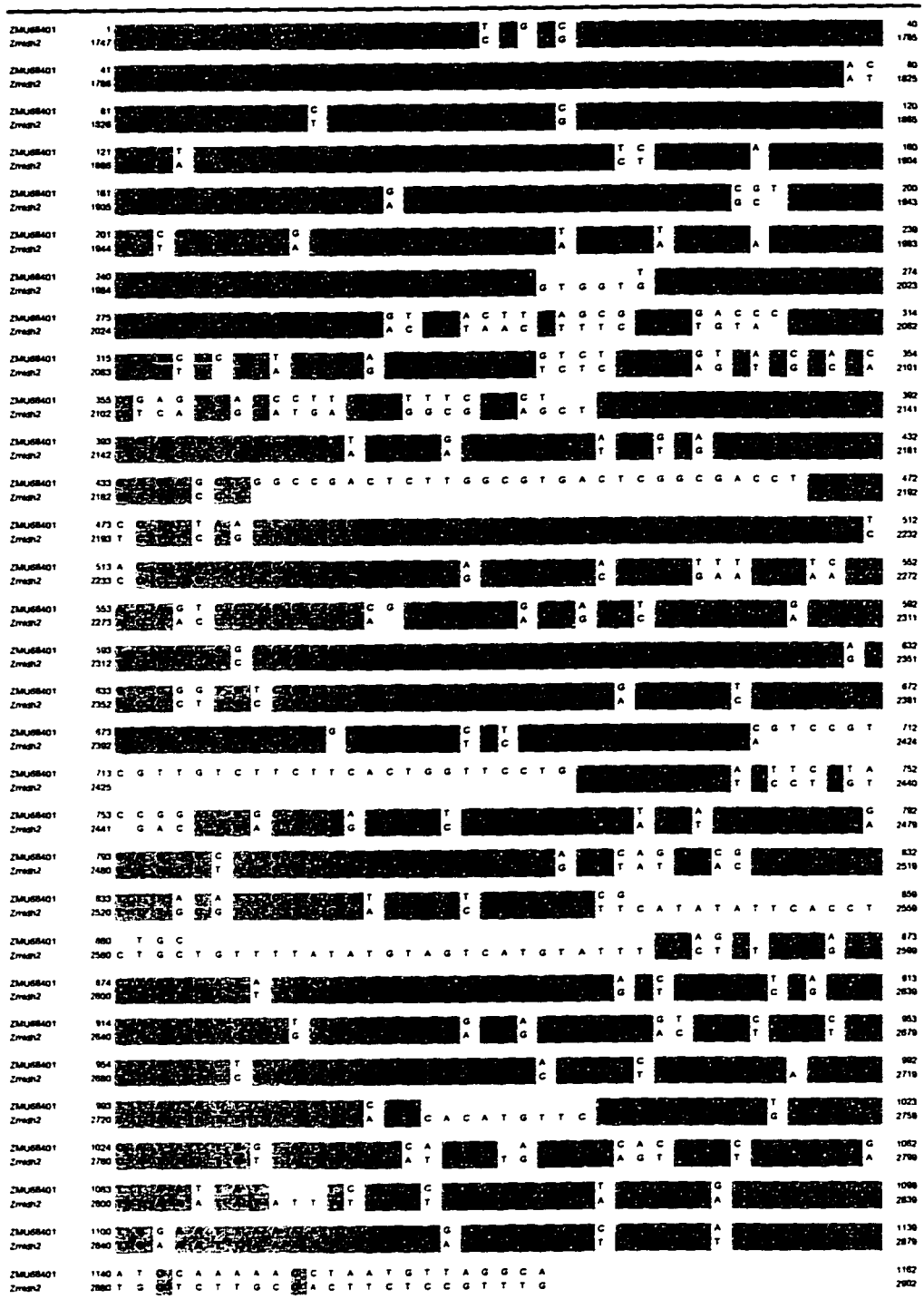
Homology of the Zmldh2 clone in the region 1 to 1039 with a 19 kDa zein protein from maize (ZMPMS2) (Quayle et al., 1989) and the maize retrotransposon Opie-2 (ZMU68408) minus strand (SanMiguel et al., 1996). Identical nucleotides are shaded. The bold line in the ZMPMS2 sequence at position 844 / 1022 indicates a gap in the region of homology.

Figure 3-13: Homology of Zmldh2 with a *Zea mays* gene encoding a zein protein (ZMPMS2)



Homology of the Zmldh2 clone in the region 1068 to 1775 with a 19 kDa zein protein from maize (ZMPMS2) (Quayle et al., 1989). Identical nucleotides are shaded.

Figure 3-14: Homology of Zmldh2 with retrotransposon Fourf 5' LTR (ZMU68401)

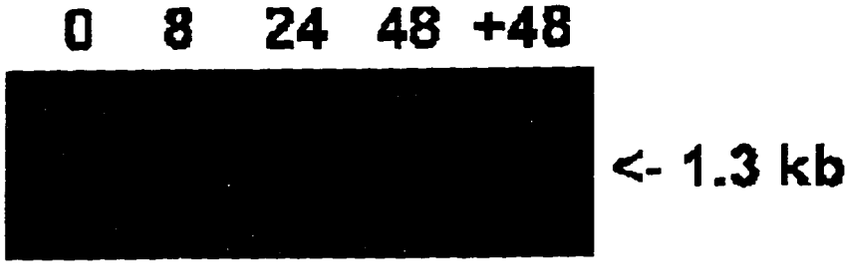


Homology of the Zmldh2 clone in the region 1747 to 2902 with the maize retrotransposon Fourf 5' LTR (SanMiguel et al., 1996). Identical nucleotides are shaded.

3.3.3 Analysis of gene specific expression

Northern analysis of hypoxically induced maize plants demonstrated the presence of two *ldh* transcripts (1.3 and 1.7 kb) with different induction profiles (Figure 2-13). This suggested that two *ldh* genes may be expressed. To determine whether the transcripts were the result of expression of a single gene, with either different transcription start sites or poly(A) sites, resulting in the production of different sized transcripts or two different genes, poly(A)$^+$ Northerns were probed with a fragment from pldh1XSB (Figure 2-3A) containing the 3' UTR of maize *ldh1*. This probe detected only the 1.3 kb transcript (Figure 3-15). Probing with the PCR product generated from the Zmldh2 clone did not produce a hybridization signal even with a two-week exposure of the northern blot (data not shown). The PCR generated probe amplified a fragment downstream of the region of *ldh* homology within the LP-like element. It did not contain any sequences in the region of homology to *pdc*.

Figure 3-15: Hypoxic induction of *ldh* expression

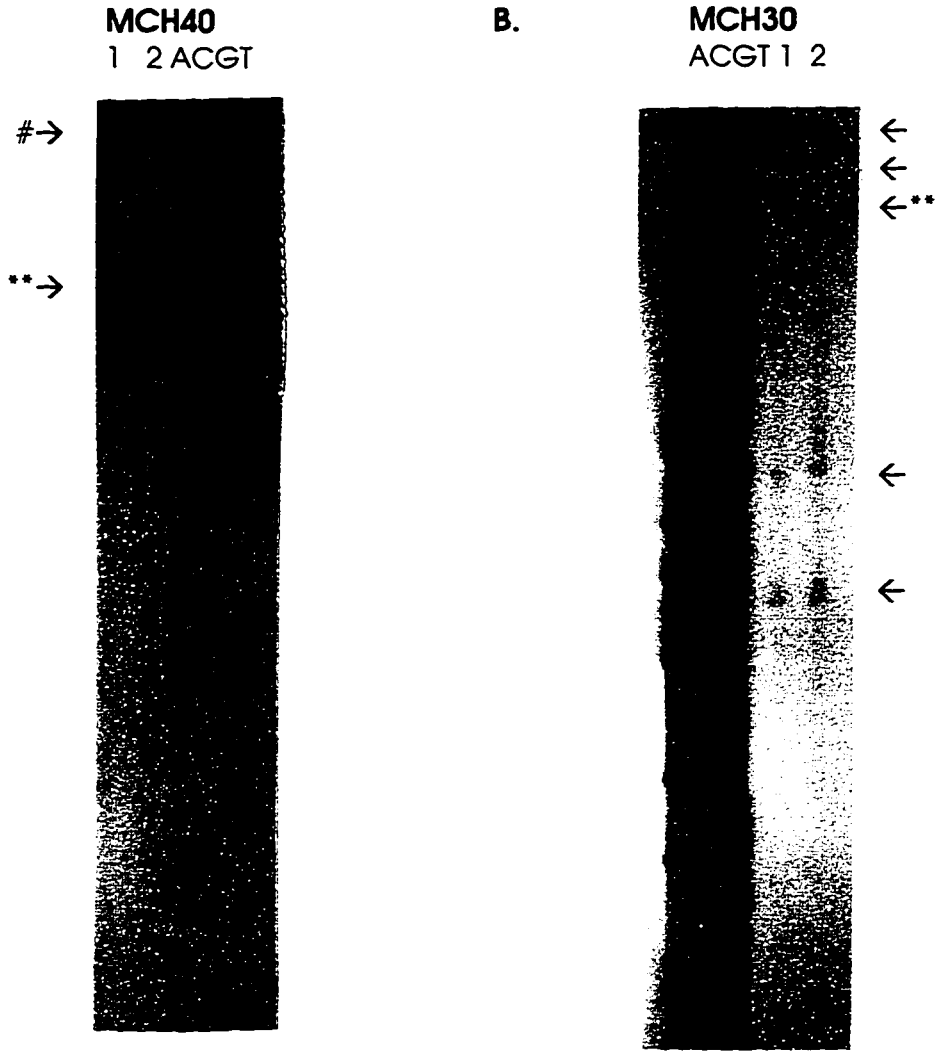


Hypoxic expression of *ldh1*. A northern blot probed previously with pldh1Sal1.0 (*ldh1* coding region) and pATc4B/E1.8 (actin) (Figure 2-13) was reprobed with *ldh1* 3' UTR (pldh1XSB) A representative northern blot is shown. Duplicate samples from three separate experiments were analyzed.

3.3.4 Determination of transcription start site of maize *Idh1*

To map the 5' end of the transcript, primer extension was performed using three different primers (Figure 3-1). MCH30 is a 32-mer corresponding to the region 108 bp downstream of the AUG start codon and 257 bp downstream of the predicted +1 transcription start site. MCH40 is an 18-mer from the region 105 bp upstream of the predicted AUG codon, and is 44 bp downstream of the predicted transcription start site. No primer extension product was observed with primer MCH39, a 22-mer located 83 bp downstream of the predicted transcription start site. Figure 3-16 shows the primer extension results together with sequencing reactions using the same primer. The extension reaction using the MCH40 primer (Figure 3-16A) gave one major band ending at an A residue in the predicted TATA box and a shorter band ending at a G residue 8 bases downstream of the predicted start site. The MCH30 primer (Figure 3-16B) extension reaction gave several bands. One of them is in the region of the predicted transcription start site (C residue 5 bases downstream). This extension product is 285 nucleotides and thus difficult to resolve well on the sequencing gel. The other bands are likely due to either the premature termination of reverse transcriptase or the presence of shorter incomplete primers in the synthetic oligonucleotide preparation. Both primers MCH 30 and MCH40 yield extension products in close proximity to each other, thus the strong band observed with primer MCH40 is likely the transcription start site. Based on these results, it can be concluded that the 5' end of the *Idh1* transcript is at the site indicated in Figure 3-17 as +1. The RNA was isolated from *Zea mays* cv. W22, the same line that was used in construction of the maize genomic library, and thus the difference between the predicted and the observed transcription start sites is not due to the analysis of different maize lines.

Figure 3-16: Primer extension of maize *Idh1* with primers MCH40 and MCH30



Primer extension mapping of the 5' end of the maize *Idh1* transcript using (A) primer MCH40 (Table 3-2 and Figure 3-2) which is complementary to the *Idh1* transcript from +61 to +44 and (B) primer MCH30 (Table 3-2 and Figure 3-1) which is complementary to the *Idh1* transcript from +288 to +257. Two different RNA samples were analyzed. The primer extension lanes are indicated as 1 and 2 while A, C, G and T indicates sequencing reactions performed using the same primer. The products of the reaction were analyzed by electrophoresis on a 6% polyacrylamide-urea sequencing gel. The arrows indicate primer extension products. (A) The starred arrow indicates the product corresponding to the transcription start site. The # arrow indicates the primer extension product terminating in the TATA box. (B) The starred arrow indicates the product corresponding to the "C" in the region around the predicted transcription start site.

Figure 3-17: Transcription start site of maize *Idh1*

Sequence of *Idh1* around the predicted transcription start site. The TATA box is boxed with a double line. The predicted transcription start site is boxed and heavily shaded. The primer extension product obtained with MCH30 is outlined with a dotted line and those obtained with MCH40 are lightly shaded. The transcription start site experimentally determined is indicated with +1 and outlined with a thick line.

3.4 *Discussion*

Grass genomes are mostly colinear but vary greatly in size primarily as the result of retroelements (Voytas, 1996). The maize nuclear genome consists of between 60 and 80% repetitive DNA (Flavell et al., 1974; Hake and Walbot, 1980) and is organized into three dispersion patterns (Hake and Walbot, 1980). These are: unique sequences interspersed with mid-repetitive sequences, mid-repetitive sequences interspersed with highly repetitive sequences and unique sequences greater than 5 kb from repetitive units. The highly repetitive sequences have a high proportion of their cytosine methylated (at CG and CNG), whereas the lower copy number repetitive units tend to be unmethylated (Bennetzen et al., 1994).

Analysis of a yeast artificial chromosome containing 280 kb of maize genomic DNA including the *adh1-F* and *u22* genes demonstrated a complex intergenic organization of nested retrotransposons (SanMiguel et al., 1996). Half the retrotransposon elements were inserted into another element. The authors detected very few data base hits when maize sequences were examined for the retrotranspon sequences Cinful, Grande, Huck, Ji and Opie unless the sequence was continued for several kb 5' or 3' to the analyzed locus suggesting that these elements are not found near active genes. The analysis of the 8.2 kb Zmldh2 fragment sequenced in this work is consistent with the presence of nested retrotransposons in maize genome organization. Promoter analysis of the hypoxically inducible maize *GapC4* gene, encoding GAPDH, also detected regions with homology to the LTRs of Cin1 and Zeon-1 retrotransposons (Köhler et al., 1995).

Plant genes are usually small and can only tolerate insertions by small elements i.e. Mu1 (SanMiguel et al., 1996). The retroelement Bs1, a low

copy number copia-like retrotransposon, was detected in the maize *adh1* gene following infection of the plant by barley stripe mosaic virus (Johns et al., 1985). This element (Jin and Bennetzen, 1989) is similar to the LP-like element in respect to the size of the direct repeat (302 vs 226 bp) and the distance between repeats (2599 vs 2609 bp).

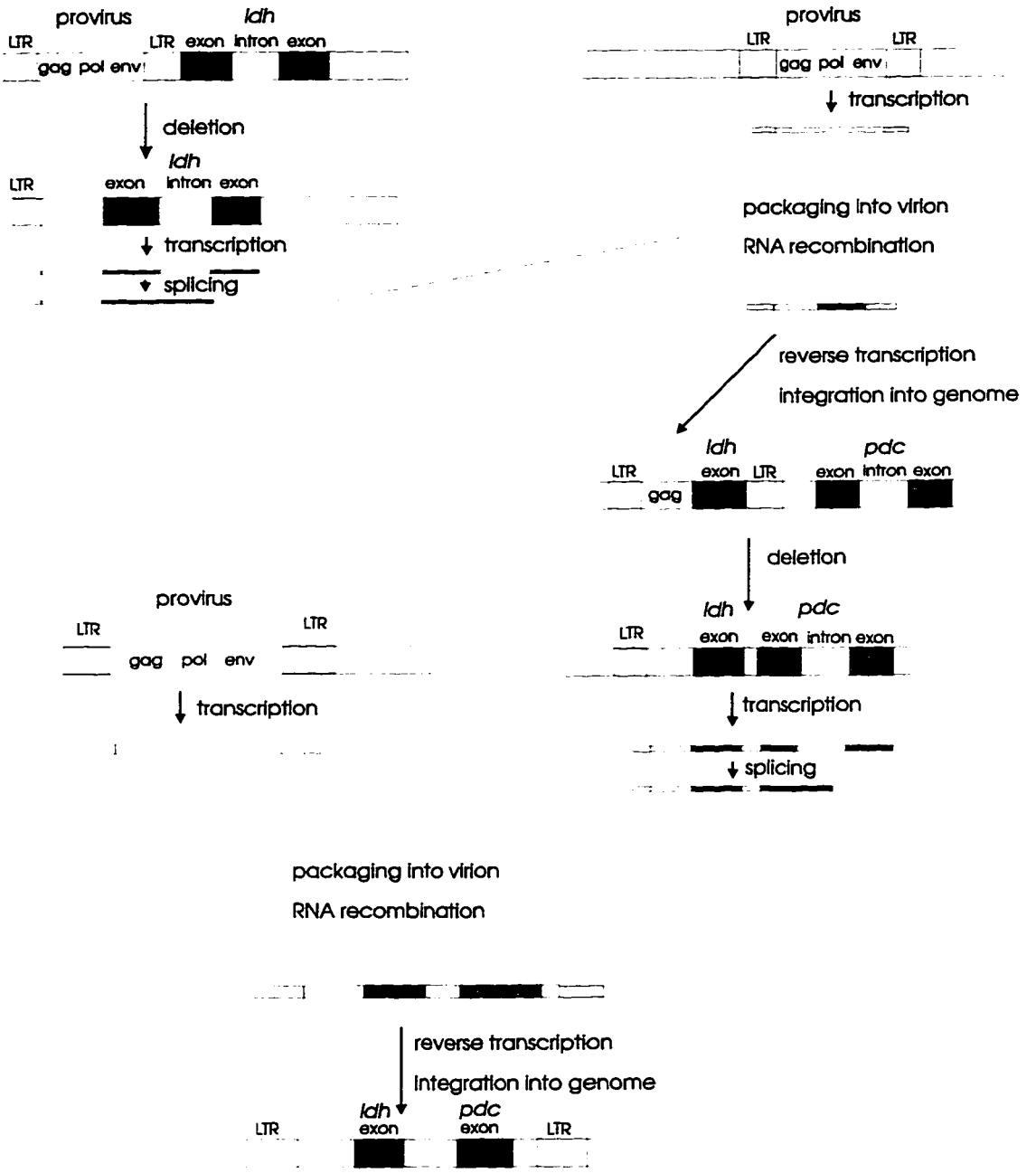
An absolute identity between the two LTRs of a given retrotransposon copy indicates that this element is (or was recently) functionally active and able to transpose. Retrotransposon RNAs contains one part of the LTR at their 5' end (R and U5 regions) and the other part of the LTR at their 3' end (U3 and R regions). During the transposition cycle these parts are used as templates for reconstruction of both LTRs (Arkhipova et al., 1995). The LP-like element also possesses the PBS in addition to absolute identity between the LTRs. This suggests that the LP-like element could transpose if the required viral elements (reverse transcriptase) were supplied in *trans*. Studies of the highly repetitive sequences surrounding *adh1* indicated that many are transcribed and that some exhibit tissue specificity in their expression (Avramova et al., 1995). The presence of regions with homology to *ldh1* and *pdc1* within the LP-like element demonstrated that retrotransposons can transfer fragments of host genes during the transposition event. A possible sequence of events leading to the presence of small regions of *ldh* and *pdc* homology residing within the LP-like element is illustrated in Figure 3-18. Maize possesses at least three *pdc* genes (Peschke and Sachs, 1993). Southern blots probed with the 3' UTR of each of the genes detected a single band, whereas probing with the coding region detected at least five bands. This work suggests that at least one of the *pdc* hybridizing bands detected in Southern blots probed with the *pdc* coding region is a nonfunctional pseudogene.

The λ clone isolated does not contain a functional *ldh* gene. Pseudogenes have been detected for a number of the hypoxically induced genes including *adh* in tomato (Longhurst et al., 1994) and *pdc* in rice (Hossain et al., 1996). Screening of the maize genomic library twice (this work and Good and Paetkau, 1992) for *ldh* has yielded only one functional gene. Comparisons of W22 and λ genomic Southern blots (Figure 3-2 and Table 3-3) digested with three different restriction enzymes demonstrated, with each restriction enzyme, the presence of two additional *ldh* hybridizing bands which could not be explained by either the *ldh1* or 39-121 λ clones. This suggests that maize possesses one or two *ldh* genes in addition to the functional *ldh1* gene (Good and Paetkau, 1992) and the psudogene (this work). Northern analysis demonstrated the presence of two *ldh* transcripts (Figure 2-13) only one of which hybridized to a *ldh1* 3' UTR probe (Figure 3-15). As library screening has not detected a second functional gene, it suggests that either the two genes have diverged significantly or that the DNA region harbouring the second *ldh*

gene is underrepresented in the genomic library. The latter is more likely as evidenced by the hybridization, to a *ldh* probe, of multiple DNA fragments and transcripts on Southern (Figure 3-2) and Northern (Figure 2-13) blots, respectively.

Southern analysis of W22 genomic DNA digested with HindIII and probed with maize *ldh1* coding region detected fragments of ~14.0 and ~8.9 kb. Neither of these fragments were detected by Southern analysis of the *ldh1* or the *ldh* pseudogene λ clones. To facilitate cloning of maize *ldh2*, W22 genomic DNA digested with HindIII could be subjected to agarose gel electrophoresis. Gel slices containing DNA fragments of the appropriate size could be cut out of the gel, the DNA eluted and then the DNA fragments could be cloned into a λ vector to generate two maize genomic libraries containing either ~14.0 or ~8.9 kb fragments. This should then enhance the likelihood of the second *ldh* gene being represented in the library. These libraries could then be screened with the maize *ldh1* coding region according to standard procedures.

Figure 3-18: Possible mechanism for the generation of the LP-like element



A provirus located within the maize genome adjacent to a *ldh* gene could undergo a deletion event. Following transcription, processed RNA could be packaged into a virion particle along with viral RNA where it would undergo RNA recombination. If the reverse transcribed product inserted into the genome adjacent to a *pdc* gene followed by another deletion event, transcription, packaging, viral RNA recombination and reverse transcription, it could result in an element with direct repeats and portions of *ldh* and *pdc* genes.

3.5 *Bibliography*

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4. Determination of components in the lactate dehydrogenase signaling pathway

4.1 *Introduction*

Stress responses induce the synthesis of proteins that function to increase survival or decrease the effectiveness of the stress agent (Ho and Sachs, 1989). The hypoxic stress response is similar to a number of other stress responses which exhibit transcriptional and translational control, rapid dissociation of polyribosomes, and decreased total protein synthesis (Hahn and Walbot, 1989; Ho and Sachs, 1989). Mitochondrial respiration is limiting under hypoxic conditions (Morard and Silvestre, 1996) and its activity is affected by other stresses. High temperatures inhibit AOX pathway activity (Vanlerberghe and McIntosh, 1992) whereas chilling, nutrient deprivation and salt stress reduce the activity of the cytochrome pathway (Vanlerberghe et al., 1997). As reduction of electron transport through the cytochrome pathway significantly reduces ATP production (Vanlerberghe et al., 1997) it is possible that, in addition to stimulation of the AOX pathway, an increased glycolytic flux to lactate and ethanol may occur.

The responses of several abiotic stresses have been shown to overlap. Exposure of *Arabidopsis thaliana* to drought stress increases freezing tolerance (Mäntylä et al., 1995), and transgenic tobacco and *A. thaliana* plants expressing a cold inducible promoter-GUS fusion directs GUS expression in response to ABA, dehydration and osmotic stress (Wang et al., 1995). Some heat shock genes are induced by desiccation, salt, and anaerobic stress, albeit at much reduced levels relative to heat stress (Czarnecka et al., 1984). Ethylene is produced in response to a variety of stresses including hypoxic (Drew et al., 1994), osmotic (Czarnecka et al., 1984; Rodríguez et al., 1997) and heat stress (Czarnecka et al., 1984). Ca^{2+} has been implicated in the hypoxic (Subbaiah et al., 1994 a and b), aerenchyma (He et al., 1996) and cold (Monroy and Dhindsa, 1995) signal transduction pathways. Additionally, DNA binding proteins of the 14-3-3 class associate with DNA-protein complexes on hypoxically induced genes (Lu et al., 1994) and salt-regulated genes (Chen et al., 1994). Together, these observations suggest some similarity in signal transduction between abiotic stresses. *A. thaliana adh* appears to be a more general stress responsive gene than maize *adh* and maize *ldh* as it is induced by hypoxia, dehydration, salt and cold (5°C) (Jarillo et al., 1993; Dolferus et al., 1994). This emphasizes that, although many components of stress responsive pathways are consistent between species, the study of the response exclusively in one species will lead to inaccurate generalities

being made. Thus it was of interest to determine whether *Idh* induction was specific to the hypoxic response or whether it was part of a more general stress response. The abiotic stresses examined were salt, polyethylene glycol (PEG), chilling and heat.

Ionic (salt) and nonionic (PEG) osmotica have profoundly different effects on bioenergetically important metabolites (Spickett et al., 1992). Salt (NaCl) stress decreases concentrations of glucose-6-phosphate, uridine diphosphate glucose and γ-nucleotide triphosphate, metabolites which are unaffected by nonionic osmotica (Spickett et al., 1992). The decrease in glucose-6-phosphate and uridine diphosphate glucose may reflect attempts to increase cellular energy by increasing glycolytic flux or may result from differential sensitivity of the primary metabolic pathways to NaCl concentrations (Spickett et al., 1992). Barley seedlings exposed to 400 mM NaCl for 3 d have impaired cytochrome pathway activity and lower phosphorylation efficiency with only a slight decrease in oxidation rates as a result of increased activity of the alternative pathway (Jolivet et al., 1990).

Salt stress, which imposes both a water deficit and ion imbalance (Niu et al., 1995) is becoming more prevalent as a result of irrigation (Ho and Sachs, 1989). Exposure of maize roots to 100 mM NaCl for 24 h decreases root extension as a result of cell wall hardening; however, extension rates remain similar to controls if 10 mM Ca^{2+} is present in the external solution (Rodríguez et al., 1997). Similar effects have been observed in barley (Katsuhara and Kawasaki, 1996) and wheat (Galvez et al., 1993) seedlings. Ca^{2+} is speculated to act by displacing Na^+ from the plasmalemma of salt-stressed root cells, thereby decreasing Na^+ influx into the roots (Galvez et al., 1993; Katsuhara and Kawasaki, 1996). Salt stressed tomato plants differentially accumulate the catalytic subunit of the H^+-ATPase in the tonoplast (expanded leaves) and plasma membrane (roots and expanded leaves) (Binzel, 1995). No increase in message for either the tonoplast or plasma membrane H^+-ATPase is observed when tomato plants are drought or PEG stressed, suggesting that this response is associated with the ionic component of NaCl stress (Binzel, 1995).

PEG treatment is used to imitate desiccation stress. The inhibitory effect of PEG on whole plant growth exceeds that of other osmolytes and can be ascribed primarily to decreased root water transport (Chazen et al., 1995). PEG treatment negatively affects cellular membranes (Lopez-Carbonell et al., 1994) and results in cytosolic and vacuolar alkalization (Spickett et al., 1992), effects opposite to those seen in hypoxia (Saint-Ges et al., 1991; Roberts et al., 1992).

Chilling stress decreases pH_c in mung bean suspension cultured cells, with actively growing cells being more adversely affected (Yoshida, 1994). This is similar to the hypoxic response (Roberts et al., 1984 a and b). Decreasing temperatures affect membrane fluidity properties, which

directly affect membrane-bound metabolic processes such as respiration (Jarillo et al., 1993). Discontinuities in respiration rates of chilling sensitive plants occur at membrane phase transition temperatures of 10 to 12°C, suggesting that physical changes in membranes disrupt mitochondrial activity (Christie et al., 1991). It has been proposed that compensation for impaired mitochondrial function occurs by shifting metabolism from aerobic to anaerobic respiration (Christie et al., 1991).

Dissection of the hypoxic signal transduction pathway is still in its infancy. A number of transcription factors such as GBF1 (de Vetten and Ferl, 1995), ARF-B2 (Ferl, 1990) and GF14ϖ (Lu et al., 1994), and signaling molecules such as Ca^{2+} (Subbaiah et al., 1994 a and b), have been identified, although their mechanism of activation and location in the signaling pathway has not been determined. Studies on the rice aldolase gene *AldC1* demonstrated that inhibitors of both protein phosphatases and protein kinases increase *AldC1* mRNA abundance. This suggests that, in rice, at least two proteins subject to reversible phosphorylation / dephosphorylation are required for the signaling pathway (Nakamura et al., 1996). Two interacting *Arabidopsis thaliana* proteins, GBF1 (Klimczak et al., 1992) and GF14ϖ, a Ca^{2+} binding protein, (Lu et al., 1994) are stimulated by phosphorylation. While complete analysis of the signal transduction pathway leading to increased *ldh* expression is beyond the scope of this thesis, the role of the signaling molecule Ca^{2+} and new protein synthesis was examined.

The purpose of the experiments described in this chapter was to address three hypotheses: (1) *ldh* induction is specific to the hypoxic response, in that oxygen deprivation and not inhibition of mitochondrial respiration is the trigger for the hypoxic signal transduction pathway, (2) new transcription factor synthesis is required during hypoxia to facilitate hypoxic *ldh* expression, and (3) Ca^{2+} is a component of the *ldh* signaling pathway.

4.2 *Materials and methods*

4.2.1 Plant material and growth conditions

Plant growth conditions were as described in Section 2.2.1.

4.2.2 Cycloheximide and chloramphenicol treatment

For 2 d prior to testing whole plants were incubated aerobically in 0.5X Hoagland's solution containing 0.2 mg mL^{-1} carbenicillin (Sigma) and

0.005% (v/v) No-Damp (Plant Products, Brampton, Ontario) to minimize bacterial and fungal contamination, respectively. Cycloheximide (CHX, Sigma) and chloramphenicol (CAP, Sigma) stock solutions were dissolved in ethanol and all plants treated with CHX, CAP or ethanol were exposed to a final concentration of 0.36% (v/v) ethanol. On the day of testing whole plants were aerobically pretreated with ethanol, 150 μg mL^{-1} CHX, 40 μg mL^{-1} CAP, both CHX and CAP, or water (control plants) for 1.5 h. Half the plants from each test condition were maintained aerobically and half were transferred to hypoxic conditions for an additional 6.5 h, at which time samples were analyzed for LDH activity and *ldh* expression. Duplicate samples from eight experiments and triplicate samples from three experiments were analyzed for *ldh* expression and LDH activity, respectively.

To determine whether the CHX concentration used affected the response, plants were exposed to 0, 50, 100 and 150 μg mL^{-1} CHX and tested as above. For the CHX titration experiments the control plants (0 μg mL^{-1} CHX) were treated with 0.36% (v/v) ethanol. Three plants were analyzed from each time point and the experiment was replicated three times.

4.2.3 *In vivo* labeling

In vivo labeling was performed to confirm the effectiveness of CHX and CAP. Plants pretreated for 1.5 h with either ethanol, CHX or CAP were incubated with 30 μCi of (^{35}S)Met (Amersham) (Gowri et al., 1992). After 4.5 h roots were washed, harvested and analyzed for incorporation of radiolabeled Met into protein. Duplicate aliquots (5-10 μL) of each root extract were placed on glass-fiber filters (GF/C, Whatman). One filter was dried and its radioactivity measured in aqueous counting scintillant (Amersham) for determination of total radioactivity. The other filter was used to determine incorporation of the radiolabel into protein. After drying, the filter was washed with cold 10% (w/v) TCA, heated at 100°C for 15 min in 10% (w/v) TCA, rewashed with cold 10% (w/v) TCA, and then washed again with cold 95% (v/v) ethanol and dried. The radioactivity was measured in aqueous counting scintillant (Amersham) and counted on a liquid scintillation analyzer (model 1600TR, Canberra Packard, Mississauga, Ontario). Washing efficiency was monitored by spiking extracts with (^{35}S)Met. In all experiments, less than 1% of the spike was recovered on the washed filter. Three samples were analyzed in each experiment and the experiment was replicated eight times.

Root protein extracts were subjected to 12% (w/v) SDS-PAGE to determine the effects of protein synthesis inhibitors on the protein profile.

The gel, loaded with 10,000 cpm per lane of denatured protein extract, was electrophoresed at 150 volts for 60 min, fixed in 30% (v/v) methanol, 10% (v/v) acetic acid for 60 min, washed twice in EN^{3}HANCE (DuPont) for 60 min each and in water for 5 min, dried at 60°C for 2 h and autoradiographed at -80°C for 16 to 24 h. A duplicate gel was silver stained according to the manufacturers protocol (Bio-Rad).

4.2.4 Calcium chloride and ruthenium red treatment

On the day of testing three-week-old seedlings were aerobically pretreated with 5 mM $CaCl_2$, 25 μM ruthenium red (RR, Sigma), both or neither (control plants) for 1 h. Half the plants were maintained aerobically and half were transferred to hypoxic conditions for an additional 4.5 h, at which time samples were analyzed for LDH activity and *ldh* expression. To determine the effect of RR on the early hypoxic response, plants pretreated with RR and maintained in RR for the first h of hypoxia were transferred to $CaCl_2$ for the final 3.5 h of hypoxic induction. To determine whether the Ca^{2+} concentration used affected the response, plants were exposed to 0, 0.25, 0.5, 1, 2.5 and 5 mM $CaCl_2$ and tested as above. Three plants were analyzed from each time point in three separate experiments.

4.2.5 Stress treatment

Control plants for the stress experiments were maintained at 20°C in 0.5X Hoagland's solution and exposed to either aerobic or hypoxic conditions for the same length of time as plants exposed to the various abiotic stresses. All plants were grown in the same growth chamber except for those exposed to cold stress.

4.2.5.1. *Cold*

Studies of 3-d-old dark-grown maize seedlings demonstrated a significant decrease in survival when seedlings were grown at 4°C for 7 d compared to those grown at 6°C (Anderson et al., 1994), Thus 4°C was selected as the temperature of study. Four-week-old seedlings were transferred to hydroponic tanks prechilled to 4°C. Plants were maintained at 4°C for up to 6 d and were bubbled with either air or 100% N_2. The light / dark cycle was the same as for the control plants maintained at 20°C. The plants were maintained in 0.5X Hoagland's solution throughout the

course of the experiment. Three plants were analyzed from each time point in two separate experiments.

4.2.5.2. *Heat*

Four-week-old seedlings grown in 0.5X Hoagland's solution were transferred to 1 L beakers containing 500 mL Hoagland's solution which had been prewarmed to 38°C. The beakers, maintained at 38°C, were either bubbled with air or 100% N_2 for 8, 24, or 48 h. Two plants were analyzed from each time point in three separate experiments.

4.2.5.3. *Salt*

Four-week-old seedlings were transferred to beakers containing Hoagland's solution supplemented with 100 mM NaCl and tested as in section 4.2.5.2.

4.2.5.4. *Desiccation*

Four-week-old seedlings were transferred to beakers containing Hoagland's solution supplemented with 20% (w/v) PEG 8000 and tested as in section 4.2.5.2. This concentration of PEG 8000 should give an osmotic pressure of approximately 0.5 mPa (Money, 1989). PEG with molecular weights >6000 are non-ionic, water-soluble polymers which are not expected to penetrate intact plant tissues readily or to be rapidly metabolized (Chazen et al., 1995). Additionally, they do not result in accumulation of inorganic ions and the water relations in plants are similar to those of plants grown in the soil with the same water potential (Lopez-Carbonell, 1994).

4.2.6 Relative water content

Plants exposed to heat, salt and desiccation stress were analyzed for water content in the leaf tissue. Leaf tissue was weighed (fresh weight, FW), rehydrated in water for 24 h (turgid weight) and dried to a constant weight in a 65°C oven (dry weight). Relative water content (RWC) is calculated as described in Equation 4-1.

Equation 4-1: Relative water content

$$RWC = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Turgid Weight} - \text{Dry Weight}}$$

4.2.7 Enzyme assays and protein determination

LDH assays were performed as described in Section 2.2.3. ADH assays were performed as described in Section 2.2.4 and protein concentrations were performed as described in Section 2.2.5.

4.2.8 Root bacterial cultures

To monitor bacterial contamination, maize plants were incubated for 5.5 h in either water or 0.2 mg mL^{-1} carbenicillin plus 0.005% (v/v) No-Damp following which the roots were ground in extraction buffer or extensively washed with running water prior to grinding. Dilutions of the extract in Luria broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) were plated on Luria broth plates supplemented with 15 g L^{-1} agar and incubated for 4 d at 28°C.

4.2.9 Northern blots

Poly(A)$^+$ RNA, isolated as described in Section 2.2.14, was subjected to Northern analysis (Section 2.2.16). Northern blots, hybridized to both maize *Idh1* and actin probes (Section 2.2.15), were analyzed by densitometry (Section 2.2.17). Blots selected for densitometric analysis were those in which the autoradiographic film was not saturated. Blots shown in the figures were selected to allow visualization of the weakly hybridizing transcripts. Thus, the bands corresponding to transcripts present at elevated levels appeared to be saturated on the autoradiogram.

4.2.10 Plasmid constructs for future studies

Examination of the maize *Idh1* promoter demonstrated the presence of a sequence similar to the ARE, located at -111 to -69 (Figures 1-10 and 4-1). *In vitro* mutagenesis was performed according to the manufacturers protocol (Bio-Rad Muta-Gene Phagemid *in vitro* mutagenesis kit) to generate promoter deletions. The mutagenic primers (Table 4-1) generated a HindIII restriction site to facilitate subcloning. The mutagenized plasmids were digested with HindIII and PvuII and ligated into pBI221 (Clontech) which had previously been digested with HindIII and SmaI to remove the CaMV 35S promoter. Plasmid pBI221 contains the CaMV 35S promoter ligated to the coding region for β-glucuronidase (GUS) and the terminator region, NOS. The constructs generated (Figure 4-1) were confirmed by sequence analysis.

Table 4-1: Primers used for *in vitro* mutagenisis of the maize *Idh1* promoter

Primer	Sequence (5' → 3')	mer	% G+C	T_m
MCH1	CTCC**AAGCTT**GTGAAGTGGTTTGG	24	50%	72°C
MCH2	GGCC**AAGCTT**TTAATGGAATTG	22	41%	62°C
MCH3	GTG**AAGCTT**TAAACCGCACCT	21	48%	62°C

Mutagenized nucleotides are bolded and the HindIII site is underlined. Primers were synthesized by the Molecular Biology Service Unit.

Figure 4-1: Maize *Idh1* promoter-GUS fusions

Deletion constructs of maize *Idh1* generated by *in vitro* mutagensis followed by ligation into the promoterless GUS-NOS plasmid, pBI221. The location of the primer binding sites for MCH1, MCH2 and MCH3 are indicated as 1, 2 and 3, respectively. The AREI, AREII and TATA box are shown.

4.3 *Results*

4.3.1 Effect of protein synthesis inhibitors on LDH activity and *Idh* expression

CHX and CAP inhibit protein synthesis by inhibiting the peptidyl transferase activity of cytosolic and organellar ribosomes, respectively (Pestka, 1977). Other effects of CHX include inhibition of DNA polymerase, RNA polymerase I and tyrosine hydroxylase (Pestka, 1977). CAP can also inhibit mitochondrial respiration (Pestka, 1977). Plants were treated with CHX to determine whether new protein synthesis was required for hypoxic induction of *Idh*, and with CAP to determine if there was a mitochondrial or plastid component to the *Idh* signal transduction pathway.

In vivo labeling was performed to monitor CHX and CAP uptake by the root. Bacterial contamination can significantly affect the results of *in vivo* labeling, therefore bacterial colony forming units (CFU) were determined. Bacterial CFUs were very low on washed roots and those exposed to carbenicillin and No-damp (Table 4-2). All *in vivo* labeling experiments were therefore performed in 0.2 mg mL^{-1} carbenicillin and 0.005% (v/v) No-damp with the roots being extensively washed prior to grinding. This treatment should minimize bacterial and fungal uptake of the (^{35}S)Met.

CHX can be absorbed through the root system to inhibit protein synthesis *in vivo* (Table 4-3) (Gowrie et al., 1992). Hypoxia had no effect on LDH enzyme activity in control (no CHX) plants after 6.5 h of treatment (Table 4-3), although the 1.3 kb *ldh* transcripts abundance was increased 2 fold at this time (Figure 4-2). Treatment with CHX at 100 µg mL^{-1} had no effect on LDH enzyme activity in either aerobic or hypoxically treated plants. Plants treated with 150 µg mL^{-1} of CHX under aerobic conditions had a slight increase in LDH activity, which was not observed in hypoxically treated plants. Treatment with CHX increased *ldh* mRNA expression in both aerobic and hypoxic conditions relative to the aerobic control plants (Table 4-3 and Figure 4-2). The experiment was not continued for longer periods as the plants were extremely stressed after 12 h of CHX treatment and thus reliable interpretations could not have been made. Analysis of silver stained and *in vivo* labeled protein extracts subjected to SDS-PAGE detected no observable differences in the protein profiles (Figure 4-3). Differences in protein profiles are more likely to be detected in two-dimensional electrophoresis rather than in a single phase, such as SDS-PAGE.

Table 4-2: Bacterial growth on roots used for *in vivo* labeling

Supplement	Treatment	CFU	# of Types of Colonies
none	none	52×10^6	4
none	washed	$<10^6$	
carbenicillin + No-damp	none	$<10^6$	

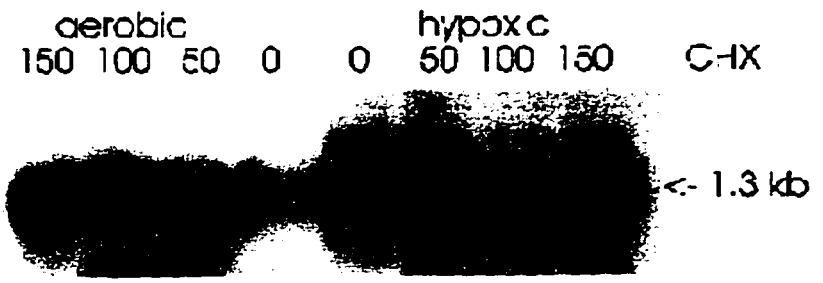
Maize plants were incubated for 5.5 h in either water or 0.2 mg mL^{-1} carbenicillin plus 0.005% (v/v) No-Damp after which the roots were ground in extraction buffer or extensively washed with running water prior to extraction. Dilutions of the extract in Luria broth were plated on Luria broth plates supplemented with agar and incubated for 4 d at 28°C. CFU = colony forming units.

Table 4-3: Effect of CHX on *in vivo* protein synthesis, LDH activity and *ldh* transcript abundance

CHX concentration	(^{35}S)Met incorporated into protein		LDH enzyme activity				1.3 kb *ldh* transcript intensity			
			Aerobic		Hypoxic		Aerobic		Hypoxic	
µg mL^{-1}	Relative %		µmol min^{-1} g^{-1} fresh weight				Relative intensity			
0	100.0	(3.3)	74.9	(5.9)	79.2	(5.1)	1.0		3.0	(1.0)
50	66.8	(4.6)	NT		NT		2.0	(0.7)	6.5	(0.2)
100	55.9	(2.8)	80.1	(7.1)	84.1	(2.0)	6.2	(1.0)	3.0	(0.6)
150	64.8	(2.3)	97.4	(3.2)	76.0	(5.4)	3.8	(0.4)	4.7	(0.5)

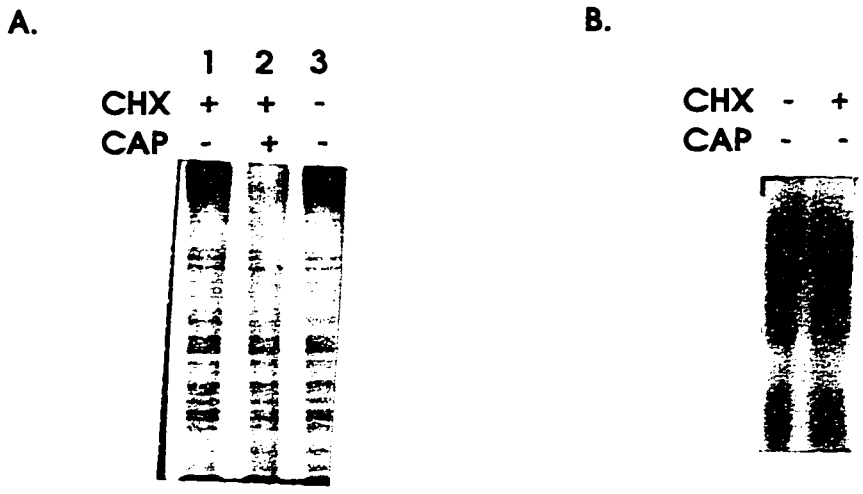
Maize plants were pretreated with 0, 50, 100 or 150 µg mL^{-1} CHX for 1.5 h prior to addition of 30 µCi (^{35}S)Met. Ethanol, 0.36% (v/v), was present in all treatments. *In vivo* labeling continued for 4.5 h, at which time soluble root proteins were extracted and analyzed for incorporation of (^{35}S)Met into protein. LDH enzyme activity and *ldh* induction were determined after 6.5 h of treatment. Three samples were analyzed in each experiment and the experiment was replicated 8 times (*in vivo* labeling) and 3 times (LDH enzyme assays and *ldh* Northerns) (+SE). Densitometric analysis for *ldh* expression was performed on autoradiograms which were not saturated A representative Northern blot is shown in Figure 4-2. NT = not tested.

Figure 4-2: Effect of CHX concentration on *ldh* expression



Maize plants were aerobically pretreated with 0, 50, 100 or 150 µg mL^{-1} CHX for 1.5 h prior to transfer to either aerobic or hypoxic conditions (CHX concentrations were the same as in the pretreatment) for an additional 6.5 h. Ethanol, 0.36% (v/v), was present in all treatments. One µg poly(A)$^+$ RNA was analyzed by Northern blotting and probing with *ldh1* and actin. A representative Northern blot is shown. Three samples were analyzed in each experiment and the experiment was replicated 3 times. Results from densitometric analysis are reported in Table 4-3.

Figure 4-3: Effect of CHX and CAP on protein synthesis



Effect of CHX and CAP on (A) total protein and (B) *in vivo* labeled protein profiles. Maize plants were pretreated with 0, 150 μg mL^{-1} CHX, or 150 μg mL^{-1} CHX and 40 μg mL^{-1} CAP for 1 h prior to addition of 30 μCi (^{35}S)Met. Ethanol, 0.36% (v/v), was present in all treatments. *In vivo* labeling continued for 4 h, at which time root extracts were prepared. Equal counts were loaded in each lane. (A) Silver stained gel. (B) Autoradiographic exposure.

Northern blot analysis of CHX treated plants demonstrated that CHX had a significant effect on the 1.3 kb *Idh* transcript (Figure 4-2 and Figure 4-4). Previous analysis of *Idh* transcript profiles during hypoxia (Figure 2-13) demonstrated that the 1.3 kb transcript was of higher abundance than the 1.7 kb transcript at all time points tested. After 8 h of hypoxic treatment the 1.3 kb transcript abundance had increased ~30 fold relative to the aerobic control, whereas the 1.7 kb transcript was only induced ~10 fold (Figure 2-13). In the experiments with CHX, RNA was isolated after 6.5 h of aerobic or hypoxic treatment and thus the lack of detection of the 1.7 kb transcript can be attributed to its lower relative abundance. After 6.5 h of hypoxia the relative intensity of the 1.3 kb *Idh* transcript increased ~10 fold in the control plants (Figure 4-4). As CHX stocks were dissolved in ethanol, plants treated with 0.36% (v/v) ethanol were analyzed. In aerobic conditions ethanol had a slight effect on *Idh* transcript abundance, but in hypoxic conditions, treatment with ethanol increased *Idh* transcript levels ~40 fold. CHX treated, hypoxic plants had approximately the same amount of the 1.3 kb transcript as the ethanol treated, hypoxic plants regardless of whether aerobic pretreatment was in water or CHX. It is interesting that CHX treatment of plants maintained

aerobically resulted in a dramatic increase in *ldh* transcript abundance (~120 fold, Figure 4-4). The difference in the fold increase following exposure to 6.5 h of hypoxia in plants exposed to 150 μg mL^{-1} CHX appears to be lower in Figure 4-2 than in Figure 4-4. In Figure 4-2, control plants were exposed to the same concentration of ethanol as the test plants whereas in Figure 4-4 the control plants were not exposed to ethanol. Since exposure to ethanol resulted in a 5 fold increase in aerobic *ldh* expression compared to plants not exposed to ethanol the relative abundance of *ldh* transcripts would be lower in the control plants shown in Figure 4-4 than in those shown in Figure 4-2.

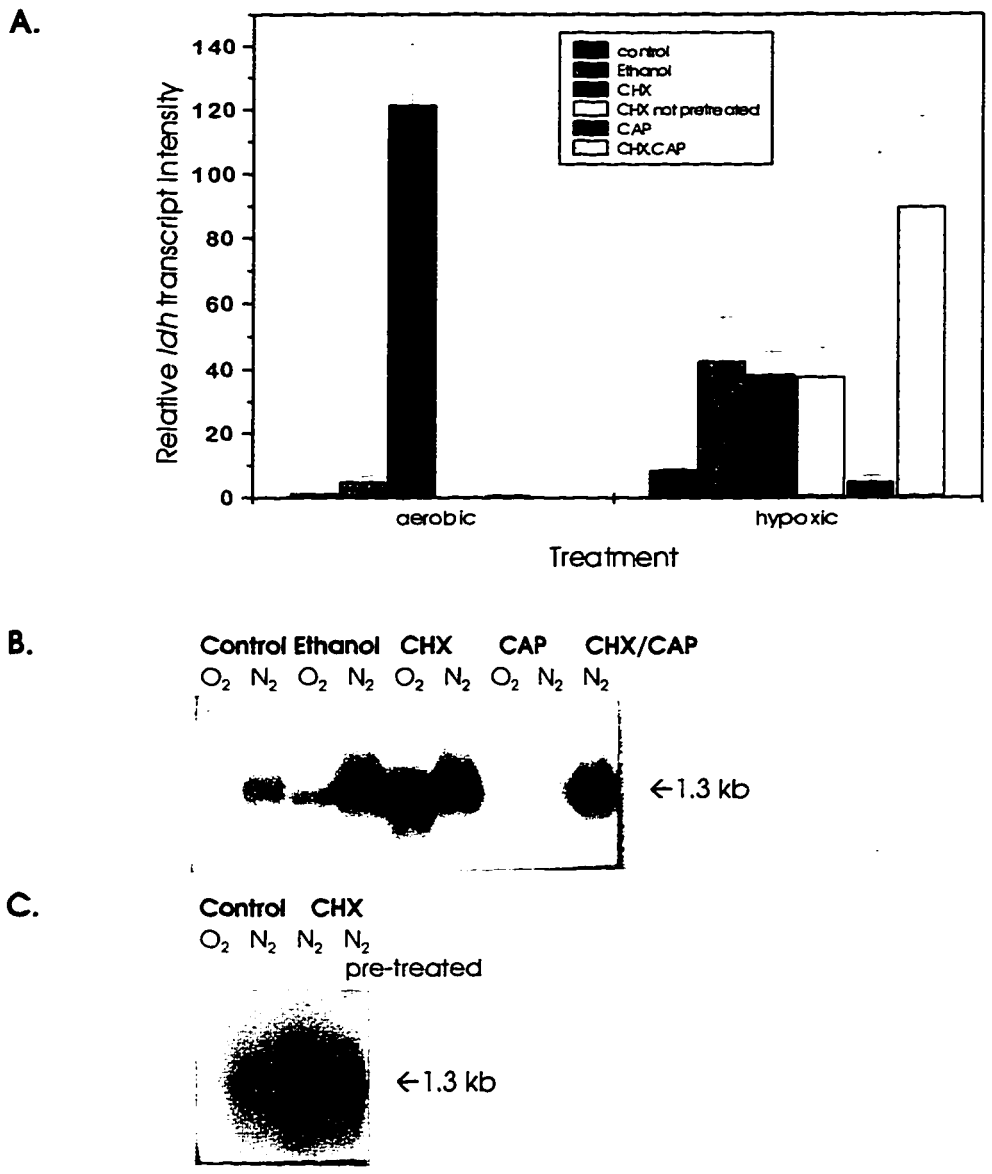
Exposure of maize roots to CAP decreased the incorporation of (^{35}S)Met into protein (Table 4-4) and the hypoxic induction of *ldh* relative to control plants (Figure 4-4). This decrease in *ldh* induction is most noticeable when *ldh* transcript intensities are compared between CAP and ethanol treated plants.

Table 4-4: Comparison of CHX and CAP on *in vivo* protein synthesis

Treatment	Relative % (^{35}S)Met incorporated into protein	(+SE)
control	100	(3.3)
CHX	70.1	(4.2)
CAP	43.7	(3.5)
CHX + CAP	17.7	(3.8)

Maize plants were pretreated with 0, 150 μg mL^{-1} CHX and / or 40 μg mL^{-1} CAP for 1.5 h prior to addition of 30 μCi (^{35}S)Met. Ethanol , 0.36% (v/v), was present in all treatments. *In vivo* labeling continued for 4.5 h, at which time soluble root proteins were extracted and analyzed for incorporation of (^{35}S)Met into protein. Three samples were analyzed in each experiment and the experiment was replicated 6 times (+SE).

Figure 4-4: Effect of CHX and CAP on *Idh* transcripts



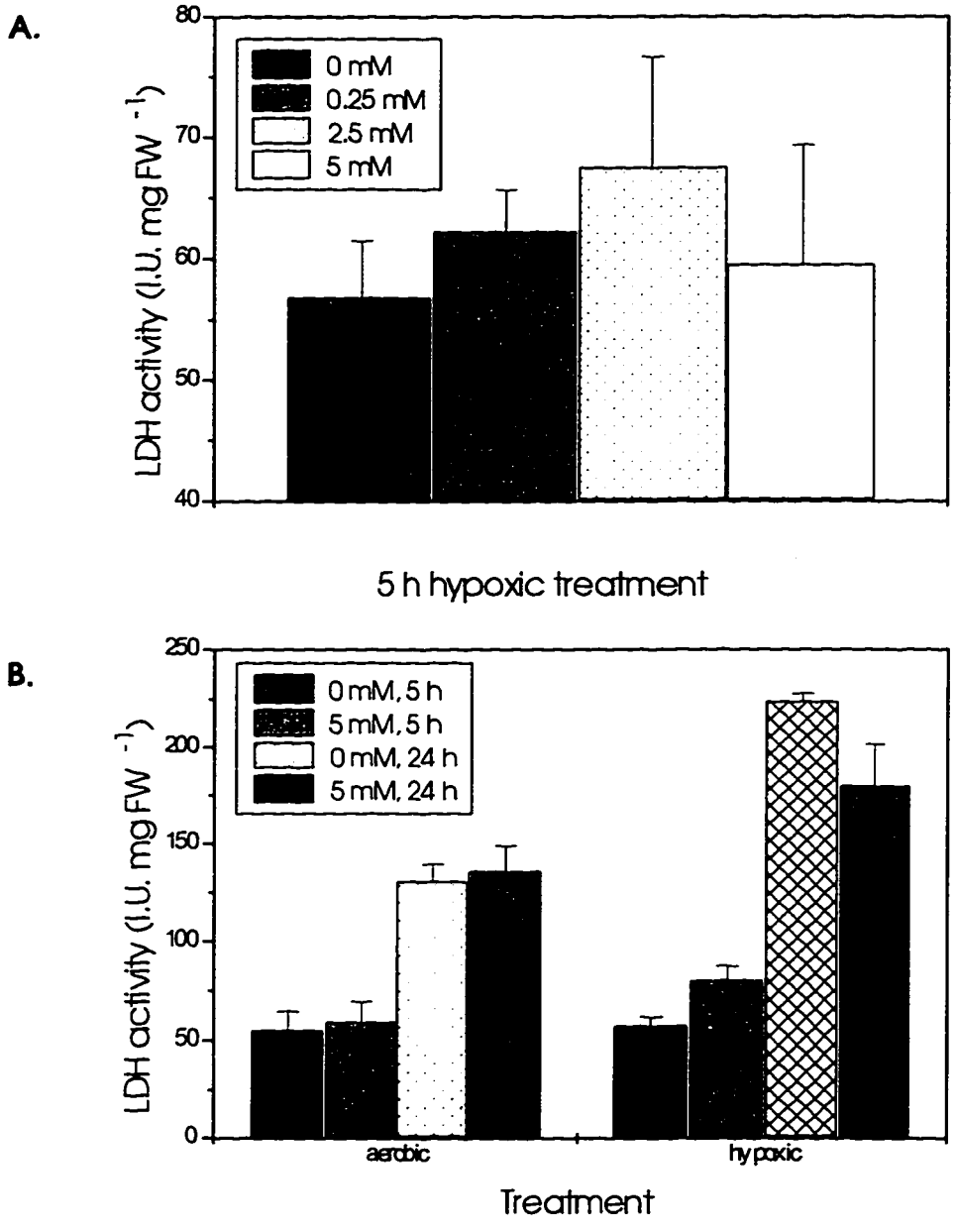
Analysis of the effect of CHX and CAP on *Idh* transcripts. (A) Densitometric analysis, (B and C) Northern blots. (A and B) Plants were aerobically pretreated with water (control), 0.36% (v/v) ethanol, or 0.36% (v/v) ethanol plus 150 μg mL^{-1} CHX or 40 μg mL^{-1} CAP for 1.5 h and then either maintained aerobically or transferred to hypoxic conditions for an additional 6.5 h. (C) Comparison of CHX and non-CHX pretreated plants. Conditions were aerobic, hypoxic, hypoxic CHX without pretreatment and hypoxic CHX with pretreatment, for the same times as in A and B. One μg poly(A)$^+$ RNA was analyzed by Northern blotting and probing with *Idh1* and actin. Representative Northern blots are shown. Densitometric analysis for *Idh* expression was performed on autoradiograms which were not saturated. Duplicate samples from 8 separate experiments were analyzed (+SE).

4.3.2 Effect of manipulation of extracellular calcium and intracellular calcium release on LDH activity and *ldh* expression

Exposure of maize plants to increased Ca^{2+} during 5 h of hypoxia resulted in a slight but insignificant increase in LDH activity with increasing concentrations of Ca^{2+} (Figure 4-5A). Longer exposure to 5 mM Ca^{2+} (24 h) resulted in decreased hypoxic LDH activity relative to hypoxic controls (Figure 4-5B) suggesting that the maize plants were not initially Ca^{2+} deficient. Northern analysis also demonstrated a general increase in *ldh* expression with increasing Ca^{2+} concentrations (Figure 4-6). Ca^{2+} treatment also dramatically increased the expression of the 1.7 kb transcript (Figure 4-7) so that, even with short periods of hypoxic treatment, both *ldh* transcripts were detectable.

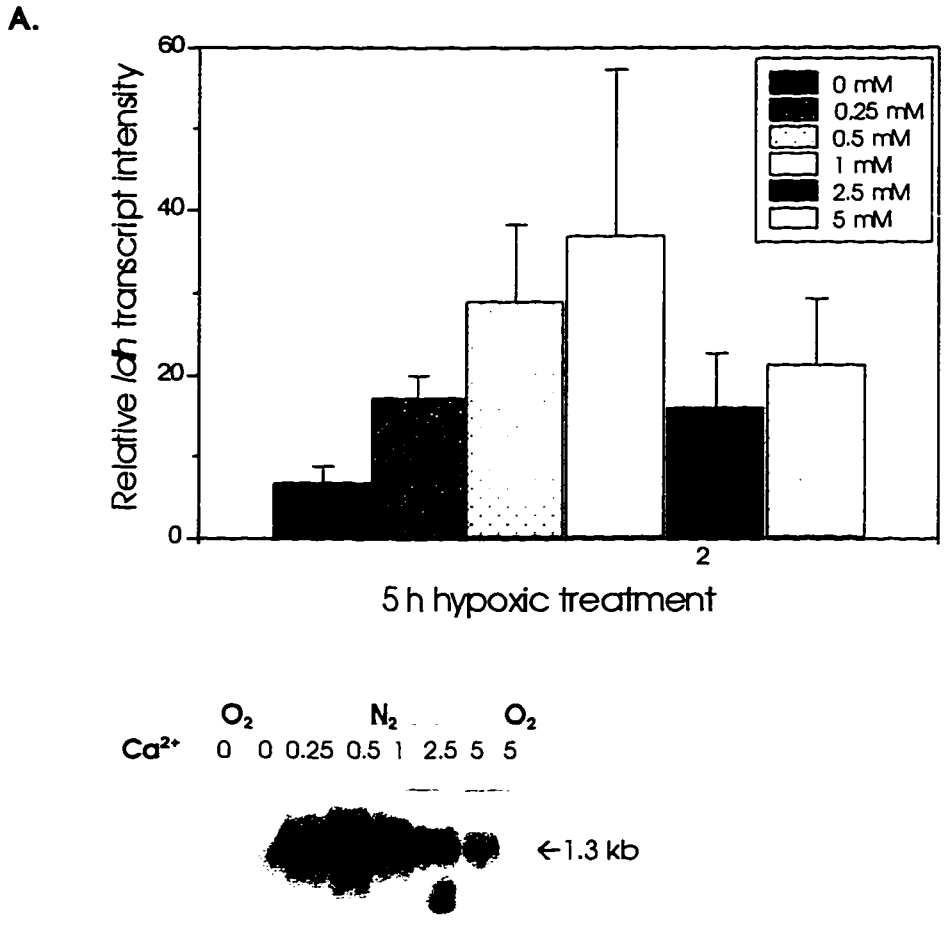
RR, an inhibitor of organellar Ca^{2+} release (Subbaiah et al., 1994a) interacts with Ca^{2+}-binding proteins including calmodulin and the endoplasmic reticulum proteins calsequestrin and Ca^{2+}-ATPase (Charuk et al., 1990). RR depressed the expression of the 1.3 kb *ldh* transcript 67 fold relative to the hypoxic control, but had less of an effect on the 1.7 kb transcript (Figure 4-7). This suggests a role for organellar Ca^{2+} release in *ldh* regulation. Plants pretreated with, and exposed to, RR for the first h of hypoxic treatment and Ca^{2+} for the remainder of the experiment had increased expression of the 1.3 kb *ldh* transcript relative to the control. This suggests that extracellular Ca^{2+} may also have a role in *ldh* expression. A possible role for extracellular Ca^{2+} is also suggested by the comparison of *ldh* transcript abundance in plants treated with Ca^{2+} alone (100 fold increase) with plants treated concurrently with Ca^{2+} and RR (45 fold increase).

Figure 4-5: Effect of Ca^{2+} concentration on LDH enzyme activity



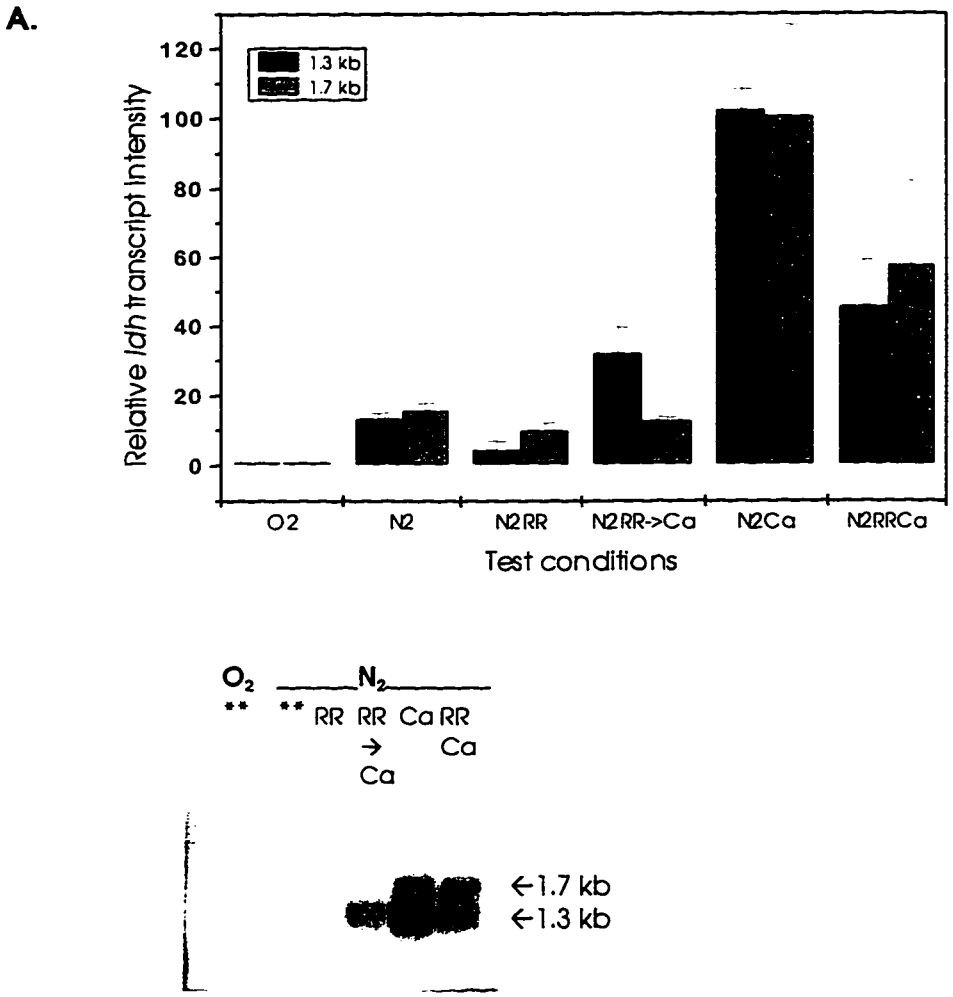
Effect of Ca^{2+} concentration on LDH enzyme activity in maize roots. (A) Plants were aerobically pretreated with 0, 0.25, 2.5 or 5 mM $CaCl_2$ for 1 h and then transferred to hypoxic conditions for 5 h. (B) Plants were aerobically pretreated with 0 or 5 mM $CaCl_2$ and then either transferred to aerobic or hypoxic conditions for 5 or 24 h. Three samples were analyzed from three separate experiments (+SE).

Figure 4-6: Effect of Ca^{2+} concentration on *ldh* expression



Effect of Ca^{2+} concentration on *ldh* expression in maize roots. (A) Densitometry, (B) Northern blot. Plants were aerobically pretreated with 0, 0.25, 0.5, 1, 2.5 or 5 mM $CaCl_2$ for 1 h and then transferred to hypoxic conditions for 5 h. Plants pretreated with 0 or 5 mM $CaCl_2$ were also maintained aerobically. One μg of poly(A)$^+$ RNA was analyzed by Northern blotting and probing with *ldh1* and actin. A representative Northern blot is shown. Duplicate samples were analyzed from three separate experiments (+SE).

Figure 4-7: Effect of Ca^{2+} and RR on *Idh* expression



Effect of Ca^{2+} and RR on *Idh* transcripts in maize roots. (A) Densitometry, (B) Northern blot. Plants were aerobically pretreated with water (control), 25 µM RR and / or 5 mM $CaCl_2$ for 1 h and then either maintained aerobically or transferred to hypoxic conditions for an additional 4.5 h. For the RR → Ca treatment, pretreatment and the first h of hypoxia was with RR followed by transfer to $CaCl_2$ for the remaining 3.5 h of hypoxia. One µg of poly(A)$^+$ RNA was analyzed by Northern blotting and probing with *Idh1* and actin. A representative Northern blot is shown. ** indicated control plants. Duplicate samples were analyzed from 5 separate experiments (+SE).

4.3.3 Effect of abiotic stresses on *ldh* expression and hypoxic induction

The hybridization of transcripts to a *ldh* probe was used to assess whether other environmental (abiotic) stress agents could induce *ldh* expression in order to determine if LDH was a general or specific stress responsive protein. This approach was used as the primary evaluation tool, but was supplemented by assays for LDH activity. The effect of concurrent abiotic and hypoxic stress was also examined to determine conditions in which the hypoxic response could be mitigated.

Treatment with heat (38°C), cold (4°C) or salt (100 mM NaCl) had no visible affect on the appearance of maize plants after 2 d of treatment; however the plants were visibly stressed (wilted) after 8 h of desiccation (20% (w/v) PEG) and looked extremely stressed after 48 h of treatment. The wilting was likely the result of the abruptness of the water deficit imposed on the plants (Mansour et al., 1993).

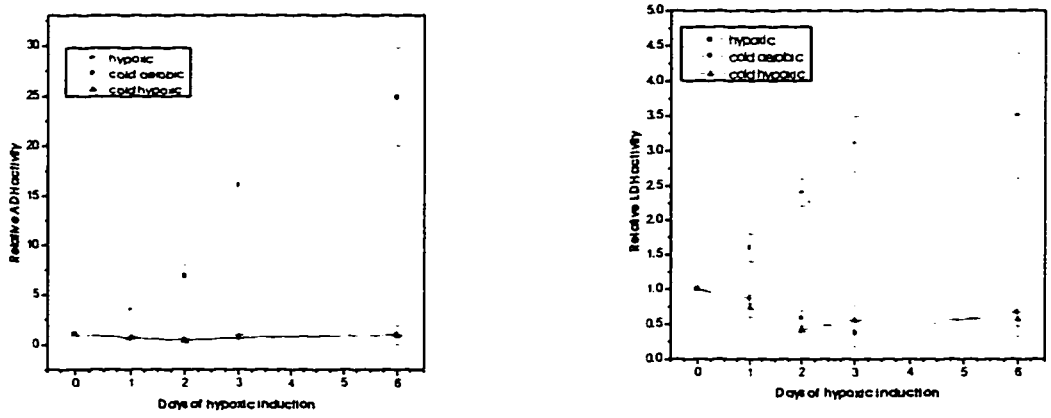
In maize, ADH is induced by cool temperatures (10°C) (Christie et al., 1991) but not by temperatures near freezing (0.5 to 1°C) (Peschke and Sachs, 1994). This suggests that there is a temperature threshold below which ADH can no longer be induced. Maize seedlings have diminished survival at 4°C relative to growth at 6°C (Anderson et al., 1994); thus, 4°C was selected as the temperature of study to determine if temperatures within the range of 0.5 to 10°C had a mitigating effect on the hypoxic induction response. ADH is not induced at 4°C (Figure 4-8A) and this temperature completely suppressed the hypoxic induction of ADH enzyme activity. This cold mediated repression of the hypoxic response had not previously been demonstrated. LDH was also not induced by cold, with a similar repression of the hypoxic response by cold temperatures (Figure 4-8B). Plants maintained aerobically at 20°C and then transferred to either aerobic or hypoxic conditions at 4°C had a 40 to 60% decrease in LDH activity within 2 d (Figure 4-8B). The impairment of the hypoxic response is mirrored by *ldh* transcript abundance (Figure 4-9). A slight induction of *ldh* transcripts in cold hypoxically treated roots is present at 3 d, but this induction is of significantly lower intensity relative to hypoxic 20°C control plants. Only the 1.3 kb transcript was detected in Northern analysis because of the relative abundance of the two transcripts. At 48 h of hypoxic induction (at 20°C) the 1.3 and 1.7 kb transcripts were induced 12.5 and 5 fold, respectively, and both transcripts were reduced to aerobic levels by 96 h of hypoxia (Figure 2-13).

The abiotic stresses, salt and PEG, act as osmotica; therefore, it was of interest to determine their effect on the water status of the stressed plants. Neither stress resulted in a significant difference in RWC relative to

control plants (Figure 4-10). It should be noted that the results from the PEG treated plants are likely inaccurate as the leaf tissue was extremely wilted and would not rehydrate.

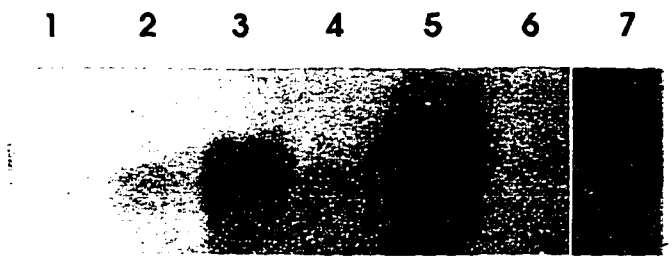
Figure 4-8: Effect of cold temperatures on the hypoxic response

A. **B.**



Effect of concomitant cold stress on the hypoxic response of (A) ADH and (B) LDH activity in maize roots exposed to 0, 1, 2, 3 and 6 d of hypoxia at 20°C or 4°C and of plants exposed to aerobic conditions at 4°C for 1, 2, 3 and 6 d. Relative values are given as, although the trend was the same in both experiments, the actual activity values varied. Each time point is the mean of data obtained from a single experiment (n=3; +SE). The experiment was replicated twice.

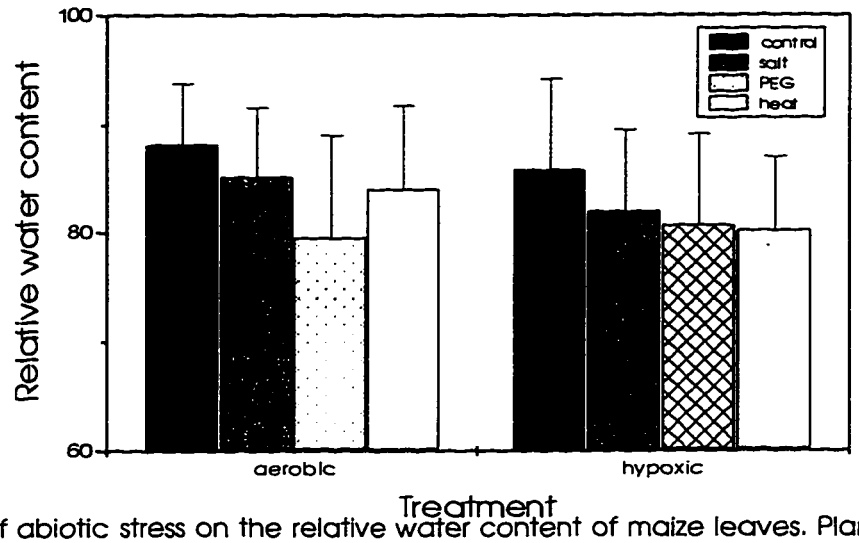
Figure 4-9: Effect of cold temperatures on *ldh* expression



Effect of concomitant cold stress on the hypoxic response of *ldh* transcripts. One μg of poly(A)$^+$ RNA isolated from maize roots exposed to 4°C aerobic conditions for 3 d (lane 1) or 6 d (lane 2), 4°C hypoxic for 3 d (lane 3) or 6 d (lane 4), 20°C hypoxic for 3 d (lane 5) or 6 d (lane 6) and 20°C aerobic (lane 7) was probed with maize *ldh1* and actin. A representative Northern is shown. The lanes were reordered for ease of visualization. Duplicate samples from two separate experiments were analyzed.

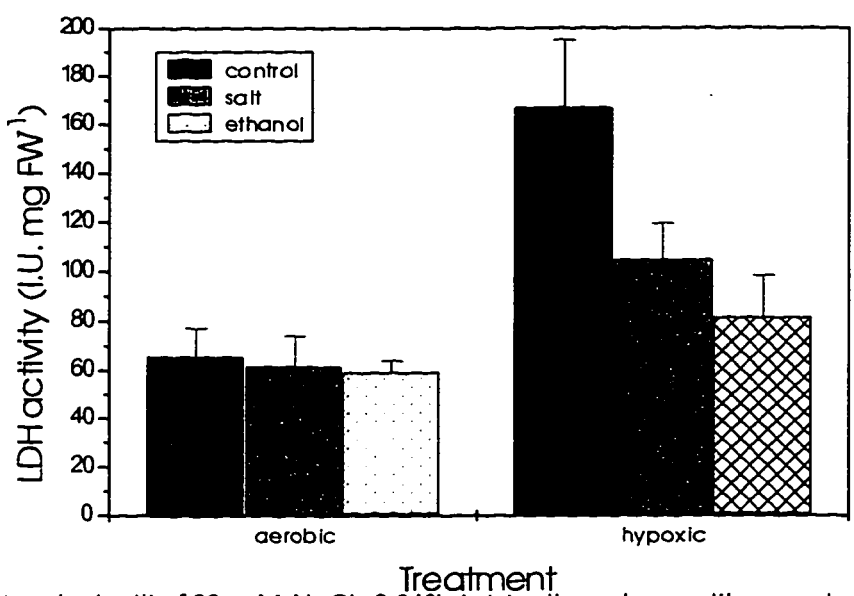
Analysis of enzyme activity following 24 h treatment of salt and 0.36% (v/v) ethanol did not result in increased LDH activity in aerobic conditions, but both treatments reduced LDH activity in hypoxically treated plants (Figure 4-11). It was previously shown that a 5 h exposure to ethanol in aerobic conditions results in ~5 fold increase in *ldh* expression (Figure 4-4). An increase in aerobic *ldh* expression was also observed in 24-h aerobically-maintained plants exposed to ethanol and salt (Figure 4-12). Concurrent hypoxic treatment had no effect on *ldh* expression in plants treated with ethanol, but a synergistic increase was observed in salt stressed plants. PEG and 38°C treatment had a slight inductive effect on aerobically maintained plants but both stresses, when present concomitantly with hypoxia, reduced the hypoxic expression of *ldh*, 38°C more so than PEG (Figure 4-12).

Figure 4-10: Relative water content of maize leaves exposed to abiotic stress



Effect of abiotic stress on the relative water content of maize leaves. Plants exposed to 100 mM NaCl, 20% (w/v) PEG, 38°C or 22°C (control) were maintained for 24 h under either aerobic or hypoxic conditions. The shoots were weighed, rehydrated for 24 h and then dried at 65°C to a constant weight. Duplicate samples were analyzed from 3 separate experiments (+SE).

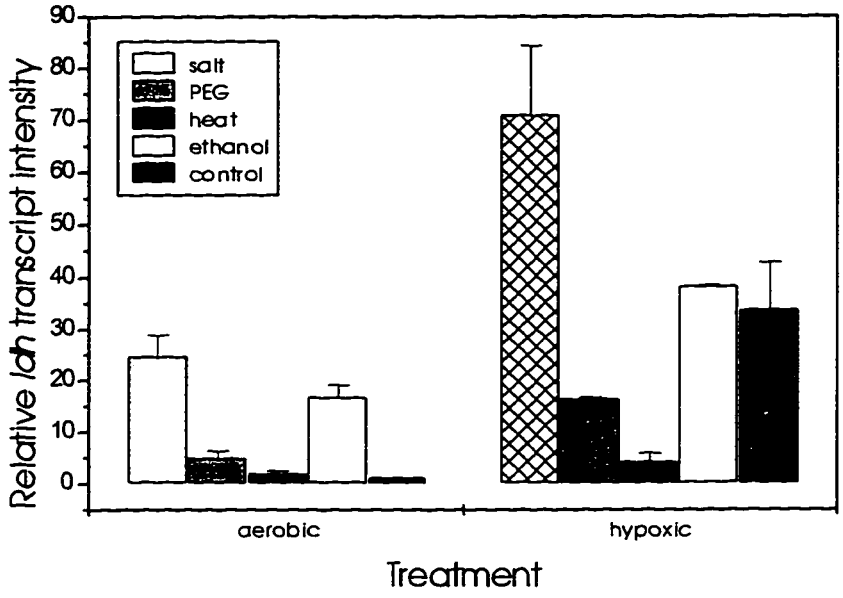
Figure 4-11: Effect of abiotic stress on LDH enzyme activity



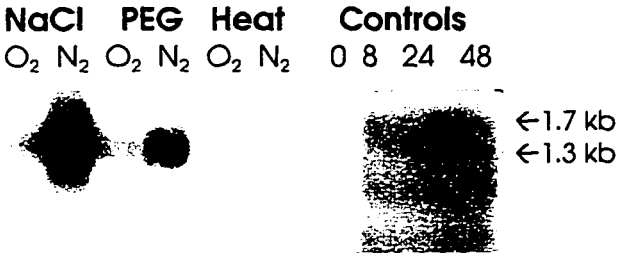
Plants were treated with 100 mM NaCl, 0.36% (v/v) ethanol or neither and maintained aerobically or hypoxically for 24 h. Three samples were analyzed in each experiment and the experiment was replicated three times (+SE).

Figure 4-12: Effect of abiotic stress on *Idh* expression

A.



B.



Effect of abiotic stress on *Idh* expression. (A) Densitometry, (B) Northern. Plants were treated with 100 mM NaCl, 20% (w/v) PEG, 38°C, 0.36% (v/v) ethanol or Hoagland's solution and maintained aerobically or hypoxically for 24 h. One µg of poly(A)$^+$ RNA was subjected to Northern analysis and probed with *Idh1* and actin. A representative Northern is shown. Duplicate samples were analyzed from three separate experiments (+SE).

4.4 *Discussion*

In order to gain some preliminary insights into the *ldh* signal transduction pathway, maize plants were exposed to several abiotic stresses, protein synthesis inhibitors and conditions that manipulated Ca^{2+} concentrations. Maize LDH does not appear to be a general stress responsive protein as neither *ldh* expression nor LDH activity were stimulated by exposure to low (4°C) or high (38°C) temperature or nonionic osmotic (PEG) stress (Figures 4-8 to 4-9 and 4-11 to 4-12). Additionally, concurrent exposure of these stresses with hypoxia reduced hypoxic *ldh* expression. These observations suggest that, in the experimental regime utilized, temperature and desiccation stress were more severe stresses to the plant than hypoxic stress.

The impairment of hypoxic induction by PEG is not surprising as PEG treatment results in desiccation and is more typically associated with drought conditions than with flooding. The impairment of hypoxic expression by low temperatures is more surprising as a late spring snowfall can result in plants being exposed to hypoxic conditions. Low temperatures (10°C) have been reported to induce ADH in maize (Christie et al., 1991) although, at temperatures near freezing (0.5 to 1°C), maize ADH is not induced (Peschke and Sachs, 1994). Maize *adh1* and *sh1* are not induced by 40°C temperatures (Peschke and Sachs, 1994). These observations, together with the results presented in this thesis could be the result of a general depressive effect of temperature extremes on transcription. However, they do suggest that there is a temperature range in which the hypoxic response can be induced.

In contrast, ionic osmotic (NaCl) stress resulted in increased *ldh* expression in aerobically maintained plants and a synergistic increase in expression in hypoxically treated plants (Figure 4-12). LDH enzyme activity, however, remained at control values during aerobic treatment and decreased with hypoxic treatment (Figure 4-11). As nonionic osmotica did not result in increased *ldh* expression (Figure 4-12), it suggests that these effects are the result of the ionic component of salt stress. Thus it appears that although *ldh* expression is up regulated by saline environments, that either translation or enzyme activity are impaired under these conditions. Impaired translation could be distinguished from reduced enzyme activity by comparison of immunoprecipitated radiolabeled *in vivo* and *in vitro* synthesized LDH. If *ldh* transcripts were not translated then radiolabeled LDH protein concentrations would be lower *in vivo* than *in vitro* assuming that the *in vitro* translation system can translate all mRNAs present. Whereas, if *ldh* was translated but the enzyme was inactive then radiolabeled LDH concentrations would be approximately the same *in vivo* and *in vitro*.

In carrot, induction and translation of heat shock mRNAs is tissue specific (Apuya and Zimmerman, 1992). Heat shocked callus cells and globular embryos accumulate comparable amounts of heat shock proteins; however, callus cells produce significantly more heat shock transcripts than globular embryos. This suggests that the mechanism by which mRNAs are recruited for translation during heat shock is different and more stringent in globular embryos than in callus cells. An analogous scenario may occur in salt stressed maize plants in order to maintain LDH protein concentrations at a specific level in root tissue. As salt stress reduces the activity of the cytochrome pathway (Jolivet et al., 1990; Vanlerberghe et al., 1997) it may be a survival advantage to increase glycolytic flux to lactate and ethanol thereby partially compensating for the reduced ATP production of the AOX pathway. Consistent with this idea is the observation that maize *adh1* and *sh1* transcripts are induced by NaCl, however *pdc* is not (Peschke and Sachs, 1994).

In order to obtain preliminary information on the *ldh* signal transduction pathway, maize plants were treated with protein synthesis inhibitors to determine whether new protein synthesis was required for *ldh* induction, and with Ca^{2+} and RR to determine if Ca^{2+} was involved in signal transduction.

When maize plants treated with CHX were hypoxically stressed, *ldh* expression was approximately the same as ethanol treated hypoxic plants (Figure 4-4), despite the presence of concentrations of CHX that inhibited cytoplasmic protein synthesis by 30 to 35% (Table 4-3 and 4-4). This suggests that transcription factors required for ethanol induced *ldh* hypoxic expression might be present prior to hypoxic treatment. The maize G-box binding factor (GBF1), which binds the maize *adh1* G-box, is expressed at low levels in aerobic conditions and at elevated levels during hypoxic treatment (de Vetten and Ferl, 1995). *ldh* transcript abundance increases dramatically during aerobic treatment in the presence of CHX (Figure 4-4). The reasons for this are unclear but it may be mediated by:

- inhibition of synthesis of a labile transcriptional repressor;
- inhibition of synthesis of an RNAse;
- prevention of ribosome movement, with subsequent loading of additional ribosomes onto the mRNA protecting the mRNA from RNAse activity; and
- a direct effect of CHX on chromatin bound proteins.

Transcripts for soybean *SAURs* (Li et al., 1994), barley *HVA22* (Shen et al., 1993), *Arabidopsis thaliana acs2* (Liang et al., 1996) and the transcription factors *c-fos* and *c-jun* (Greenberg et al., 1986; Mahadevan and Edwards, 1991) are induced by CHX alone and are superinduced when both CHX and the appropriate inducers are present. In studies of this phenomenom it has been observed that CHX and anisomycin

(another protein synthesis inhibitor), as well as the growth factors that normally induce *c-fos* and *c-jun*, can stimulate phosphorylation of the chromatin-associated phosphoproteins pp33 and pp15 (histone H3) (Mahadevan and Edwards, 1991). The rat *ldha* promoter contains a cAMP regulatory element which is also found in *c-fos* and *PEPCK*, both of which can effectively compete with *ldha* cAMP regulatory element in gel shift assays (Short et al., 1991). CHX induces *Arabidopsis thaliana acs2* and *acs5*, genes encoding ACC synthase, with different induction profiles suggesting that CHX affects these two genes by different mechanisms (Liang et al., 1996).

The presence of elevated *ldh* transcript levels in ethanol (0.36% (v/v)) treated, hypoxic plants compared to hypoxic plants not exposed to ethanol, suggests that the sequential activity of PDC and ADH to produce ethanol may function as a positive regulator of *ldh* expression by an as yet unknown mechanism. Ethanol has been shown to have a stimulatory effect on the germination of two cultivars of dehusked rice germinated under aerobic conditions (Miyoshi and Sato, 1997). It was speculated that ethanol could break dormancy by modifying membrane properties, acting as a respiratory substrate or inducing conditions in the embryo that are physiologically similar to those induced by anaerobiosis (Miyoshi and Sato, 1997). The presence of ethanol in the external media at a final concentration of 5 $\mu L\ L^{-1}$ (0.05% (v/v)) had no effect on maize root tips (He et al., 1996) whereas this work, using a higher ethanol concentration, demonstrates a positive effect on *ldh* expression. This difference could be the result of differences in the genes under investigation or in the ethanol concentration utilized.

Germinating seeds of *Erythrina* sp., the only dicot known to germinate anaerobically, has a decreased respiratory capacity when germinated aerobically in the presence of CHX and CAP. Germination under hypoxic conditions, in the presence of CHX decreases respiratory capacity, whereas germination in the presence of CAP stimulates respiratory capacity (Kemp and Small, 1994). The presence of CAP in both aerobic and hypoxic conditions reduced *ldh* expression whereas when present conjointly with CHX during hypoxia, *ldh* expression increased (Figure 4-4). CAP inhibits mitochondrial respiration, in addition to organellar protein synthesis (Pestka, 1977). The observation that *ldh* transcript abundance did not increase during aerobic treatment with CAP suggests that the hypoxic *ldh* signaling pathway is not the direct result of inhibition of mitochondrial respiration. Alternatively, if a mitochondrially encoded protein is required to transfer the signal to the cytosol then inhibition of its synthesis would prevent aerobic induction of *ldh* in the presence of CAP. The observation that *ldh* transcript abundance in CAP treated hypoxic plants is decreased relative to control plants suggests a possible role for a mitochondrially encoded

protein in either detection or passage of the signal. The large increase in *ldh* transcript abundance observed when both CHX and CAP were present may reflect the stimulating effect that CHX has on *ldh* expression.

It has been reported that CHX can trigger apoptosis (Vaux, 1993). Aerenchyma formation has many similarities to apoptosis (He et al., 1996) and thus induction of *ldh* expression by CHX may reflect activation of a common pathway between aerenchyma formation and increased glycolytic flux.

Ca^{2+} is required for aerenchyma formation and antagonists of IP_3, calmodulin and Ca^{2+}-dependent protein kinase inhibit aerenchyma formation suggesting roles for these molecules in the aerenchyma pathway (He et al., 1996). The interaction, if any, of the signaling pathways for aerenchyma formation and the modification of glycolytic flux is unknown. However, inhibitors of ethylene biosynthesis, which is required for aerenchyma formation, have no effect on *adh* induction (Saab and Sachs, 1996) suggesting that the hypoxic signal transduction pathway consists of either two separate pathways or a branched common pathway. Ca^{2+} has been demonstrated to have a role in the induction of *adh* in maize (Subbaiah et al., 1994 a and b). In BHK-21 fibroblasts, Ca^{2+} release from internal stores is regulated specifically by cytosolic ATP concentrations, such that as cellular ATP concentrations decrease, intracellular Ca^{2+} stores increase (Hofer et al., 1996). ATP concentrations also decrease during hypoxic treatment (Johnson et al., 1989).

Increasing Ca^{2+} concentration in the external environment had no effect on LDH enzyme activity (Figure 4-5) but increased *ldh* expression (Figure 4-7) suggesting a role for extracellular Ca^{2+} in the early (4.5 h) *ldh* signal transduction pathway. This contrasts with studies on ADH enzyme activity and *adh* expression (Subbaiah et al., 1994 a and b) (Tables 4-5 and 4-6).

Although the experimental protocols were different between the experiments (anoxic shock versus hypoxic treatment) there is clearly a difference in the response of *adh1* and *ldh1* at both the transcript and enzyme activity levels. In barley aleurone layers anoxic and hypoxic treatment stimulated similar responses with *adh1*, whereas *ldh1* was induced by hypoxia but not by anoxia (Hanson and Jacobsen, 1984; Taylor et al., 1994).

Table 4-5: Comparison of the response of *adh1*, *sh1* and *ldh1* to RR and Ca^{2+} treatment

	Relative transcript abundance		
Treatment	*adh1*[a]	*sh1*[a]	*ldh1*[b]
control	100	100	100
RR	5	20	32
Ca	95	65	770
RRCa	70	25	340

a = Subbaiah et al., 1994b. Maize roots were submerged in flooding buffer containing 25 µM RR and / or 2 mM $CaCl_2$ for 2 h following a 1 h aerobic pretreatment in the same buffer. Transcript intensities in the control conditions were set to 100%. *sh1* transcript values compared to the *adh1* anoxic control (100%) were 12%, 4%, 8% and 5% for control, RR, Ca and RRCa, respectively. These values were converted, for the purpose of this table, to set the control value, from 12% of the *adh1* transcript abundance to 100% *sh1* abundance to facilitate comparison of the three tanscripts.
b = this work. Maize roots were hypoxically treated with 25 µM RR and / or 5 mM $CaCl_2$ for 4.5 h following a 1 h aerobic treatment in the same buffer. Transcript intensity of the control was set at 100%.

Table 4-6: Comparison of the response of ADH and LDH enzyme activity to Ca^{2+} treatment

	Relative enzyme activity (% of N_2 control)				
	ADH activity[a]		ADH activity[b]	LDH activity[c]	
Treatment	O_2	N_2	N_2	O_2	N_2
control	49	100	100	96	100
Ca	36	105	90	105	140

a = Subbaiah et al., 1994b. Maize roots were submerged in flooding buffer containing 0 or 2 mM $CaCl_2$ for 16 h following a 1 h aerobic pretreatment in the same buffer.
b = Subbaiah et al., 1994a. Maize cell suspension culture line P3377 were treated aerobically or anoxically with 0 or 5 mM $CaCl_2$ for 24 h.
c = this work. Maize roots were treated with 0 or 5 mM $CaCl_2$ aerobically or hypoxically for 5 h following a 1 h aerobic pretreatment.

RR depressed the expression of *ldh* transcripts (Figure 4-7), suggesting a role for organellar Ca^{2+} release in *ldh* regulation. The addition of Ca^{2+} to RR treated plants after 1 h of hypoxia relieved the repression mediated by RR suggesting a role for extracellular Ca^{2+} also. These results are in opposition to those observed with ADH where the addition of Ca^{2+} to RR treated seedling after 1 h of submergence failed to reverse the repression

of ADH activity (Subbaiah et al., 1994b). RR treatment of maize suspension cultures resulted in low resting levels of Ca^{2+}, even in aerobic conditions. Treatment with 5 mM $CaCl_2$ in conjunction with RR resulted in the maintenance of normal intracellular Ca^{2+} concentrations (Subbaiah et al., 1994a). Concurrent Ca^{2+} and RR treatment increased *ldh* hypoxic expression above that of control plants (Figure 4-7), again suggesting a role for extracellular Ca^{2+} in *ldh* regulation.

Studies on maize suspension cultures confirmed that oxygen depletion results in an immediate (within 1 to 2 min) increase in intracellular Ca^{2+}, which is reversible within a few sec of reoxygenation (Subbaiah et al., 1994a). Similar observations were observed in *Arabidopsis thaliana* cotyledons and leaves, which also possess a second prolonged Ca^{2+} peak lasting from 1.4 to 4 h (Sedbrook et al., 1996). RR partially inhibits the first Ca^{2+} response, promoting a larger and earlier second response and partially inhibiting anaerobic *adh* induction in leaf tissue (Sedbrook et al., 1996). This suggests that different intracellular Ca^{2+} stores are responsible for the two Ca^{2+} peaks. *Arabidopsis thaliana* roots lack a Ca^{2+} response but increase *adh* transcript expression during hypoxia (Sedbrook et al., 1996). The maize root response to anoxia is thus more similar to that of *Arabidopsis thaliana* leaf tissue in that Ca^{2+} signaling appears to be important in both of those tissues. Unpublished observations suggest that treatments abolishing Ca^{2+} fluxes in maize cells also prevent the occurrence of pH_c changes during anoxia (Subbaiah et al., 1994a).

The anoxic Ca^{2+} response is decreased by a concurrent cold treatment in *Arabidopsis thaliana* (Sedbrook et al., 1996). If a similar situation occurs in maize roots during cold treatment, it could partially explain the lack of induction of ADH and LDH during cold hypoxic treatment.

In summary, work in this chapter demonstrated that:

- increased LDH enzyme activity is specific to the hypoxic response;
- abiotic stresses inhibit the hypoxic *ldh* response;
- ethanol production during the hypoxic stress may be involved in a positive feedback loop to stimulate *ldh* expression;
- new protein synthesis is not required for hypoxic *ldh* expression;
- *ldh* expression is significantly enhanced in aerobically maintained plants treated with CHX;
- a mitochondrial protein may be involved in sensing or passing on a signal for hypoxic *ldh* expression;
- extracellular and organellar Ca^{2+} fluxes may be involved in the *ldh* signaling pathway; and
- maize *adh* and *ldh* are regulated differently.

4.5 *Bibliography*

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5. Alanine aminotransferase in the hypoxic response in maize[1]

5.1 *Introduction*

Soil nitrogen is utilized within 3 d of flooding (Ernst, 1990). In barley, 2 d of flooding severely inhibits the uptake of nitrogen, phosphate and potassium leading to abrupt decreases of these elements in the shoot (Drew and Sisworo, 1979). Nitrogen is transported to the younger leaves and tillers at the expense of older leaves, consistent with the degradation of proteins and redistribution of amino acids to younger, growing tissues (Drew and Sisworo, 1979).

In vivo labeling studies of hypoxic barley root tissue demonstrated that 47% of the label from ^{14}C-glucose is found in alanine, an amount equal to that of ethanol (46%) (Hoffman et al., 1986). Alanine formed during hypoxic metabolism results from the incorporation of nitrogen and carbon. The carbon is derived from pyruvate and the nitrogen is derived from either protein degradation, deamination of aspartate or other amino acids, or continued ammonium assimilation, such as occurs in the green alga *Selenastrum minutum* (Ricard et al., 1994). Alanine is retained within the vacuole and it has been speculated that this results in conservation of pyruvate carbons which, if they were converted to ethanol, would diffuse out of the cell (Good and Crosby, 1989).

AlaAT reversibly catalyzes the conversion of pyruvate and glutamate to alanine and α-ketoglutarate (Figure 1-5 and Equation 5-1). Increased AlaAT activity following 5 d of hypoxia has been observed in roots of barley, rye, maize and wheat (Good and Crosby, 1989), and in white spruce cell suspension cultures (Good et al., 1990).

Aerenchyma formation is an important adaptive feature of flood stressed plants (Section 1.3.1) and is also produced in nitrate stressed plants. In aerated solutions high nitrate (KNO_3 or $Ca(NO_3)_2$) concentrations (>2 mM) and ammonium ions decrease aerenchyma formation in maize roots, whereas nitrate concentrations <1 mM stimulate aerenchyma formation (Konings and Verschuren, 1980; Drew et al., 1994). Plants grown for 13 d in aerated nutrient solutions and then transferred to aerated nitrate-free conditions develop symptoms similar to water logging (leaf chlorosis, lower concentrations of shoot nitrogen and lower accumulation of dry matter) which can be alleviated by calcium nitrate treatment (Drew et al., 1979). This suggests that nitrate deficiency is a

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[1] A portion of the work in this chapter has been submitted for publication. Muench DG, Christopher ME, Good AG (1997) Plant Mol Biol (submitted)

major contributor to these flood-mediated effects. Hypoxic maize root tips, pretreated with nitrate, have increased NTP concentrations, lower lactate and better recovery (Ricard et. al., 1994). This may be due to nitrate respiration that results from the utilization of nitrate as an alternative electron acceptor.

Increased AlaAT activity during hypoxia may thus be a consequence of nitrogen deficiency rather than hypoxia per se. To examine this hypothesis AlaAT was examined in conditions similar to those analyzed for LDH (Chapters 2 and 4), and in the response of AlaAT to nitrogen deficiency and relief from nitrogen deficiency.

5.2 *Materials and methods*

5.2.1 Plant material and growth conditions

Plant growth conditions were as described in Section 2.2.1. Four-week-old plants tested for the effect of re-exposure to nitrogen were grown in nitrogen-free Hoagland's solution (Table 2-2) for 5 d and then were transferred to hydroponic tanks containing regular Hoagland's solution for 0, 12, 24, 48, and 72 h. One set of plants was maintained in regular Hoagland's solution as a control. All conditions were aerobic and the oxygen concentration in the tanks, as determined by an oxygen meter (YSI Instruments, Yellow Springs, OH, USA), was 9.1 mg L^{-1}. Three samples were analyzed for each timepoint and the experiment was replicated five times.

5.2.2 Maize cell cultures

The maize cell culture line, BMS, was maintained as described in Section 2.2.2.

5.2.3 Stress treatment

Plants were treated with 100 mM NaCl, 5 mM $CaCl_2$ or 0.36% (v/v) ethanol for 24 h under both aerobic and hypoxic conditions as described in Section 4.2.5. Three samples were analyzed in each experiment and the experiment was replicated three times.

5.2.4 Treatment with protein synthesis inhibitors

Plants were treated with both chloramphenicol and CHX as described in Section 4.2.2. Duplicate samples from eight separate experiments and triplicate samples from three separate experiments were analyzed for *alaat* expression and AlaAT enzyme activity, respectively.

Plants were tested for their dose response to CHX treatment under both aerobic and hypoxic conditions as described in Section 4.2.2. Three samples were analyzed in each experiment and the experiment was replicated three times.

5.2.5 *In vivo* labeling

In vivo labeling to determine the effectiveness of the protein synthesis inhibitor treatment was as described in Section 4.2.3.

5.2.6 Treatment with calcium chloride and ruthenium red

Plants were treated with 5 mM $CaCl_2$ and / or 25 μM RR as described in Section 4.2.4. Two samples were analyzed in each experiment and the experiment was replicated four times.

Plants were tested for their dose response to $CaCl_2$ treatment during hypoxic treatment as described in Section 4.2.4. Three samples were tested in each experiment and the experiment was replicated three times.

5.2.7 AlaAT enzyme assays

Proteins were extracted by grinding root tissue in a mortar and pestle in an extraction buffer (described in Section 2.2.3). AlaAT was measured spectrophotometrically as previously described (Good and Crosby, 1989). The reaction mixture contained 5 mM α-ketoglutarate, 0.1 mM NADH, 100 mM Tris-HCl (pH 8.0), 5 units of LDH (Sigma L1254), and 50 μL of root extract in a final volume of 1 mL. The reaction was carried out at 22°C and was started by the addition of alanine to a final concentration of 25 mM. Nonspecific NADH consumption was measured by following the decrease in absorbance at 340 nm prior to the addition of substrate (alanine). The decrease in NADH absorbance following the addition of alanine reflects AlaAT activity plus nonspecific

dehydrogenase activity. AlaAT activity was determined by subtracting nonspecific enzyme activity from the activity observed following substrate addition. All enzyme activities were linear with respect to time. All activities are reported as μmol min^{-1} and all assays were done in duplicate.

The AlaAT reaction is a coupled reaction (Equation 5-1) containing an assay reaction (aminotransferase reaction with the formation of an oxoacid) and an indicator reaction (dehydrogenase reaction which quantitates the oxoacid formed in the initial reaction).

Equation 5-1: AlaAT reaction assayed

assay reaction (AlaAT):
α-ketoglutarate + L-alanine $\xrightarrow{pH\,8.0}$ pyruvate + L-glutamate

indicator reaction (LDH):
pyruvate + NADH + H^+ $\xrightarrow{pH\,8.0}$ L-lactate + NAD^+

5.2.8 AlaAT western blotting

Root extract (10 μg protein) was subjected to SDS-PAGE and electroblotting as described in Section 2.2.13. Antibody staining was performed using a 1:40,000 dilution of anti-AlaAT antibody (Muench and Good, 1994) and chemiluminescent detection, performed according to the manufacturer's protocol (Lumi GLO Chemiluminescent Substrate Kit, Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA).

5.2.9 Northern analysis

Total and poly(A)$^+$ RNA were isolated as described in Section 2.2.14 and Northern blots were performed as described in Section 2.2.16. In some cases poly(A)$^+$ RNA was analyzed by slot blotting. Poly(A)$^+$ RNA (0.5 μg) was heated at 67°C in 7.5X SSC and 4.6 M formaldehyde for 15 min, prior to loading onto the slot blotter (Minifold II, Schleicher and Schuell). The wells were prewashed with DEPC-treated water and 10X SSC.

Following loading of the RNA samples, a vacuum was applied to filter the samples rapidly through the Gene Screen Plus membrane (DuPont). The wells were washed with 400 µL of 10X SSC and the membrane was dried at 80°C for 2 h. *alaat* expression was monitored by probing with a Kpn1/EcoR1 fragment isolated from pAlaKpn2.1, a maize *alaat* genomic subclone (Muench et al., submitted). *alaat* Northern blot data was normalized by probing replicates with the *Arabidopsis* actin genomic clone pATc4 (Figure 2-3) (Nairn et al., 1988). Blots were scanned and analyzed by densitometry as described in Section 2.2.17. Densitometric results from poly(A)$^+$ Northern and slot blots correlated well with each other.

5.2.10 Primer extension

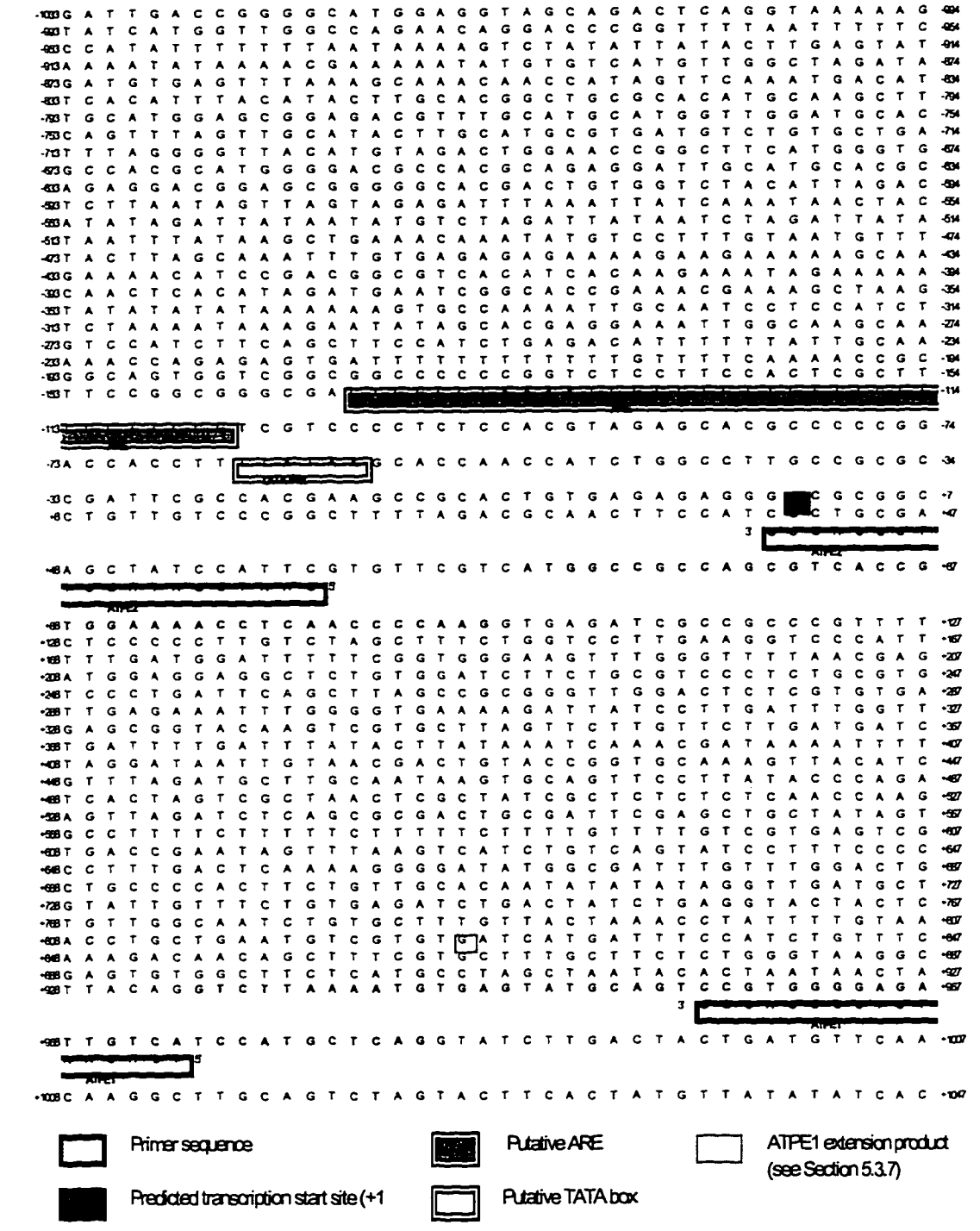
Twenty mer and 17 mer nucleotide primers (Table 5-1) specific to different 5' regions of the *alaat* mRNA (Figure 5-1) were kinase labeled, reverse transcribed and analyzed as described in Section 3.2.4.

Table 5-1: Primers used for primer extension of maize *alaat*

Primer	Sequence (5' → 3')	mer	% G+C	T_m
ATPE1	TGACAATCTCCCCACGG	17	59%	54°C
ATPE2	GAATGGATAGCTTCGCAGCG	20	55%	62°C

Primers were synthesized by the Molecular Biology Service Unit.

Figure 5-1: Maize *alaat* promoter and first two introns



Bolded nucleotides represent intron sequences.
From Muench et al., submitted

5.3 *Results*

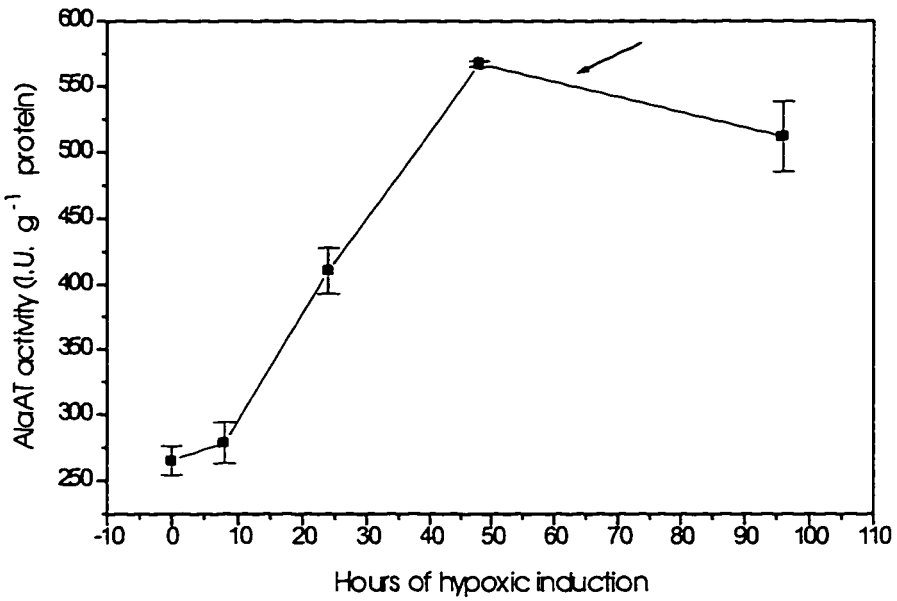
5.3.1 Hypoxic induction of AlaAT enzyme activity

AlaAT enzyme activity increased 1.5 and 2 times that of control plants after 24 and 48 h of hypoxic treatment, respectively (Figure 5-2A). The increase in AlaAT was delayed relative to both LDH and ADH, both of which had a detectable increase in activity after 8 h of hypoxic treatment (Figure 2-9). Return of plants to aerobic conditions for 48 h following 48 h hypoxic treatment did not result in a significant decrease in AlaAT enzyme activity as was observed with LDH enzyme activity. Enzyme activity continued to increase for 6 d at which time AlaAT enzyme activity was 5 fold higher than in aerobic plants (Figure 5-2B). The results in Figure 5-B are expressed as relative AlaAT enzyme activity as opposed to absolute values since the number of assays performed required the experiment to be completed over a 2 d period. Slight differences in assay buffer composition affected the absolute values obtained but did not affect the relative differences between groups of samples. Aerobic and 24-h hypoxically-treated plants were assayed on both days to monitor reproducibility of results.

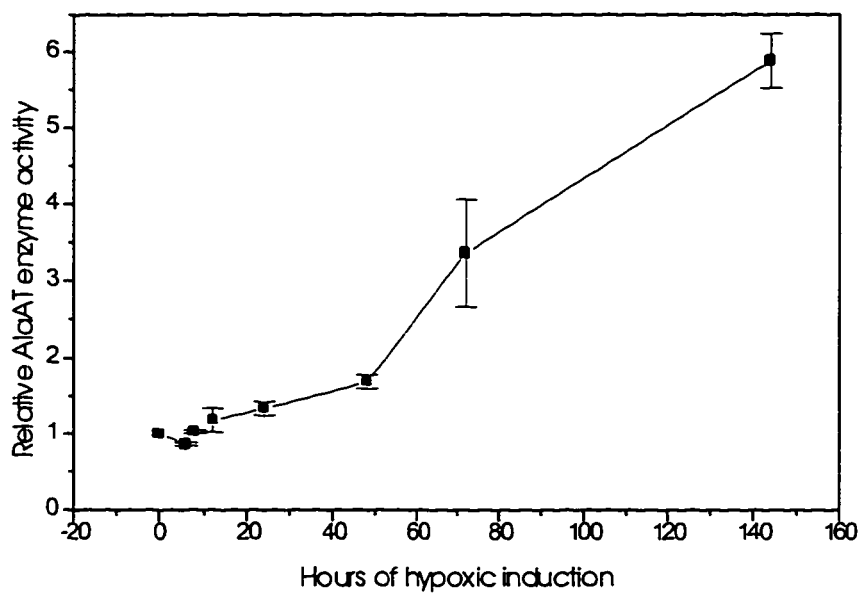
Maize cell cultures exhibited an increase in AlaAT enzyme activity, only when exposed to hypoxic (2% O_2, 98% N_2) conditions (Figure 5-3). AlaAT activity remained stable in cell cultures maintained in aerobic conditions, increased 1.7 times that of aerobically maintained cultures in 24 h hypoxically induced cultures and decreased slightly in cultures exposed to anoxia (100% N_2). These results differed from those of both LDH and ADH (Figure 2-10). LDH was not induced under either condition, whereas ADH was induced by both hypoxic and anoxic conditions.

Figure 5-2: Hypoxic induction of AlaAT

A.

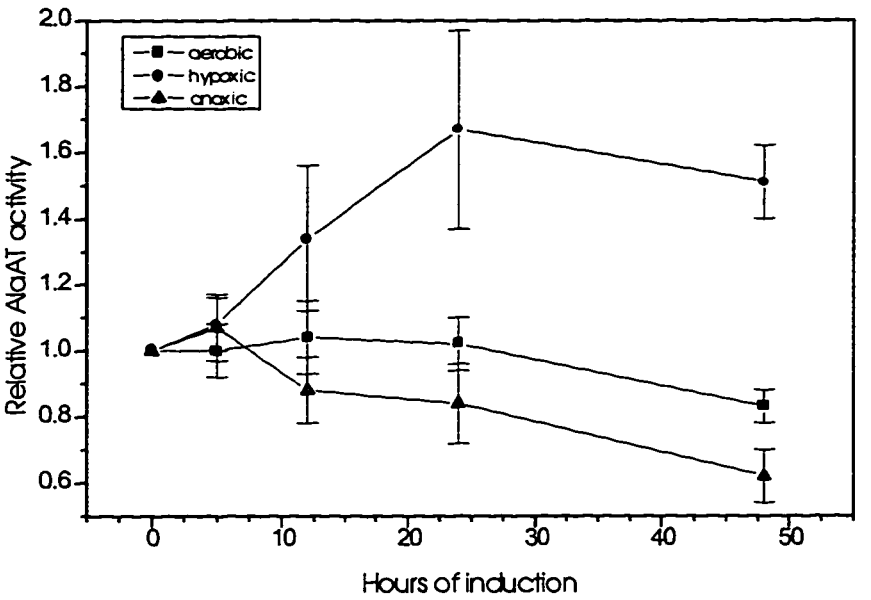


B.



Effect of hypoxia on AlaAT activity in maize roots. (A) Plants were exposed to 0, 8, 24 and 48 h of hypoxic stress, or aerobically recovered for 48 h after 48 h of hypoxic treatment. Each time point is the mean from data obtained from a single experiment (n=5; +SE). The arrow indicates return to aerobic conditions. (B) Plants were exposed to 0, 6, 8, 12, 24, 48, 72 and 144 h of hypoxic stress. As the experiment required 2 d to complete, AlaAT activity in the induced plants was compared to that of aerobic plants tested on each day of the experiment, thus relative AlaAT activity is reported instead of actual AlaAT activity. The experiments were replicated four times (n=5; +SE).

Figure 5-3: AlaAT activity in maize cell suspension cultures



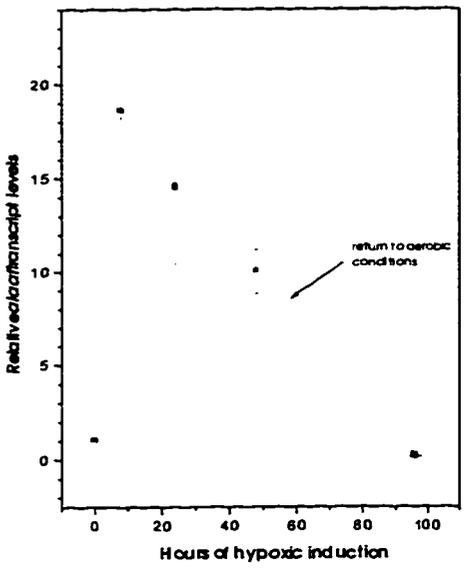
Effect of hypoxia (2% O_2, 98% N_2) and anoxia (100% N_2) on AlaAT activity in maize suspension cultures. Logarithmically growing cells were bubbled with air for 2 d prior to transfer to flasks which were bubbled with either air (control), 2% O_2, 98% N_2 (hypoxia) or 100% N_2 (anoxia). Each time point is the mean from data obtained from a single experiment (n=4; +SE). The experiment was replicated four times.

5.3.2 Hypoxic accumulation of *alaat* mRNA

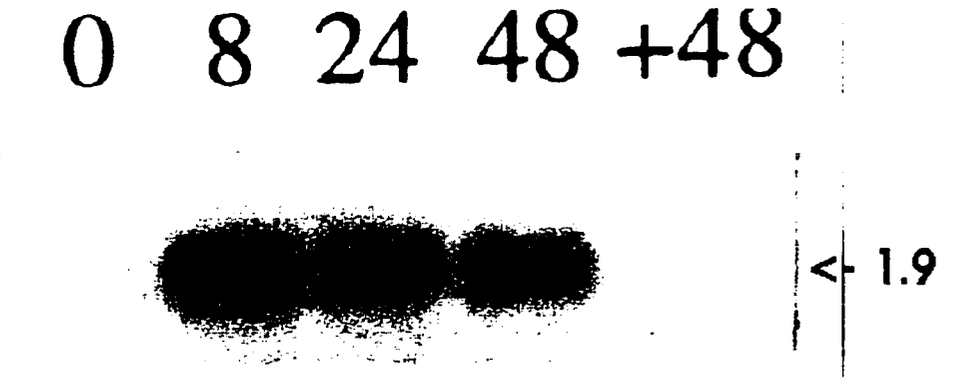
Northern analysis demonstrated the strong induction of a single *alaat* transcript of ~1.9 kb peaking at 8 h of hypoxia (Figure 5-4). Transcript abundance decreased with longer hypoxic exposure. Return to aerobic conditions for 48 h following 48 h of hypoxia resulted in transcript levels similar to those of the aerobic control. The *alaat* transcript profile differed from that of both *ldh* transcripts (Figure 2-13), which remained elevated for 24 h before decreasing.

Figure 5-4: Hypoxic induction of *alaat*

A.



B.



Effect of hypoxia on *alaat* expression in maize roots. (A) Densitometric analysis; (B) Northern blot. Northern blots previously probed with pIdh1Sal1.0 and pAtC4B/E1.8 were reprobed for *alaat* expression. Poly(A)$^+$ RNA (1 µg) isolated from maize roots exposed to 0, 8, 24 and 48 h of hypoxic stress and from roots aerobically recovered for 48 h after 48 h of hypoxic treatment was probed with pAlaKpn2.1. A representative Northern blot is shown. Duplicate samples from three separate experiments were analyzed (+SE). The arrow indicates the return to aerobic conditions.

5.3.3 Effect of nitrogen starvation on AlaAT activity and *alaat* expression

Three week old plants were grown in nitrogen-free Hoagland's solution for 5 d and then returned to regular Hoagland's (high nitrogen) solution. It has previously been shown, in barley root tissue, that 4 d of nitrogen starvation eliminated the hypoxically induced increase in alanine (Good and Muench, 1993). AlaAT activity was low in plants maintained in nitrogen-free Hoagland's solution and increased to the activity seen in plants maintained in high nitrogen Hoagland's solution after 3 d of re-exposure to nitrogen (Figure 5-5A). AlaAT protein concentrations were unaffected by nitrogen stress and relief from nitrogen stress (Figure 5-5B and C). *alaat* expression was very low in nitrogen stressed plants (Figure 5-6) and relief from nitrogen stress resulted in a rapid induction of *alaat* transcript expression (14 fold increase) within the first 12 h, followed by a rapid decline.

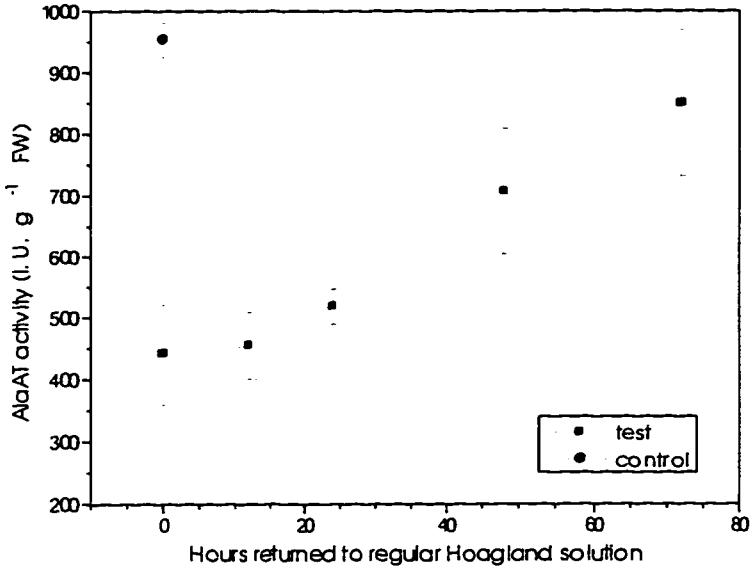
5.3.4 Effect of stress on AlaAT activity and *alaat* expression

AlaAT is induced in response to hypoxic stress in maize (Figure 5-2). To investigate whether other stresses also affect AlaAT, the response to salt (NaCl), calcium and ethanol were examined. Plants maintained aerobically and exposed to either NaCl (100 mM) or Ca^{2+} (5 mM $CaCl_2$) for 24 h had a slight increase in AlaAT enzyme activity, whereas exposure to ethanol (0.36% (v/v)) had no effect (Figure 5-7A). AlaAT activity in hypoxically stressed plants concurrently exposured to Ca^{2+} or ethanol was the same as hypoxic control plants. Concurrent hypoxic and salt exposure resulted in an inhibition in the AlaAT hypoxic response, similar to that observed with LDH (Figure 4-11). Plants maintained aerobically and treated with salt, Ca^{2+} or ethanol had a slight increase in *alaat* expression (Figure 5-7B and C). The presence of ethanol during 24 h of hypoxic treatment reduced *alaat* expression, but Ca^{2+} and salt had no effect on hypoxic induction of *alaat*.

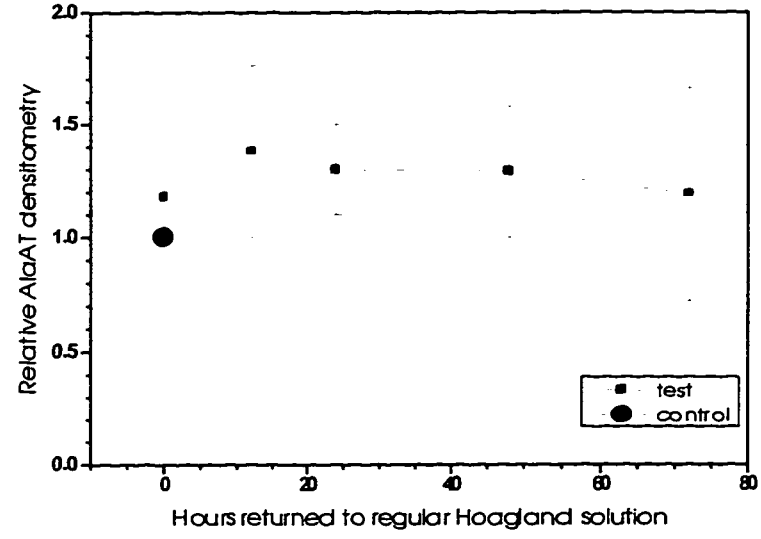
Cold temperatures (4°C) prevented the hypoxically-induced increase in AlaAT activity (Figure 5-8A), results similar to those observed with LDH and ADH (Figure 4-8). Hypoxic plants maintained at 4°C had elevated *alaat* transcript levels compared to aerobic plants maintained at the same temperature (Figure 5-8B). However, the transcript levels of the hypoxic plants maintained at 4°C was similar to those of aerobic control plants maintained at 20°C, and significantly less than those of hypoxic plants maintained at 20°C.

Figure 5-5: Effect of relief from nitrogen starvation on AlaAT activity and protein concentration

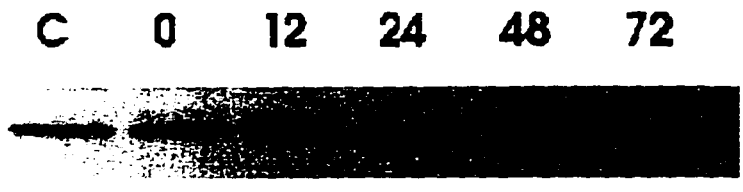
A.



B.



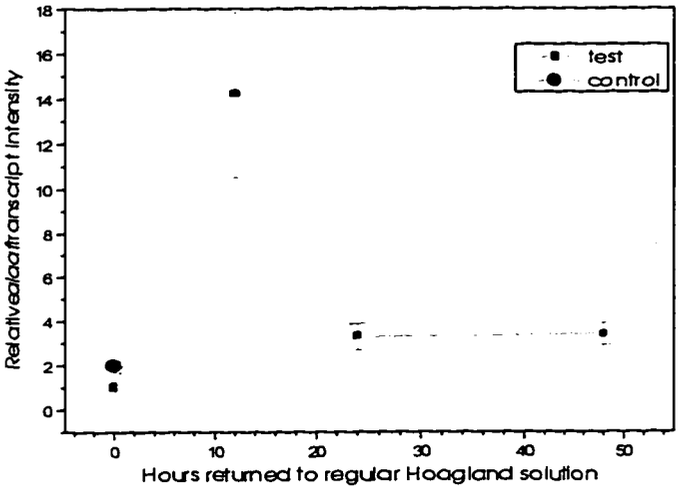
C.



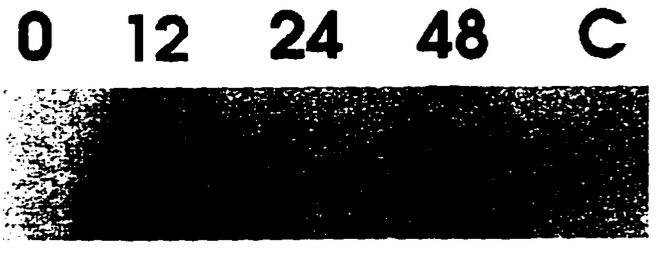
Effect of returning maize plants to high nitrate conditions following nitrogen starvation. (A) AlaAT enzyme activity; (B) AlaAT protein densitometric analysis; (C) AlaAT western blot. Plants were analyzed for AlaAT enzyme activity and protein concentration following re-exposure to nitrogen for 0, 12, 24, 48 and 72 h after being nitrogen starved for 5 d. Control plants were maintained with nitrogen. (B and C) Ten micrograms of protein, subjected to SDS-PAGE, was probed with barley anti-AlaAT antiserum. The experiment was replicated three times (n=3; +SE).

Figure 5-6: Effect of relief from nitrogen starvation on *alaat* expression

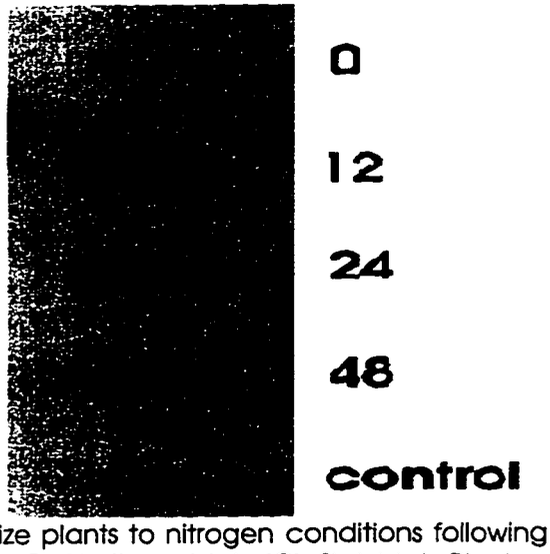
A.



B.



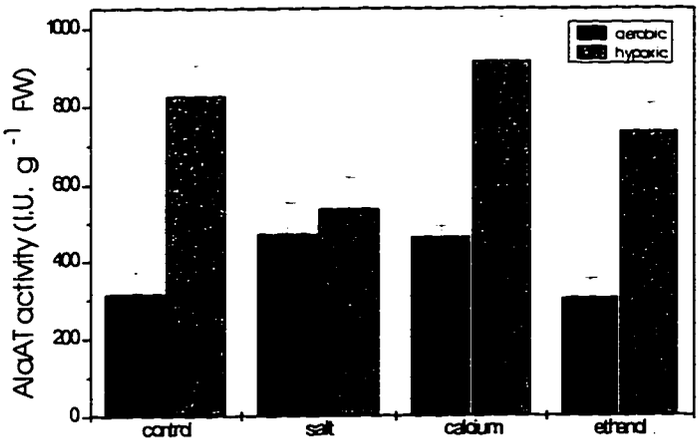
C.



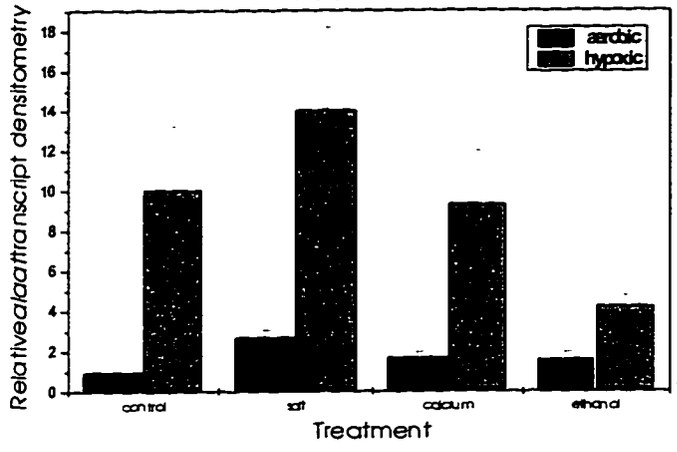
Effect of returning maize plants to nitrogen conditions following nitrogen starvation. (A) Densitometric analysis; (B) Northern blot; (C) Slot blot. Plants were analyzed for *alaat* expression following re-exposure to nitrogen conditions for 0, 12, 24 and 48 h after being nitrogen starved for 5 d. Control plants were maintained with nitrogen. Poly(A)$^+$ RNA (1 μg for Northern blots and 0.5 μg for slot blots) was probed with pAlaKpn2.1. Densitometric results were normalized to actin expression. Northern and slot blot analysis gave similar results. The experiment was replicated three times (n=2; +SE).

Figure 5-7: Effect of stress treatment on AlaAT enzyme activity and *alaat* expression

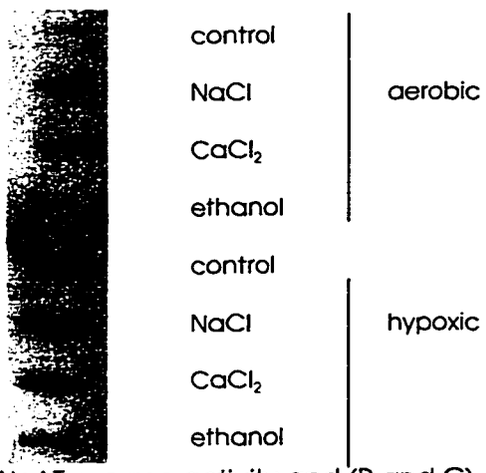
A.



B.



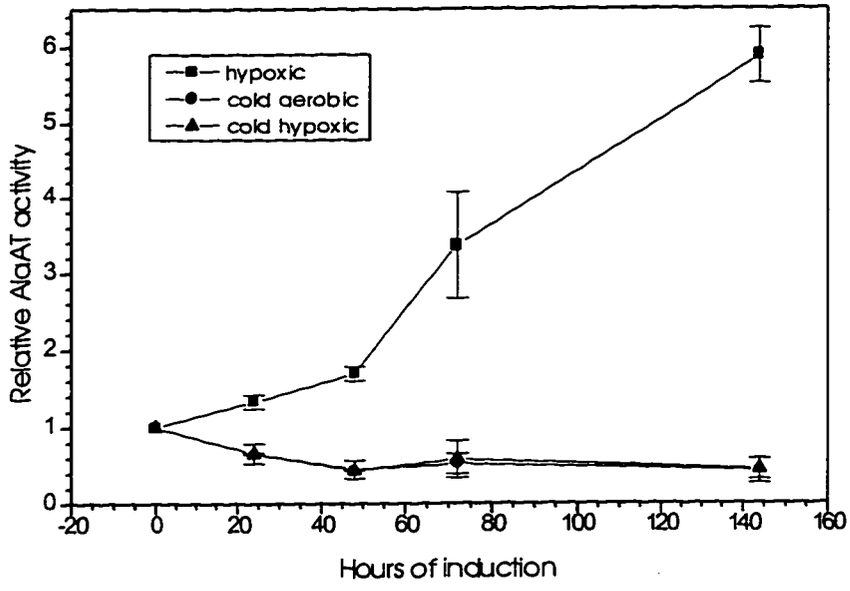
C.



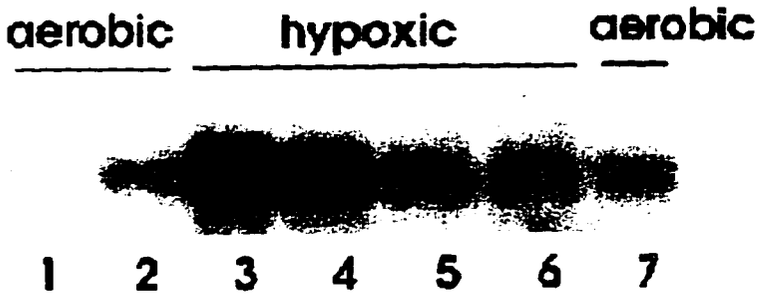
Effect of stress on (A) AlaAT enzyme activity and (B and C) *alaat* expression. Maize plants were exposed to 100 mM NaCl, 5 mM $CaCl_2$, 0.36% (v/v) ethanol supplemented or unsupplemented (control) Hoagland's solution for 24 h under both aerobic and hypoxic conditions. (A) AlaAT enzyme activity; (B) Relative *alaat* transcript intensity; (C) Slot blot. Three samples were analyzed in each experiment (n=3) and the experiment was replicated three times (+SE). The slot blot was reprobed with actin.

Figure 5-8: Effect of cold temperatures on AlaAT activity and *alaat* expression

A.



B.



Effect of concomitant cold stress on the hypoxic response of (A) AlaAT enzyme activity and (B) *alaat* expression in maize roots. (A) Plants were exposed to 0, 1, 2, 3 and 6 d of hypoxia at 20°C or 4°C, or exposed to aerobic conditions at 4°C for 1, 2, 3 and 6 d. Relative AlaAT activity values are given as, although the trend was the same in both experiments, the actual AlaAT activity values varied. Each time point is the mean of data obtained from a single experiment (n=3; +SE). The experiment was replicated twice. (B) One μg of poly(A)$^+$ RNA isolated from maize roots exposed to 4°C aerobic conditions for 1 d (lane 1), or 2 d (lane 2), 20°C hypoxic for 1 d (lane 3) or 2 d (lane 4), 4°C hypoxic for 1 d (lane 5) or 2 d (lane 6) or 20°C aerobic (lane 7) was probed with pAlaKpn2.1. A representative northern is shown. Duplicate samples from two separate experiments were analyzed.

5.3.5 Effect of protein synthesis inhibitors on AlaAT activity and *alaat* expression

Maize plants treated with CHX, to inhibit cytosolic protein synthesis (Table 4-3), exhibited a slight increase in AlaAT activity after 6.5 h of exposure when maintained in aerobic conditions (Table 5-2) relative to aerobic control plants. A slight increase in *alaat* transcript intensity was observed in aerobic CHX treated plants (Figures 5-9 and 10). This increase (2.6 fold in the presence of 150 μg mL^{-1} CHX) is significantly less than the 120 fold increase seen with *ldh* transcripts maintained under the same conditions (Figure 4-4). Hypoxically exposed CHX treated plants had an increase in *alaat* transcript abundance relative to aerobic controls. The presence of ethanol slightly impaired *alaat* transcript accumulation after 6.5 h of hypoxia (Figure 5-10), similar to the results observed following 24 h of exposure to ethanol (Figure 5-7). CAP had no effect on aerobic *alaat* transcript accumulation, but resulted in a slight increase in hypoxic transcript accumulation.

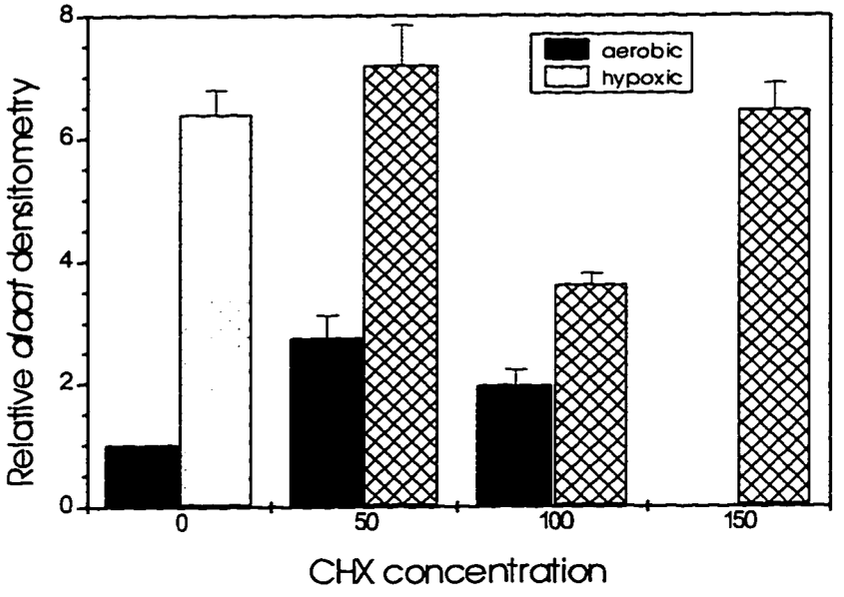
Table 5-2: Effect of CHX on AlaAT activity

CHX concentration	Condition	AlaAT activity ($\mu mol\ min^{-1}\ g^{-1}$ fresh weight)	(+SE)
0	Aerobic	637.0	(+78.8)
0	Hypoxic	822.3	(+0.7)
100	Aerobic	766.4	(+56.3)
100	Hypoxic	682.3	(+46.6)
150	Aerobic	812.2	(+19.9)
150	Hypoxic	703.1	(+55.1)

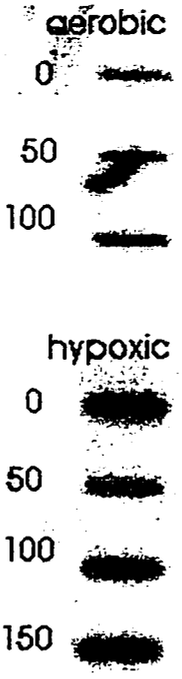
Effect of CHX concentration on AlaAT enzyme activity. Maize plants were pretreated with 0, 100 or 150 μg mL^{-1} CHX for 1.5 h. Ethanol, 0.36% (v/v), was present in all treatments. Samples were analyzed after 6.5 h of aerobic or hypoxic treatment. The experiment was replicated three times (n=3; +SE).

Figure 5-9: Effect of CHX concentration on *alaat* expression

A.



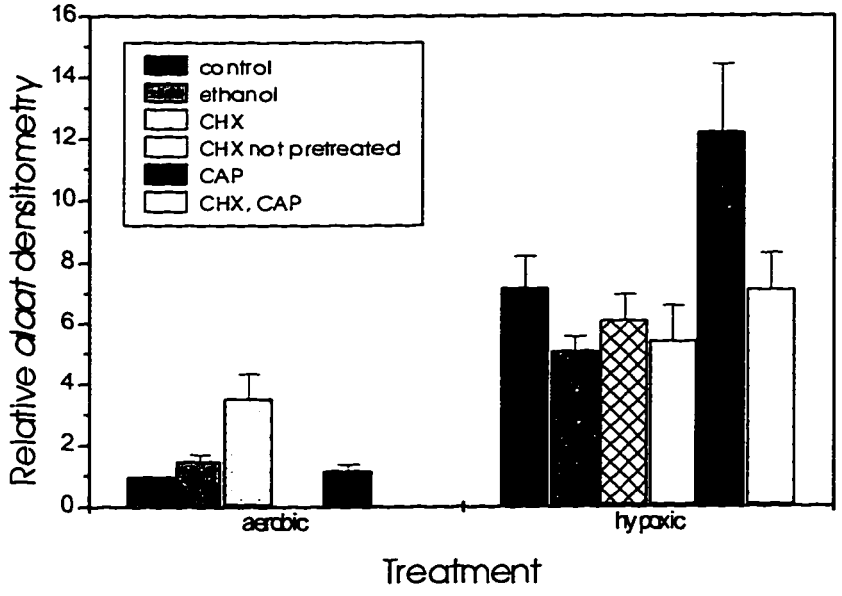
B.



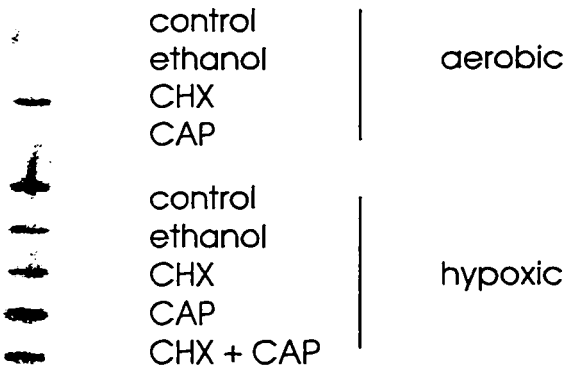
Effect of CHX concentration on *alaat* expression. (A) Densitometric analysis, (B) Slot blot. Maize plants were pretreated with 0, 50, 100 and 150 μg mL^{-1} CHX for 1.5 h. Ethanol, 0.36% (v/v), was present in all treatments. Samples were analyzed after 6.5 h of additional aerobic or hypoxic treatment. Poly $(A)^+$ RNA (0.5 μg), loaded onto a slot blot, was probed with pAlaKpn2.1. RNA loading was normalized by comparison with hybridization to an actin probe. The experiment was replicated three times (n=2; +SE).

Figure 5-10: Effect of CHX and CAP on *alaat* expression

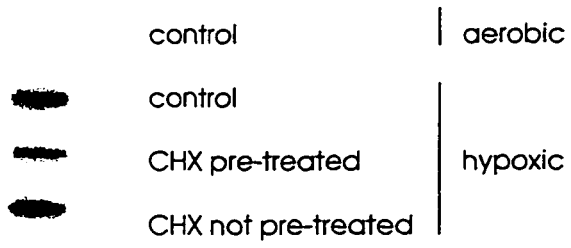
A.



B.



C.



Analysis of the effect of CHX and CAP on *alaat* transcripts. (A) Densitometric analysis, (B and C) Slot blots. (A and B) Plants were aerobically pretreated with water (control), 0.36% (v/v) ethanol plus 150 μg mL^{-1} CHX or 40 μg mL^{-1} CAP for 1.5 h and then either maintained aerobically or transferred to hypoxic conditions for an additional 6.5 h. (C) Comparison of CHX and non-CHX pretreated plants. Conditions were aerobic, hypoxic, hypoxic CHX without pretreatment and hypoxic CHX with pretreatment. Poly(A)$^+$ RNA (0.5 μg) was analyzed by probing with pAlaKpn2.1. Representative slot blots are shown. Samples from three separate experiments were analyzed (n=2; +SE).

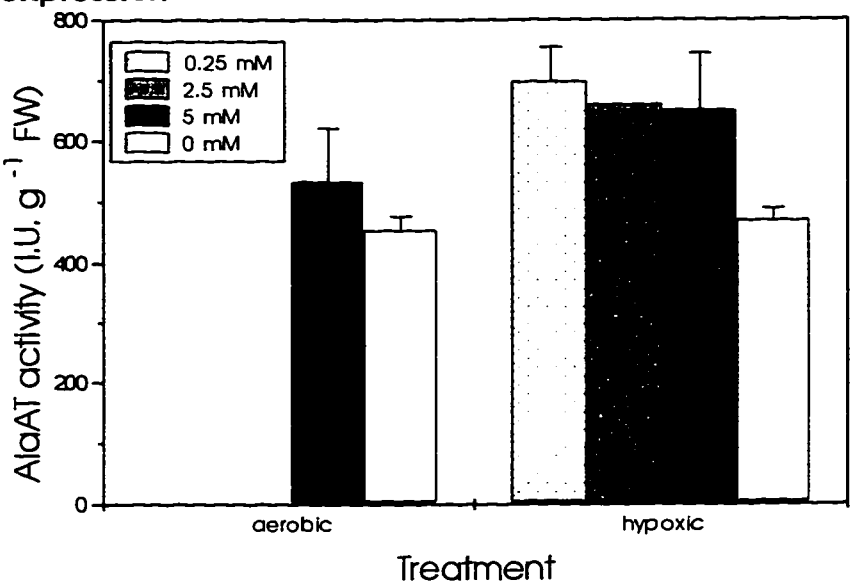
5.3.6 Effect of manipulation of extracellular calcium and intracellular calcium release on AlaAT activity and *alaat* expression

To investigate if calcium has a role in *alaat* expression, plants were exposed to various concentrations of Ca^{2+} and either maintained aerobically or transferred to hypoxic conditions for 5 h. AlaAT enzyme activity was unchanged in aerobically maintained plants exposed to 5 mM Ca^{2+} relative to the control plants (Figure 5-11A). Five h of hypoxic treatment did not result in a detectable increase in AlaAT activity in the control plants, but an increase in enzyme activity was observed in plants treated with Ca^{2+} regardless of the concentration used. Ca^{2+} had no effect on *alaat* transcript intensity in plants maintained aerobically but did increase *alaat* transcript abundance under hypoxic conditions (Figure 5-11B), consistent with the observed increase in enzyme activity.

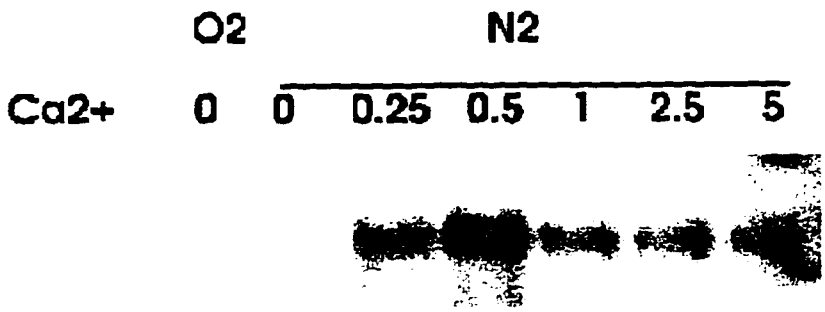
Experiments to investigate the possible role of extracellular Ca^{2+} and organellar Ca^{2+} were performed using 5 mM Ca^{2+} as this was the concentration used in the analysis of the *ldh* response, thus allowing direct comparison between the *ldh* and *alaat* responses. Plants exposed to hypoxic conditions had a significantly higher transcript intensity when extracellular calcium was present (53.6 fold increase) versus its absence (7.6 fold increase) (Figure 5-12). RR, an inhibitor of organellar calcium release (Subbaiah et al., 1994a), had no effect on the intensity of *alaat* transcripts following 4.5 h of hypoxia relative to the hypoxic control but when present concurrently with 5 mM calcium, the large increase in transcript intensity mediated by calcium alone was mitigated. *alaat* transcript abundance in plants exposed to RR for the first h of hypoxia and then transferred to 5 mM Ca^{2+} for the remaining 3.5 h, remained at levels similar to that of plants maintained in RR throughout the experiment. The effect of calcium on *alaat* transcript intensity was only observed with short-term hypoxic induction (4.5 h) (Figure 5-12). After 24 h of hypoxic induction the relative *alaat* transcript intensity between plants treated with and without calcium was the same (Figure 5-7).

Figure 5-11: Effect of calcium concentration on AlaAT enzyme activity and *alaat* expression

A.



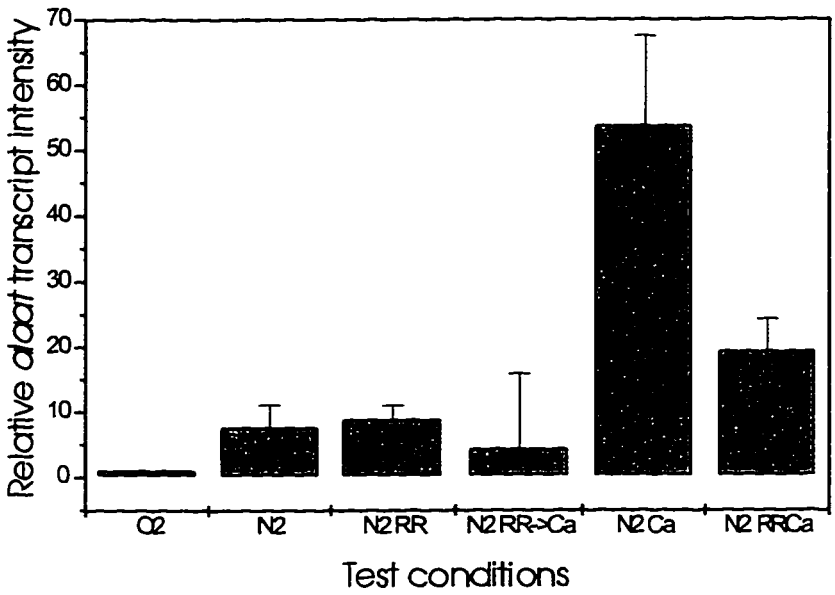
B.



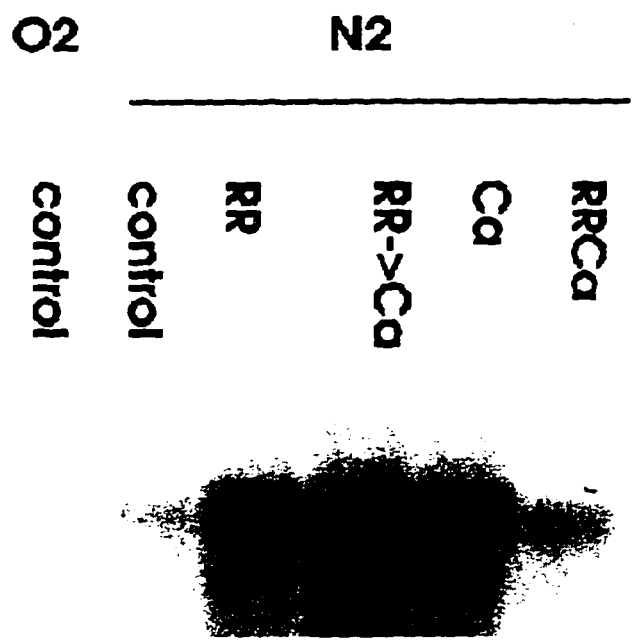
Effect of Ca^{2+} concentration on (A) AlaAT enzyme activity and (B) *alaat* expression. (A) Maize plants were pretreated with Hoagland solution supplemented with 0, 0.25, 2.5, and 5 mM $CaCl_2$ for 1 h prior to transfer to hypoxic conditions in the same medium for an additional 5 h. Control plants (0 and 5 mM $CaCl_2$) were maintained aerobically throughout. The experiment was replicated three times (n=3; +SE). (B) One µg of poly(A)$^+$ RNA isolated from roots of plants, aerobically pretreated with 0, 0.25, 0.5, 1, 2.5 or 5 mM $CaCl_2$ for 1 h and then transferred to hypoxic conditions for 5 h, was analyzed by Northern blotting and probing with pAlaKpn2.1. Plants pretreated with 0 or 5 mM $CaCl_2$ were also maintained aerobically. Duplicate samples were analyzed from three separate experiments.

Figure 5-12: Effect of Ca^{2+} and RR on *alaat* expression

A.



B.



Effect of Ca^{2+} and RR on *alaat* transcripts (A) Densitometric analysis, (B) Northern blot. Plants were aerobically pretreated with water (control), 25 µM RR and / or 5 mM $CaCl_2$ for 1 h and then either maintained aerobically or transferred to hypoxic conditions for an additional 4.5 h. Treatment RR → Ca was pretreatment and the first h of hypoxia in RR followed by transfer to $CaCl_2$ for the remaining 3.5 h. One µg of poly(A)$^+$ RNA was analyzed by Northern blotting and probing with pAlaKpn2.1. A representative Northern blot is shown. Duplicate samples were analyzed from 4 separate experiments (+SE).

5.3.7 Determination of transcription start site of maize *alaat*

To map the 5' end of the *alaat* transcript, primer extension was performed using two different primers (Figure 5-1). ATPE1 is a 17-mer corresponding to a region in exon two 63 bp downstream of the AUG start site and 131 bp downstream of the predicted +1 transcription start site in the spliced transcript. This primer in exon 2 was selected due to the small size of exon 1 (39 bp). ATPE2 is a 20-mer from the region just upstream of the translation start site and 39 bp downstream of the predicted +1 transcription start site. The extension reaction using the ATPE2 primer gave a single band, which was readily detectable with overexposure of the autoradiographic film. This was necessary because of the shortness of the extension product (59 nucleotides including the 20 nucleotides of the primer). The extension product was consistent with that predicted for the transcription start site (Figures 5-1 and 5-13). The ATPE1 primer extension reaction generated one strong band corresponding to a product of 147 nucleotides including the 17 nucleotides of the primer. Comparison of the band location with the sequencing reaction performed using the same primer and a maize genomic clone as a template indicated a termination product of a size equivalent to the G-residue located 107 nucleotides upstream of exon 2 and within the intron (Figure 5-1). As the intron would be spliced out of the mature transcript, calculations were performed to determine the location of the primer extension product. Exon1 is 39 bp and when subtracted from the 107 nucleotides of the ATPE2 product upstream of exon 2, it indicates that the transcription start site should be 68 nucleotides upstream of the translation start codon. This corresponds with the primer extension product observed with primer ATPE2. Based on these results, it can be concluded that the 5' end of the *alaat* transcript is at the site indicated in Figure 5-13 as +1.

Figure 5-13: Transcription start site of maize *alaat*

TTATAAGCACCAACCATCTGGCCTTGCCGC

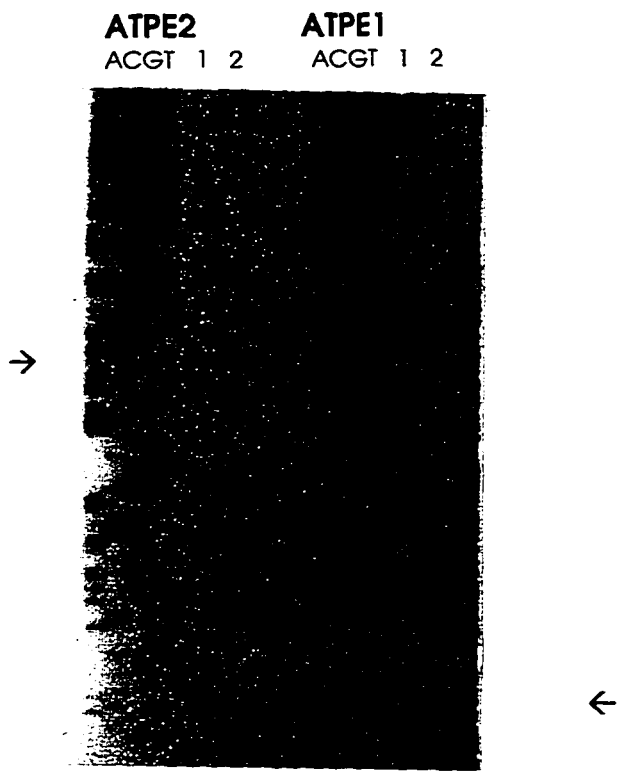
GCCGATTCGCCACGAAGCCGCACTGTGAGA

GAGGGACGCGGCCTGTTGTCCCGGCTTTTA

+1

Sequence of *alaat* around the predicted transcription start site. The TATA box is outlined with a double line. The calculated primer extension product for primer ATPE2 and the observed primer extension product for primer ATPE1 is the same and is shaded. The transcription start site is indicated with +1 and is outlined with a heavy line.

Figure 5-14: Primer extension of maize *alaat*



Primer extension mapping of the 5' end of the maize *alaat* transcript. Primer extension was performed using oligonucleotides ATPE1 and ATPE2 (Table 5-1 and Figure 5-1), both of which are complementary to the *alaat* transcript and correspond to bases +148 to +131 for ATPE1 and +59 to +39 for ATPE2. Numbering is based on the nucleotide sequence of the spliced transcript. Two different RNA samples were analyzed. The primer extension lanes are indicated as 1 and 2 while A, C, G and T indicates sequencing reactions performed using the same primer. The products of the reaction were analyzed by electrophoresis on a 6% polyacrylamide-urea sequencing gel. Arrows indicate the major primer extension product generated with each primer.

5.4 *Discussion*

Hypoxic expression of AlaAT has previously been characterized in barley (Muench and Good, 1994). Barley differs from maize in that a detectable increase in AlaAT activity is observed within the first 12 h of hypoxic treatment in barley whereas the increase in AlaAT activity is somewhat delayed in maize (Figure 5-2). After 48 h of hypoxic treatment maize exhibited a 2 fold increase in AlaAT activity whereas barley exhibited a 3.5 fold increase. Transcript profiles also differ slightly between the two species. In barley, the elevated expression of *alaat* transcripts remained stable between 12 and 24 h of hypoxia (~4 fold increase) followed by a gradual decrease (Muench and Good, 1994). In maize, the increase in *alaat* transcript expression was of greater magnitude (~18 fold increase), peaking at 8 h of hypoxia followed by a sharp decline (Figure 5-4).

The hypoxic induction profile for AlaAT activity also differed from maize LDH and ADH (compare Figure 5-2 with Figure 2-9) in respect to the timing of the first observable increase in enzyme activity. Transcript expression for both *ldh* and *alaat* declined rapidly after peaking (compare Figure 5-4 with Figure 2-13); however, *alaat* transcript abundance peaked earlier than *ldh* transcript abundance. Analysis of maize suspension cultures also demonstrated differences between the regulation of AlaAT, LDH and ADH (compare Figure 5-3 with Figure 2-10). AlaAT activity was induced only in hypoxic conditions, ADH activity was induced in both hypoxic and anoxic conditions and LDH was not induced with either. This demonstrates that these genes, although they are all hypoxically inducible in root tissue, have unique regulatory features.

AlaAT activity was very low in nitrogen starved maize plants (Figure 5-5). Relief from nitrogen stress gradually increased AlaAT activity over a 3 d period and rapidly increased *alaat* expression which peaked at 12 h followed by an equally rapid decrease (Figure 5-6). The increase in *alaat* expression was not translated into a significant increase in AlaAT protein (Figure 5-5). Thus, it is likely that post-translational mechanisms are involved in increasing AlaAT activity in plants recovering from nitrogen stress.

Utilization of absorbed nitrate by higher plants involves reduction of NO_3^- to NH_4^+ via the sequential action of nitrate reductase (NR) and nitrite reductase (NiR), and incorporation of NH_4^+ onto carbon skeletons via the glutamine synthetase / glutamate synthase cycle (Ivashikina and Sokolov, 1997). Nitrate uptake rates increase following nitrogen starvation. Studies indicate that modulation of NO_3^- uptake is mediated by metabolites downstream in the NO_3^- assimilation pathway as well as phloem-translocated amino acids (Ivashikina and Sokolov, 1997). Alanine is

involved with nitrogen uptake and AlaAT activity has been shown to increase in *Panicum miliaceum* plants during the recovery from nitrogen stress (Son and Sugiyama, 1992). Alanine synthesis may also be the major pathway of nitrogen assimilation when plants are subjected to simultaneous nitrogen and hypoxic stress (Vanlerberghe et al., 1991).

If the increase in AlaAT activity during hypoxic treatment was the result of nitrogen deprivation, then it would be predicted that AlaAT activity would be high in nitrogen stressed plants. As this was not observed, it suggests that the increased AlaAT activity observed during hypoxia was a direct result of reduced oxygen availibility. Consistent with this is the presence of a putative ARE in the *alaat* promoter region (Figure 5-1) (Muench et al., submitted).

Exposure of maize plants to simultaneous hypoxia and cold temperatures (4°C) inhibited the hypoxic induction of AlaAT, a response similar to those found for both LDH and ADH (Figure 4-8). Exposure to salt and ethanol decreased hypoxic AlaAT activity with ethanol treatment resulting in decreased *alaat* mRNA expression and salt treatment resulting in increased *alaat* mRNA expression relative to plants exposed to hypoxia alone. Hypoxic expression of *ldh* increased significantly in hypoxically treated plants exposed to ethanol (Figure 4-4). This suggests a positive regulatory role for ethanol in *ldh* regulation. This is opposite to the results observed with *alaat*, which suggest that ethanol has a negative regulatory role for *alaat* expression. This is consistent with the hypoxic induction profile of *alaat* expression, with transcript abundance peaking at 8 h of hypoxic treatment followed by a steep decline. ADH activity increases with prolonged hypoxia, with the ethanol produced primarily being transported out of the cell, increasing ethanol concentrations in the surrounding medium (Hoffman et al., 1986).

When hypoxic maize plants were treated with CHX, *alaat* mRNA expression was approximately the same as in hypoxic control plants despite the presence of CHX at concentrations which inhibited protein synthesis by 35% (Table 4-3). This suggests that new protein synthesis may not be required to stimulate transcription from *alaat* during hypoxic conditions. It has been determined that the maize G-box binding factor (GBF1) which binds the maize *adh1* G-box, is expressed in both aerobic and hypoxic conditions albeit at significantly lower levels in aerobic conditions (de Vetten and Ferl, 1995). Exposure to CHX in aerobically treated plants resulted in a slight increase in *alaat* mRNA relative to the aerobic control. This differed significantly from *ldh* whose transcripts dramatically increase in CHX treated aerobic plants (compare Figure 4-4 with Figure 5-10). This difference, together with the slight increase in *alaat* transcript levels in aerobic CHX treated plants could indicate that a labile transcriptional repressor or an RNAse is no longer being translated. These factors could function to maintain *alaat* and *ldh* mRNA at the low levels

normally seen in aerobic plants; however, *ldh* may be more sensitive to these factors than *alaat*.

A role for calcium in the *alaat* signal transduction pathway is suggested by the observation that short term exposure of plants to an external calcium source dramatically increased *alaat* mRNA expression (Figure 5-12). If plants were slightly calcium deficient, then it would be predicted that longer calcium treatment (24 h) would also result in a dramatic increase in *alaat* mRNA expression. This was not observed (Figure 5-7), suggesting that import of external calcium may have a role in *alaat* signal transduction. This differs from maize *adh1* which was unaffected by an external calcium source (Subbaiah et al., 1994 a and b). RR treatment had no effect on *alaat* mRNA expression during hypoxia. RR when present together with calcium during hypoxia decreased the relative increase in *alaat* transcript level relative to calcium treatment alone, however, the transcript levels were still higher than control plants. This suggests that release of calcium from intracellular stores may also have a role in *alaat* expression.

In summary, work in this chapter demonstrated that:

- AlaAT activity increased in response to hypoxic stress and relief from nitrogen stress in maize, confirming work done earlier in barley;
- abiotic stresses (cold and salt) inhibited the hypoxic increase in AlaAT activity;
- ethanol production during the hypoxic stress might be involved in a negative feedback loop to inhibit *alaat* expression;
- new protein synthesis was not required for hypoxic *alaat* expression;
- extracellular and organellar Ca^{2+} fluxes might be involved in the *alaat* signaling pathway; and
- maize *alaat* and *ldh* were regulated differently.

5.5 *Bibliography*

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6. General discussion

The major goal of this thesis was to characterize the hypoxic response in maize with regard to *ldh* expression. Prior to beginning this thesis work, LDH had been subjected to limited study although it was speculated to play a pivotal role in initiating ethanolic fermentation. LDH enzyme activity, isozyme profiles and northern analysis have been examined in barley at 2 and 4 d of hypoxia (Hondred and Hanson, 1990). Since LDH activity is thought to be important in the early hypoxic response it was essential to obtain detailed information on LDH at the protein and RNA levels at earlier time points (Chapter 2). Although all the work on LDH had been performed in barley, maize is a better model system because detailed characterizations of *adh*, and other hypoxically induced genes, have been performed in maize. This facilitates comparisons of similarities and differences in the regulation of *ldh* with other hypoxically induced genes.

Several hypoxically induced proteins are encoded by more than one gene. These genes can have different hypoxic induction profiles (Dennis et al., 1985; Peschke and Sachs, 1993; Peschke and Sachs, 1994; Köhler et al., 1995) and be expressed in a variety of tissues in addition to the root (Dennis et al., 1985). Examination of the hypoxic response of genes only expressed in root tissue (i.e. LDH) would lead to a clearer understanding of the hypoxic response. Transcript profiles indicated the presence of two *ldh* genes, which were regulated slightly differently; therefore, the second goal of this thesis was to isolate the second *ldh* gene. Although this goal was not met, the work in Chapter 3 adds to the understanding of maize genome organization.

During the course of this thesis work, several transcription factors involved in the hypoxic response were identified (Lu et al., 1992; de Vetten and Ferl, 1995). Ca^{2+} was also determined to be involved in the signaling pathway (Subbaiah et al., 1994). The understanding of the hypoxic signal transduction pathway is still in its infancy and work presented in Chapter 4 aimed to enhance the understanding of the *ldh* signal transduction pathway.

Comparisons between *adh*, the most well studied hypoxically induced gene, and *ldh* indicated differences in their regulation. Thus it was of interest to compare *ldh* with another hypoxically induced gene, *alaat* (Chapter 5). *alaat* makes a particularly interesting comparison as it is involved in amino acid metabolism rather than glycolysis although it uses a substrate produced by glycolysis.

6.1 *Hypoxic expression of LDH and AlaAT*

Chapters 2 and 5 describe the response of LDH and AlaAT, respectively, to hypoxia in maize roots and maize suspension cultures. Hypoxia results in induction of both enzymes in maize roots, with the increase in LDH activity being detectable earlier than AlaAT. Enzyme activity continued to increase for 6 d with the slope of increase in LDH activity being maximal in the first 2 d and the slope for AlaAT activity being maximal after 2 d of hypoxia. The earlier response of LDH is consistent with the Davies-Roberts pH stat hypothesis (Davies et al., 1974; Roberts et al., 1984) which predicts that enzymes with acidic pH optimums become activated as pH_c decreases. LDH, with an alkaline pH optimum, converts pyruvate to lactate (an acidic product) resulting in lowered pH_c and activation of PDC and ADH, both of which have acidic pH optimums. This results in enhanced ethanol production and stabilization of pH_c. In animal cells, ADH functions primarily to detoxify ethanol and as a result, during hypoxia, pH_c continues to decline until cell death occurs (Dolferus et al., 1994).

Hypoxia decreased the *in vitro* stability of LDH, which may reflect a change in the *in vivo* stability of the enzyme. Experimental results suggest that this could be the result of maize LDH being intrinsically unstable. This feature could be advantageous to the plant in that it would limit lactate production and thus minimize further declines in pH_c. An inhibitor of ADH activity has been demonstrated in maize (Ho and Scandalios, 1975) and *Erythrina caffra* (Small et al., 1993) which serves to decrease ADH activity following germination. No inhibitor against LDH activity was isolated and experimental results for the presence of one were confusing, either reproducibly demonstrating the possibility of an inhibitor (early experiments) or reproducibly demonstrating its absence (later experiments).

Transcripts for *ldh* and *alaat* increased several fold during hypoxic treatment with *alaat* transcripts peaking earlier than *ldh* transcripts. Comparisons between transcript profiles and enzyme activity profiles suggest that post-transcriptional regulation is important for both genes. LDH activity and *ldh* transcript abundance increased during the first 24 h of hypoxia, following which *ldh* transcript abundance decreased whereas LDH activity continued to increase. LDH protein levels decreased after 24 h of hypoxia suggesting that the specific activity of the enzyme changed during the course of hypoxic treatment. AlaAT enzyme activity increased after 12 h of hypoxia, at which time *alaat* transcript expression had already peaked. The delayed increase in AlaAT activity, relative to

alaat expression, may reflect preferential translation of *alaat* transcripts during prolonged hypoxic treatment or changes in the specific activity of the enzyme during hypoxia. Discrimination between these possibilities would entail comparisons of *in vivo* and *in vitro* translation products (preferential translation) or examination of the K_m of the pure enzyme (change in specific activity).

In maize cell suspension cultures, LDH activity was not induced by either hypoxia or anoxia, AlaAT activity increased with hypoxia but not with anoxia whereas ADH activity increased with both hypoxia and anoxia. This could reflect features unique to cell suspension cultures. Alternatively, it could indicate the tissue specificity of the hypoxic response and the complexity of induction of ANPs depending on the prevailing oxygen concentration. It does suggest that some form of oxygen sensing mechanism must be operative. Studies on intact maize plants also indicate that AlaAT activity is not induced in anoxic root tissue (Muench et al., submitted).

6.2 *The role of LDH and AlaAT in the hypoxic response*

As alluded to in Section 6.1, LDH is speculated to be involved in the early hypoxic response. The observation that LDH activity remains elevated for up to 6 d also suggests a role in the long-term response. Lactate is exported form the cell (Hanson and Jacobsen, 1984; Xia and Saglio, 1992; Rivoal and Hanson, 1993; Rivoal and Hanson, 1994); thus, LDH activity may serve to regenerate NAD^+, which can be utilized by enzymes of the stem glycolytic pathway, without having a detrimental effect on pH_c. Alternatively it may be utilized in other reactions such as the production of glycolate which also would result in the production of NAD^+ (Bais et al., 1989). Prolonged hypoxia results in the production of a number of metabolites, such as GABA, which contribute to the stabilization of pH_c (Roberts et al., 1992). Elevated concentrations of these metabolites could raise pH_c, thus inhibiting ethanol production. GABA concentrations, after 8 h of hypoxia, are highest in plant species least tolerant of hypoxia (Menegus et al., 1989). LDH activity could therefore be required in the long-term response to maintain pH_c low enough to facilitate continued ethanolic fermentation.

The role of AlaAT in the hypoxic response is unclear. Nitrogen is an essential nutrient and is taken up by the root in the form of either nitrate (NO_3) or ammonium (NH_4) (Fernandes and Rossiello, 1995). In the plant, the nitrogen is assimilated, in an energy dependent process, to produce NH_4^+ which can be used in amino acid synthesis (Ivashikina and Sokolov, 1997). In the alga, *Selanastrum minutum*, alanine is important in hypoxic

nitrogen assimilation (Vanlerberghe et al., 1991). Thus it appears that plants have developed a strategy for nitrogen assimilation through the synthesis of alanine. Alanine produced by a plant under hypoxic conditions obtains its carbon skeleton from pyruvate. Early in hypoxia malic enzyme, which has an acidic pH optimum, catalyzes the conversion of malate to pyruvate which can then be transaminated to alanine (Roberts et al., 1992). Following consumption of malate, glycolytically derived pyruvate is used for alanine synthesis. Alanine production during hypoxia allows for low levels of nitrogen assimilation with alanine functioning as a nitrogen reservoir for protein synthesis.

6.3 *The hypoxic signal transduction pathway*

Analysis of the effects of protein synthesis inhibitors, Ca^{2+} and several abiotic stresses demonstrated the complexity of the *ldh* signal transduction pathway. Results in Chapters 4 and 5 indicate that the transcription factors required for hypoxic *ldh* induction are present during aerobic conditions, that a mitochondrially encoded protein may be required for detection or passage of the signal and that Ca^{2+} is an important signaling molecule in the pathway. Additionally, ethanol appears to act as a positive regulator of *ldh* and a negative regulator of *alaat*.

Concurrent exposure to several abiotic stresses inhibited the *ldh* and *alaat* hypoxic responses suggesting the presence of negative regulatory factors. In both aerobic and hypoxic conditions, the presence of salt increased *ldh* and *alaat* transcript expression. This increase in transcript abundance was not translated into increased enzyme activity indicating that post-transcriptional regulatory mechanisms were operative. Post-translation regulation was demonstrated in this thesis by the differing stability of LDH in aerobic and hypoxic conditions and by the observation that LDH protein concentration decreased after 24 h, whereas LDH activity continued to increase for up to 6 d.

A model for the hypoxic signal transduction pathway is shown in Figure 6-1. This model proposes that, in aerobic conditions, there is a basal level of expression from the genes encoding ANPs, transcription factors (TF1 and TF2) and a repressor. TF1 is active and can enter the nucleus to facilitate transcription from the genes encoding ANPs, TF1 and TF2. TF1 is normally present in low concentrations in the cytosol where it is complexed with a labile repressor protein resulting in cytosolic retention. In this model the mitochondrial membrane contains a mitochondrially encoded protein which functions as an oxygen sensor. This model proposes that reduced oxygen availability is detected by the sensor

protein, possibly resulting in a conformational change of the protein. This, directly or indirectly, results in Ca^{2+} release from the mitochondria, possibly by the opening of a Ca^{2+} channel. Hypoxia results in phosphorylation of TF2. Binding of Ca^{2+} to phosphorylated TF2 results in its activation, nuclear localization and the formation of a DNA / TF1 / TF2 transcription complex which is active in recruiting RNA polymerase and increasing transcription of the genes encoding ANPs, TF1 and TF2. As the concentration of TF1 in the cytosol increases, it dilutes out the effect of the repressor protein. In addition, repressor concentrations decrease because its RNA lacks the signal recognized by ribosomes for translation during hypoxia.

This model is consistent with several observations from this thesis work and from work of others.

- Maize GBF1, which binds the maize *adh1* G-box, is expressed at low levels in aerobic conditions and at elevated levels during hypoxic treatment (de Vetten and Ferl, 1995). This is similar to the role played by both TF1 and TF2 in the model.
- CHX treatment during aerobic conditions resulted in increased *ldh* expression. If TF1 was a stable protein whereas the repressor was labile, then the ratio of TF1: repressor would increase during CHX treatment, resulting in increased transcription from the genes to which TF1 bound.
- CAP treatment inhibits mitochondrial respiration in addition to organellar protein synthesis (Pestka, 1977). If inhibition of mitochondrial respiration was the initiating signal for the hypoxic signal transduction pathway, then it would be predicted that CAP treatment of aerobically maintained plants would increase *ldh* expression. If a mitochondrially encoded protein acted as an oxygen sensor to initiate the signaling pathway, then it would be predicted that inhibition of its synthesis would eliminate hypoxic *ldh* expression. CAP treatment reduced the aerobic and hypoxic expression of *ldh*, consistent with the proposed oxygen sensor protein present in mitochondria.
- RR inhibits organellar Ca^{2+} release (Subbaiah et al., 1994) and RR treatment depressed hypoxic *ldh* expression. This is consistent with a mitochondrial Ca^{2+} channel being opened in hypoxic conditions. The presence of a sensor protein in chloroplasts has also been suggested. Hexokinases, present in the outer chloroplast membrane, are proposed to function as the sensor and signal transmitter for sugar repression of photosynthesis-related genes (Barbier-Brygoo et al., 1997).
- Ethanol treatment resulted in increased *ldh* expression in aerobic and hypoxic conditions. It has been suggested that ethanol can modify membrane properties (Miyoshi and Sato, 1997). If ethanol modified the properties of the mitochondrial membrane it could affect the oxygen sensor molecule resulting in a conformational change, triggering Ca^{2+} release and initiation of the hypoxic signaling pathway. The observation that *ldh* transcript expression was higher in ethanol

treated hypoxic plants than ethanol treated aerobic plants also suggests that another pathway must be operative. The observation that *alaat* transcript expression was reduced in hypoxic plants treated with ethanol, relative to the hypoxic controls, also suggests the presence of a second signaling pathway.

- GF14ϖ binds Ca^{2+} and is phosphorylated at serine residues. The phosphorylated form has a higher affinity for Ca^{2+} than the nonphosphorylated form (Lu et al., 1994). This is similar to TF2 in the model.
- GF14ϖ interacts with GBF1 to form a DNA / GBF1/ GF14ϖ complex (Lu et al., 1992). In this model TF2 requires the binding of Ca^{2+} in order for it to form a complex with TF1, thus facilitating increased transcription from genes which have TF1 bound to the promoter region.
- Analysis of maize *adh1* detected four regions in the promoter which were footprinted. Proteins were bound constitutively to both subregions of the ARE, with the footprint changing upon hypoxic induction (Ferl and Nick, 1987; Paul and Ferl, 1991). This is consistent with the idea of TF1 being present at low concentrations in aerobic conditions to facilitate basal expression of specific genes. Because the ARE footprint changes during hypoxia it could indicate that another protein binds to the TF1-DNA complex. The presence of other footprinted regions suggests that other proteins (transcription factors) must also be involved in hypoxic induction.

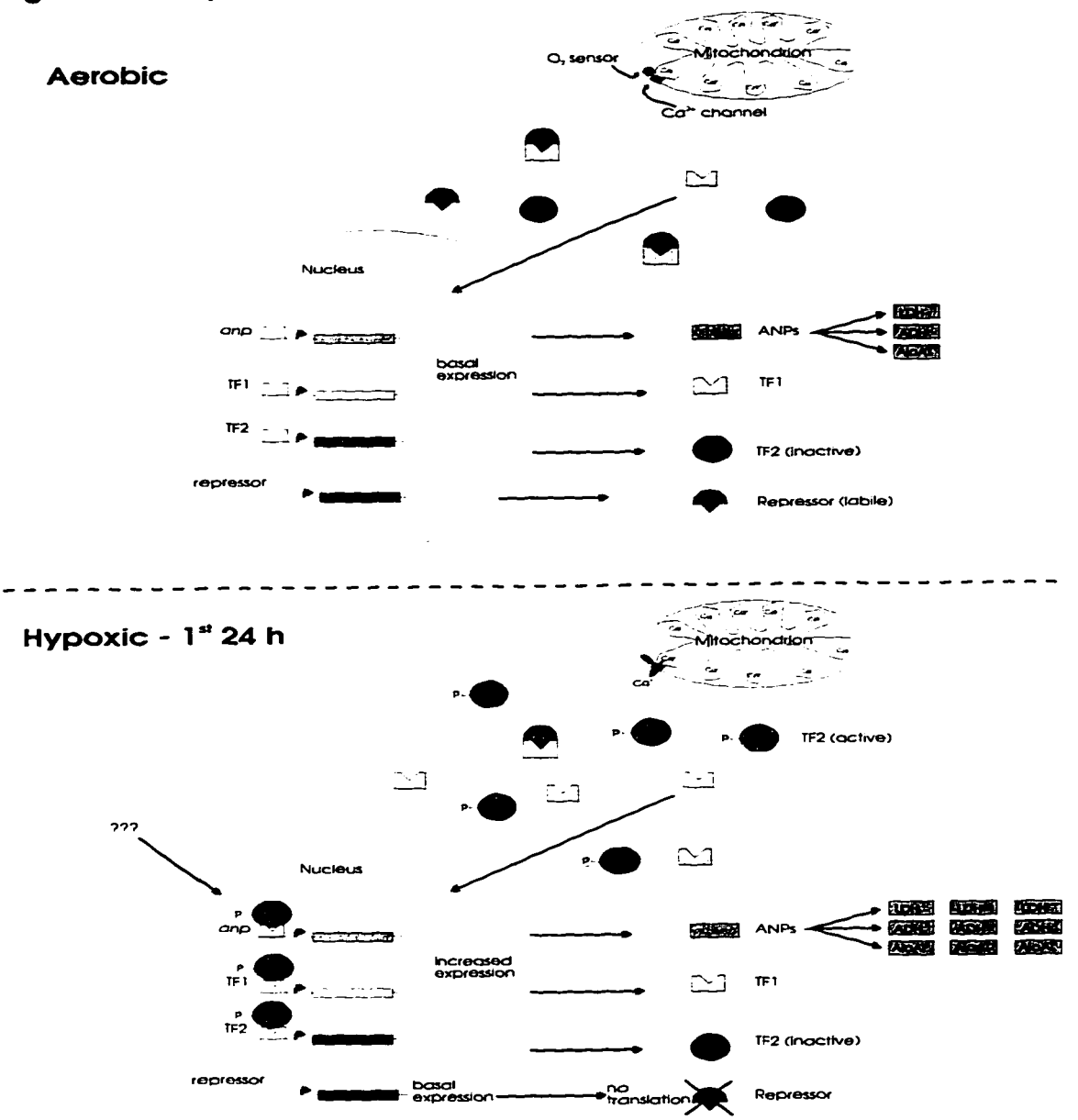
This model does not address the pathway by which TF2 is phosphorylated, nor does it address the role of ethanol acting as a positive regulator of *ldh* and a negative regulator of *alaat*.

6.4 *Future work on LDH in the hypoxic response*

As with most research, the work in this thesis generates more questions than it answers.

- Why does *in vitro* stability of LDH change in hypoxic conditions? Does this reflect an *in vivo* change or is it an artifact of the test conditions? This is difficult to answer considering the difficulties involved in the purification of LDH and production of a high titred anti-LDH antibody. Examination of other maize lines or mutants may be useful.

Figure 6-1: Proposed model for ANP signal transduction



The mitochondria contains an oxygen sensor molecule which undergoes a conformational change in hypoxic conditions. This transmits a signal to a Ca^{2+} channel resulting in the release of Ca^{2+} from the mitochondria. Phosphorylated TF2 binds Ca^{2+} enabling it to enter the nucleus and interact with TF1 previously bound to the promoter region of genes encoding ANPs, TF1 and TF2. TF1 can bind the promoter in both aerobic and hypoxic conditions, but high expression of genes to which it is bound does not occur in the absence of interactions with TF2. In aerobic conditions, TF1 activity is mitigated by its binding to a repressor protein resulting in its cytosolic retention. The repressor is not translated during hypoxic conditions thus allowing TF1 to enter the nucleus.

- How is the transcription of *ldh* regulated? Analysis of reporter genes, under the control of an intact and deleted maize promoter, would be useful to ascertain regions within the promoter that are important in the hypoxic response. Several constructs for this analysis have been generated. As LDH is not induced in maize cell cultures, promoter analysis would have to be performed in a transgenic system. *Arabidopsis thaliana* and tobacco are relatively easy to transform and have been used extensively in analysis of transgenes. However, they are both dicots and since maize is a monocot it would generate an additional layer of complexity. Alternatively, regions bound by proteins in both aerobic and hypoxic conditions could be determined by gel shift analysis using PCR generated radiolabeled promoter fragments and nuclear protein extracts. Isolation of the second *ldh* gene would be required in order to analyze sequences responsible for the different induction profiles of the two *ldh* transcripts.
- Why does CHX treatment generate such a dramatic increase in the aerobic expression of *ldh*?
  - If synthesis of a labile repressor protein is prevented by CHX treatment, then analysis of two-dimensional gels could detect proteins that are present in control conditions but are absent in the presence of CHX. These proteins could be compared to two-dimensional gels of protein extracts isolated from aerobic and hypoxic root tissue to determine if any correspond to proteins present in aerobic but not hypoxic root tissues.
  - If CHX prevented synthesis of an RNAse or protected the *ldh* transcript from RNAse activity by increasing ribosome density, it would be predicted that the $T_{1/2}$ of the transcript would increase. Comparisons of mRNA levels with nuclear run-on transcription assays should yield information regarding a change in stability of the mRNA.
  - If ribosome loading to protect mRNA from RNAse activity occurred during CHX treatment, then a shift in association of *ldh* with larger polysomes should be evident with CHX treatment.
  - If CHX had a direct effect on phosphorylation of chromatin bound proteins, as has been demonstrated in mammalian cell culture lines (Mahadevan and Edwards, 1991), then differences in the phosphorylation status of histones in aerobic CHX, aerobic or hypoxic plants should be detected.
- If there is a role for a mitochondrially encoded protein in the signal transduction pathway, as suggested by the experiments with CAP, what is this protein and what is its function? This would be most easily analyzed using cell suspension cultures. One approach would be to expose mutagenized cell culture lines to inhibitors of both cytochrome and AOX respiratory pathways. This should result in aerobic

fermentation. Mitochondria from lines that did not respond could then be compared to mitochondria from lines that underwent aerobic ethanolic fermentation.

- Is the mitochondrion the site of organellar Ca^{2+} release or are other organelles the source of the Ca^{2+} spike during hypoxia? Roots treated with chemicals that inhibit both Ca^{2+} release and *Idh* expression can be examined by electron microscopy to determine the organelle affected.
- Salt stress increases *Idh* expression in aerobic conditions and synergistically in hypoxic conditions, yet LDH activity remains unchanged. How is this regulation achieved? If salt treatment impairs translation of the *Idh* mRNA, then immunoprecipitation of *in vitro* translated LDH should be greater than that of *in vivo* synthesized LDH.
- Why do temperature extremes eliminate the hypoxic response? This would best be addressed by first determining the upper and lower ranges of temperatures in which the hypoxic response can still occur. Temperatures affect membrane function, therefore membrane function and integrity could be examined at the upper and lower limits of the temperature responsive range, especially with respect to the mitochondrial membrane.

6.5 *Maize genome organization*

Maize is a diploid (2n=10) with a genome size of 1 C equaling 3.2 X 10^9 bp (Croy et al., 1993). The large genome, relative to species such as *Arabidopsis thaliana* is the result of extensive duplication (Lapitan, 1992) and a large number of retroelements (Voytas, 1996) which account for between 60 to 80% of the maize genome size (Flavell et al., 1974; Hake and Walbot, 1980). The retroelements are located within intergenic regions and are organized in a nested fashion (SanMiguel et al., 1996). Attempts to isolate a second *Idh* gene were unsuccessful (genomic southern blots suggest the presence of 4 *Idh* genes) but it did yield information regarding maize genome organization. Within a small portion of the genome (8.2 kb) there appears to be three retrotransposon elements. Within the direct repeats of one element (LP-like element) are regions with homology to *Idh* and *pdc*. This region accounts for some of the bands on southern blots probed with the *Idh1* coding region; thus, maize contains at least one pseudogene in addition to the functional gene previously cloned. Genomic southern blots probed with the *pdc* coding region indicated the presence of more than three genes (Peschke and Sachs, 1993), whereas probing with the 3' UTR of each of the three genes isolated thus far detected a single band. The additional

bands detected on the genomic southern blots probed with *pdc* may represent *pdc* pseudogenes.

The direct repeats of the LP-like element are completely identical. This suggests that this element has, or recently had, the ability to transpose. No sequences with homology to the direct repeats were obtained in a database search. It would be interesting to estimate the copy number of this element and to determine if any functional elements exist.

6.6 *Concluding remarks*

The maize hypoxic response is complex, with many layers of regulation. At least 20 ANPs have been identified in maize, with additional ANPs being detected in other species. The understanding of the key regulatory elements is far from complete. The long term goal of studies in the hypoxic response, aside from an understanding of the system itself, is the generation of commercially valuable crop species with improved flood tolerance. This appears to be a long way off, although ADH has been demonstrated to be essential for viability under hypoxic conditions.

6.7 *Bibliography*

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