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Manipulation of the levels of pyruvate
decarboxylase and alcohol dehydrogenase
for submergence tolerance in rice

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A thesis submitted for the degree of Doctor of Philosophy
Department of Biochemistry and Molecular Biology
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In the name of Allah
The Most Gracious and The Most Merciful
Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, the thesis contains no material previously being published other than that listed below, nor is the result of any work by another person, except where due reference is given in the text.

Publication:


The research described in this thesis is my own original work with the following exceptions: Dr. Marc H. Ellis, CSIRO, PI, Canberra, prepared the *ADH* constructs and introduced the *UBIQUITIN1-ADH2* into rice and Dr. Anil Grover, Department of Plant Molecular Biology, University of Delhi, made *PDC* constructs. These constructs were used throughout this study. Dr. Stuart Craig and Ms. Celia Miller, CSIRO Microscopy Department, Canberra, took microscopic pictures of promoter-*GUS* fusions and Ms. Judy Gaudron did some of the rice transformation described in chapter 4 and Dr. Lorraine Tonnet operated gas chromatography (GC) and collected data from GC for the quantification of acetaldehyde and ethanol by GC.

Musrur Rahman
Abstract

Rice plants are partially or completely submerged when fields are flooded. During submergence, rice plants encounter anaerobic conditions, and suffer severe injury, often death, leading to major crop losses in countries affected by monsoonal flooding. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) catalyse ethanol fermentation (EF), the major energy-producing pathway under conditions of low oxygen and are among the anaerobic polypeptides induced under such conditions. The importance of EF is emphasised by the reduced survival and germination of the ADH null mutants of maize, barley, rice and Arabidopsis under anaerobic conditions.

The research described in this thesis has taken a transgenic approach to manipulate the levels of PDC and ADH to determine whether altering the EF pathway can affect anaerobic tolerance in rice. It was found that one antisense ADH1 (rice gene) line had decreased levels of both ADH1 and ADH2 polypeptides, and greatly reduced ADH activity of 6% of that of wild type (WT- untransformed Taipei). This antisense ADH1 line showed reduced ethanol production and coleoptile growth under anoxia, and mature plants exhibited reduced survival when submerged in anaerobic water, suggesting ADH plays a role in seed germination and plant survival under anoxia. One sense ADH2 (cotton gene) line had significantly increased levels of ADH activity compared to WT and a flooding tolerant rice variety FR13A in air and under hypoxia. No significant increase in ethanol production was observed in the line which over-produced ADH by 439% of WT. Similar levels of anoxia tolerance were found in mature plants of the line which over-produced ADH and WT whereas in the flooding tolerant variety, anoxia tolerance was much higher. This suggests that over-production of ADH increases neither ethanol production nor anaerobic survival. Three independently transformed lines of the rice PDC1 driven by an anaerobically inducible promoter contained an increased level of PDC1 polypeptides. A moderate increase in PDC activity and ethanol production compared to WT was also observed in these lines under anaerobic conditions. Effects of anoxia on seed germination were assayed in these lines over-producing PDC and neither retardation nor acceleration of germination was observed. However, mature plants showed decreased survival under anaerobiosis. On the contrary, hybrid plants over-expressing both PDC and ADH were found to have
better anaerobic tolerance than plants over-producing PDC alone. These results indicate that over-producing PDC plants suffered from some kind of toxicity which was counterbalanced and/or neutralised in plants over-producing ADH along with PDC. Acetaldehyde levels were appreciably higher in the plants over-producing PDC compared to WT and hybrid plants over-producing both PDC and ADH indicating that acetaldehyde might cause early senescence in plants over-producing PDC alone under anaerobic conditions. No transformed lines with either over-producing PDC, ADH or both PDC and ADH had increased submergence tolerance relative to the WT, however lines often had different metabolic rates and demonstrated the versatility of a molecular approach to evaluating metabolic controls affecting plant growth and survival.

A second objective of this research was to study the expression of the rice PDC1 and PDC3 promoters in various tissues of rice by GUS histochemical analysis. Translational fusion of the PDC1 promoter-GUS gave positive blue staining in embryos, endosperm, shoots, and roots and showed strong anaerobic induction in shoots and roots. GUS staining was found in anthers but absent in pollen. In immunoblotting analysis using an antibody raised against the rice PDC1 polypeptide, the PDC band corresponding to PDC1 was also absent in pollen of untransformed Taipei, suggesting that the rice PDC1 gene is not expressed in pollen. Nine independently transformed lines of the rice PDC3 promoter-GUS fusion (translational) did not express GUS in any vegetative tissues even under anaerobic conditions. GUS staining was seen in the pollen of three independently transformed lines of PDC3-GUS. A novel PDC band with a MWt of approx. 62 kDa was found in immunoblots of pollen of untransformed Taipei using an antibody generated against the rice PDC1, indicating that the rice PDC3 has pollen specific expression. Expression of PDC3 was seen after the first mitosis of microspores and increased with maturation, implying that it may have a role during pollen germination.

The final objective was to study the cis-acting regulatory elements required for anaerobic induction in the rice PDC1 promoter. GUS histochemical analysis of transcriptional fusions of the various lengths of 5' truncated PDC1 promoter-GUS revealed that the regulation of the GUS reporter gene did not mirror that of the endogenous PDC1 and the PDC1 promoter acted in a constitutive manner.
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<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>acetyl CoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>ACS</td>
<td>acetyl-CoA synthetase (protein)</td>
</tr>
<tr>
<td>ACT1</td>
<td>actin 1</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase (gene)</td>
</tr>
<tr>
<td>adh</td>
<td>mutant of ADH gene</td>
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<tr>
<td>ADH</td>
<td>alcohol dehydrogenase (protein)</td>
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<td>AIE</td>
<td>anaerobically inducible early gene</td>
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<td>aldehyde dehydrogenase (gene)</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase (protein)</td>
</tr>
<tr>
<td>ANP</td>
<td>anaerobically-induced polypeptide</td>
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<tr>
<td>ARE</td>
<td>anaerobic responsive element</td>
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<td>6XARE</td>
<td>promoter with six tandem copies of the ARE</td>
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<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<tr>
<td>EF</td>
<td>ethanol fermentation</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine diphosphate (oxidised form)</td>
</tr>
<tr>
<td>FADH₂</td>
<td>flavin adenine diphosphate (reduced form)</td>
</tr>
<tr>
<td>FWt</td>
<td>fresh weight</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GDH</td>
<td>glutamate dehydrogenase (protein)</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase (protein)</td>
</tr>
<tr>
<td>GUSA or GUS</td>
<td><em>Escherichia coli</em> β-glucuronidase (gene)</td>
</tr>
<tr>
<td>GUS</td>
<td><em>Escherichia coli</em> β-glucuronidase (protein)</td>
</tr>
<tr>
<td>He</td>
<td>helium</td>
</tr>
<tr>
<td>Hg</td>
<td>mercury</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa/ KD</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>L</td>
<td>length</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase (protein)</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>MWt</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>N_2</td>
<td>nitrogen</td>
</tr>
<tr>
<td>NAA</td>
<td>naphthalene acetic acid</td>
</tr>
<tr>
<td>Na_2EDTA</td>
<td>(ethylenedinitrolo)-tetraacetic acid disodium salt</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>β-nicotinamide adenine dinucleotide (oxidised form)</td>
</tr>
<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>O_2</td>
<td>oxygen</td>
</tr>
<tr>
<td>O_2</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>.O_2⁻</td>
<td>superoxide radical</td>
</tr>
<tr>
<td>.OH</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDC</td>
<td>pyruvate decarboxylase (gene)</td>
</tr>
<tr>
<td>pdc</td>
<td>mutant of PDC gene</td>
</tr>
<tr>
<td>PDC</td>
<td>pyruvate decarboxylase (protein)</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
</tbody>
</table>
RT  room temperature
SDS  sodium dodecyl sulphate
SOD  superoxide dismutase (protein)
T  thymine
TCA  tricarboxylic acid cycle
TEMED  N, N, N', N', tetramethylethylenediamine
TES  N-tris(hydroxymethyl) methyl-2-aminoethane-sulfonic acid
tNOS  nopaline synthase termination signal
TP  transition polypeptide
TPP  thiamine pyrophosphate
Tris  tris(hydroxymethyl)aminomethane
UBI1  ubiquitin 1
UTR  untranslated region
UV  ultraviolet
V  voltage
v/v  volume by volume
W  width
WT  wild type
w/v  weight by volume
X-gluc  5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Notes

All homozygous families of ADH or PDC transgenic lines were determined by comparing the levels of ADH and PDC activity of wild type (WT- untransformed Taipei) and transformants. Homozygous families had a higher (over-producing line) or lower (under-producing line) level of ADH or PDC activity than WT. Similarly, azygous families were also determined from each of these transgenic lines by assaying ADH and PDC enzyme activity and had similar level of ADH and PDC as WT.
Chapter 1

General Introduction and Review of Literature
1. GENETIC ENGINEERING FOR SUBMERGENCE TOLERANCE IN RICE:
General introduction

1.1 Importance of submergence tolerance

Flooding occurs in many areas, adversely affecting agricultural crops. It is a major limitation to rice production in many countries and particularly reduces rice production in Bangladesh, Northeastern India, and Southeast Asia (De Datta, 1981). Submergence ranks as the third greatest constraint to rice production in the rainfed lowland ecosystems of Eastern India (Widawsky and O'Toole, 1990). Partial or complete submergence usually occurs with a rapid increase in water levels (flash flood) when plants may be under water for several days to a few weeks. There are varying extents of crop damage depending on the growth stage of the plants at the time of flooding (Fakuda and Tsutsui, 1968) and on the depth and duration of submergence (Palada and Vergara, 1972). Direct adverse effects of flooding on plants are reduced growth, impeded flowering, poor seed production (Blom et al., 1994), reduced dry weight (Mazaredo and Vergara, 1982), chlorosis (Jackson et al., 1987) and death (Palada and Vergara, 1972).

Deepwater rice, with its remarkably high growth rate of up to 25 cm a day (Vergara et al., 1976) is able to emerge from the rising flood waters and avoid submergence, but its yield is far below that of improved rice varieties, which lack the capacity to elongate (Mannan, 1988).

Submergence also occurs when rice is directly seeded into the flooded soil. Direct seeding is preferable to the traditional method of transplanting, which, in spite of its high yield, is costly and labour intensive. However, direct seeding causes poor seedling establishment when the seed settles below the flooded soil surface.
Finally, submergence tolerance is a valuable strategy for weed management in areas where rice is seeded directly into standing water. In this system, deeper water levels (20 cm) at seeding can provide effective weed control (Williams et al., 1990).

1.2 Types of different flooding regimes in rice fields

Rice can be grown in three main types of flood prone environments. These are classified according to the depth and duration of flooding:

1. The stagnant deepwater rice ecosystem, where water depth rises slowly (<30 cm d⁻¹) and remains at the depth of 50 to 400 cm for 2 to 4 months of the year (HilleRisLambers and Seshu, 1982; Catling, 1992).

2. The areas where varieties called ‘floating rice’ are grown and where maximum water depths exceed those for deepwater rice and range from 1-6 m (Huke, 1982; Metraux and Kende, 1983; Khush, 1984).

3. The flash flood affected areas of the rainfed lowland rice ecosystem where water levels are usually up to 50 cm (Maurya et al., 1988) and sometimes higher depending on the size of the flood. In these regions, flooding usually occurs during monsoonal seasons. Flash floods can also affect large areas of deepwater and floating rice where water depths may reach 1-2 m (HilleRisLambers and Seshu, 1982; Setter et al., 1987a).

Flooding also occurs in irrigated areas throughout the world, but little work has been done on the frequency of occurrence and the extent of damage in such ecosystems. According to the International Rice Research Institute’s estimation in 1993, the world’s total area sown to rice is 148 million ha, of which 10 million ha is used to grow deepwater and floating rice. Twenty million ha of the world’s total rice growing area is in the category of flash-flood prone lowland rice (Maurya et al., 1988).
Chapter 1 / General Introduction

1.3 Mechanisms of flooding avoidance by deepwater and floating rice and genetics of submergence tolerance

The mechanisms for successful adaptation to flooding depend on the water regime. Deepwater rice is planted in the stagnant deepwater ecosystem: the rice elongates with the slow rise of floodwater and can maintain its shoot above water level. Deepwater rice survives through stem elongation via internodal growth. The adaptive response of floating rice to flooding is also by stem elongation under stagnant deepwater conditions; floating rice can reach 5 m in height.

In flash-flood prone areas, submergence of rice plants usually occurs because of the rapid increase in water level and plants may be under water for a few days or weeks. In these areas, the level of water rises so rapidly that rice plants can not elongate quickly enough to keep their shoots above water. As the floods are of relatively short duration, elongation is not an appropriate strategy, since when the floodwaters recede, plants that have elongated will tend to lodge. Therefore, the stress avoidance strategy by stem elongation used by deepwater and floating rice can be a useful strategy in some areas but is ineffective in the flash-flood prone lowland, and varieties grown in these areas need to tolerate submergence. The work presented in this thesis relates to this latter adaptation.

Among cereals rice has the extraordinary capacity to tolerate short-term submergence and germplasm with enhanced submergence tolerance exists. A few varieties such as FR13A, Kurkaruppan, Thavalu and Goda Heenati are adapted to short-term submergence (IRRI, 1978; Mazaredo and Vergara, 1982; Setter et al., 1989c). Complete submergence is tolerated for a limited time by both 'intolerant' and 'tolerant' varieties. However, intolerant varieties are destroyed more rapidly than tolerant ones (HilleRisLambers and Vergara, 1982). For instance, seedlings of FR13A can survive complete submergence after over two weeks while seedlings of intolerant varieties like the high yielding IR42 are completely destroyed (Mazaredo and Vergara, 1982). Furthermore, most rice varieties cannot survive complete submergence of the shoot and root systems for a long time. The majority (94%) of 3156 rice varieties tested at the age
of 30d at Huntra Rice Experiment Station, Thailand, in 1983 were intolerant to 10d of complete submergence (Setter et al., 1987a). There are no varieties having both submergence tolerance and good agronomic traits such as high yield, disease resistance, and good grain quality. Efforts have been made to develop submergence tolerant elite lines by conventional breeding using FR13A as a donor for submergence tolerance (Mackill et al., 1993), but progress in combining tolerance with improved plant type and high yield has been slow.

Genetic research involving crosses between two submergence tolerant varieties and between submergence tolerant- and intolerant variety, and analysis of the inheritance of submergence tolerance, suggested that this trait is under the control of a single dominant gene (Mazaredo and Vergara, 1982; Mohanty and Chaudhary, 1986; Mishra et al., 1996). FR13A, Kurkaruppan, and Thavalu submergence tolerant varieties possess the same tolerance gene in the same locus whereas Goda Heenati, a submergence tolerant variety, does not (Mazaredo and Vergara, 1982; Setter et al., 1997). One major locus for submergence tolerance, SUBI, has been mapped to chromosome 9 (Xu and Mackill, 1995, 1996; Nandi et al., 1997). Within a population of double haploid lines obtained from a cross between a tolerant and an intolerant variety, 69% of the phenotypic variance for submergence tolerance is contributed by the SUBI locus (Xu and Mackill, 1996). Nandi et al. (1997) also detected quantitative trait loci (QTLs) associated with submergence tolerance on chromosomes 6, 7, 11, and 12. The QTL on chromosome 11 was in the vicinity of the ADH1 and ADH2 genes and it was proposed that this QTL might correspond to the ADH genes (Nandi et al., 1997).

1.4 Adverse effects of submergence on rice

Submergence causes a range of adverse environmental conditions for rice plants including:

(a) The turbidity of floodwater restricts plants from getting enough light for photosynthesis; photosynthesis slows down resulting in a block in the synthesis of carbohydrates and generation of molecular oxygen (Heckman, 1979; Setter et al.,
1987b; Witton and Rother, 1988). Palada and Vergara (1972) showed that the survival of rice seedlings after complete submergence in water is decreased with increased turbidity of water, increased with high light intensity and decreased with the depth of water. This reduced tolerance to submergence under low light conditions has also been observed in various species of the genus *Rumex* and other riverside species (Blom *et al.*, 1994).

(b) Turbulence of floodwater during flooding causes mechanical damage to the plants.

(c) One important factor that affects survival of rice during submergence is limited gas diffusion which is $10^4$ times slower in water than in air (Armstrong, 1979). The relevance of low O$_2$ to field conditions was demonstrated by measurements of O$_2$ concentrations in several locations in Thailand (Heckman, 1979; Setter *et al.*, 1988a) and Bangladesh (Witton and Rother, 1988) during flooding. The O$_2$ concentration in floodwater in the deepwater and floating rice areas decreases during the night to 0.18 mol m$^{-3}$ and increases during the day to 0.13-0.28 mol m$^{-3}$ (0.28 mol m$^{-3}$ is 120% of the O$_2$ concentration of air saturated water at 30 °C) (Setter *et al.*, 1987a, 1988a). Similar diurnal changes in O$_2$ concentration were observed in floodwater (0.06 m water depth) of lowland, rainfed rice fields (Heckman, 1979), and in lakes (Van *et al.*, 1976). Setter *et al.* (1988b) demonstrated that the increase in O$_2$ concentration of floodwater during the day is due to O$_2$ evolution by photosynthesis and that the evolved O$_2$ is more than the amount needed by the plant for respiration. These diurnal changes became less distinct with the depth of floodwater (Setter *et al.*, 1988a). Setter *et al.* (1987a) also found that the O$_2$ concentration in floodwater and inside the internodal lacunae (gas spaces in shoots in higher plants) of floating rice growing at 1-2 m water depths decreased with the depth of water; there were no diurnal changes in O$_2$ concentration inside the internodal lacunae. It has been found that stagnant flooding is generally worse than when floodwater is flowing, where rapid mixing of air with floodwater occurs during the turbulence of flooding.
Ellis and Setter (1999) have shown that when anoxia is imposed during submergence, death of rice plants occurs in less than 24h.

(d) Post-submergence photo-oxidative damage: when anaerobically either hypoxically (reduced oxygen partial pressure than atmospheric pressure 0.23 mole m\(^{-3}\)) or anoxically (total absence of oxygen) treated tissues brought to re-aeration, tissue decay occurs. Tissue damage might result from the build up of potentially toxic free radicals (Hendry and Brocklebank, 1985; for a review see Armstrong et al., 1994; Crawford et al., 1994). Tissue injury and cell death after anoxia might also result from the rapid conversion of ethanol to acetaldehyde, which is a very strong cell toxin (He and Lambert, 1990; Perata and Alpi, 1991; Perata et al., 1992; Grafstrom et al., 1994; Koivisto and Salaspuro, 1998). This conversion is probably caused by catalase (Monk et al., 1987a; for a review see Armstrong et al., 1994; Pfister-Sieber and Brandle, 1994). Ethylene may also be formed from anaerobically accumulated 1-aminocyclopropane-1-carboxylic acid (ACC) and can promote early senescence (Armstrong et al., 1994).

1.5 Response of plants to low oxygen conditions

Tolerance to submergence in plants is achieved by strategies which improve gas exchange, as well as various metabolic features which help plants to produce sufficient energy to sustain cell integrity and avoid irreparable damage under low oxygen stress. Wetland vegetation gradients along riversides are established through the ability of plants to change their anatomy, morphology and metabolism for surviving both temporary flooding and prolonged submerged periods (for a review, see Blom, 1999). Among higher plants, rice is unusual in that it can tolerate low oxygen conditions remarkably well, partly due to its morphological and metabolic adaptations. The adaptive responses that plants have developed for some degree of tolerance against these adverse environmental conditions are outlined in the following section.
1.5.1 Avoidance of low oxygen stress by morphological adaptations

1.5.1.1 Aerenchyma

Aerenchyma is interconnected gas-filled intercellular spaces and allows the diffusion of oxygen internally, especially to root tissue. Such diffusion reduces the risk of asphyxiation under soil flooding (for a review, see Jackson and Armstrong, 1999). Aerenchyma is found in roots, rhizomes, stems and leaves and is categorised by the way it is formed: lysigeneous aerenchyma is formed by cell collapse and some degree of cell separation, and shizogeneous aerenchyma results only from cell separation. A cDNA with homology to xyloglucan endotransglycosylase was isolated from anoxic maize roots, and may have a cell-wall loosening function in aerenchyma formation (Sachs et al., 1996). Aerenchyma is present constitutively in many wetland species world wide, although some dry land plants also possess this specialised structure (for a review, see Jackson and Armstrong, 1999). In some species, such as maize, barley and wheat, aerenchyma is produced in response to flooding, whereas in rice it is an almost constant feature of the root system and may become more extensive under flooding (Justin and Armstrong, 1991). Studies on rice and other species showed that O₂ in internodes may be evolved during photosynthesis by submerged tissues or diffused from the air into the submerged shoots via aerenchyma, or along the gas envelope around the surface of leaves (Raskin & Kende, 1983; Setter et al., 1987a; Laan and Blom, 1990). However, plants with aerial parts submerged have blocked shoot to root oxygen diffusion from air and cannot obtain any long-term benefit from aerenchyma.

Ethylene plays an important role in aerenchyma formation in flooding tolerant species as well as dry land cereals, such as maize, wheat and barley. Armstrong et al. (1994) demonstrated that norbornadiene, a volatile inhibitor of ethylene action, arrests aerenchyma formation.

1.5.1.2 Root extension

Root extension is strongly inhibited by oxygen shortage in many species (Menegus et al., 1992). An adaptation to flooding of many plant species, including maize and rice, is the increased formation of adventitious roots, which are developed
particularly well when plants are submerged and often contain more aerenchyma than primary roots (Drew et al., 1979; Justin and Armstrong, 1987, 1991). Riverside species, which are often submerged for prolonged periods, develop larger numbers of adventitious roots than species that grow on seldom flooded river habitats (Blom et al., 1994). Adventitious root formation may be triggered by low oxygen conditions in flooding tolerant *Rumex palustris*, a species from frequently flooded areas of river foreland and the well developed aerenchyma in these roots facilitate oxygen diffusion from the emerged shoots to the roots (Blom et al., 1994).

Root formation is stimulated by naphthalene acetic acid (NAA) and ethylene applications in *Rumex palustris* (Blom et al., 1994), maize (Drew et al., 1979; Jackson et al., 1981) and *Arabidopsis* (Bleecker et al., 1988) under non-flooded conditions. The formation of adventitious roots may be triggered by the build-up of a high concentration of auxin at the shoot-root junction upon submergence (Blom, 1999). An application of both auxin and ethylene induces more adventitious root formation in *Rumex palustris* than applications of them separately (Blom, 1999).

1.5.1.3 Stem elongation

Another morphological escape from anaerobiosis arises from the rapid elongation of stems and petioles, enabling aquatic and flooding-tolerant terrestrial species to emerge from the water (Raskin and Kende, 1984b; Voesenek and Blom, 1996). Under complete submergence, many amphibious and aquatic plants extend their internodes or petioles rapidly, thereby shortening the duration of asphyxiation caused by submergence conditions. Floating and deepwater rice plants also respond to submergence by a considerable increase in rates of internodal elongation (Vergara et al., 1976).

Low oxygen conditions stimulate the biosynthesis of ethylene which is accumulated within the internodes of rice (Raskin and Kende, 1983, 1984a, 1984b) and promotes shoot elongation (Musgrave et al., 1972). The *Arabidopsis* *ETRI* (ethylene response) gene encodes an ethylene receptor (Chang et al., 1993; Schaller and Bleecker, 1995) and is up-regulated by flooding in *Rumex palustris* (Vriezen et al., 1997). *ETRI* is essential for ethylene action since the mutant *etr1-1* of *Arabidopsis* lacks almost all ethylene response, although the ethylene synthesis rate in this mutant is similar to wild
type (Bleecker et al., 1988). Other plant hormones, often in combination with ethylene, play an essential role in this adaptive response. It is generally accepted that application of auxin increases ethylene production in plants (Kelly and Bradford, 1990). Ethylene increases responsiveness of cells to the immediate growth-promoting hormone gibberellic acid (GA) by reducing the level of endogenous abscisic acid (ABA), a potent inhibitor of growth in rice (Hoffmann-Benning and Kende, 1992). GAs levels are increased by submergence in rice (Azuma et al., 1990) and act by enhancing cell elongation and cell division activity in the intercalary meristem (Sauter and Kende, 1992; Sauter et al., 1993). Blom et al. (1994) demonstrated that the application of gibberellin-biosynthesis inhibitor paclobutrazol caused an inhibition of petiole elongation of *Rumex palustris*. Moreover, if the production of GAs is blocked with the inhibitor tetecyclasis neither submergence nor ethylene can promote growth (Armstrong et al., 1994).

Expansins are newly identified proteins which have been recognised as cell wall-loosening factors that can promote long-term extension of isolated cell walls (for a review, see McQueen-Mason and Cosgrove, 1995; Cosgrove, 1996). Cho and Kende (1997c) found a link between expansin and submergence, such that the accumulation of *Os-EXP4* (expansin 4) mRNA in deepwater rice is induced by submergence or treatment with GA. In addition to rice, expansin gene families have also been found in cucumber and *Arabidopsis* (Shcherban et al., 1995; Cho and Kende, 1997a). The plant cell wall is principally a network of microfibrils interconnected by hemicelluloses (Carpita and Gibeaut, 1993). Expansins might disrupt hydrogen bonds between cellulose microfibrils and matrix polymers (McQueen-Mason and Cosgrove, 1994) and facilitate cell elongation. However, as discussed in section 1.3, rapid elongation is not an appropriate trait for submergence tolerance in rice.

### 1.5.2 Tolerance involving metabolic adaptations

Anoxia may occur during submergence and is usually preceded by less harmful hypoxic conditions. Under laboratory conditions, hypoxic pre-treatments dramatically increase a plant’s ability to tolerate subsequent anoxia. This acclimation process has
been demonstrated in rice (Ellis and Setter, 1999), *Arabidopsis* (Ellis *et al*., 1999), tomato (Germain *et al*., 1997), wheat (Waters *et al*., 1991), and maize (Saglio *et al*., 1988; Johnson *et al*., 1989). These observations demonstrate that hypoxic pre-treatment induces some response in plants that gives them the ability to survive longer under anoxia. The mechanisms for adaptation are discussed below.

### 1.5.2.1 Induction of anaerobic polypeptides (ANPs)

Plants exposed to low oxygen conditions stop the synthesis of proteins present under normal conditions and induce the synthesis of a new set of proteins, the anaerobic polypeptides (ANPs). In roots of maize seedlings, 20 ANPs were detected which account for 70% of protein synthesis (Sachs *et al*., 1980). This alteration in protein profile during anaerobiosis has also been found in *Arabidopsis* (Dolferus *et al*., 1985), soybean (Russell *et al*., 1990), rice (Bertani *et al*., 1981; Ricard and Pradet, 1989) and cotton (Millar and Dennis, 1996). Most of the ANPs are enzymes either involved in glycolysis, including glucose phosphate isomerase (Kelly and Freeling, 1984b), cytosolic fructose-1, 6-biphosphate aldolase (Kelly and Freeling, 1984a) and glyceraldehyde-3-phosphate dehydrogenase (Ricard *et al*., 1989; Russell and Sachs 1989; 1992) or in the ethanol fermentation pathway e.g. alcohol dehydrogenase (ADH) (Hageman and Flesher, 1960; Sachs and Freeling, 1978 (ADH1); Ferl *et al*., 1979 (ADH2)) and pyruvate decarboxylase (PDC) (Wignarajah and Greenway, 1976; Laszlo and St Lawrence, 1983; Hossain *et al*., 1996). Springer *et al*. (1986) identified the starch mobilising enzyme sucrose synthase 1 isoenzyme as an ANP. Bailey-Serres *et al*. (1988) measured the enzyme activities of other glycolytic enzymes and found that enolase and phosphoglucomutase are also induced by anaerobic stress.

The importance of ANPs for anaerobic survival has been demonstrated using chemical inhibitors. Subbaiah *et al*. (1994a) found that the application of ruthenium red inhibited anaerobic gene expression and greatly reduced anaerobic survival. Vartapetian and Poljakova (1994) and Ellis *et al*. (1999) found a reduction in anoxia tolerance by inhibiting ANP production in rice and *Arabidopsis* respectively by using cycloheximide. The simplest ANP profile of four polypeptides was found in soybean (Russell *et al*.,...
1990), a flooding intolerant species, compared to 20 ANPs in maize (Sachs et al., 1980). ANPs may be important in flooding tolerant species.

1.5.2.2 Changes in energy metabolism

1.5.2.2.1 Shift of energy metabolism from aerobic to anaerobic fermentation

Under normal aerated conditions, higher plants require molecular oxygen to oxidise glucose for energy production. Under such conditions, glucose enters glycolysis and produces pyruvate with the concomitant formation of ATP and NADH (fig. 1.1).

Figure 1.1: This is a schematic diagram which qualitatively, but not quantitatively, reflects the catabolism of carbohydrates to CO$_2$, water, or ethanol and ATP.
Pyruvate is oxidised in the citric acid cycle and through the electron transport chain and generates energy. However, in the absence of oxygen, higher plants initially use lactic acid fermentation and subsequently EF. During low oxygen conditions or under anoxia, the activity of lactate dehydrogenase (LDH) is increased and NADH is oxidised to NAD$^+$ to maintain glycolysis. Consequently, the cytoplasmic pH is decreased from an alkaline pH of 7.4 to an acidic pH due to the accumulation of lactate. LDH has an alkaline pH optimum and is inhibited by low cytoplasmic pH (Davies et al., 1974; Davies, 1980; Roberts et al., 1984a, 1984b, 1985). The low cytoplasmic pH inhibits LDH activity and activates pyruvate decarboxylase (PDC). PDC has an acidic pH optimum and catalyses the first step of EF (Davies, 1980). Further lowering of cytoplasmic pH can be prevented by compartmentation of newly synthesised lactate inside a vacuole (Kennedy et al., 1992). EF is thought to play a vital role during anoxia. EF, unlike lactate fermentation, does not result in severe cytoplasmic acidosis which is a determinant of flooding intolerance in some plants (Davies, 1980; Roberts et al., 1984b). Prolonged anaerobiosis causes protons to leak from vacuoles and results in an increase in vacuolar pH, and at the same time, a decrease in cytoplasmic pH (Roberts et al., 1984b). Menegus et al. (1991) also found a more alkaline vacuolar pH in rice under anoxia.

Tadege et al. (1999) proposed that the concentration of pyruvate, the substrate of both PDH (pyruvate dehydrogenase) and PDC, is more important than pH in regulating the entry of pyruvate to EF pathway. In kinetic studies, PDCs showed high Michaelis-Menten constants ($K_m$) in the range of mM, such as 3.0 mM for wheat germ PDC (Zehender et al., 1987), 1.7-1.8 mM for yeast PDC (Ullrich and Donner, 1970) and 0.25 mM in rice (Rivoal et al., 1990) whereas PDHs have $K_m$ values in the µM range (Tadege et al., 1999). Under aerobic conditions pyruvate preferentially enters the TCA cycle and is metabolised by PDH, as the pyruvate concentration in the cytosol is between 0.1 and 0.4 mM which is too low for PDCs to successfully compete with PDHs (Davies et al., 1974; Rivoal et al., 1990). PDC has a higher $K_m$ value at a higher pH. This may be due to the subunit dissociation of PDCs, since at pH 7.0 and a concentration of 33 mM pyruvate, pea PDC exhibits only 85% of its activity at pH 6.0 (Mucke et al., 1995). PDC metabolises pyruvate under oxygen deprivation (Laber and
Amrhein, 1987) or in conditions when respiration is blocked by inhibitors (Good and Muench, 1993), since under such conditions, the pyruvate concentration increases considerably. At this higher pyruvate concentration, there is no competition between PDC and PDH for pyruvate. It appears that both pH and pyruvate concentration play roles in the regulation of ethanol fermentation under anoxia and the Davies-Roberts' pH stat hypothesis (Davies et al., 1974; Roberts et al., 1984a) alone might not be sufficient to explain this regulation. For example, LDH was induced by long-term hypoxia in roots of barley and other species (Hoffman et al., 1986; Hanson et al., 1987; Rivoal et al., 1991). Lactate fermentation also continues during prolonged anoxia in barley aleurone layers (Hanson and Jacobsen, 1984) and roots of some Limonium species (Rivoal and Hanson, 1993). Furthermore, in anaerobic wheat seedlings, cytoplasmic acidification stops after 2h in spite of the continuation of lactate accumulation for more than 10h (Menegus et al., 1989) and in rice shoots, cytoplasmic pH drops immediately although lactate production is low (Menegus et al., 1991). Moreover, hypoxia stimulates a drop in cytoplasmic pH long before the lactate concentration reaches a steady-state level and subsequent re-oxygenation brings the pH to normal value long before the reduction in lactate concentration (Saint-Ges et al., 1991).

1.5.2.2.2 Importance of ethanol fermentation in plants under anaerobic conditions

One of the metabolic adaptive responses of plants under anaerobic stress caused by flooding is a switch from the high energy yielding oxidative energy metabolism to the less efficient fermentative pathway. Immediately after anoxia, cessation of oxidative phosphorylation typically results in a marked decrease in ATP levels (Raymond et al., 1985). Only 2 molecules of ATP are produced per molecule of glucose under anaerobic conditions whereas 36 molecules of ATP are produced under oxidative conditions (Davies, 1980). Anaerobiosis induces glycolysis and ethanol fermentation (EF) and therefore, enhances carbohydrate channelling through this less energy efficient pathway ('Pasteur effect', Beevers, 1961; Davies, 1980; Raymond et al., 1985). The importance of EF in plants under anaerobic conditions is emphasised by the following observations:
1. The reduced survival of the root tips, the failure to germinate and the reduced anaerobic tolerance were observed in adh null mutants of *Arabidopsis* (Jacobs et al., 1988; Ellis et al., 1999), barley (Harberd and Edwards, 1982), maize (Schwartz, 1969; Freeling and Bennett 1985) and rice (Matsumura et al., 1995, 1998).

2. The majority of the ANPs are enzymes involved in glycolysis and EF (Dennis et al., 1984, 1985; Bailey-Serres et al., 1988; Russell and Sachs, 1992; Sachs, 1993). At least one functional ADH isoenzyme is present in anaerobic tissues in all plants (Ho and Sachs, 1989). Hypoxic pre-treatment prolongs survival under anoxia by increasing glycolysis and fermentation (Saglio et al., 1988).

3. Stimulating glycolysis with exogenous sugars can prolong survival under oxygen deprivation (Webb and Armstrong, 1983).

### 1.5.2.3 Protection against post-anoxic injury

Tissue damage occurs when plants are exposed to air immediately following anoxic treatment. This may be caused by the generation of extremely reactive superoxide radicals (\( \cdot O_2^- \)), hydrogen peroxide (\( H_2O_2 \)), hydroxyl radicals (\( \cdot OH \)) and singlet oxygen (\( ^1O_2 \)) (Hendry and Brocklebank, 1985; Scandalios, 1993; Crawford et al., 1994). All these reactive oxygen species can produce mutations, dysfunction of enzymes and peroxidative damage to lipids and membranes by reacting with unsaturated fatty acids. The intracellular damage causes dysfunction of organelles and cells with plasmalemma damage leak their solutes, desiccate and die quickly (Scandalios, 1993). The rhizomes of marsh plants such as *Acorus calamus* and *Schoenoplectus lacustris* exhibit very slow lipid peroxidation and stable membranes during recovery following 50-70 days of anoxic treatment. In contrast, rhizomes of the less tolerant *Iris germanica* show high peroxidation after only 7-10 days anoxia, and membranes completely disintegrate within 2-3 weeks (Henzi and Brandle, 1993). Another protective system involves superoxide dismutase (SOD) which converts \( \cdot O_2^- \) radicals to \( H_2O_2 \), which is then reduced to water by peroxidases or catalases. SOD is an ANP and is induced following low oxygen conditions (Monk et al., 1987b). The level of
SOD is increased 13-fold during 28d of anoxia in anoxia tolerant rhizomes of *Iris pseudacorus* but fails to increase in *Iris germanica* and *Glyceria maxima*, which can only tolerate anoxia for much shorter periods (Armstrong *et al.*, 1994; Monk *et al.*, 1989). Transgenic tobacco plants over-expressing SOD are better protected against oxidative stress than non-transgenic control plants (Herouart *et al.*, 1993; Sen Gupta *et al.*, 1993). The activity of SOD in the transgenic plant is enough to scavenge all the \( \cdot O_2^- \) so that \( \cdot OH \) (derived from a reaction of \( \cdot O_2^- \) with \( H_2O_2 \)), the most potent oxidant known, can not be formed.

### 1.6 Perception of oxygen shortage and signal transduction events that lead to the anaerobic response

The molecular mechanisms by which a plant cell perceives anoxia and transduces these signals are only partially understood. Recent observations suggest the involvement of transcriptional, post-transcriptional and translational controls of gene expression, and the role of \( Ca^{2+} \) as a second messenger for signal transduction leading to anaerobic responses in plants.

#### 1.6.1 Transcriptional control

**1.6.1.1 DNA motifs for anaerobic induction**

The DNA sequences responsible for the anaerobic induction of ANPs have been investigated and a motif called the anaerobic responsive element (ARE) was identified in the maize *ADHI* promoter (Walker *et al.*, 1987). Anaerobic induction increases with the number of ARE regions, with six copies giving 16-fold induction of a reporter gene (Olive *et al.*, 1990). Two sub-regions are present in the ARE, both of which are necessary for activity. Each of these two sub-regions contains a GT-motif (5'-[T/C]CGGTTT-3') and a GC motif (5'-GCC[G/C]-3'). This element gave anaerobic induction when placed in either orientation adjacent to a minimal 35S promoter (Olive *et al.*, 1990). These motifs were later found to be widespread in the promoter region of many anaerobically inducible genes, such as in the maize *ADH2* and aldolase, pea *ADH*
and *Arabidopsis ADH1, LDH1*, and *PDC1* (Dennis et al., 1987, 1988a; Llewellyn et al., 1987; Dolferus et al., 1994b; Hoeren et al., 1998). These motifs are also found in the rice *ADH* and *PDC* promoters (Xie and Wu, 1990; Hossain et al., 1996; Hoeren et al., 1998; Huq and Hodges, 1999). Disruption of this element in the *ADH1* promoter of maize and *Arabidopsis* abolished expression and anaerobic induction of a reporter gene (Walker et al., 1987; Dolferus et al., 1994b). While many of the internal features of the ARE elements may be conserved, the number of elements and the spatial arrangement is different between species and genera (Dennis et al., 1987, 1988a; Llewellyn et al., 1987; Dolferus et al., 1994b; Hoeren et al., 1998).

### 1.6.1.2 Proteins binding to the ARE region

Regulation of transcription is mediated through proteins binding to specific cis-acting regulatory sequences in promoters. Putative protein binding sites have been identified in the maize *ADH1* promoter by using in vivo dimethyl sulfate (DMS) protection and two sites have been foot-printed that coincide with the two sub-regions of the ARE (Ferl and Nick, 1987; Paul and Ferl, 1997). Importantly, the *Arabidopsis* MYB transcription factor AtMYB2 binds specifically to the GT-motifs of the *ADH1* promoter. When driven by a constitutive promoter, AtMYB2 is able to transactivate *ADH1* expression in transient assays in both *Arabidopsis* and *Nicotiana plumbaginifolia* protoplasts, and in particle bombardment of pea leaves (Hoeren et al., 1998).

Soon after the onset of anaerobiosis, plants produce a small set (approximately four) of 33 kDa proteins, known as the transition polypeptides (TPs) which disappear long before ANPs are synthesised (Sachs et al., 1980). The significance of the transition polypeptides is unknown at the moment, as their structure and the genes coding for them have yet to be characterised. These might be the transcription factors involved in the induction of genes for anaerobic response (Sachs et al., 1980). However, soybean does not appear to have TPs (Russell et al., 1990). Huq and Hodges (1999) isolated a full-length cDNA which encodes a 14 kDa putative protein and two cDNA fragments, and called them collectively the anaerobically inducible early (*AIE*) gene family. Huq and Hodges (1999) found the *AIE2* gene to be induced earlier than *PDC* and *ADH* genes.
in rice. The transcript levels of \textit{AlE} peaked after 1.5h to 3h of anoxia and were still at high levels after 72h of anoxia, suggesting that they possibly play roles in plant metabolism under anaerobic conditions.

1.6.2 Post-transcriptional and translational controls

Translation of mRNA is an important mode of gene regulation in plants. Environmental stimuli modulate components of protein synthesis for global repression of translation and selective translation of mRNAs that encode stress proteins (Butler \textit{et al.}, 1990; for a review see Bailey-Serres, 1999). Genes that encode ANPs are transcribed at elevated levels and effectively translated in O$_2$-deprived tissues. Genes that encode proteins under normal conditions continue to be transcribed, but the translation of their mRNAs is inefficient (Fennoy and Bailey-Serres, 1995; Fennoy \textit{et al.}, 1998). Bailey-Serres and Dawe (1996) demonstrated that the interaction between the 5' and 3' ends of the maize \textit{ADHI} mRNA enhances translation. Other modifications include hypoxia induced phosphorylation of components of the mRNAs 5' cap-binding complex, ribosome and mRNA binding proteins (Webster \textit{et al.}, 1991; Bailey-Serres \textit{et al.}, 1997; Manjunath \textit{et al.}, 1999), reduced levels of large polyribosome (≥5 ribosomes) (Bailey-Serres and Freeling, 1990) and differential sequestration of mRNAs on messenger ribonucleoproteins (mRNPs) (Fennoy \textit{et al.}, 1998; for a review see Bailey-Serres, 1999).

1.6.3 Ca$^{2+}$/cGMP as second messengers

Recent evidence suggests that Ca$^{2+}$ may be involved as a second messenger in transducing the anoxic signal into a physiological response (reviewed by Poovaiah and Reddy, 1993, and Sachs \textit{et al.}, 1996; Sedbrook \textit{et al.}, 1996; Subbaiah \textit{et al.}, 1998). Ca$^{2+}$ is mobilised from the intracellular stores, possibly from mitochondria, under anoxia (Subbaiah \textit{et al.}, 1994a, 1994b, 1998). The changes in cytosolic Ca$^{2+}$ under anoxia are inhibited by ruthenium red (RR), a blocker of organellar Ca$^{2+}$ channels, and RR inhibition is prevented by the addition of exogenous Ca$^{2+}$ to the medium (Subbaiah \textit{et al.}, 1994a, 1994b). RR inhibits anaerobic gene expression and post stress survival of
maize seedlings. Manjunath et al. (1999) demonstrated that phosphorylation of the mRNA cap-binding protein occurs in oxygen-deprived roots in maize and is mediated by Ca$^{2+}$.

cGMP may also involved in the regulation of anaerobic metabolism in plants. Reggiani (1997) found a rapid (2 min) and transient increase in cGMP content in roots and coleoptiles of rice seedlings under anoxia. The increase in cGMP concentration is a recognised signal in the response to ischaemia and anoxia in mammalian cells (Depre and Hue, 1994).

1.7 Background work on the rice ADH and PDC genes

ADH has been extensively studied in higher plants with the maize ADH system being the most characterised (Dennis et al., 1985; for a review, see Freeling and Bennett, 1985). Most plants have multiple ADH genes, and commonly have two genes and three isoenzymes derived from random dimerisation (Schwartz, 1973; Tanksley and Jones, 1981; Xie and Wu, 1989; Gregerson et al., 1991). Some plants eg. wheat and barley (Hanson and Brown, 1984) have a third ADH gene and Arabidopsis is the only plant known to have a single ADH gene (Dolferus and Jacobs, 1984). In rice two ADH genes have been reported and three isoenzymic forms are present (Xie and Wu, 1989, 1990). Expression is organ specific and ADH1-ADH1 is found predominantly in leaves whereas the ADH1-ADH2 heterodimer and the ADH2-ADH2 homodimer are present in roots. Levels of the translatable mRNAs of both ADH1 and ADH2 genes are induced by anaerobic conditions and ADH activity increases under anaerobiosis in rice (Ricard et al., 1986; Xie and Wu, 1989). The expression of ADH genes in maize and Arabidopsis is regulated at both transcriptional and post transcriptional levels (Walker et al., 1987; Dolferus et al., 1994b; Fennoy et al., 1998; Hoeren et al., 1998).

PDCs has been found in fungi, higher plants and to a lesser extent in prokaryotes but is absent in animals (for a review see Candy and Duggleby, 1998). All plants studied so far have more than one PDC gene. In rice, the presence of four to five PDC genes has been suggested and PDC1, PDC2 and PDC3 genes have been isolated and
characterised (Hossain et al., 1994a, 1994b, 1996; Huq et al. 1995). A partial clone of PDC4 was also isolated (Rivoal et al., 1997). PDC1, PDC2 and PDC4 are anaerobically inducible but their expression levels are different (Hossain et al., 1996; Rivoal et al., 1997). PDC1 is the most highly induced in shoots and 2d old seedlings of rice whereas PDC4 is the least induced (Hossain et al., 1996; Rivoal et al., 1997). The anoxic induction of PDC1 was found to be three-fold higher than PDC2 after 3h anoxia (Hossain et al., 1996; Rivoal et al., 1997). The rice PDC holoenzyme is a tetramer of α and β subunits having MWt 64 kDa and 62 kDa, respectively (Rivoal et al., 1997).

ADH is present in higher amounts than PDC in rice (John and Greenway, 1976; Rivoal et al., 1989; Ellis and Setter, 1999) and in wheat (Waters et al., 1991) and in normoxic conditions, the level of PDC ranges from very low to undetectable. It is thought that PDC catalyses the rate-limiting step of ethanol fermentation to produce acetaldehyde from pyruvate.

1.8 Aims of this thesis

As plants appear to depend on ethanol fermentation for survival under low O2 conditions, genetic manipulation of the levels of PDC and ADH appears to be a feasible strategy for attempting to improve submergence tolerance. A molecular genetic approach could substantially help in understanding the significance of the ADH and PDC enzymes in anaerobic tolerance. Overproduction of these enzymes by introducing extra copies of their genes might allow a greater capacity for ethanol fermentation under anaerobic stress and enhance survival during submergence. Alternatively, underexpression of these two enzymes by employing an antisense strategy may decrease ethanol fermentation and reduce anoxia tolerance in plants.

Outline of this thesis:

The aim of the work presented in this thesis was to investigate and evaluate the roles of ADH and PDC in submergence tolerance in rice by manipulating their levels by genetic engineering. Additionally, the spatial and temporal distribution of the rice PDCI
and PDC3 promoters and the study of the cis-acting regulatory elements for the anaerobic induction of the rice PDC1 promoter were investigated.

At the start of these studies, constructs for the over- and under-expression of PDC and ADH genes were available. Constructs for the over- and under-expression of PDC and ADH were made by Dr. Anil Grover (Grover et al., 1995) and Dr. Marc H. Ellis, respectively, using the rice ADH1, the cotton ADH2 and the rice PDC1 cDNAs. Constitutive (the rice ACTIN1 and the maize UBIQUITIN1) and anaerobically inducible (6XARE) promoters were used to express these genes in sense or antisense orientations in rice. The rice PDC1 and PDC3 promoter-GUS fusions and constructs of various lengths of the truncated rice PDC1 promoter fused to the GUS reporter gene were also available and were made by Dr. Anil Grover (Grover et al., 1995). The detailed configuration of these constructs as well as all methods and materials used in this research are given in chapter 2.

Results for the manipulation of the levels of ADH enzyme are given in chapter 3. The effects of the over- and under-expression of ADH on ethanol fermentation were determined. The anaerobic survivals of these over- and under-producing ADH transgenic lines relative to the wild type plants (untransformed) and an azygous family (which had not inherited the transgene) of the line were analysed and discussed in terms of their ADH levels.

Chapter 4 includes the analysis of the spatial and temporal expression of the rice PDC1 and PDC3 promoters. The correspondence of the PDC1 and PDC3 genes with PDC1 and PDC3 polypeptides was determined. The investigation of the cis-acting element in the rice PDC1 promoter for anaerobic induction is also described in this chapter.

Chapter 5 describes the manipulation of the levels of PDC. The over-expression traits of PDC and ADH were brought together by crossing the over-expressing lines of PDC1 and ADH2. The effects of these manipulations on ethanol production under
anoxia and the effects of the increased level of PDC alone and both PDC and ADH on anaerobic survival were investigated and discussed.

Chapter 6 focuses mainly on the general discussion of the effects of over- and under-expression of PDC and ADH genes on ethanol production and anaerobic survival. Future research directions for improving submergence tolerance are also outlined in this chapter.
Chapter 2

Materials and Methods
2. MATERIALS AND METHODS

2.1 Plasmids used for rice transformation

The rice ADH1 cDNA clone (Genbank accession number: X16296) was a kind gift of Dr. Ray Wu, Cornell University, U.S.A. (Xie and Wu, 1989). The rice PDC1 (pGEMPDC7Zf(+); accession number: U26660; Hossain et al., 1994a) and PDC3 (pUC19-2.8 kb; accession number: U07338; Hossain et al., 1994b) genomic clones and a PDCJ cDNA clone (pSPORT-PDCJ; accession number: U07339; Hossain et al., 1996) were a kind gift of Dr. Thomas K. Hodges, Purdue University, U.S.A. These were employed for making the constructs shown in figures 2.1 to 2.6. The cotton ADH2 cDNA (Millar et al., 1994; accession number: U49061) was employed to make the pUBI-ADH2 construct. Plasmids of ADH were constructed by Dr. Marc H. Ellis and plasmids of PDC were constructed by Dr. Anil Grover (Grover et al., 1995).

2.1.1 Sense and antisense constructs of the rice ADH1 and PDC1 cDNAs driven by the rice ACTIN1 promoter

The rice ADH1 cDNA used is not a full-length cDNA and lacks 119 bp including the start codon ATG. This partial cDNA of the rice ADH1 was subcloned as an EcoRI fragment from pKr Bluescript SK(-) into pACT1-cassette (derived from pCOR113; McElroy et al., 1991). The rice ADH1 sequence is therefore only used in the reverse (antisense) orientation relative to the rice ACTIN1 promoter (fig. 2.1A) which gave good constitutive expression in rice (McElroy et al., 1991).

The rice PDC1 cDNA sequence (derived from pSPORT-PDC1; Hossain et al. 1994a, 1996) was subcloned as an XbaI fragment from pKr Bluescript SK (-) into pACT1-cassette. The rice PDC1 sequence is in the sense orientation relative to the rice ACTIN1 promoter (fig. 2.1B). pPDC40 (fig. 2.1C) was obtained in the same way as pPDC38 but contains the XbaI fragment of the rice PDC1 cDNA sequence in the antisense orientation.
A. rice ACTIN\textsuperscript{1} promoter rice ADH\textsubscript{1} cDNA (antisense)

B. rice ACTIN\textsuperscript{1} promoter rice PDC\textsubscript{1} cDNA (sense)

C. rice ACTIN\textsuperscript{1} promoter rice PDC\textsubscript{1} cDNA (antisense)

**Figure 2.1**
Maps of plasmids A, pTC6; B, pPDC38; C, pPDC40.

\textit{ACTIN\textsuperscript{1}}: the rice \textit{ACTIN\textsuperscript{1}} promoter and the first intron.

\textit{tNOS}: \textit{nopaline synthase} termination signal.

2.1.2 Sense construct of the cotton \textit{ADH\textsubscript{2}} cDNA driven by the maize \textit{UBIQUITIN\textsuperscript{1}} promoter

An EcoRI fragment containing the cotton \textit{ADH\textsubscript{2}} cDNA was cloned into pCh\textsuperscript{bl} Bluescript cloning vector, then as a Sacl-Kpnl fragment into the corresponding sites of pZLUBI-cassette (a kind gift of Dr. Zhongyi Li) (fig. 2.2). It was found that the maize \textit{UBIQUITIN\textsuperscript{1}} promoter worked better than the rice \textit{ACTIN\textsuperscript{1}} promoter (Dr. Narayana Upadhyaya and Dr. Marie-Francoise Jardinuad, unpublished) and cotton \textit{ADH\textsubscript{2}} cDNA was shown to increase the rate of EF in transgenic cotton (Ellis et al., manuscript in preparation).

**Figure 2.2** Map of plasmid pUBI-AD\textsubscript{m}.

\textit{UBIQUITIN\textsuperscript{1}}: maize \textit{UBIQUITIN\textsuperscript{1}} promoter and the first intron.

\textit{tNOS}: \textit{nopaline synthase} termination signal.
2.1.3 Sense construct of the rice PDC1 cDNA driven by an anaerobically inducible promoter 6XARE

The synthetic promoter 6XARE containing six tandem copies of the ARE region upstream of a truncated maize ADH1 promoter and the first intron of the ADH1 was used to drive the transcription of PDC1.

The maize ADH1 intron-multiple cloning site (mcs)-tNOS was cut as an EcoRI/BamHI fragment from the plasmid pEMU-mcs-tNOS (Last et al., 1991) and was cloned into plasmid 6X464/s 99/92 IGN (Olive et al., 1990), replacing the homologous EcoRI/BamHI fragment of intron-GUS-tNOS. The resulting pTC1 contains the 6XARE promoter fused with the maize ADH1 intron. The rice PDC1 cDNA sequence (derived from pSPORT-PDC1; Hossain et al., 1994a, 1996) was subcloned as an XbaI fragment from pKr Bluescript SK (-) into pTC1 (fig. 2.3). The resulting plasmid (pPDC42) contains the rice PDC1 cDNA sequence in the sense orientation relative to the 6XARE promoter.

![Figure 2.3](image.png)

Map of plasmid pPDC42.

6XARE: six tandem copies of the anaerobic responsive element (ARE) from the maize ADH1 promoter; pADH1: truncated maize ADH1 promoter and first intron; tNOS: nopaline synthase termination signal. The translation start site ATG is shown at +1 in the rice PDC1 cDNA.
2.1.4 The rice *PDC1* and *PDC3* promoter-GUS fusions

An *XhoI*-NcoI fragment representing the promoter region (1201 bp) of *PDC1* and 5’ UTR (122 bp) (Hossain et al., 1994a, 1996) was fused to the *Escherichia coli* β-glucuronidase gene *UIDA* (GUSA) and the *nopaline synthase* 3’ region (tNOS) from pDMC202 (a gift of Dr. David McElroy) to make pPDC55 (translational fusion) (fig. 2.4A). This translational as well as transcriptional (described in section 2.1.5) promoter-GUS fusions were used to study the functions of the *PDC1* promoter and the role of its 5’UTR.

An *EcoRI*-SmaI fragment (1240 bp) from the putative promoter region of *PDC3* including 180 bases downstream from the putative ATG (Hossain et al., 1994b, 1996) was cut from pUC19-2.8 kb and subcloned into pEM3 (a gift of Dr. David McElroy) to yield pPDC25 (translational fusion) (fig. 2.4B).

A.

![Diagram A](image_url)

B.

![Diagram B](image_url)

**Figure 2.4:**
Maps of plasmids A, pPDC55; B, pPDC25. The translation start ATG is numbered +122 in *PDC1*. The rice *PDC1* has two transcription start sites at +1 and at +3 (Hossain et al., 1996). The ATG shown in the rice *PDC3* at position +1 is the putative translation start site since no transcript was detected from this gene and this is the first ATG after TATA box.

**GUSA:** *Escherichia coli* β-glucuronidase gene *UIDA*; tNOS: the *nopaline synthase* termination signal.
2.1.5. 5'-deleted rice \textit{PDCI} promoter-GUS fusions

To identify the cis-acting elements responsible for the anaerobic inducibility of the rice \textit{PDCI} promoter, the full length and a series of 5'-truncated promoter fragments, including a region of +35 bp downstream the first transcription initiation site (Hossain \textit{et al.}, 1996), were fused with the \textit{GUS} reporter gene (fig. 2.5; Grover \textit{et al.}, 1995). In these transcriptional fusions, fragments of \textit{PDCI} promoter were subcloned upstream of an \textit{ACTIN1} 5' intron-GUSA-tNOS fragment (obtained from pDMC205, McElroy \textit{et al.}, 1995) into pKJ km. These plasmids are called pPDC59, pPDC72, pPDC73, pPDC78, and pPDC80 (fig. 2.5).

\textbf{Figure 2.5:}
Maps of plasmids A, pPDC59; B, pPDC72; C, pPDC73; D, pPDC78; E, pPDC80. GUSA: \textit{Escherichia coli} \(\beta\)-glucuronidase gene \textit{UIDA}; tNOS: the nopaline synthase termination signal. The first transcription start site out of two (Hossain \textit{et al.}, 1996) is numbered +1 and the other is at +3 in \textit{PDCI} region.
2.2 Rice transformation

2.2.1 Tissue culture

The details of the culture conditions and rice transformation have been described by Upadhyaya et al. (1996).

Media:

NB solid medium:

<table>
<thead>
<tr>
<th>N6 macro-elements</th>
<th>B5 micro-elements</th>
<th>B5 vitamins</th>
<th>FeEDTA</th>
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<td>mg/L</td>
</tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td></td>
<td>CoCl₂·6H₂O</td>
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</tr>
<tr>
<td></td>
<td>KI</td>
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<td></td>
</tr>
</tbody>
</table>

2 mg/L 2,4-D
30 g/L sucrose
500 mg/L proline
500 mg/L glutamine
300 mg/L casein hydrolysate
2.5 g/L phytagel
pH 5.8-5.85

NBO solid medium:

NB medium
30 mg/L mannitol
30 mg/L sorbitol
Chapter 2/ Materials and methods

NB30 solid medium:

NB medium
30 mg/L hygromycin B (Boehringer Mannheim)

NB50 solid medium:

NB medium
50 mg/L hygromycin B

Preregeneration medium (PRH50):

NB medium without 2,4-D
50 mg/L hygromycin B
2 mg/L benzyl amino purine
1 mg/L naphthalene acetic acid
5 mg/L abscisic acid

Regeneration medium (RNH50):

NB medium without 2,4-D
50 mg/L hygromycin B
3 mg/L benzyl amino purine
0.5 mg/L naphthalene acetic acid

Rooting medium: MS medium (Murashige and Skoog, 1962).

<table>
<thead>
<tr>
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<th>Half strength of MS micro-elements</th>
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</thead>
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</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Half strength of B5 Vitamins
10 g/L sucrose
0.05 mg/L naphthalene acetic acid

2.2.2 Callus induction

Mature seeds of the Japonica rice variety Taipei 309 were dehusked and surface sterilised with 30% bleach for 30 min and washed with sterile distilled water four times. Sterile seeds were placed on NB medium for 5d (fig. 2.6A) and the 5d old embryos were used for callus induction in NB solid medium. The newly budded (1-2 mm size) callus was transferred to fresh NB medium every two weeks. Embryogenic calli at a stage between two to four subcultures were selected for transformation.

2.2.3 DNA delivery

Embryogenic calli (1-2 mm in size) were used for transformation by particle bombardment (Biolistic PDS-1000/He particle delivery system, BIO-RAD). Approximately 120 embryogenic calli were placed close together in a circle 2 cm in diameter at the middle of NBO solid medium (fig. 2.6B), 4h prior to shooting with gold particles coated with DNA according to the manufacturer’s instructions (under 1100-psi He pressure and 26° Hg vacuum).

2.2.4 Selection of transgenic calli and regeneration

For selection of transformants, a plasmid with a hygromycin resistance gene (pTRA151) (Zheng et al., 1991) was used for co-transformation. During tissue culture all selection for the transformed calli and plantlets was done in presence of hygromycin. After shooting, calli were placed in the dark for 16h before being placed separately in NB30 medium in the dark for 2-3 weeks at 27 °C (fig. 2.6C). Hygromycin resistant calli clusters were transferred to NB50 medium for 2-3 weeks (fig. 2.6D) and propagated individually to maintain independent transformed lines. Hygromycin resistant calli were
subcultured in preregeneration medium (PRH50) for 2 weeks under indirect light and at 27 °C (fig. 2.6E). Calli were transferred to the regeneration medium (RNH50) for 16h light (130 μmol m⁻² s⁻¹) at 27 °C (fig. 2.6F). The regenerated plantlets were then transferred to rooting medium (fig. 2.6G). Plants were then transferred to a glass house with 28 °C day and 20 °C night temperatures (figs 2.6H and 2.6I).
Figure 2.6: Oryza sativa cv. Taipei 309 transformation by particle bombardment. A, 5d old embryos before excision for callus induction; B, regenerable calli in the osmotic plate just before shooting; C, calli in 30 mg/L hygromycin after shooting; D, calli transferred to 50 mg/L hygromycin 3 weeks after shooting; E, calli in the preregeneration medium with 50 mg/L hygromycin; F, regenerating plants in regeneration medium with 50 mg/L hygromycin; G, plants in rooting medium; H, T₀ plants in glass house; I, T₁ plants in glass house.
2.3 Screening of transgenic plants for the presence of the inserted gene

Screening of the transformed plants and their progeny for the presence of the inserted gene was done by Southern blotting, polymerase chain reaction (PCR) and GUS histochemical staining.

2.3.1 Southern blotting analysis

Genomic DNA was isolated from plants according to Drapper and Scott (1988) and using a modified Puragene DNA isolation protocol (N. Upadhyaya, K. Ramm and J. Gaudron, unpublished). 5-10 µg of genomic DNA was digested with restriction endonuclease, fractionated by electrophoresis on an 0.8% agarose gel and transferred to nylon membrane (Hybond N*, Amersham) according to the manufacturer’s instructions. A random priming protocol was used to label probe (³²P) (Feinberg and Vogelstein, 1983). Probe hybridisation and membrane washing were performed according to Dolferus et al. (1994a). The blot was exposed to X-ray film at -70 °C for 7d before being developed. Phosphor screens (Molecular Dynamics, Sunnyvale, CA) were also used to visualise bands.

2.3.2 Polymerase chain reaction (PCR)

Primers for the rice PDC1 and ADH1 cDNAs were designed from their published sequences (Xie and Wu, 1989; Hossain et al., 1994a, 1996), using the PRIME program of GCG (Devereux et al., 1984).

\textit{ADH1} cDNA (accession number: X16296) primers:

Forward primer (112—131): 5’-CAAGATCTTCCAGACCTC-3’
Reverse primer (429—410): 5’-CCGATGAAATGGTAAATGGG -3’
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**PDC1** cDNA (accession number: U07339) primers:

- Forward primer (215—233): 5'-'TGCTCGACTACCTCATCGC -3'
- Reverse primer (536—517): 5'-'AGGTTGTTAATGACGGCCTG -3'

**Sucrose synthase I** primers (Upadhyaya et al., 2000):

- Forward primer: MNSS1_F (58-76) (5'-'TGCCCTTGATCGAAGCTGAC -3')
- Reverse primer: MNSS1_R(604—585)
  (5'-'AGCAAGGGGTAGAGGCTC -3')

**GUS** primers:

- Forward primer (1218-1240): 5'-'AAAACCTCGTACAATTTCAGCTAG-3'
- Reverse primer (1726-1710): 5'-'TTGTACCGCGCTTTCCC-3'

**Hygromycin** primers (Upadhyaya et al., 2000):

- Forward primer: MN35S_3 (5'-'GGGATGACGCACAATCCC-3')
- Reverse primer: MNHPH5_180(5'-'GATCTTTGTAGAAACCATCGGC-3').

### 2.4 Analysis of transgenic plants

#### 2.4.1 Seed germination and hydroponic growth of seedlings

Seeds were germinated in the dark at 27 °C for 2d in a Petri dish on sterile filter paper soaked with sterile distilled water. Another piece of filter paper soaked with water was also placed on the top to prevent loss of moisture. The age of seedlings was calculated from the day of soaking. After 2d, the top filter papers were removed and germinated seeds were placed in 16h light (130 μmol m⁻² s⁻¹ PAR) at 27 °C for another 3d in enough water to submerge the roots but not the shoot. Plants were transferred to a glass house with 28 °C day and 20 °C night temperatures and placed onto Styrofoam seedbeds which were floated on nutrient solution (Hewitt, 1966). Ten day old plants
with a uniform appearance were placed between two bars with a tissue paper cushion on both without crushing the culm and were grown in nutrient solution (Ellis and Setter, 1999).

2.4.2 Anaerobic treatments of seedlings

The aquarium for anaerobic treatment of rice plants (350 mm (L) x 240 mm (W) x 300 mm (H); Ellis and Setter 1999) was filled with nutrient solution, equilibrated to glass house temperature and bars holding the plants were placed inside the aquarium. Aquariums were placed in the dark to prevent photosynthesis and during the onset of the anaerobic treatment, gas was flushed at a rate of 600 ml/min for 40 min followed by 300 ml/min until the treatment ended. Either 5% oxygen (in nitrogen) gas mixture (hypoxia) or nitrogen gas (anoxic stress) was used. Aerated samples were grown and treated the same way except for submergence and gassing treatments.

Alternatively, one-week old seedlings grown in a Petri dish were stressed inside an anaerobic jar at 27 ℃ with continuous gassing with either pure nitrogen or 5% oxygen (in nitrogen) for 30 min and sealed to avoid contamination from atmospheric oxygen. Aerated samples were kept in the dark at 27 ℃.

Treatment was ended by quickly freezing the samples in liquid nitrogen and samples were stored at −70 ℃.

2.4.3 Analysis of ADH transgenic plants

2.4.3.1 Protein extraction

Extraction buffer: 100 mM Tris-HCl pH 8.0

(Xie and Wu, 1989) 25% glycerol (v/v)

0.8% β-mercaptoethanol (v/v)

2% soluble polyvinylpyrrolidone (w/v)

5 mM DTT
Protocol:

Plant samples were ground with liquid N$_2$ and quickly transferred to eppendorf tubes containing extraction buffer as above (Xie and Wu, 1989) and mixed in a horizontal shaker for 15 min at 4 °C. Samples were centrifuged at 4 °C and the supernatant was recentrifuged if there was any debris present. Clear supernatant was used for ADH assays by spectrophotometry, native-polyacrylamide gel electrophoresis (PAGE) and starch gel electrophoresis.

2.4.3.2 ADH enzyme assay

ADH assay mixture: 47.3 mM TES buffer pH 7.0
- 0.21 mM NADH
- ADH crude extract (no more than 100 μl)
- 10.2 mM acetaldehyde

ADH enzyme activity was measured in crude extracts (Ellis and Setter, 1999) from the reduction of acetaldehyde to ethanol coupled with the concomitant oxidation of NADH to NAD$^+$ (fig. 2.9). The rate of oxidation of NADH was measured by spectrophotometer (GBC UV/VIS 920) at 340 nm from the slope of the conversion of NADH to NAD$^+$ after addition of the acetaldehyde substrate. The rate of conversion of NADH to NAD$^+$ corresponds to the rate of ADH activity. Some oxidases are also responsible for this conversion. Therefore, the net rate is calculated by subtracting the rate before addition of substrate from the rate after addition of substrate (fig. 2.7) (Ellis and Setter, 1999).
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Figure 2.7: Determination of the net rate (b-a) of ADH activity by spectrophotometry. (a), rate before addition of substrate; (b), rate after addition of substrate.

The absorption coefficient utilized for NADH was 6.22 mM$^{-1}$ cm$^{-1}$ and one unit of ADH activity was defined as the amount of enzyme required to produce 1 nmol product per min per mg protein and is calculated by using the formula:

$$\text{Net rate} \times \frac{1000}{6.22 \times \text{mg protein}}$$

The protein concentration of the crude extract was determined according to the Bradford Method (Bradford, 1976), using bovine serum albumin as a standard and a Lab system Multiskan spectrophotometer (Pathtech).

2.4.3.3 Starch isoenzyme gel

DL-histidine buffer (4.58 mM) of pH 8.0 was used for 11% horizontal starch gels [15 cm (W) x 24 cm (L)] prepared from potato starch (Sigma S-4501). Crude extracts were absorbed by paper wicks (SERVA 42987) measuring 6 (L) x 5 (W) x 1 (H) mm that were inserted into the gel (20 wicks per gel). Electrophoresis was carried out at 4°C in 0.4 M tri-sodium citrate buffer pH 8.0. A constant potential of 120V was applied for 30 min before wick removal and a constant potential of 150V was continued for 5h after wick removal. The gel was sliced with fishing line and one slice of gel was stained.
after wick removal. The gel was sliced with fishing line and one slice of gel was stained specifically for ADH activity overnight in the dark (Hanson et al., 1984). The middle slice of the gel was activity-stained with a GDH-specific staining overnight in the dark and served as a positive control. After staining, gels were removed from reaction mixture, soaked in distilled water overnight and photographed.

**Solutions:**

**ADH specific staining:**

0.2 M tris-HCl buffer pH 8.0  
1% absolute ethanol  
0.02% NBT  
0.02% β-NAD⁺  
0.005% PMS  

**Glutamate dehydrogenase (GDH) specific staining:**

0.2 M tris-HCl buffer pH 8.0  
1% lactate (lithium salt)  
0.02% NBT  
0.02% β-NAD⁺  
0.005% PMS

**2.4.3.4 Native polyacrylamide gel electrophoresis (PAGE)**

Native PAGE was performed in the same way as SDS-PAGE described in section 2.4.4.2 except for the addition of SDS. The gels were stained overnight for ADH-specific staining except when otherwise stated.
2.4.4 Analysis of PDC transgenic plants

2.4.4.1 Protein Extraction

**Extraction buffer:**
- 50 mM MES buffer pH 6.25
- 50 mM KCl
- 1 mM Na₂EDTA;
- 2 mM MgCl₂
- 3 mM DTT
- 5 mM TPP

**Protocol:**

Plant tissue was ground in liquid nitrogen. Extraction buffer was added quickly in a ratio of no more than 1 ml buffer/g tissue of shoot and leaf blade and 0.5 ml buffer/g tissue of root and leaf sheath. The extracts were vortexed and then mixed in a horizontal shaker for 15 min at 4 °C. Samples were centrifuged at 4 °C and supernatants were re-centrifuged if there was any debris present. Clear supernatants were used for spectrophotometric PDC assays and Western blotting.

Protein was extracted from endosperm according to Burkhardt *et al.* (1997) as follows: the embryo portion (1/3 of the seed) was discarded from the dry mature seed and the remaining endosperm was ground dry in the mortar and pestle. The powder was transferred to an eppendorf tube and addition of 100 μl distilled H₂O was followed by 10 min shaking at 4 °C. 27% ethanol was added to precipitate carbohydrates. After a 15 min spin at 13,000 rpm and at 4 °C, the clear supernatant was dried in a Speedvac. The protein was then dissolved in 16 μl distilled H₂O and used for Western blotting analysis.

To harvest protein from pollen, five panicles were cut into small fragments with sharp scissors into extraction buffer described above and stirred for 30 min at room temperature (RT). The mixture was strained successively through a coarse nylon mesh
undisturbed to settle at RT. The supernatant was removed and the pollen was ground in the remaining extraction buffer (1 vol. extraction buffer/1 vol. pollen) using a glass pestle (suitable for eppendorf tubes) attached to a drill. This suspension was then centrifuged for 15 min at high speed at 4 °C and the clear supernatant was used for immuno-blotting analysis.

Rice spikelets, anthers and pollen were collected from panicles at different developmental stages for protein extraction. The differentiation and development of the rice panicle is correlated fairly precisely with external development (Hoshikawa, 1989). The stages are defined according to the distance between the auricles of the last two leaves (the penultimate leaf and the flag leaf) of rice (fig. 2.8). The assumption about the nuclear stages of the pollen made here is based on studies from another japonica rice variety, *Oryza sativa* L. japonica variety “Nihonmasari” (Tamaru, 1991). The distance

![Diagram of rice panicle](image)

**Figure 2.8:** Developmental stages of rice panicles (adapted from Tamaru, 1991).

is denoted as negative when the auricle of the flag leaf is below that of the penultimate leaf, and positive when the auricle of the flag leaf is above that of the penultimate leaf. The distance is considered zero when the auricle of both leaves is at the same height. When the distance between auricles of the last two leaves is near -5 cm, meiosis of the
When the distance between auricles of the last two leaves is near –5 cm, meiosis of the pollen mother cell starts. Microspores are mainly in the unicleate stage when the distance is +6 cm. At +8 cm, most of the microspores are in the dinucleate stage. At +11 cm, both dinucleate and trinucleate microspores are present, with trinucleate microspores being more prevalent. The panicle starts to appear outside the leaf sheath when the distance is +13 cm, and at +15 cm, spikelets start anthesis.

2.4.4.2 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Solutions:

**Separating gel:**

- 8% w/v acrylamide (with a 37:1 ratio of acrylamide:bisacrylamide)
- 370 mM tris-HCl buffer pH 8.8
- 0.1% (w/v) SDS
- 0.08% (w/v) ammonium persulfate
- 0.08% (v/v) TEMED

**Stacking gel:**

- 3.9% w/v acrylamide (with a 37:1 ratio of acrylamide:bisacrylamide)
- 115 mM tris-HCl pH buffer 6.8
- 0.09% (w/v) SDS
- 0.12% (w/v) ammonium persulfate
- 0.13% (v/v) TEMED

**Electrophoresis buffer:**

- 25 mM tris-HCl buffer pH 8.4
- 1.44% (w/v) glycine
- 0.01% (w/v) SDS
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Protocol:

Protein extraction was described in section 2.4.4.1. Eight percent w/v separating and 3.9% stacking gels were prepared and electrophoresis was carried out at 15 mA current per gel for 4h according to Laemmli (1970) to separate PDC polypeptides. Spacers of 1.5 mm and Mighty Small II SE250/SE260 gel apparatus from Hoefer Scientific instruments were used. Thirty microgram of total protein extracted from embryos, endosperm, spikelets, anthers and pollen, 50 µg protein from leaf sheath, shoot and root, and 150 µg protein from leaf blades were loaded per lane for SDS-PAGE.

2.4.4.3 Immuno-detection of PDC polypeptides

Solutions:

Transfer buffer:

25 mM tris-HCl buffer pH 8.4  
1.44% (w/v) glycine  
20% (v/v) methanol

TBS-Tween:

50 mM tris buffer pH 8.0  
0.15 M NaCl  
2% (V/V) Tween

Blocking agent:

5% skim milk (Diploma) in TBS-Tween

Protocol:

A polyclonal antibody (a kind gift of Drs Danny Llewellyn and Marc Ellis) from rabbit was raised against the rice PDC1 that was purified from recombinant bacteria.
expressing pGEX-\textit{PDC}1 (a kind gift of Dr. Thomas K. Hodges, Purdue University, U.S.A.). Rabbit PDC1 antiserum was purified before use as described by Harlow and Lane (1988).

Proteins were transferred electrophoretically from the gel to a nitrocellulose membrane overnight at 4 °C using 50 mA current and tris-glycine transfer buffer pH 8.4 (Towbin \textit{et al.}, 1979). The filter was blocked with 5% non-fat milk in TBS-Tween for 1h at room temperature. After incubation with rabbit anti-PDC1 polyclonal antiserum at 1:10,000 dilution for 4h, filters were washed and incubated with 1:3000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (BIORAD 170-6518) for 2h. Immuno-detection was performed with NBT and NCIB (Promega S380 and S381) according to the manufacturer’s instructions. The protein molecular weight marker of SeeBlue™ Pre-Stained standards from Novel Experimental Technology (cat. no. LC5625) was used.

\subsection{2.4.4.4 PDC enzyme assay}

PDC assay mixture:

\begin{itemize}
\item 57.5 mM MES buffer pH 6.25
\item 0.21 mM NADH
\item 1.14 mM MgCl$_2$
\item 0.5 mM TPP
\item 50 mM sodium oxamate
\item 0.3 \textmu g/ml ADH (Sigma, activity of 100 \textmu mol/min acetaldehyde to ethanol)
\item PDC crude extract (no more than 100 \textmu l)
\item 7.0 mM sodium pyruvate as a substrate
\end{itemize}

PDC enzyme activity was measured in a reaction of the conversion of pyruvate to acetaldehyde coupled with the reduction of acetaldehyde to ethanol by ADH (Ellis and Setter, 1999) (fig. 2.9). The coupled oxidation of NADH to NAD$^+$ was measured at 340
nm and 25 °C and corresponds to the rate of PDC activity.

![Chemical Reaction](image.png)

**Figure 2.9:** Reactions catalysed by PDC and ADH.

The crude extracts were incubated for 30 min at 25 °C, as this was found to increase the activity of the extract. The net rate of PDC was calculated as per section 2.4.3.2 and the specific activity of PDC was expressed as nmol/min/mg protein.

### 2.4.5 GUS histochemical staining

Two day old germinated seeds were cut in half longitudinally using a razor blade and were stained with X-gluc solution for 24h except where otherwise stated (Jefferson *et al.*, 1987; Kyozuka *et al.*, 1994) to study GUS expression in the embryo, scutellum and endosperm. Eighty percent ethanol was used to remove chlorophyll from green tissues. Transverse sections of 8d and three week old shoots and roots were analysed to study GUS expression in the vascular region. The laema and the pallea from the spikelet were removed to stain the flower. Spikelets were collected in three stages of maturation stated below:

(a) When the panicle was still inside the leaf sheath and the distance between auricles of the last two leaves (the flag leaf and the penultimate leaf) was 11 cm (ref. fig. 2.8). At that time, the spikelet was closed; anthers were intact and reached less than half the length of the spikelet.

(b) Prior to anthesis when the spikelet was still closed; anthers covered half the length of flower and were intact and the pollen was inside anthers.

(c) During anthesis when spikelet opened and contained splitted anthers; anthers reached the length of palea and pollen was found everywhere inside flower, such as on
Pollen was collected from panicles just prior to anthesis as described in the protein extraction section (2.4.4.1) and germinated in vitro for 24h at room temperature in a sterile medium of 15% sucrose, 0.1% CaCl₂ and 0.01% H₃BO₃ (Hagimori et al., 1992). After this time interval, medium was completely removed and GUS staining was performed (Kyozuka et al., 1994).

2.4.6 Seed germination under anoxia

A 500 ml conical flask containing rice seeds was filled with 250 ml of 0.5 mM CaSO₄ in tap water, flushed with nitrogen gas and continuously agitated for 5d in the dark. Another flask containing seeds was flushed with air and served as a control. The lengths of roots and shoots were measured and seeds and seedlings were transferred to 16h light (130 μmol m⁻² s⁻¹) at 27 °C for 4d in air and were then grown in a glass house with 28 °C day and 20 °C night temperatures.

2.4.7 Assay of acetaldehyde and ethanol production

Plants of 26d old were treated hypoxically (5% O₂ in N₂) inside an anaerobic jar for 16h. The root or leaf sheath was dissected and cut into three pieces of approximately equal length. These were weighed quickly then placed inside a 10 ml syringe (approximately 0.5 g tissue per syringe) containing 10 ml of sterile medium (0.1 M glucose, 0.5 M CaSO₄, pH 6.5 in tap water) which was flushed prior to use for 10 min with argon gas to exclude O₂. The plunger was inserted and a rubber septum was fitted to the needle of the syringe. Samples were incubated at 27 °C with continuous horizontal shaking on a rotor. One millilitre samples were collected over various time intervals and ethanol and acetaldehyde contents were analysed by gas chromatography on a Varian 3700 GC equipped with a Varian 800 series autosampler and a flame ionisation detector. The column was a 2 m x 2.16 mm ID stainless steel column packed with Porapak QS. GC conditions were: Injector T, 170 °C; Detector T, 250 °C with the
column programmed from 110 °C to 130 °C; nitrogen carrier gas flow, 30 ml/min. Calibration was carried out using a standard solution of acetaldehyde and ethanol in water with isopropanol added as an internal standard. Data collection and processing were carried out using a Varian Star Chromatographic Workstation.

2.4.8 Assay for anaerobic tolerance

Plants were grown for 26d and then transferred to an aquarium filled with nutrient solution as described in sections 2.4.1 and 2.4.2. Fifteen to 18 plants with a uniform appearance were selected and placed between a set of two bars. Each aquarium accommodated 30 to 40 plants from each variety of rice tested. Three aquariums were connected in parallel and attached to the same gas cylinder.

Plants were stressed with approximately 24h hypoxic pretreatment followed by 66h anoxia inside the aquarium in a dark room as described in section 2.4.2, and transferred to glass house after the treatment. The anaerobic treatment was performed in complete darkness to eliminate evolution of O₂ from photosynthesis under water. The anaerobic survival assay conditions were maintained strictly since reports showed that submergence tolerance is highly related to the age of the rice plants (Adkins et al., 1990; Mallik et al., 1995; Chaturvedi et al., 1995) and carbohydrate content (see Setter et al., 1997 for a review). Scoring of live and dead plants was performed after a 10d recovery period.
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3. MANIPULATION OF THE LEVEL OF ALCOHOL DEHYDROGENASE IN RICE AND EVALUATION OF ITS ROLE IN ANAEROBIC TOLERANCE

3.1 Introduction

Rice alcohol dehydrogenase (ADH; alcohol: NAD⁺ oxidoreductase, EC 1.1.1.1), is a cytosolic enzyme encoded by two genes (Ricard et al., 1986) both of which have been cloned (Xie and Wu, 1989, 1990). Like the maize ADH enzyme, rice ADH is active only as a dimer and exists as three isoenzymes. ADH1-ADH1 is the major cathodic migrating homodimer, ADH2-ADH2 is the weaker anodic migrating homodimer and the ADH1-ADH2 heterodimer migrates in the middle of these two homodimers (Xie and Wu, 1989).

The rice ADH genes are expressed in different developmental and organ-specific patterns. In 3-week old seedlings, the ADH2-ADH2 homodimer is present predominantly in roots, whereas the ADH1-ADH1 homodimeric isoform is present predominantly in leaf blades, leaf sheaths and pollen (Xie and Wu, 1989). The rice ADH genes are expressed constitutively in seeds and ADH activity has been found at all stages of seed development, from the grain filling stage to the mature seed (Xie and Wu, 1989). Although all three isoforms are present in the grain filling stage, the ADH1-ADH1 isoenzyme becomes predominant towards seed maturation (Xie and Wu, 1989). ADH1-ADH1 is present predominantly in embryos of dry seeds and seeds germinated in aerobic conditions. The levels of ADH1-ADH2 and ADH2-ADH2 increase in embryos germinated under anaerobic conditions (Ricard et al., 1986). ADH activity is rapidly induced in various organs of rice in response to O₂ deprivation and the increase of ADH activity is accompanied by the synthesis of two ADH polypeptides (Ricard et al., 1986; Kadowaki et al., 1988; Xie and Wu, 1989). The anaerobic induction of ADH activity has been found in roots, embryos, leaves and endosperm of rice (Xie and Wu,
1989). ADH activity is induced in response to anaerobiosis even in mature rice leaves (Xie and Wu, 1989) unlike in maize where ADH shows no induction in mature leaves (Okimoto et al., 1980).

ADH plays a major physiological role during anaerobic stress when a plant shifts its energy metabolism from the oxidative to the fermentative mode (Davies, 1980). ADH catalyses the conversion of acetaldehyde to ethanol and uses NADH as a cofactor. It is the terminal enzyme in the ethanol fermentative pathway that operates in the absence of oxygen and yields 2 molecules of ATP per molecule of glucose. Plants mainly carry out ethanol fermentation during anaerobiosis (Smith and Ap Rees, 1979; Alpi and Beevers, 1983); however, they differ greatly in their ability to tolerate such conditions. The demonstration of the importance of ADH for anoxia tolerance came from the reduced anaerobic tolerance of the *adh* null mutants of maize (Schwartz, 1969; Freeling and Bennett, 1985; Johnson *et al.*, 1994), barley (Harberd and Edwards, 1982), *Arabidopsis* (Jacobs *et al.*, 1988; Ellis *et al.*, 1999) and rice (Matsumura *et al.*, 1995, 1998). These results reinforce the view that ADH enzyme activity is important for the survival of plants and raise the hope that over-production of ADH might increase anoxia tolerance in plants.

In wheat, the increased ability of hypoxically pretreated (HPT) plants to tolerate subsequent anoxia is associated with higher activities of pyruvate decarboxylase (PDC) and ADH (Waters *et al.*, 1991). Similar responses to HPT and the augmentation of PDC and ADH activities have been found in roots of maize seedlings (Johnson *et al.*, 1994). Ricard *et al.* (1986) found that the levels of ADH1-ADH2 and ADH2-ADH2 increase in rice embryos after an anaerobic treatment of at least 6h. Bucher and Kuhlemeier (1993) also showed a marked difference in ADH isoenzyme pattern in *Acorus calamus*, a monocotyledonous wetland plant that can survive extremely long periods of anoxia, when plants were subjected to two months anoxia compared to control plants grown aerobically.

ADH activity was greatly increased by submergence in four varieties of 10d old rice seedlings; FR13A, the most tolerant, had the highest increase of ADH activity and
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IR42, the least tolerant, had the lowest increase in ADH activity under anaerobic treatment (Setter, 1992). Ellis and Setter (1999) also demonstrated a positive correlation between the level of ADH activity during anaerobiosis and anoxia tolerance. One submergence tolerant inbred variety, IR49830 (Mackill et al., 1993), showed high basal ADH activity and tolerated direct exposure to anoxia, whereas IR36 and IR42, having low ADH activity, did not tolerate anoxia (Ellis and Setter, 1999). However, the basal ADH activity of the submergence tolerant variety FR13A was lower than that of the two flood intolerant varieties IR36 and IR42 (Ellis and Setter, 1999). In contrast, maize lines with ADH activities differing over about a 200-fold range failed to demonstrate a positive correlation between the increased level of ADH activity and increased anaerobic tolerance (Roberts et al., 1989).

The aim of this chapter was to generate transgenic rice with altered levels of ADH, examine whether the rate of ethanol fermentation changed and to see if there was any correlation with respect to tolerance of anaerobic conditions. An antisense strategy was adopted to down-regulate ADH levels by using the rice $ADH1$ cDNA driven by the rice $ACTIN1$ promoter, and a sense strategy was used to over-produce ADH by using the cotton $ADH2$ cDNA driven by the maize $UBIQUITIN1$ promoter.

3.2 Results

3.2.1 Temporal expression pattern of ADH isoenzymes in various tissues of untransformed rice

The temporal expression pattern of ADH isoenzymes in rice was studied by isoenzyme gels. The intensities of bands of all three isoenzymes were similarly high in roots of one-week old seedlings (figs 3.1 and 3.2A). The intensities of the ADH1-ADH1 and ADH1-ADH2 bands decreased with age and the intensity of the ADH2-ADH2 band remained the same until the plants were at least 3 weeks old (fig. 3.2A). The ADH1-ADH1 band was predominant in shoots of rice and its intensity also decreased with age (figs 3.1 and 3.2B). Therefore, ADH isoenzymes were highly expressed in shoots and roots of younger plants compared to those of older plants. In 2d old germinating rice
endosperm all three isoforms were present, but the ADH1-ADH1 isoenzyme was predominant (Fig. 3.1).

---

**Figure 3.1:** Starch isoenzyme gel to study the distribution of ADH isoenzymes in 2d old germinating endosperm and 7d old roots and shoots of rice. The gel was stained specifically for ADH activity following electrophoresis. Different wells may not contain the same amount of protein.
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3.2 B

Figure 3.2: Effects of development on distribution of ADH isoenzymes in A, roots (starch isoenzyme gel; different wells may not contain the same amount of protein) and B, shoots of rice (25 μg protein was loaded per lane in the native PAGE gel). Both gels were stained for ADH specific activity following electrophoresis.

3.2.2 Antisense ACTI-ADH1 transformants

3.2.2.1 Detection of the antisense ACTI-ADH1 transformants and study of the expression of the transgene in transformants

A plasmid (pTC6 in section 2.1.1) containing the rice ADH1 cDNA fused in the reverse orientation to the promoter of the rice ACTIN1 gene was used to transform rice. Five independent transgenic lines were selected for hygromycin tolerance from tissue culture after co-transforming the antisense ACTI-ADH1 construct with a construct pTRA151 which confers hygromycin resistance (section 2.2). Three transgenic lines AA101, AA108 and AA121 contained the transgene as determined by PCR. Primers (section 2.3.2) amplified the region from 112 to 429 bases of the rice ADH1 cDNA, producing a 318 bp fragment (fig. 3.3). As a control for the PCR, primers for the amplification of the rice sucrose synthase 1 (SS-1) gene were synthesised (section 2.3.2) and ADH1 and SS-1 genes were amplified in parallel. The size of the SS-1 PCR product is 547 bp. When ADH1 PCR products were Southern blotted, a 318 bp fragment hybridised with the ADH1 cDNA probe (data not shown).
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Figure 3.3: Screening of the antisense ACT1-ADH1 T₀ transformants by PCR for the ADH1 gene. Each sample of plant DNA was PCR-amplified in duplicate or triplicate.

Lane a: SSP-1 DNA/EcoRI fragments (Bresatec).
   b: plasmid pTC6 control
   c: line AA101
   d: line AA108
   e: line AA121
   f: lines without transgene ADH1
   g: WT (untransformed Taipei) control

Expression of the transgene in the antisense ACT1-ADH1 transformants was detected by comparing ADH enzyme levels in transgenic (T₁ generation) and WT (untransformed) plants. The levels of ADH activity in lines AA101, AA121 and WT ranged from 704 to 793 units (nmol/min/mg protein) in shoots and from 353 to 398 units in roots (fig. 3.4). Additional T₁ plants of lines AA101 and AA121 were tested for ADH activity to confirm that they had a similar level of ADH activity as WT plants (data not shown). In line AA108, some T₁ plants had similar levels of ADH as the WT; presumably these did not inherit the transgene. Other plants had greatly reduced levels of ADH activity.
Figure 3.4: *In vitro* ADH activity of individual plants measured spectrophotometrically in extracts from 8d old aerated shoots of segregating T₁ plants of three independently transformed antisense *ACTI-ADH* lines AA101, AA108, and AA121 each carrying the transgene, and of untransformed Taipei as a WT control. ADH activity was also estimated in roots from the same plants used for the ADH assay of shoots. Three spectrophotometric measurements were taken using the same extract and specific activities were calculated. Standard errors were calculated from values of specific activities.

In order to test the effect of the antisense *ADH* expression in floral tissues, anther and pollen tissues of line AA108 and WT plants were analysed on ADH isoenzyme gels (fig. 3.5). Two heterozygous families AA108-7 and AA108-11 (segregating with respect to ADH activity) and one azygous family AA108-17 (which had a similar level of ADH as WT), were analysed. In WT plants, a prominent band was observed in the anther extract. This band co-migrated with the band found in
untransformed shoots, suggesting that it is the ADH1-ADH1 homodimer. A fainter band migrating at the same position was also observed in pollen. The azygous family AA108-17 had an ADH1-ADH1 band with a similar intensity as WT in anthers and pollen. In AA108-7 and AA108-11 plants, the intensity of the band in anthers was greatly reduced and no band was observed in pollen. These results suggest that antisense ADH expression led to decreased ADH activity in floral tissues.

Figure 3.5: ADH activity-stained native PAGE gel analysing ADH levels in anthers and pollen of antisense ADH1 line AA108 and WT plants. Total protein extracts were fractionated by electrophoresis and the gel was stained for several hours specifically for ADH activity.

- AA108-17: an azygous family of line AA108 which had the same level of ADH activity as WT.
- WT: untransformed Taipei.
- WT shoot: 60 µg protein of 7d old shoot of WT was included as a control.

3.2.2.2 Detection of homozygous and azygous families of the antisense ACT1-ADH1 line AA108

The determination of the homozygous and azygous families was done by assaying the expression of the transgene rather than by the mere presence of the transgene itself. The effect of the expression of the transgene antisense ADH1 was monitored by in vitro
spectrophotometric enzyme assays, native-PAGE and starch isoenzyme gel analysis. Twelve T₁ plants were grown in the glass house and seeds were harvested separately from each plant. Thirteen T₂ progeny from 4 T₁ plants (AA108-7, AA108-11, AA108-12, and AA108-17) and WT plants were analysed for ADH activity using starch isoenzyme gel electrophoresis (fig. 3.6). Two putative homozygous families (AA108-7 and AA108-11) were identified in line AA108. All 13 samples of these two families showed reduced ADH activity as evident from the faint bands in gels compared to those of wild type controls, and were considered homozygous. However, later ADH assays in additional plants revealed that some plants of these families had the same level of ADH as WT plants, suggesting that they were still segregating. This error occurred due to the analysis of too few plants.

In line AA108, one family AA108-17 had ADH activity the same as WT and was confirmed as an azygous family by assaying additional samples from the same seed lot.

![Figure 3.6: Starch isoenzyme gel assaying four families AA108-7, AA108-11, AA108-12 and AA108-17 of line AA108 for the determination of homozygous and azygous families. Total protein extracts from 16d old aerated shoots were fractionated by electrophoresis under non-denaturing conditions. Afterward, two slices of the same gel were stained specifically for ADH and glutamate dehydrogenase (GDH) activity as a control.](image-url)
3.2.2.3 Measurement of ADH activity in the antisense \textit{ADH} line AA108 in air and under hypoxia

ADH activity was compared between WT and line AA108 (T\textsubscript{1} plants). The levels of mean ADH activity were 704 units (nmol/min/mg protein) and 40 units in air (fig. 3.4) and 1499 units and 123 units under hypoxia (fig. 3.7) in one week old shoots of AA108 and WT, respectively. The ADH activities in shoots of one week old plants of AA108 were 6\% that of WT in air and 8\% that of WT under hypoxia. Again, the effect of the expression of the antisense \textit{ADH} was to reduce the level of ADH in air and under hypoxia.

![Bar chart showing ADH activity under Hypoxia and WT](image)

Figure 3.7: \textit{In vitro} ADH activity measured spectrophotometrically in shoots of 6d old individual T\textsubscript{1} seedlings (segregating) of line AA108 and WT treated under 16h hypoxia (5\% O\textsubscript{2} in N\textsubscript{2}). Three spectrophotometric measurements were taken using the same extract and specific activities were calculated. Standard errors were calculated from values of specific activities.
3.2.2.4 Comparison of the levels of ADH1 and ADH2 polypeptides in the antisense ACT1-ADH1 transformants (AA108) and WT plants

In order to investigate the effect of the antisense ADH1 on the level of the endogenous ADH1 and ADH2 polypeptides, total protein was extracted from one-week old shoots and roots of AA108 (segregating population) and WT plants and fractionated by ADH starch isoenzyme gel electrophoresis (fig. 3.8). The intensity of the ADH1-ADH1 band was strong in shoots of WT and of three plants (numbers 2, 4, and 5) of AA108. ADH1-ADH1, ADH1-ADH2, and ADH2-ADH2 isoenzymes were also present in similar intensities in roots of these plants. However, in two plants (numbers 1 and 3) of AA108, the ADH1-ADH1 isoenzyme in shoots and all three isoforms in roots were absent under the assay conditions suggesting that suppression of ADH1 as well as ADH2 had occurred in AA108 plants.

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**Figure 3.8:** ADH activity-stained starch isoenzyme gel analysing the levels of ADH1 and ADH2 in the antisense ADH1 line AA108 and WT. Total protein was extracted from roots and shoots of individual 8d old air grown seedlings and fractionated by electrophoresis.
- WT: untransformed Taipei.
- AA108 (T1): five seedlings in T1 generation (segregating population) of the antisense ACT1-ADH1 line AA108.
3.2.2.5 Effect of the reduction of ADH level on ethanol production in the antisense ACT1-ADHI line AA108

Ethanol production in excised roots from hypoxically treated plants of WT and two heterozygous families (AA108-7 and AA108-11) of the antisense ACT1-ADHI line was compared. WT plants and one azygous family (AA108-17) of line AA108 which was shown previously to have a similar level of ADH activity as WT were included as negative controls. ADH activity level was not determined in the T3 plants of AA108-7 and AA108-11 used for the ethanol measurement assay. These two families were segregating and hence, the level of ethanol produced might be either from plants having lower ADH activity compared to WT plants or could be an average of some plants having lower ADH activity and some similar to WT plants. Roots of WT plants produced 138 nmol ethanol/g FWt after 6h (fig. 3.9). There were no significant differences in ethanol content between the azygous family AA108-17 and WT. However, a significant reduction in ethanol production was observed in the two heterozygous families AA108-7 and AA108-11, compared to the wild type and the negative control AA108-17. Family AA108-7 produced only 49 nmol ethanol/g FWt after 6h, only 36% of that produced by the WT and the azygous family. These results suggest that decreasing ADH expression decreases ethanol production. Leaf sheaths of AA108-7 and AA108-11 also showed reduced level of ethanol production compared to azygous family AA108-17 and WT leaf sheaths (data not included).
Figure 3.9: Ethanol production in excised roots of 26d old plants of the antisense ADH1 transformants, WT (untransformed Taipei), and a family (AA108-17) which had a similar ADH level as WT. Ethanol contents were measured by GC. Each point is the mean ± standard error calculated from ethanol contents produced over various time intervals by three replicates of roots.

- (+)ADH antisense: two heterozygous families AA108-7 and AA108-11 of line AA108.

3.2.2.6 Anaerobic Survival

3.2.2.6.1 Effects of anoxia on germination and coleoptile growth of the antisense ACT1-ADH1 line AA108

Seeds of both T1 and T2 generations of line AA108 and of wild type were submerged in conical flasks containing 0.5 mM CaSO4 in tap water and flushed with N2 gas for 5d. The anoxic condition inside the flasks was maintained by keeping them in the dark, to prevent O2 generation through photosynthesis of the germinated seedlings.
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Under such conditions, etiolated coleoptiles of wild type and antisense ADH transformants emerged but root growth was completely inhibited. Each of the 48 seeds of WT Taipei germinated, having on average 18 mm long coleoptiles (figs 3.10A and 3.10B). The length of coleoptiles of 23 T1 seeds of line AA108 were in the range of 0 to 4 mm (called 'retarded') and of 25 T1 seeds were 9 to 17 mm (called 'non-retarded'). The retarded to non-retarded germination ratio of the segregating T1 seeds of AA108, was 1:1 instead of 3:1 and the reason for this has not been investigated.

Figure 3.10A: Comparison of the effects of anoxia on germination (for 5d) of seeds of WT (untransformed Taipei) and the transgenic line AA108 of the antisense ACT1-ADH1.

One family (AA108-3) of line AA108 tested had retarded coleoptile growth (0 to 4 mm long) of all the 39 T2 seeds under anoxia, and is potentially homozygous. Emergence and elongation of coleoptiles occurred within 2d when 'retarded seeds' were transferred to light at 27 °C in normoxia and the variations of the phenotype (retarded germination or elongation of coleoptiles) between antisense ADH1 seedlings and wild type disappeared.
Figure 3.10B: Comparison of coleoptile length of WT, AA108 (T₁) and AA108 (T₂) seeds germinated for 5d under anoxia.

Under continuous aeration in the dark for 5d, all seeds from antisense ADH₁ transformants and WT plants germinated, and possessed roots of 44 mm long and etiolated coleoptiles of 36 mm long on average. There was no difference in phenotype under aerated conditions (data not shown).

Antisense ADH rice seeds remained viable after at least 5d anoxia and germinated and grew upon return to air. Plants were grown in the glass house after anoxic germination experiments and total protein was extracted from leaf blades of 50d old plants showing retarded and non-retarded germination, and WT plants. Total protein
was assayed spectrophotometrically for ADH activity (fig. 3.11). The ADH activity in leaf blades of plants from the retarded T\textsubscript{2} seeds of family AA108-3 was 35% of WT, and the ADH activity of retarded T\textsubscript{1} plants was 13% of non-retarded and WT plants (fig. 3.11).

![Figure 3.11: In vitro ADH activity measured spectrophotometrically in the leaf blade of individual plants (50d old) of line AA108 and WT rescued after 5d germination under anoxia (fig. 3.10). Three spectrophotometric measurements were taken using the same extract and specific activities were calculated. Standard errors were calculated from values of specific activities.

- WT: untransformed Taipei.
- Non-retarded: transgenic plants of line AA108 rescued from seeds of non-retarded coleoptile growth after 5d anoxic germination.
- Retarded (T\textsubscript{1}): transgenic plants (T\textsubscript{1} generation) of line AA108 rescued from seeds of retarded coleoptile growth after 5d anoxic germination.
- Retarded (T\textsubscript{2}): transgenic plants (T\textsubscript{2} generation) of line AA108 rescued from seeds of retarded coleoptile growth after 5d anoxic germination.
Extracts used in the above experiment were also fractionated by starch gel electrophoresis. One slice of the gel was stained for ADH specific activity and another slice of the same gel was stained for GDH activity as a control (fig. 3.12). Extracts from leaf blades of WT plants and plants from non-retarded germination gave very intense bands. However, band intensity in the extracts of leaf blades of plants from retarded germination was faint, suggesting again that ADH activity was low in plants from the retarded T1 seeds of AA108 compared to plants of WT and from the non-retarded seeds.

**Figure 3.12:** Starch isoenzyme gel of total protein extracted from the leaf blade of 50d old plants of line AA108 and WT rescued after 5d germination under anoxia. One slice of the gel was stained for ADH specific activity and another slice of the same gel was specifically stained for glutamate dehydrogenase (GDH) and served as a control.

- **WT:** untransformed Taipei.
- **Non-retarded:** transgenic plants of line AA108 rescued from seeds of non-retarded coleoptile growth after 5d anoxic germination.
- **Retarded:** plants of line AA108 rescued from seeds (T1 generation) of retarded coleoptile growth after 5d anoxic germination.

### 3.2.2.6.2 Analysis of mature plants of the antisense ACT1-ADH line AA108 for anoxia tolerance

Plants transformed with antisense *ADH* showing decreased levels of ADH were tested for their ability to withstand anaerobic conditions. To assay their ability to tolerate
anaerobiosis, plants were submerged in aquariums and treated anaerobically (fig. 3.13). It was found that the anaerobic survival of rice plants was highly variable in terms of plant’s age, the rate of gas flow through the aquariums, duration of treatment and type of treatment (i.e. time length of hypoxia and anoxia treatments). One notable constraint was the control of gas flow through the interconnected three aquariums and it was found that a slight variation of the flow rate and duration of treatments caused different levels of anaerobic survival within the same genotype. To amend this situation, each experiment was performed as a complete set including WT control, negative (azygous family) control, intolerant control and tolerant control besides over- and under expressing plants and data was interpreted by comparing the survival with controls. Each survival experiment in this PhD work was repeated three times except the one presented in section 5.2.4.2 which was repeated twice.

**Figure 3.13:** Aquariums used for submergence of rice seedlings.

Mature plants of the antisense *ADH1* were investigated for their ability to withstand anoxia. The percent survival was calculated from the number of dead and alive plants after a 10d long recovery period following the anaerobic treatment.
and IR42 were included as submergence-tolerant and submergence-intolerant varieties, respectively, in addition to the WT control (untransformed Taipei). One azygous family AA108-17 had a similar level of ADH activity as WT and was also included as a negative control. Individual plants of two segregating families AA108-7 and AA108-11 included in the anaerobic survival assay were analysed spectrophotometrically for ADH activity in leaf blades prior to submergence. Plants with ADH activity of 13% or less of WT were scored as (+)ADH antisense (fig. 3.14). In figure 3.14, the ‘azygous’ variety comprised plants of families AA108-7 and AA108-11 having a similar level of ADH activity as plants of WT and azygous family AA108-17.

Figure 3.14: Comparison of anoxia tolerance among mature plants of antisense ADH1, wild type, and azygous plants. Percent survival was estimated after a 10d recovery following anoxia and 30-40 plants were tested for each variety in each aquarium. Standard errors were calculated from three aquariums’ values for each variety.

- WT: untransformed Taipei as control.
- Azygous: plants from an azygous family (AA108-17) and two heterozygous families (AA108-7 and AA108-11) with a similar level of ADH activity as WT plants.
- (+)ADH antisense: plants of AA108-7 and AA108-11 families with reduced ADH activity.
- IR42: flooding intolerant control.
- FR13A: flooding tolerant control.
Hundred percent survival was found in the flooding tolerant variety FR13A. Eighty three percent survival was recorded in IR42 under the assay conditions. Twenty five percent of Taipei plants and 23% of azygous plants survived and both showed similar anoxia tolerance. Significantly reduced anoxia tolerance was found in plants (of which 8% survived) of AA108-7 and AA108-11 which possessed reduced ADH activity compared to WT plants

### 3.2.3 Sense UBIQUITIN1-ADH2 transformants

#### 3.2.3.1 Detection of UBIQUITIN1-ADH2 transformants by ADH enzyme assay

A plasmid (pUBI-ADH2) containing the cotton ADH2 cDNA fused with the maize UBIQUITIN 1 gene promoter (section 2.1.2) was used for rice transformation to over-produce ADH. ADH activity was measured spectrophotometrically in vitro in the leaf blade of 4 months old glass house grown T₀ plants. ADH activity was very low (47 units (nmol/min/mg protein)) in the leaf blade of WT control plants at this age (fig. 3.15). Six independent transgenic lines showed 25 units ADH activity on average. However, three lines, AS3, AS8 and AS9 had much higher ADH activity (5218, 10927 and 5760 units, respectively) compared to control plants. Line AS9 was sterile. When T₁ seeds of line AS3 were tested for ADH activity, no significant difference in ADH level was found compared to WT. This line was therefore not studied further.

![Graph showing ADH activity in different lines](image)

**Figure 3.15:** In vitro ADH activity measured spectrophotometrically in leaf blades of four months old T₀ plants of nine independently transformed lines of UBIQUITIN1-ADH2 and untransformed controls.
ADH activity was measured in shoots of 4 week old plants of Taipei, FR13A, IR42 and T1 plants (expressing the transgene) of lines AS8 and AA108 (fig. 3.16). The over-expressing line AS8 showed ADH specific activities of 917 units (nmol/min/mg protein) in air and 1718 units under hypoxia, which were 439% and 213% more than the ADH activities (209 units in air and 805 units under hypoxia) found in WT. The ADH activity in the flooding intolerant variety IR42 was 189 units in air and 647 units under hypoxia. The flooding tolerant variety FR13A had slightly higher ADH activity (272 units in air and 884 units under hypoxia) than IR42 which corresponded to 144% (in air) and 136% (under hypoxia) of that of IR42, respectively. The under-expressing antisense ADH line AA108 showed a very low level of ADH activity of only 11 units in air, which was 4% of the ADH level of WT. The measurement of ADH activity of line AA108 was not done under hypoxia.

![Graph](image)

**Figure 3.16:** *In vitro* ADH activity measured spectrophotometrically in shoots of four week old plants of untransformed Taipei (WT), FR13A, IR42 and T1 plants (expressing the transgene) of antisense *ADH1* line AA108 and sense ADH line AS8. Standard errors were calculated from ADH activities of three independent plants of each variety.
A homozygous and azygous family (similar level of ADH activity as WT) of line AS8, AS8-10 and AS8-2 respectively, were raised according to section 3.2.2.2 and were detected by analysing *in vitro* ADH activity of 24 T₂ plants. The level of ADH activity in two week old leaf sheaths of family AS8-10 was 637% more than WT plants (fig. 3.17). Family AS8-2 had a similar level of ADH activity (103 units) as WT plants.

![Graph](image)

**Figure 3.17:** Determination of homozygous family AS8-10 from the over-producing ADH line AS8 by assaying ADH activity in leaf sheaths of two-week old plants of transformants and WT plants. *In vitro* ADH activity was measured by spectrophotometer as per section 2.4.3.2.

Total soluble protein was extracted from shoots and roots of two-week old plants of WT and homozygous family AS8-10 of line AS8, and was separated by starch isoenzyme gel electrophoresis (fig. 3.18). The gel was stained for ADH activity. rADH1-rADH1 (‘r’ stands for rice) was the only isoenzyme present in the shoot of wild type plants whereas three isoenzymes, rADH1-rADH1, rADH1-cADH2 and cADH2-cADH2 (‘c’ stands for cotton), were detected in shoots of AS8-10. The level of rADH1-rADH1 is lower in transgenic plants; presumably the rice ADH1 polypeptide forms a dimer with the cotton ADH2 polypeptide. Similarly, the cotton ADH2 polypeptide
forms a dimer with both of the rice ADH1 and ADH2 polypeptides in transgenic roots, consequently lowering the levels of rADH1-rADH1 and rADH2-rADH2. A total of six electrophoretically distinct isoforms were present in the roots of transgenic plants, compared to three isoforms in WT plants.

Figure 3.18: Comparison of different ADH isoenzymes in shoots and roots of two week old plants of *UBIQUITIN1-ADH2* transformants and WT plants by starch isoenzyme gel electrophoresis. Total soluble protein was fractionated by electrophoresis on a starch isoenzyme gel and the gel was stained specifically for ADH activity.

- WT: untransformed Taipei
- AS8-10: a homozygous family of the over-expressing ADH line AS8.

### 3.2.3.2 Ethanol production in line AS8

Excised leaf sheaths of hypoxically treated plants of a homozygous family (AS8-10), an azygous family (AS8-2 of line AS8) and WT were incubated in anoxic medium in the presence of 0.1 M glucose, and ethanol content was measured in the medium by gas chromatography. Over-expressing plants (family AS8-10) showed a very slight increase in ethanol production compared to the wild type plants and the azygous family AS8-2 which had a similar level of ADH activity as WT (fig. 3.19). Line AS8-10 was also tested together with the over-expressing *PDC* line and both *PDC* and *ADH* lines
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(fig. 5.17). Ethanol contents in AS8-10 were 51, 269, 627, 1046, 7009 and 8364 units and those in WT were 49, 253, 607, 1085, 6787 and 7802 units after 2, 4, 6, 8, 24 and 32h incubations respectively (fig. 3.19). AS8-2 and WT produced a very similar amount of ethanol throughout the experiment.

![Graph of Ethanol production vs Incubation time]

**Figure 3.19:** Ethanol production in excised leaf sheaths of family AS8-10, WT and an azygous family AS8-2. Ethanol contents were measured by GC. Standard errors were calculated from ethanol contents produced by three replicates of leaf sheaths.

- WT: untransformed Taipei.
- (+)ADH transgene: a homozygous family AS8-10 of the over-producing ADH line AS8.
- Azygous family: family AS8-2 of line AS8 which had a similar ADH level as WT.

### 3.2.3.3 Anoxia tolerance of the over-producing ADH line AS8

To simulate submergence, hydroponically grown seedlings (26d old) were submerged in aquariums (fig. 3.13) and treated anaerobically. Transgenic plants of homozygous family AS8-10 over-expressing ADH (10% ± 6 survived) did not show improved anoxia tolerance compared to WT (17% ± 10 survived) or azygous AS8-2
plants (16% ± 4 survived) (fig. 3.20). All plants of FR13A survived as before (fig. 3.14) and IR42 survival was 16% ± 7 under the assay conditions.

Figure 3.20: Assay of anoxia tolerance in mature plants of the over-expressing ADH homozygous family AS8-10, WT and an azygous family AS8-2. 30-40 plants were tested in each aquarium for each variety. Standard errors were calculated from three aquariums' values of each variety.

- WT: untransformed Taipei.
- (+)ADH transgene: a homozygous family AS8-10 of the over-expressing ADH transgenic line AS8.
- Azygous family: family AS8-2 of the line AS8 which had a similar level of ADH activity as WT.
- A flooding tolerant variety (FR13A) and an intolerant variety (IR42) were included as controls.

3.3 Discussion

In the ADH overproducing transgenic line AS8 of rice, the cotton ADH2 was ectopically expressed whose product formed novel dimers with the rice ADH1 and ADH2 (fig. 3.18). In the ADH underproducing transgenic line AA108 of rice, the rice antisense ADH1 was able to reduce the level of both ADH1 and ADH2 (fig. 3.8). Since
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the rice ADH1 and ADH2 genes have 80.1% homology overall and several stretches of 100% homology at the nucleotide level (Xie and Wu, 1989, 1990), it is likely that the antisense ADH1 is effective in reducing the expression of both ADH1 and ADH2.

Hypoxia can occur in germinating seeds due to inadequate access to atmospheric oxygen, since the outer layers of the seed coat reduce the seed’s permeability to gaseous exchanges (Nutbeam and Duffus, 1978; Duffus and Duffus, 1984). But there are a few species, such as rice, four Echinochloa species and the African legume, Erythina caffra that are able to extend their coleoptiles or leaves under anoxia (see Armstrong et al., 1994 for a review). Among higher plants, rice is unusual in that its seeds can germinate in the total absence of molecular O2 and even under vacuum (Vartapetian et al., 1978; Cobb and Kennedy, 1987), presumably by using energy produced by the fermentation pathway (Mocquot et al., 1981; Cobb and Kennedy, 1987). Rice seeds and seedlings increase ethanol production for at least the first 2d under anoxia (Raymond et al., 1985). In addition, rice is able to degrade the starchy endosperm under anoxia by the concerted action of the complete set of amylolytic enzymes of α-amylase, β-amylase, debranching enzyme and α-glucosidase (Perata et al., 1997; Loreti et al., 1998).

Seeds remained viable after a long period of germination under anoxia (Zhang et al., 1994). In rice under anoxia and in the dark, early growth of embryos and etiolated coleoptiles occurs but root growth is inhibited (Kordan, 1977a, 1977b). Further development of coleoptiles is O2 dependent. Setter and Ella (1994) found that the extent of coleoptile growth in IR42, a flooding intolerant variety, and Calrose, a flooding tolerant variety, was highly dependent on the rate of ethanol synthesis (RE) under anoxia. Coleoptiles of IR42 were shorter with a lower RE whereas those of Calrose were longer with a higher RE under anoxia (Setter and Ella, 1994). Importantly, the extent of coleoptile growth of rice seeds germinating under anoxia has been correlated to submergence tolerance in established seedlings (Setter et al., 1994). The elongation of the coleoptile of an adh1 null mutant of rice (rad) was strongly suppressed under anoxia (Matsumura et al., 1995, 1998). This ‘retardation’ of germination and coleoptile elongation under anoxia was also observed in the antisense ADH line AA108 in this study. This demonstrates the reduced anoxia tolerance of line AA108 during germination (fig. 3.10). ADH enzyme assays of the T1 plants of line AA108 after anoxic
germination experiment showed that plants rescued from retarded germination under anoxia possessed a reduced level of ADH activity compared to wild type and plants of AA108 with non-retarded germination (figs 3.11 and 3.12). This clearly indicates a correlation between reduced ADH activity and the suppression of coleoptile emergence and the need for ADH for germination of rice seeds under anoxia. There was no retardation of germination in air of line AA108, however, the requirement of ADH for rice seeds germination in O2 can not be ruled out since aerobic EF has been observed during germination (Setter and Ella, 1994). Moreover, although the levels of both ADH1 and ADH2 polypeptides are reduced and total ADH activity was greatly lowered in line AA108 compared to wild type, there was still some ADH activity which might be enough to carry out EF at the level for germination in air. Additionally, the adh1 null mutant of rice also contains a reduced but appreciable level of ADH activity (Matsumura et al., 1998).

Mature plants of the antisense ADH line AA108 with reduced levels of in vitro ADH activity and EF, also exhibited reduced survival to anoxia compared to wild type and azygous plants of line AA108 (fig. 3.14). Maize root tips and rice seedlings of adh mutants similarly showed reduced survival under anoxia (Schwartz 1969; Freeling and Bennett, 1985; Johnson et al., 1994; Matsumura et al., 1998). The reduced tolerance of the antisense ADH line AA108 and adh mutants raises an important question: does the low rate of ethanol fermentation in these plants alone cause their reduced anaerobic tolerance? There are at least three possible explanations for the reduced anaerobic tolerance of the antisense ADH transformants and adh mutants. The first could be due to the reduced rate of NAD+ regeneration from NADH as a product of the reaction from acetaldehyde to ethanol catalysed by ADH. In the antisense ADH transformants and the adh mutants, the flux of glycolysis may be slowed down due to the shortage of NAD+. It has been found that cultured maize cells over-expressing barley haemoglobin showed no effect on the energy status under low oxygen conditions, but WT cells and cells that were under-expressing (antisense) haemoglobin had ATP levels decreased by 61% and 27%, respectively (Sowa et al., 1998). The authors suggested a haemoglobin-mediated oxidation of NADH to NAD+ and hence a promotion of glycolytic flux and substrate level phosphorylation under low oxygen conditions. A second explanation might be inadequate energy production due to the low rate of EF caused by the lowered level of
ADH activity in these mutants and transformants. A third cause for the reduced anaerobic survival could be the accumulation of the potential cell toxin acetaldehyde (Davies et al., 1974; Tadege et al., 1998a), resulting from the activation of PDC at low pH, which would produce acetaldehyde. Due to the low ADH levels, elevated levels of acetaldehyde might exert toxic side effects by forming acetaldehyde-protein adducts (Perata et al., 1992; Braun et al., 1995). The level of acetaldehyde is greatly increased after submergence in most rice varieties examined; the highest increase has been found in the least flooding tolerant variety IR42 and the lowest increase occurred in FR13A, the most tolerant variety tested (Kundu et al., 1992). During seed germination under anoxia, an increased level of acetaldehyde was also associated with flooding intolerant varieties among six varieties of rice tested which differ in submergence tolerance (Setter et al., 1994). However, Patnai 23, a moderately tolerant variety, is an exception and showed no significant increase in acetaldehyde concentration after submergence (Kundu et al., 1992). Without comparison of the levels of acetaldehyde and NADH/NAD+ in wild type and antisense ADH line AA108, it is not possible to pinpoint exactly which of the three potential factors is responsible for the reduced tolerance in the antisense ADH line AA108.

The basal ADH activity in the homozygous family AS8-10 was increased by 439% in shoots and 423% in roots compared to WT (ADH activity was estimated in 3 week old plants). The ADH activity level in shoots of line AS8-10 was four fold higher in air and two-fold higher under hypoxia compared to that of Taipei, FR13A and IR42. However, the effect of the high ADH level on EF and anaerobic survival was insignificant (figs 3.19 and 3.20). In contrast, a positive correlation was found between anoxia tolerance and increased levels of ADH activity under anoxia in different rice genotypes (Ellis and Setter, 1999). Many researchers have found a correlation between improved anoxic survival of acclimated root tips by hypoxic pre-treatment (HPT) and shoots and enhanced level of ADH activity in HPT treated plants (see Drew, 1997 for review). However, it might not be appropriate to compare HPT acclimated plants with the over expressing ADH transformants. HPT induces the activity of other enzymes along with PDC and ADH causing improved energy metabolism. HPT may also induce a lactate transport mechanism and consequently improve control of cytoplasmic acidosis for better survival than no hypoxic pretreatment (NHPT) plants (for a review
Chapter 3/ Evaluation of the role of ADH in anaerobic tolerance

see Drew, 1997). This raises the following questions: is ADH rate limiting in EF, and is the ADH level in excess of that required for increasing the rate of EF in rice plants? It has been found that ADH also accumulates in maize root tips under hypoxia to levels much greater than those required for survival (Roberts et al., 1989; Johnson et al., 1994), which clearly indicates that ADH is not limiting the rate of EF. In the transgenic line AS8, increasing the level of ADH activity did not increase the rate of EF. However, the level of PDC is lower than that of ADH by a factor of 8-65 (Morrell and Greenway, 1989) and its activity is very close to the rate of EF in vivo (Morrell et al., 1990; Waters et al., 1991; for a review see Drew, 1997). In this study, ADH levels were higher by 3 fold in leaf sheaths and by 671 fold in leaf blades of WT (untransformed Taipei) plants compared to their PDC levels (sections 3.2.3.1 and 5.2.2.3). Due to the growing concern about PDC limitation in the EF pathway, transgenic rice plants over-expressing PDC and both PDC and ADH have been developed and the results of these lines are presented and discussed in chapter 5.

Several questions can be asked about these findings. Is the elevated level of ADH activity in the flooding tolerant rice varieties responsible for their tolerance against flooding? If not, what is the role of the increased production of ADH under anaerobic conditions? Within the scope of the results presented here, the significance of the high basal level of ADH, the excessive induction of ADH under anaerobiosis and the genotypes with better anoxic survival containing higher levels of induced ADH remain unknown. Does such an apparently “excessive” response have survival value under stressed conditions? Cytoplasmic acidosis might be a factor for the selective synthesis of ADH (for reviews: Drew, 1997; Bailey-Serres, 1999) as it was found that low pH increased the in vitro translation of certain proteins. It appears from genetic analysis of rice for flooding tolerance that the ADH gene belongs neither to the major submergence tolerance locus (SUBJ) nor to the four QTLs associated with this character in rice (Xu and Mackill, 1996; Nandi et al., 1997).

In conclusion, over-expression of ADH did not improve anoxia tolerance in this study, but a minimal level of ADH is a prerequisite under adverse conditions to protect plants from energy deficiency and/or toxicity from anaerobic metabolism. The latter is supported by significantly reduced anaerobic metabolism in transformants with antisense ADHI (fig. 3.14).
Chapter 4

Spatial and Temporal Distribution and Anaerobic Induction of the Rice PDCs
4. REGULATION OF PYRUVATE DECARBOXYLASE GENES IN RICE

4.1 Introduction

Pyruvate is a major branch point between aerobic and anaerobic energy metabolism. In the presence of oxygen, pyruvate is imported into mitochondria and oxidised by the pyruvate dehydrogenase (PDH) complex. In the absence of oxygen, there are at least three fates of pyruvate: it can be converted to ethanol through the ethanol fermentation pathway, to lactate by lactate dehydrogenase or to alanine by alanine aminotransferase. Ethanol is the major product of anaerobic glycolysis although lactate and alanine also accumulate (Smith and ap Rees, 1979; Saglio et al., 1980; Roberts et al., 1982; Hoffman et al., 1986). Pyruvate decarboxylase (PDC, EC 4.1.1.1) together with alcohol dehydrogenase (ADH, EC 1.1.1.1) catalyse the fermentation of ethanol which is the major energy-producing pathway in plants under low oxygen conditions. PDC non-oxidatively decarboxylates pyruvate to acetaldehyde and acetaldehyde is reduced to ethanol by ADH. These two enzymes are among the twenty "anaerobic polypeptides" (ANPs) that are induced under anaerobic stress in maize (Sachs et al., 1980).

Hossain et al. (1996) suggested that in rice, PDC is encoded by four or five genes. Rice PDC1, PDC2 and PDC3 have been cloned and characterised (Hossain et al., 1994a, 1994b, 1996; Huq et al. 1995). PDC3 was suggested to be a pseudo-gene as it does not contain introns and there was no evidence of transcription either by Northern blotting or by probing a cDNA library made from anaerobically induced suspension cells (Hossain et al., 1994b, 1996). A partial cDNA clone of a PDC4 gene has been reported (Rivoal et al., 1997). The rice PDC genes are closely related (Hossain et al., 1996; Rivoal et al., 1997). PDC1 shows similarity to PDC2, PDC3, and PDC4 of 79%, 81% and 96% at the nucleotide level and PDC2 is 79% identical to PDC3. PDC1,
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PDC2, and PDC4 mRNAs are induced by anoxia in rice seedlings with PDC1 being the most highly induced (Hossain et al., 1996; Rivoal et al., 1997).

The subunit compositions of the PDC enzyme have been studied and two subunits (α and β) have been detected in yeast, maize, wheat, peas and rice (Rivoal et al., 1997; Konig et al., 1998; see Candy and Duggleby, 1998 and Furey et al., 1998 for reviews). However, the identity of the structural genes encoding α and β subunits is still unclear. In particular, there is much speculation about whether a separate gene encodes the smaller subunit β or whether proteolysis during purification steps is responsible for the generation of β from a possible precursor- the larger subunit α. The MWt of the rice α and β subunit is 64 kDa and 62 kDa respectively (Rivoal et al., 1990, 1997). The identity of the PDC genes encoding the α and β subunits is not known in rice. However, the α and β subunits are products of two separate PDC genes; so the possibility of a precursor/product relationship between α and β subunits has been eliminated (Rivoal et al., 1990, 1997). The MWt of the rice PDC1, PDC2 and PDC3 polypeptides deduced from the cDNA sequence are 65, 64 and 62 kDa respectively (Hossain et al., 1996).

The relationship between the four or five PDC genes and the two types of PDC polypeptides (α and β) has not been established. Previous studies have focussed on the analysis of the polypeptides in young rice seedlings. One aim of the work in this chapter was to study the distribution of the PDC polypeptides in different tissues at various ages and identify individual PDC polypeptides encoded by particular PDC genes. Anaerobic induction of rice PDCs was also investigated. The cis-acting regulatory elements for anaerobic induction of the rice PDC1 promoter were also studied.
4.2 Results

4.2.1 Study of the differential expression of the PDC polypeptides in various tissues of rice

Total protein was extracted from different tissues of untransformed Taipei in order to analyse the differential expression of various PDCs. A polyclonal antibody raised against the bacterially expressed rice PDC1 was used to probe Western blots. As the rice PDC polypeptides are closely related (78%-95% identity) (Hossain et al., 1996; Rivoal et al., 1997), the antibody can be expected to cross-react with all the PDC gene products.

A total of 6 PDC polypeptide bands with different mobilities were detected in Western blots (fig. 4.1). These bands were numbered according to increasing mobility and had apparent MWt ranging from 66 kDa to 58 kDa. PDC polypeptides were found in all tissues tested including embryos, endosperm, leaf blades, leaf sheaths, roots, spikelets, anthers and pollen. However, each tissue type contained a different set and number of PDC bands. Shoots and leaf sheaths had a total of 5 PDC bands, and embryos and leaf blades had 4 bands (fig. 4.1 and table 4.1). Endosperm, roots, spikelets and anthers contained 3 bands and pollen was the only tissue found to have 2 bands. A novel band (band 3) with an approximate MWt of 62 kDa was found in floral tissues of spikelet, anther and pollen.

The abundance of PDCs also varied in different tissues such that the intensities of PDC bands were very strong in embryos, anthers and pollen and faint in the leaf blade. Bands 2 and 4 were the major bands found in vegetative tissues of rice. Band 2 with an approximate MWt of 64 kDa was the only band found ubiquitously and was present at the highest level in embryos, endosperm, leaf sheaths, and spikelets (collected before the first mitosis of microspores; 6 cm in fig. 4.2). Band 4 was abundant in embryos and roots. Bands 1, 5, and 6 were present in low levels in some of the vegetative tissues and were absent in floral tissues.
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Figure 4.1: Immunoblot analysis to study the spatial distribution of the rice PDC polypeptides in untransformed Taipei using the antibody raised against the rice PDC1. The amount of total protein loaded per lane is 30 µg for embryo, endosperm, spikelet, anther and pollen, 50 µg for shoot, leaf sheath and root; 150 µg for leaf blade.

- Lane a, molecular weight marker; b, embryo; c, endosperm; d, 5th foliage leaf blade; e, leaf sheath; f, root; g, spikelet; h, anther; i, pollen.

4.2.2 Effects of development on the distribution of PDCs in different tissues of rice

A novel band (band 3) was detected only in spikelets, anthers and pollen (fig. 4.1) and was studied further to investigate at exactly which developmental stage it is expressed. The various developmental stages of rice panicles were calculated from the distance between auricles of the penultimate leaf and the flag leaf (section 2.4.4.1; fig. 2.8). Total protein was extracted from spikelets of Taipei collected at the stage of uninucleate microspores (+6 cm) and during anthesis (+15 cm). Proteins was also extracted from anthers and pollen collected at stages of dinucleate microspores (+8 cm), trinucleate microspores (+11 cm) and during panicle appearance outside the leaf sheath (+13 cm) and anthesis (+15 cm) (figs 4.2A and 4.2B).

Band 3 was absent in spikelets at the developmental stages prior to mitosis in uninucleate microspores (+6 cm in fig. 4.2) but is present in anthers and pollen at the dinucleate stage (+8 cm in figs 4.2A and 4.2B). The intensity of band 3 increased in
spikelets, anthers and pollen towards maturation and the highest intensity in pollen was observed after anthesis (+15 cm in fig. 4.2B). These data show that band 3 appeared in floral tissues after the first mitosis of the microspores. The expression of band 3 increased whereas the expression of band 2 decreased with maturation in anthers and pollen. It should be mentioned here that the same samples in fig. 4.2 were also fractionated in gels run for a longer time to confirm the identity of each band.

Figure 4.2: Immunoblot analysis to study the temporal distribution of PDCs in spikelets, anthers and pollen of untransformed Taipei. Two vegetative tissues, leaf sheath and root, were included as a control along with the floral tissues.

- Lane M, marker.
- Spikelets were collected at +6 cm and +15 cm distances.
- Anthers and pollen were collected at +8, +11, +13 and +15 cm distances.
The effects of age and hypoxia on the expression of the rice PDCs in vegetative tissues were also investigated. Plants of untransformed Taipei were grown aerobically. Half of the air grown plants aged 1, 2, 3 and 4 weeks were subjected to hypoxia in an anaerobic jar. Shoots, roots, leaf sheaths, and leaf blades were harvested from the air grown and hypoxically treated plants and total protein was extracted. The change in PDC bands during development is summarised in table 4.1. The intensity of band 2 decreased with age in shoots and leaf sheaths and the intensity of bands 4 and 5 also decreased with age in shoots and leaf blades (figs 4.3 and 4.4).

**Figure 4.3:** Immunoblot analysis to study the temporal distribution and hypoxic induction of rice PDCs in A, shoot; B, root; C, leaf sheath of four, three, two and one-week old untransformed Taipei using an antibody raised against the rice PDC1. The amount of total protein loaded per lane was 50 μg.
- Lane M: molecular weight marker
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Figure 4.4: Immunoblot analysis to study the temporal distribution of PDCs in leaf blades of untransformed Taipei under aeration: 5th, 4th and 3rd foliage leaf blades were collected from the four-week old plant and 4th, 3rd and 2nd foliage leaf blades were collected from the three-week old plant.

- Lane 1, 1st leaf blade (incomplete) and a, coleoptile of two-week old plants, respectively; M, molecular weight marker.

Table 4.1: Summary of the distribution of PDCs in various tissues of untransformed Taipei.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Band number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Endosperm</td>
<td>absent</td>
</tr>
<tr>
<td>Embryo</td>
<td>very low level</td>
</tr>
<tr>
<td>Shoot</td>
<td>very low level</td>
</tr>
<tr>
<td>Root</td>
<td>absent</td>
</tr>
<tr>
<td>Leaf sheath</td>
<td>very low level</td>
</tr>
<tr>
<td>Leaf blade</td>
<td>low level</td>
</tr>
<tr>
<td>Anther</td>
<td>absent</td>
</tr>
<tr>
<td>Spikelet</td>
<td>absent</td>
</tr>
<tr>
<td>Pollen</td>
<td>absent</td>
</tr>
</tbody>
</table>

* ↓ with maturation in anthers and ↑ with maturation in spikelets.
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Anaerobic induction of bands 2, 4, 5, and 6 was found in various tissues (fig. 4.3). The results are presented in table 4.2. Band 4 was strongly induced in roots (fig. 4.3B) and leaf sheath (fig. 4.3C) under anaerobic treatment while band 2 was slightly induced in roots (fig. 4.3B).

Table 4.2: Summary of the hypoxic induction of rice PDCs in shoots, roots and leaf sheaths of untransformed Taipei.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Expression in air</th>
<th>Induction under hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>bands 1, 2, 4, 5 and 6</td>
<td>bands 2, 4 and 5</td>
</tr>
<tr>
<td>Root</td>
<td>bands 2, 4 and 5</td>
<td>bands 2, 4 and 5</td>
</tr>
<tr>
<td>Leaf Sheath</td>
<td>band 1, 2, 4, 5 and 6</td>
<td>bands 2 (?), 4, 5 and 6</td>
</tr>
</tbody>
</table>

4.2.3 Study of the rice PDC1 promoter for its spatial and temporal expression and anaerobic induction

A translational fusion (pPDC55 - section 2.1.4) of the rice PDC1 promoter with the GUS reporter gene was introduced into rice. The tissue specific and developmentally regulated expression of the PDC1 promoter was investigated in vegetative and floral tissues of the PDC1-GUS transformants.

One-week old air grown seedlings of PDC1-GUS transformants (line no. P1G123) were treated hypoxically (5% O2 in N2) and anoxically (N2) for investigating the anaerobic induction of the PDC1 promoter. PDC1-GUS transformants exhibited GUS staining in roots (figs 4.5G, H and I). Intense staining was observed in the root cap and meristem of aerated, hypoxic and anoxic samples (figs 4.5G, H and I) and the whole root stained blue under anoxia. Under aerobic conditions, the PDC1-GUS transformants showed a low level of staining in the vascular region and epidermal layer of the shoot (fig. 4.5D). Very high expression was found in the entire shoot section under hypoxia and anoxia (figs 4.5E and F). Intense staining was seen in the scutellum, shoot and root meristems of embryos from 2d old germinated seeds either under air (fig. 4.5A) or anaerobic stresses (figs 4.5B and C) but very little staining was seen in the endosperm in all conditions. These results suggest that the rice PDC1 promoter is expressed constitutively in the root tip, the vascular regions of shoot, scutellum, shoot and root
meristems of embryos in germinating seeds and is induced throughout the root and the shoot under anaerobic conditions.

Figure 4.5: Expression pattern of the PDC1 promoter in various tissues of rice transformed with a translational fusion of PDC1-GUS (T1/T3 plants of line P1G123) at different developmental stages and under anaerobic stresses and aerated conditions. GUS staining was performed overnight in longitudinal halves of 2d old germinated seeds under A, aeration; B, hypoxia and C, anoxia. Staining in the cross-sections of 7d old shoots under D, aeration; E, hypoxia and F, anoxia. Staining in the 7d old roots under G, aeration; H, hypoxia and I, anoxia.
Spikelets of *PDC1-GUS* transformants (line no. P1G123) were collected at different stages of maturation as described in section 2.4.5. *PDC1-GUS* showed staining in anthers but not in pollen (fig. 4.5M). Staining of anthers increased with the age of flowers and most staining was observed in anthers just prior to anthesis (figs 4.5J, K and L). Anther filaments exhibited little staining and no staining was found in the rachilla, stigma or ovary tissues.

Figure 4.5:

GUS staining in spikelets of T₃ plants of the translational fusion of *PDC1-GUS* line P1G123. J, collected when the panicle was inside the leaf sheath; K, harvested one day before anthesis; L, harvested after anthesis. M, pollen and anther collected after anthesis.
4.2.4 Study of the rice PDC3 promoter for its spatial and temporal expression and anaerobic induction

A translational fusion (pPDC25 - section 2.1.4) of the rice PDC3 promoter fused to the GUS reporter gene was introduced into rice. The tissue specific and developmentally regulated expression of the PDC3 promoter was investigated in vegetative and floral tissues of the PDC3-GUS transformants.

Two day old germinated seeds, roots and shoots of one week old (figs 4.6A, B and C) and six week old (data not shown) plants of nine independently transformed lines of PDC3-GUS were analysed by GUS histochemical staining following aerobic, hypoxic (5% O2 in N2) or anoxic (N2) treatments. No staining was observed in any of the vegetative tissues in all lines following hypoxic and anoxic treatments. Intense staining was seen in pollen (figs 4.6D, E, F, G and H) of three independently transformed lines (P3G5, P3G7 and P3G10) out of nine. No GUS staining was seen in anthers, anther filaments, rachilla, stigma or ovary tissues of lines P3G5, P3G7 and P3G10 (figs 4.6D, E and F). The number of stained pollen increased with the age of the spikelets and the highest frequency of pollen staining was found on the day of flowering (figs 4.6D, E and F). The pollen tube germinated in vitro (section 2.4.5) was also stained (fig. 4.7).
Figure 4.6: GUS histochemical analysis of the expression of the rice PDC3 promoter in vegetative and floral tissues of T₁/T₃ plants of the translational fusion of PDC3-GUS line P3G5 at different developmental stages. No GUS staining was found in A, longitudinal halves of 2d old germinated seeds; B, cross-sections of 7d old shoots; C, 7d old roots under aeration, hypoxia and anoxia. GUS staining was seen only in the pollen. Spikelets D, collected when panicle was inside leaf sheath; E, harvested one day before

**Figure 4.7:** GUS histochemical analysis of the expression of the *PDC3* promoter in *in vitro* germinating pollen tubes of *PDC3-GUS* transgenic line P3G5.

### 4.2.5 Study of the anaerobic regulatory element (ARE) in the rice *PDC1* promoter for its anaerobic induction

The ARE sequence which consists of GT-motif (5’- (T/C)(T/C)GGTTT-3’) and GC-motif (5’-GCCCC-3’) has been shown to be responsible for anaerobic induction in both the *Arabidopsis* and maize *ADH1* genes (Walker *et al*., 1987; Olive *et al*., 1990; Kyozuka *et al*., 1994; Hoeren *et al*., 1998). The rice *PDC1* gene is induced by anaerobic conditions (Hossain *et al*., 1996) and its promoter contains 8 copies of the GT-motif and 3 copies of the GC-motif (Hossain *et al*., 1994a).

The regulatory effect of these cis-acting GT- and GC-motifs for anaerobic induction in the *PDC1* promoter was investigated. Five constructs of various lengths of
5’-truncated \textit{PDC1} promoter-\textit{GUS} fusions (transcriptional) (section 2.1.5 and fig. 2.5) were transformed into rice. These constructs contain different numbers of the GT and GC motifs (fig. 4.8).

\begin{itemize}
\item All gene fusions contain the \textit{PDC1} promoter (hatched box) and the rice \textit{ACTIN1} first intron (empty box) placed upstream of \textit{GUSA} gene and nopaline synthase transcription termination sequence (PIGN). The 5’ end of each gene construct is given relative to the first transcription start site (+1 in fig.) of the rice \textit{PDC1} gene (Hossain \textit{et al.}, 1996). The \textit{PDC1} gene has another transcription start site at +3 (Hossain \textit{et al.}, 1996).
\end{itemize}

Seven \textit{T\textsubscript{2}} seedlings (segregating) of one-week old transformants of each construct were treated anoxically and sections of shoot and root of aerated and anoxic seedlings were stained for GUS activity. Shoots and roots of 2-3 independent transgenic lines of each construct were stained and the line with the strongest staining in air was selected for each construct for comparison. The aerated and anoxic samples of plants carrying PIGN construct were stained for GUS activity for 40 min. However, the lines selected for \textit{\Delta1015PIGN}, \textit{\Delta770PIGN}, \textit{\Delta186PIGN} and \textit{\Delta82PIGN} transformants gave very intense staining within one hour in air and hence, to investigate the comparison between aerobic and anoxic expression, GUS staining was performed for 10 min in aerated and anoxic

\textbf{Figure 4.8:} \textit{GUS} fusions (transcriptional) containing full length (PIGN) and a series of various lengths of 5’ truncated rice \textit{PDC1} promoter (\textit{\Delta1015PIGN}, \textit{\Delta770PIGN}, \textit{\Delta186PIGN} and \textit{\Delta82PIGN}) used for rice transformation and their GT and GC motifs.

\begin{itemize}
\item All gene fusions contain the \textit{PDC1} promoter (hatched box) and the rice \textit{ACTIN1} first intron (empty box) placed upstream of \textit{GUSA} gene and nopaline synthase transcription termination sequence (PIGN). The 5’ end of each gene construct is given relative to the first transcription start site (+1 in fig.) of the rice \textit{PDC1} gene (Hossain \textit{et al.}, 1996). The \textit{PDC1} gene has another transcription start site at +3 (Hossain \textit{et al.}, 1996).
\end{itemize}
samples. A substantial level of GUS expression in air was found in shoots (fig. 4.9A) and root tips (fig. 4.9B) of all transformants including the one containing the minimal promoter (Δ82PIGN) suggesting that 82 bp of sequence of the PDC1 promoter is able to confer aerobic expression. However, the intensity of the blue stain in aerated shoots and roots was less in Δ186PIGN and Δ82PIGN than in PIGN, Δ1015PIGN and Δ770PIGN. The quantitative study of the copy number of these motifs on the extent of aerated expression may not be accurate because of the limited number of independently transformed lines for each construct (1 transgenic line of PIGN, 2 of Δ1015PIGN, 2 of Δ770PIGN, 2 of Δ186PIGN and 3 of Δ82PIGN).

Surprisingly, no anoxic induction was found in shoots (fig. 4.9A) of any of these transcriptional promoter-GUS fusions of the rice PDC1 promoter except PIGN. Shoots and roots of plants carrying PIGN construct showed more staining under anoxia than in air. Slight anoxic induction was found in roots (fig. 4.9B) of plants carrying the shorter version of the promoter-GUS fusions (Δ770PIGN, Δ186PIGN and Δ82PIGN) and no significant induction was observed in roots of Δ1015PIGN. GUS staining was also performed in pollen of transformants of all PDC1-GUS fusions (transcriptional) and blue staining was observed in all transformants (fig. 4.9C).
Figure 4.9A: GUS histochemical staining in shoot sections of 7d old aerated and anoxic T2 plants (segregating) expressing GUS fusions (transcriptional) of full length (PIGN) and a series of various lengths of 5' truncated rice PDC1 promoter (Δ1015PIGN, Δ770PIGN, Δ186PIGN and Δ82PIGN). Staining for GUS activity was performed for 40 min in shoots of PIGN and 10 min in shoots of Δ1015PIGN, Δ770PIGN, Δ186PIGN and Δ82PIGN.
Figure 4.9B: GUS histochemical staining in root sections of 7d old aerated and anoxic T₂ plants (segregating) expressing GUS fusions (transcriptional) of full length (PIGN) and a series of various lengths of 5' truncated rice PDC1 promoter (Δ1015PIGN, Δ770PIGN, Δ186PIGN and Δ82PIGN). Staining for GUS activity was performed for 40 min in roots of PIGN and 10 min in roots of Δ1015PIGN, Δ770PIGN, Δ186PIGN and Δ82PIGN.
Figure 4.9C: GUS histochemical staining in pollen of T2 plants (segregating) expressing GUS fusions (transcriptional) of full length (PIGN) and a series of various lengths of 5' truncated rice PDC1 promoter (Δ1015PIGN, Δ770PIGN, Δ186PIGN and Δ82PIGN). Staining for GUS activity was performed over night.
4.2.6 Identification of the PDC1 polypeptide among six PDC bands found in Western blots

It was previously shown that six bands were detected when various rice tissues were immuno-blotted using a polyclonal antibody raised against the rice PDC1 (fig. 4.1). To identify the PDC1 polypeptide among 6 bands, the patterns of the PDC polypeptides in the immunoblot of untransformed plants were compared with those of plants transformed with a construct expressing the rice PDC1 cDNA (chapter 5). Of 15 independently transformed lines of 6XARE-PDC1 (construct details in section 2.1.3) containing the introduced PDC1 gene, three lines PS26, PS29 and PS64 showed an increased level of bands 4 and 5 under hypoxic conditions (figs 5.4A, B and C) suggesting that bands 4 and 5 correspond to PDC1.

![Western blot image](image)

**Fig. 4.10:** Bacterially expressed rice PDC1 polypeptide co-migrating with band 4 of plant samples. The other band is of GST, since the antibody used here was raised against the thrombin digest of PDC1-GST.

- **GST-PDC1:** a thrombin digest separating the GST moiety from rice PDC1.

The expression vector (pGEX-PDC1) of the rice PDC1 was transformed into bacteria and PDC1 fused with the glutathione S-transferase (GST) (PDC1-GST) was isolated. To separate the GST portion of the fusion protein, PDC1-GST was digested by
thrombin. This digest and endosperm from untransformed Taipei were fractionated by SDS-PAGE and immuno-blotted. The bacterially expressed PDC1 (from thrombin digest) co-migrated with band 4 (fig. 4.10) again indicating that band 4 is likely to be PDC1.

### 4.3 Discussion

A total of six PDC bands were found in immuno-blots using a polyclonal antibody raised against the rice PDC1. Bands 3 and (4 and 5) are identified as the products of the PDC3 and PDC1 genes, respectively. That band 4 is indeed PDC1 is supported by the findings that (1) purified PDC1 from the bacterially expressed protein co-migrates with band 4 of the plant sample (fig. 4.10), (2) band 4 together with band 5 are increased in immunoblots of three 6XARE-PDC1 lines (figs 5.4A, B and C), (3) the lack of GUS expression in pollen of PDC1-GUS transformants (translational fusion-line P1G123, fig. 4.5M) correlates with the absence of band 4 in pollen of the immunoblot of wild type (figs 4.1 and 4.2B). Band 5 could be a breakdown or processed product of band 4. Since transgenic lines produce much more PDC1 than wild type, more of band 4 might be converted to band 5. However, neither band 4 nor band 5 migrates at the expected 65 kDa molecular mass of PDC1 but rather at 60 kDa. PDC is a cytosolic enzyme and is not likely to have an N-terminal signal sequence. Therefore, the difference is not likely to be cleavage of an N-terminal signal sequence. The likely reason for the discrepancy in apparent MWt which is supported by the co-migration of purified PDC1 with band 4, is that PDC1 migrates as a peptide with an apparent molecular mass of 60 kDa. Many peptides do not migrate at the expected MWt on SDS-PAGE; for instance, subunit 6 of the yeast cytochrome bc1 complex migrates with an apparently higher molecular mass (Trumpower, 1990).

Consistent with the pollen specific GUS expression of the PDC3-GUS transformants, a novel band (band 3) of 62 kDa was detected in pollen (figs 4.1 and 4.2B). Based on these results, band 3 is likely to be the PDC3 polypeptide. The pollen specific expression explains the failure to detect the PDC3 transcript in 3d old seedlings or anaerobically induced suspension cells and demonstrates that PDC3 is not a pseudo-
gene as has been suggested (Hossain et al., 1994b, 1996). The PDC3 promoter does not contain any of the cis-acting elements so far identified as responsible for pollen specific expression (Hamilton et al., 1998).

The PDC1-GUS transformants (translational fusion) show that the rice PDC1 promoter is anaerobically inducible (fig. 4.5) like the maize ADH1 promoter when it was introduced into rice fused to the GUS reporter gene. PDC1-GUS appears to be expressed in only some of the tissues where the maize ADH1 promoter is expressed (Kyozuka et al., 1991). Notably, the latter showed high expression in endosperm and pollen whereas rice PDC1 does not, suggesting that PDCs other than PDC1 are active in these tissues. It is shown in this thesis that rice PDC3 is highly expressed in pollen and from immuno blots of untransformed Taipei, that band 2 is present at a high level in endosperm. This suggests that PDC3 and the PDC gene coding for band 2 are operative in pollen and endosperm, respectively. The identity of the PDC gene encoding band 2 remains to be determined.

Analysis of PDC1-GUS (transcriptional fusions) transformants showed an intense GUS staining within 10-40 min in aerated shoots of transgenic plants containing a full length or the various lengths of truncated PDC1 promoter (fig.4.9A). In contrast, very little staining was seen in aerated shoots of plants containing the translational fusion of PDC1-GUS even after overnight incubation (fig. 4.5D). This suggests that there could be a loss of repression of the PDC1 promoter under aerobic condition in transformants with the transcriptional fusions. The rice PDC1 promoter (transcriptional fusion) in shoots of Δ1015PIGN, Δ770PIGN, Δ186PIGN and Δ82PIGN transformants and roots of Δ1015PIGN transformants was not further induced by anoxia but had the same high levels as in the aerated samples (fig. 4.9A and B) suggesting that PDC1 promoter functions like a constitutive promoter. In contrast, the translational fusion of PDC1-GUS and the full-length transcriptional fusion of PDC1-GUS (PIGN) gave anoxically induced expression in shoots and roots. Another anomaly is the GUS expression in pollen of transformants of all the transcriptional fusions of PDC1-GUS (fig. 4.9C). The PDC1-GUS translational fusion did not show pollen expression (fig. 4.5M) which is consistent with the absence of the rice PDC1 polypeptide (band 4 in figs 4.1 and 4.2B)
in pollen of untransformed WT. Therefore, three different kinds of misexpression took place in the transcriptional fusions. These were a high level of expression in air, a failure to increase expression under anoxia and strong expression in pollen.

Anaerobic induction is mediated by the GC and GT motifs in the ARE sequence and the extent of anaerobic induction is increased with the copy number of this ARE sequence (Walker et al., 1987; Olive et al., 1990; Hoeren et al., 1998). Several copies of these motifs are present in the rice PDC1 promoter (Hossain et al. 1994a). When the rice PDC1-GUS transcriptional fusions were introduced into rice protoplasts by electroporation, a progressive decline of GUS activity was observed with the reduction in the length of the PDC1 promoter (Grover et al., 1995). These conditions are likely to be somewhat aerobic, again suggesting a lack of repression in air.

There are two differences between the PDC1-GUS transcriptional and translational fusions. The first one is the presence of the rice ACTIN1 5’ intron of 500 bp in the transcriptional fusions and its absence in the translational fusion. The second is the absence of 87 bp of 5’UTR in the transcriptional fusions and its presence in the translational fusion (figs 2.4A and 2.5). One or other of these changes might be responsible for the disparities. It was shown that the incorporation of the rice ACTIN1 intron into the foreign gene transcription unit increased gene expression in transformed cereal cells and transgenic rice plants (McElroy et al., 1991, 1995; Xu et al., 1993). The use of the ACTIN1 intron in the potato pin2 promoter-GUS construct conferred increased GUS expression in transgenic rice without altering the expected pattern of pin2 promoter activity for wound responses (Xu et al., 1993), but there was no assessment of changes in expression in tissue types. Hence, the increased GUS expression in shoots of transcriptional fusions might be due to the presence of the rice ACTIN1 5’ intron. Another reason for the lack of regulation might be the lack of the 5’UTR of 87 bp in the transcriptional fusions or the upstream region in the truncated promoter-GUS fusions. The 87 bp region contains one GT-motif which could be important for aerobic repression and deletion of this may lead to unregulated expression in air. Alternatively, the 87 bp 5’UTR and upstream regions might be essential for the assembly of the anaerobic machinery of protein and DNA which represses the
expression under aerobic conditions. The expression of the transcriptional fusions in the pollen could be explained by the presence of a repressor which normally binds to the 5’UTR of the rice PDC1 in pollen and inhibits its expression. An alternative interpretation is that the 5’UTR may cause attenuation of transcription in pollen. When the 5’UTR sequence of the pollen-specific LAT59 (late anther tomato) gene was fused with reporter genes, gene expression in somatic cells and pollen was repressed by 20 to 300-fold both in transient expression experiments and stable transformation in tomato and tobacco (Curie and McCormick, 1997). It was shown that this sequence forms a putative stem-loop structure (calculated stability ΔG= -60 kcal/mol) that transcriptionally inhibits the expression of genes. RNA of the 5’UTR of rice PDC1 forms a stem-loop structure with Gibb’s free energy of only -11 kcal/mol (using the PC Gene RNA folding program, according to the Zucker algorithm) suggesting that the ‘attenuation of transcription’ interpretation is unlikely. In conclusion, the mechanisms for the misexpression of the transcriptional fusion are little understood; however, the expression pattern of the translational fusion of the PDC1 promoter is consistent with the immuno-blotting studies of the expression of the rice PDC1 gene in WT plants.

Besides constitutively expressed genes, there are at least two sets of genes expressed at different times according to their activities during pollen development (reviews; Mascarenhas, 1989, 1990; McCormick, 1993). The first set is expressed soon after meiosis is completed; mRNA reaches the maximum level by late pollen interphase and then decreases gradually by the stage of anthesis. The other set of genes, called pollen-specific genes, are expressed at pollen mitosis I. The levels of their mRNAs increase and reach a peak at maturity, implying possible roles in germination and pollen tube formation. The rice PDC3 gene showed pollen-specific expression commencing after the first mitosis of the microspores, since spikelets collected before microspore mitosis (6 cm in fig. 4.2) do not contain PDC3 (band 3). Anthers and pollen at the dinucleate stage (8 cm in fig. 4.2) expressed PDC3, which reached its maximum level during anthesis, suggesting a role for PDC3 during pollen development.

Tadege and Kuhlemeier (1997) suggested a role for PDC after observing aerobic fermentation in tobacco pollen. According to their hypothesis, acetyl-CoA may form
indirectly from pyruvate through the so-called "PDH bypass" which consists of three steps catalysed sequentially by PDC, aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS). Acetyl-CoA then may be utilised for lipid biosynthesis and used by the glyoxylate and TCA cycles to meet the high demand for intermediates for biosynthesis of the cell components during the germination and fast growth of pollen tubes. Observations that support this hypothesis are (1) ALDH cloned from tobacco pollen, is highly expressed in pollen and uses acetaldehyde as a substrate (Camp and Kuhlemeier, 1997); (2) when labelled acetaldehyde, acetate and ethanol were added to germinating pea and excised tissues of pea and Eastern cottonwood, the majority of the label was found in isocitrate, α-ketoglutarate, glutamate and succinate and some label was also incorporated into amino acids and proteins (Cossins and Turner, 1963; Cameron and Cossins, 1967; MacDonald and Kimmerer, 1993). Tadege and Kuhlemeier (1997) demonstrated that in tobacco pollen, the ethanol flux is regulated by sugar concentration rather than oxygen availability. In glucose limited medium, PDC cannot compete with PDH for pyruvate as a substrate since the $K_m$ value of the former is much higher than the latter. However, in a high metabolic state and with an excess of carbohydrates such as occurs in rice seeds and pollen, pyruvate concentration can be high, and both oxidative metabolism and ethanol fermentation can take place simultaneously in presence of oxygen (Tadege and Kuhlemeier, 1997).

The results presented here support this interpretation since in rice pollen, the expression of $PDC3$ is highest after anthesis when the environment is fully aerobic, suggesting the involvement of PDC in ethanol fermentation is unlikely and the assimilating PDH bypass may be operating at this stage. The constitutively high expression of $PDC1$ and band 2 (which is likely to be encoded by $PDC2$ or $PDC4$) in embryos during germination under air might have a role in supplying intermediates for biosynthesis needed for the emergence of shoots and roots. An antisense construct of rice $PDC3$ could be used to test the new role of PDC in pollen but the knockout of PDC in pollen may cause pollen sterility. If PDC is essential to pollen germination and growth, this antisense approach might give rise to male sterile plants and could be very useful for hybrid rice biotechnology.
Chapter 5

Evaluation of the Role of PDC in Anaerobic Tolerance
5. MANIPULATION OF THE LEVEL OF PYRUVATE DECARBOXYLASE IN RICE
AND EVALUATION OF ITS ROLE IN ANAEROBIC SURVIVAL

5.1 Introduction

Higher plants utilise mainly EF under anaerobic conditions. However, they vary widely in their ability to tolerate anaerobiosis. Following the onset of anaerobiosis, plants stop synthesising normal proteins and induce novel polypeptides, the ANPs, providing evidence that ANPs play a major role in a plant’s survival under low oxygen conditions (Sachs et al., 1980; Bertani et al., 1981; Dolferus et al., 1985; Dennis et al., 1987; Ricard and Pradet, 1989; Russell et al., 1990). The two EF enzymes, PDC and ADH have attracted attention in the past for their possible role in anoxia tolerance. The reduced anoxia tolerance of adh null mutants and the correlation of the induced level of ADH with anoxia tolerance has been discussed in detail in section 3.1. The importance of PDC for anoxia tolerance was supported by the increased levels of PDC and ADH activities and elevated levels of ATP and total adenylates seen in maize and wheat root tips after hypoxic pre-treatment, and the correlation of their increased activities with improved anoxia tolerance (Saglio et al., 1988; Johnson et al., 1989; Waters et al., 1991). The emphasis on PDC for limitation of anaerobic tolerance compared to other glycolytic enzymes and ADH was made because this enzyme is at the branch point of EF, and its level is lower than that of ADH by a factor of between 8-65 (John and Greenway, 1976; Wignarajah and Greenway, 1976; Morrell and Greenway, 1989). The closeness of the in vitro PDC activity to the in vivo ethanol fermentation rate stresses that PDC activity is the key regulator of EF under conditions of oxygen deprivation (Morrell et al., 1990; Waters et al., 1991; Drew, 1997).
The regulation of PDC activity is complex and is achieved in a number of ways. Being an allosteric enzyme, PDC exhibits positive co-operativity and a sigmoidal relationship between velocity and pyruvate concentration (Kenworthy and Davies, 1976; Langston-Unkefer and Lee, 1985; Lee and Langston-Unkefer, 1985; Zehender et al., 1987; Rivoal et al., 1997). Several cations and anions are reported to be competitive inhibitors, in some cases in vitro, of PDC (Lee and Langston-Unkefer, 1985; Rivoal et al., 1990).

Mg\textsuperscript{++} and thiamine pyrophosphate (TPP) (cofactor and coenzyme of PDC, respectively) are essential for the polymerisation of yeast cytoplasmic PDC subunits. However, TPP is tightly bound at the pH optimum of PDC (pH 6.5), and alkali-induced (pH 8-8.5) subunit dissociation occurs in yeast PDC with the concomitant release of the coenzyme molecules and the magnesium ions (Gounaris et al., 1971, 1975; Hopmann, 1980). Furthermore, a significant lag phase was found in PDC assays before maximum activity had been attained and therefore, PDC was called a hysteretic enzyme (Frieden, 1970, 1971; Egan and Sable, 1981; Ulrich, 1982; Langston-Unkefer and Lee, 1985; Morrell et al., 1990). It was suggested that PDC is converted from an inactive form to an active form through auto catalysis in the presence of pyruvate and low pH. This slow conversion between the two forms of the enzyme represents an in vivo time buffering regulatory mechanism to prevent the decarboxylation of pyruvate in response to transient changes in pH (Kenworthy and Davies, 1976; Hubner et al., 1978).

It was important to manipulate the levels of PDC to investigate the possibility of an increase in anaerobic tolerance. Over-expression of Zymomonas PDC in tobacco and potato has been reported to result in no significant increase in anoxia tolerance (Bucher et al., 1994; Tadege et al., 1998a, 1998b). We sought to over- and under-express PDC in rice and to determine whether the level of PDC was limiting anoxia tolerance. The manipulation of the levels of ADH has been described in chapter 3. This chapter describes the anoxia tolerance of plants over-producing PDC and plants which over-produced both PDC and ADH.
5.2 Results

5.2.1 Detection of sense and antisense \textit{PDC1} transformants by Southern blotting and PCR

Sense and antisense transformants carrying the rice \textit{PDC1} were selected in the presence of hygromycin during tissue culture. However, since the hygromycin resistance gene and the gene of interest (\textit{PDC1}) were in two separate constructs used together for transformation, PCR and Southern hybridisation were performed in the T\textsubscript{0} generation to establish the presence of the \textit{PDC1} transgene. The restriction enzyme \textit{SpeI} was used to digest genomic DNA for Southern analysis. \textit{SpeI} has a unique recognition site in the \textit{PDC1} sense and antisense constructs employed for transformation, cutting outside the \textit{PDC1} cDNA. The number of transgene incorporated (excluding concatamers) into the genome can be deduced from the Southern blot by comparing the number of bands in the transgenic plant with that of WT plants following probing with the rice \textit{PDC1} cDNA. In most transgenic lines, the insertions were present in multiple copies (fig. 5.1).

Twenty-one independent antisense \textit{ACT1-PDC1} lines out of 25 hygromycin resistant lines inherited the \textit{PDC1} transgene (table 5.1). Some of the transformants carrying \textit{6XARE-PDC1} (sense) and \textit{ACTIN1-PDC1} (sense) were identified by PCR. Primers (sequences of the primer pairs written in section 2.3.2) amplified the region from bases 215 to 536 of cDNA of the introduced rice \textit{PDC1}, yielding a 322 bp-sized fragment (figs 5.2A and 5.2B). The primer set also amplified the endogenous \textit{PDC1} gene, producing a fragment of 424 bp long due to the presence of an intron in the region between the primer pairs. As a control for the PCR results, primers for the amplification of the rice sucrose synthase 1 (\textit{SS-1}) gene were synthesised (section 2.3.2) and \textit{PDC1} and \textit{SS-1} genes were amplified in the same reaction. The \textit{SS-1} primers produced a 547 bp-sized fragment (fig. 5.2A). Two larger bands (fragments 'x' and 'y' in fig. 5.2B) were also visible when \textit{PDC} PCR products were Southern blotted, using the \textit{PDC1} cDNA as a probe. Fragments 'x' and 'y' might be the amplification products from the other endogenous \textit{PDCs} of rice which have high similarity at the nucleotide level to \textit{PDC1}.
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(Hossain et al., 1996; Rivoal et al., 1997). The PCR and Southern analysis showed that 19 independently transformed lines of sense 6XARE-PDCI and 5 lines of ACTIN1-PDCI contained the PDC1 transgene (table 5.1).

![Southern blot](image)

**Figure 5.1**: Southern blot of T<sub>0</sub> plants of independent transgenic lines of the sense and antisense PDC1. DNAs were digested with SpeI and probed with radio-labelled PDC1 cDNA.

- DNA molecular weight marker: λ DNA/ PstI fragments.
- WT: untransformed Taipei 309 as a negative control.
- Control: unlabelled probe (PDC1 cDNA) as a positive control.
- Negative antisense PDC1 lines: PA40, PA41.
- Positive antisense PDC1 lines: PA57, PA65, PA69.
- Negative sense PDC1 (6XARE-PDC1): PS44, PS48, PS60, PS61, PS62, PS66, PS68.
- Note: lines PS51, PS54, PS60 and PA65 were sterile.
Figure 5.2A: PDC PCR of DNA from T₀ plants of sense and antisense PDC1 transformants.

The copy number of the inserted transgene(s) was estimated in individual plants of three families of line PS64 and one family of line PS29 of 6XARE-PDC1 (fig. 5.3). In the Southern blot, 6 bands in line PS29, 4 bands in untransformed Taipei (WT), and 7 bands in line PS64 hybridised to the rice PDC1 cDNA. This suggests that 2 and 3 copies of the PDC1 transgene have been introduced into lines PS29 and PS64, respectively. Four to five progeny of two homozygous families PS64-3 and PS64-4 of line PS64 exhibited the same banding pattern, implying that the 3 copies are inserted at the same locus of line PS64 and have passed together into the next generation. Four progeny of one family (PS29-6) of PS29 also exhibited bands of equal size and numbers suggesting that the 2 copies of the transgene in line PS29 are linked.
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Figure 5.3: Genomic Southern of individual T\textsubscript{2} plants of families of two 6XARE-PDC\textsubscript{I} transgenic lines (PS29 and PS64) and WT plants. DNA was digested with Spe\textsubscript{I} and the radio-labelled PDC\textsubscript{I} cDNA was used to probe the blot.

- WT: untransformed Taipei 309 as a negative control.
- Families (PS64-3) and (PS64-4): two homozygous families of line PS64.
- Family (PS64-5): an azygous family of line PS64.
- Family (PS29-6): a segregating family of line PS29.
- Marker: SSP-1 DNA/ EcoRI fragments (Bresatec).

Table 5.1: Summary of the characterisation of the PDC\textsubscript{I} transgenic lines.

<table>
<thead>
<tr>
<th>Construct</th>
<th>N\textsuperscript{o} calli shot</th>
<th>Total N\textsuperscript{o} lines</th>
<th>Presence of the inserted gene</th>
<th>% frequency co-transformation (b/a)</th>
<th>N\textsuperscript{o} lines of ( T_1/T_2 ) seed</th>
<th>N\textsuperscript{o} sterile lines</th>
<th>N\textsuperscript{o} lines with altered level of PDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>6XARE-PDC\textsubscript{I}</td>
<td>462</td>
<td>24</td>
<td>19</td>
<td>5.6</td>
<td>79.2</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>ACT1-PDC\textsubscript{I}</td>
<td>281</td>
<td>5</td>
<td>5</td>
<td>5.62</td>
<td>100</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>ACT1-CDP\textsubscript{I}</td>
<td>362</td>
<td>25</td>
<td>21</td>
<td>6.9</td>
<td>84</td>
<td>18</td>
<td>3</td>
</tr>
</tbody>
</table>

\% Co-transformation frequency: c/b

CDP\textsubscript{I}: antisense PDC\textsubscript{I}
5.2.2 Sense 6XARE-PDC1 transformants

5.2.2.1 Study of the expression of the PDC1 transgene at the polypeptide level

Fifteen fertile transgenic lines were found to contain the PDC1 transgene by Southern blotted and PCR. To investigate the expression of the PDC1 transgene, the lines were analysed by Western blotting using a rabbit polyclonal antibody raised against the rice PDC1 polypeptide (section 2.4.4.3). The transgene was fused with an anaerobically inducible promoter (6XARE) and therefore, total protein was extracted from hypoxically treated plants.

Band intensities of transgenic plants were compared with those of WT in Western blots in order to detect over-producing PDC1 lines. Plants of three lines, PS26, PS29 and PS64 showed a higher intensity of bands 4 and 5 in shoots compared to WT plants (fig. 5.4A, 5.4B, 5.4C). A relative increase of PDC1 polypeptide of 14 fold in comparison with WT (using program Kodak Digital Science ID) was found in shoots of homozygous family PS64-4 of line PS64 (fig. 5.4C). Over-expression of PDC1 in shoots of each of the three lines PS26, PS29 and PS64 was studied in separate experiments. Therefore, it was not possible to compare the extent of increase of PDC1 among these three lines.
Figure 5.4 (A, B, & C): Western blotting analysis to study the over-expression of \textit{PDC1} at the polypeptide level in segregating T\textsubscript{1} population of lines PS26 and PS29 and in homozygous family PS64-4 compared to WT plants. Total protein was extracted from shoots of hypoxically treated plants and 50 \(\mu\text{g}\) of protein loaded per lane and separated by SDS-PAGE. The blot was hybridised to a polyclonal antibody raised against the rice \textit{PDC1} polypeptide.

Homozygous families expressing the rice \textit{PDC1} transgene and azygous families with a similar levels of PDC as WT plants of lines PS26 and PS64 were raised according to section 3.2.2.2. Twenty plants of each family of lines PS26 and PS64 and plants of WT were studied by Western blotting analysis to determine which were the homozygous and azygous families. Families PS26-3 and PS26-12 and families PS64-3
and PS64-4 had increased levels of PDC1 (bands 4 and 5 in fig. 5.5; see also section 4.3) compared to WT and are homozygous, whereas families PS26-8 and PS64-5 had a similar level of PDC1 as WT and are azygous.

Figure 5.5: Western blotting analysis to identify the homozygous family PS64-3. Total protein was extracted from leaf blades of two months old air grown plants and 100 μg of total protein per lane was fractionated by SDS-PAGE and the blot was hybridised with a polyclonal antibody raised against the rice PDC1.

5.2.2.2 Western blotting analysis of lines PS29 and PS64

5.2.2.2.1 Expression in air and under hypoxia

The anaerobically inducible promoter 6XARE (section 2.1.3) was fused with the rice PDC1 cDNA in order to drive its expression under anaerobic conditions. The levels of PDC1 in shoots of one homozygous family PS64-4 of the over-expressing line PS64 were compared with those of WT in air and under hypoxia by Western blotting analysis (fig. 5.6). PDCs were induced in shoots of WT and PS64-4, and PDC1 was induced at a higher level in shoots of PS64-4 than in WT under hypoxia. A higher level of PDC1 expression was also found in shoots of PS64-4 grown in air compared to WT, suggesting that the 6XARE promoter confers the PDC1 expression in air. However, the
expression of the 6XARE-PDC1 transformants was less in air than under hypoxic conditions, suggesting that the 6XARE promoter directs hypoxic induction of genes.

**Figure 5.6:** Western blotting analysis to compare the expression of the transgene PDC1 in shoots of three week old plants of the homozygous family PS64-4 of line PS64 and WT (untransformed Taipei) in air and under hypoxia. 50 µg of total protein per lane was separated by SDS-PAGE and the blot was hybridised with an antibody raised against the rice PDC1.

### 5.2.2.2.2 Expression in endosperm and roots

The PDC1 transgene was expressed in shoots, leaf sheaths and leaf blades of lines PS26, PS29 and PS64. Expression was also studied in endosperm and roots of these transformants, and their levels of PDC1 were compared with that of WT controls. A similar level of PDC1 was present in endosperm of two homozygous families PS64-3 and PS64-4, segregating T1 populations of line PS29, WT and an azygous family PS64-5 (fig. 5.7). PDCs were induced in roots of WT and PS64-4 by hypoxia (fig. 5.8). However, the levels of PDC1 found in aerated and hypoxic roots of the homozygous family PS64-4 and WT were very similar. These results suggest that the transgene PDC1 was not expressed in endosperm or roots of 6XARE-PDC1 transgenic plants in either aerated or hypoxic conditions.
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Figure 5.7: Western blotting to analyse the expression of \textit{PDC1} transgene in endosperm of non-germinated seeds of transformants and WT plants. 25 \( \mu \text{g} \) of total protein per lane was fractionated by SDS-PAGE and immuno-detected using an antibody raised against the rice PDC1.

- WT: untransformed Taipei.
- PS64-3 and PS64-4: two homozygous families of line PS64.
- PS64-5: an azygous family of line PS64.
- PS29: a segregating T\textsubscript{1} population of line PS29.

Figure 5.8: Western blotting analysis to compare the levels of PDC polypeptides in aerated and hypoxic roots of three week old plants of WT and a homozygous family (PS64-4) of 6\textit{XARE-PDC1} line PS64. 50 \( \mu \text{g} \) of total protein per lane was fractionated by SDS-PAGE and immuno-detected using an antibody raised against the rice PDC1.
5.2.2.3 Assays of the *in vitro* PDC activity of lines PS26 and PS64

PDC activity was estimated in plants of the over-producing PDC1 lines PS26 and PS64 and WT plants. The activity level of PDC was very low in rice plants in air and hence, the activity was measured in hypoxically treated plants. Older leaf blades showed virtually no PDC activity and therefore, activity was measured separately in leaf sheaths and 3rd leaf blades of hypoxically treated 19d old plants. *In-vitro* PDC activity of two homozygous families PS26-3 and PS26-12, one azygous family PS26-8 (with a similar level of PDC1 polypeptide as WT plants) and WT plants was assayed (fig. 5.9). Families PS26-3 and PS26-12 had an average PDC activity of 66 nmol/min/mg protein (66 units) in leaf sheaths and contained 218% of the PDC activity found in WT plants (30 units). The level of PDC was very low (0.07 units) in leaf blades of WT and 5 units (approx. 7000% of WT) in the two homozygous families of line PS26. No significant difference in PDC activity was found between plants of WT and the azygous family PS26-8.

![Figure 5.9: In vitro PDC activity in leaf sheaths of hypoxically treated 19d old plants of two homozygous families PS26-3 and PS26-12, WT, and an azygous family PS26-8 (same level of PDC1 as WT). Standard errors were calculated from PDC activities in leaf sheaths of three individual plants.](image-url)
Plants of two homozygous families PS64-3 and PS64-4, one azygous family PS64-5 of line PS64 and WT were also assayed by in vitro PDC activity. The levels of PDC in families PS64-3 and PS64-4 were 206% of WT in leaf sheaths and approx. 6500% of WT in leaf blades. The combined in vitro PDC activity in leaf sheaths and 3rd leaf blades in family PS64-4 was only 2 fold higher than that of WT, whereas the increase in PDC1 polypeptide level of PS64-4 in shoots was 14 fold higher than WT (fig. 5.4C).

5.2.2.4 Measurement of the levels of acetaldehyde and ethanol produced in the over-expressing 6XARE-PDC1 lines

The levels of ethanol and acetaldehyde produced by the over-expressing 6XARE-PDC1 transformants was compared with that of WT plants. Excised leaf sheaths of hypoxically treated 26d old plants of homozygous and azygous families of two over-expressing lines PS26 and PS64 and WT were incubated in an anoxic medium (0.1 M glucose). The levels of ethanol and acetaldehyde were measured at different time intervals by gas chromatography (GC).

The contents of ethanol produced by leaf sheaths of WT plants were 43 and 7412 nmol/g FWt after 2h and 32h incubation, respectively (fig. 5.10A). The azygous family PS26-8 of line PS26 produced a similar level of ethanol as WT. However, two homozygous families, PS26-3 and PS26-12 of line PS26, produced 85, 411, 1705, 5814, and 8673 nmol/g FWt ethanol on average after 2h, 4h, 8h, 24h and 32h incubations, which were 198%, 150%, 136%, 108%, and 117% of WT ethanol levels. This suggests that the level of ethanol produced by the over-producing plants was higher than WT plants; however, the ability of the over-expressing PDC1 plants to produce ethanol decreased with time compared to WT plants. The homozygous families PS64-3 and PS64-4 of another line, PS64, which over-produced PDC1, gave similar results as line PS26 (fig. 5.10B).
Figure 5.10: Levels of ethanol produced by the 6XARE-PDCI lines PS26 and PS64 compared to the wild type and an azygous family PS26-8 (similar level of PDC as WT) of line PS26. Ethanol contents were measured by GC. Standard errors were calculated from ethanol contents produced over various time intervals by three replicates of leaf sheaths.
The level of acetaldehyde was also measured in plants of PS26, PS64 and WT. Under the assay conditions, leaf sheaths of WT plants produced 6 and 10 nmol/g FWt acetaldehyde during 2h and 4h incubations, respectively (fig. 5.11A). The azygous family PS64-5 produced similar levels as WT up to 4h incubation and slightly lower levels afterwards. The homozygous families PS64-3 and PS64-4 of line PS64 produced 14, 19, 28, and 28 nmol/g FWt acetaldehyde on average after 2h, 4h, 6h, and 8h incubations, respectively. These correspond to 233%, 173%, 127%, and 112% acetaldehyde of that produced by WT. Therefore, in a similar manner to ethanol production, the ability to produce acetaldehyde by the over-producing PDC plants decreased with time compared to WT plants. In all varieties, the production of acetaldehyde decreased after 8h incubation, which might be due to the fact that acetaldehyde is very volatile and loss from the syringe may have exceeded production. Acetaldehyde loss from the syringe with time has been observed in one control experiment.

In line PS26, leaf sheaths of WT produced 7 and 17 nmol/g FWt acetaldehyde after 2h and 4h incubations, respectively (fig. 5.11B). The azygous family PS26-8 produced 157%, 118% and 106% whereas two homozygous families PS26-3 and PS26-12 produced 193%, 197% and 102% (average values) acetaldehyde of that produced by WT after 2h, 4h and 8h incubations.
5.11B. Line PS26

![Graph showing levels of acetaldehyde production](image)

**Figure 5.11**: Levels of acetaldehyde produced in leaf sheaths of two homozygous families of each the two over-expressing $PDC_{I}$ lines PS26 and PS64, compared to plants of WT and their azygous families under anoxia and in the presence of exogenous glucose. Acetaldehyde contents were measured by GC. Standard errors were calculated from acetaldehyde contents produced over various time intervals by three replicates of leaf sheaths.

5.2.2.5 Anaerobic Survival

**5.2.2.5.1 Investigation of the effects of anoxia on germination and coleoptile growth of the sense 6XARE-PDC_{I} transformants**

Effects of anoxia on germination of the two homozygous families PS64-3 and PS64-4 were compared with WT and an azygous family PS64-5 (with a similar PDC level as WT). Between 59-61 seeds of each variety were germinated in flasks for 5d
under anoxia (N₂) and in the dark (section 2.4.6). The length of coleoptiles was measured after the treatment. Root growth was inhibited completely in all seeds from all varieties under anoxia. Etiolated coleoptiles of 19-22 mm were measured in WT seedlings with two exceptions which were 2 mm and 7 mm long (fig. 5.12). Most germinated seeds of the azygous family PS64-5 and the homozygous family PS64-3 had coleoptiles of 10-12 mm long, and 58% of the germinated seeds of the homozygous family PS64-4 had coleoptiles ranging in size from 10-12 mm. Zero to 5 mm long coleoptiles were also found in PS64-3, PS64-4 and PS64-5 after 5d anoxic germination, which constituted about 42% seeds of PS64-4. However, these seeds were transferred to air, scored after a 4d recovery as 'no germination' or 'albino' (white columns in fig. 5.12) and eventually died. Experiments which involved conducting germination under anoxia were carried out using segregating T₁ seeds of the over-expressing PDC1 lines PS26 and PS29 along with WT seeds. There was no significant difference in coleoptile lengths of 19-22 mm long between WT and transgenics after 5d germination in anoxia (data not shown) suggesting that over-expression of the PDC did not have any effect during germination under 5d anoxia.

In normal conditions, plants from the two homozygous families PS64-3 and PS64-4 of the over-expressing PDC1 line PS64 were shorter at an early age compared to plants of WT and the azygous family PS64-5 (fig. 5.13). In another experiment, 20 seeds of WT, PS64-5, PS64-3 and PS64-4 were germinated under normoxia (see section 2.4.1). The number of green viable plants obtained was 20 for WT, 20 for PS64-5, 19 for PS64-3 and 10 for PS64-4. The height from the base of the shoot to the tip of the leaf blade of each 23d old hydroponically grown plant was measured and the average value (± standard error) was 35±0.4 cm for WT, 31±0.8 cm for PS64-5, 27±0.7 cm for PS64-3 and 26±0.4 cm for PS64-4. However, in 8-10 week old plants, this "shorter plant" phenotype disappeared and plants of families PS64-3, PS64-4, and PS64-5, and WT grew at the same rate and flowered nearly at the same time. In contrast, the two other over-expressing PDC1 lines PS26 and PS29 did not show this "shorter plant" phenotype under normal conditions suggesting that the phenotype in line PS64 is originated from tissue culture and transformation and not from the over-expression of the PDC1.
Figure 5.12: Comparison of coleoptile length of WT, PS64-3, PS64-4 and PS64-5 seeds germinated for 5d under anoxia.

- White columns: scored as either non-germinated or albino plants after 4d recovery following germination under anoxia.
- Green columns: scored as green and viable plants after 4d recovery following germination under anoxia.
Figure 5.13: Three week old plants of WT, an azygous family (PS64-5) and two homozygous families PS64-3 and PS64-4 of the over-expressing \textit{PDCI} line PS64 grown in the glasshouse.

5.2.2.5.2 Assay of anoxia tolerance in mature plants of the over-expressing \textit{PDCI} lines

Anaerobiosis was imposed on mature plants (26d old) of one azygous and two homozygous families from each of the two over-expressing \textit{PDCI} lines PS26 and PS64 and plants of WT. Plants were submerged in an anaerobic aquarium filled with nutrient solution (fig. 3.13) for 24h hypoxia followed by 66h anoxia. Plants were scored as either alive or dead after a 10d recovery period following the end of the anaerobic stress. 100\% and 90\% survival rate were observed in FR13A and IR42, respectively (fig. 5.14). 79\% of untransformed Taipei (WT) plants survived. The lowest survival (26\% on average) was found in plants of the two homozygous families PS26-3 and PS26-12 of line PS26 with an increased level of PDC. Therefore, transgenic plants with increased level of PDC activity had reduced anoxia tolerance compared to that of plants of WT and the azygous family PS26-8 which showed 57\% survival. The lower survival
of the azygous family compared to WT plants may be due to the effects of tissue culture and transformation procedures.

![Graph showing anaerobic survival rates](image)

**Figure 5.14:** Effects of anoxia on mature plants of two homozygous families PS26-3 and PS26-12 of the over-expressing \(PDC1\) line PS26 compared to that of WT plants and an azygous family PS26-8. Standard errors were calculated from three aquariums’ values of each variety.

The homozygous families PS64-3 and PS64-4 and azygous family PS64-5 of line PS64 showed similar kinds of survival patterns (fig. 5.15) as families of line PS26.
Figure 5.15: Effects of anoxia on mature plants of two homozygous families PS64-3 and PS64-4 of the over-expressing PDC1 line PS64 compared to that of WT plants and an azygous family PS64-5 of the same line. Standard errors were of two aquariums’ values of each variety.

5.2.3 Sense and antisense ACTIN1-PDC1 transformants

Five independently transformed and fertile lines of sense ACTIN1-PDC1 were found to contain the PDC1 transgene by PCR analysis (fig. 5.2). They were assayed for transgene expression by Western blotting analysis using the same polyclonal antibody as was used for the detection of the over-expressing 6XARE-PDC1 lines. The sense ACTIN1-PDC1 lines had PDC1 polypeptide levels which were similar to WT and were not tested further. Eighteen independently transformed and fertile lines of the antisense ACTIN1-PDC1 were found to inherit the antisense PDC1 using Southern blotting (fig. 5.1). None of the 18 lines showed reduced levels of PDC1 or other PDC polypeptides (fig. 5.16) when analysed by Western blotting.
Figure 5.16: Comparison between the level of PDCs in WT and line PA75 of the antisense ACTIN1-PDC1 by Western blotting analysis. Total protein was extracted from shoots of hypoxically treated three week old plants and 50 μg protein per lane was fractionated by SDS-PAGE. The blot was immuno-detected using an antibody raised against the rice PDC1.

5.2.4 Transformants over-producing both PDC and ADH activities

5.2.4.1 Comparison of acetaldehyde and ethanol production among WT plants and transgenic plants over-expressing PDC, ADH and both PDC and ADH

Transgenic plants of the homozygous family PS26-12 of the over-expressing 6XARE-PDC1 line PS26 were crossed to plants of the homozygous family AS8-10 of the over-expressing UBIQUITIN1-ADH2 line AS8 in order to combine these two traits, and 288 cross seeds were produced. Hybrid plants (♀ AS8-10 x ♂ PS26-12) were analysed for the expression of ADH and PDC transgenes by ADH enzyme assay and Western blotting (data not shown).

Plants of ♀AS8-10 x ♂PS26-12 which were heterozygous for both transgenes, were investigated for their ability to produce acetaldehyde and ethanol. Plants were treated hypoxically for 16h and then excised leaf sheaths were assayed for the production of these two metabolites using gas chromatography. The amount of ethanol produced by leaf sheaths of WT was 363 nmol/g FWt after 8h (fig. 5.17). Transgenic lines AS8-10, and PS26-12 and hybrid line ♀AS8-10 x ♂PS26-12 produced 405, 520
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and 458 nmol/g FWt ethanol after 8h. These correspond to 112%, 143% and 126% of the ethanol produced by WT. Therefore, leaf sheaths of the homozygous plants over-expressing PDC produced more ethanol than leaf sheaths of homozygous plants over-expressing ADH, heterozygous plants over-producing both PDC and ADH and WT plants in the presence of exogenous glucose.

![Graph showing ethanol production](image)

**Figure 5.17:** Comparison of ethanol production from excised leaf sheaths of plants over-expressing PDC, ADH, both PDC and ADH, and of WT in presence of exogenous glucose. Ethanol contents were measured by GC. Standard errors were calculated from ethanol contents produced over various time intervals by three replicates of leaf sheaths.

The acetaldehyde content was also measured. The level of acetaldehyde in WT and line AS8-10 was 21 nmol/g FWt after 8h whereas the levels in PS26-12 and hybrid line ♀AS8-10 × ♂PS26-12 were 43 and 30 nmol/g FWt, respectively (fig. 5.18). These are 205% and 143% of WT produced after 8h, respectively. In a similar manner as the ethanol level, the acetaldehyde level was higher in the over-producing PDC plants than in plants of WT, over-producing ADH or over-producing both PDC and ADH.
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Figure 5.18: Acetaldehyde production from excised leaf sheaths of plants over-expressing PDC, ADH, both PDC and ADH, and WT plants in the presence of exogenous glucose. Acetaldehyde contents were measured by GC. Standard errors were calculated from acetaldehyde contents produced over various time intervals by three replicates of leaf sheaths.

5.2.4.2 Comparison of anoxia tolerance among WT plants and over-expressing PDC, ADH and both PDC and ADH transformants

Twenty-six day old plants of WT, homozygous families AS8-10 (ADH over-producer) and PS26-12 (PDC over-producer) and hybrid plants (♀AS8-10 x ♂PS26-12: heterozygous of both PDC and ADH transgenes) were treated anaerobically in aquariums (fig. 3.13; section 2.4.8). The flooding tolerant and intolerant varieties FR13A and IR42 were also included and showed 100% and 20% survival, respectively (fig. 5.19). Plants from untransformed Taipei and line AS8-10 showed 21% and 24% survival, respectively. All 90 plants of the over-producing PDC line PS26-12 died after
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the anaerobic treatment. In contrast, plants over-producing both PDC and ADH showed 10% survival. Hence, addition of the ADH over-expression trait into the over-expressing PDC transgenic line increased tolerance to anoxia compared to transgenic plants over-expressing PDC alone.

![Graph showing anoxic tolerance of plants over-expressing PDC, ADH, both PDC and ADH and WT plants. Between 90-110 plants from each variety were tested. Standard errors were of three aquariums’ values of each variety.]

Figure 5.19. Anoxic tolerance of plants over-expressing PDC, ADH, both PDC and ADH and WT plants. Between 90-110 plants from each variety were tested. Standard errors were of three aquariums’ values of each variety.

5.3 Discussion

Mutants lacking ADH have been isolated in barley (Harberd and Edwards, 1982b), maize (Schwartz, 1969), Arabidopsis (Jacobs et al., 1988) and tomato (Wisman et al., 1991), and an adh1 null mutant has been isolated in rice with growth and fertility similar to WT (Matsumura et al., 1995). A pdc null mutant has yet to be found in any plant species. We failed to obtain any down-regulated PDC line of rice from 18 independently transformed lines using an antisense PDC1 construct driven by the rice ACTIN1 promoter. The failure to obtain down-regulated PDC transgenic lines and the
abundance of rice PDCs in embryo and pollen even under aerobic conditions (figs 4.1 and 4.2), indicate that PDC may have a crucial role in plants, particularly in embryo and pollen growth.

In the over-expressing 6XARE-PDCI line PS64, PDC1 polypeptide levels were increased by 14 fold compared to WT (fig. 5.4C) but the enzyme activity increased by only 2 fold (section 5.2.2.3). Two possibilities might explain the observed results. (1) PDC1 alone might not be able to form the tetrameric holoenzyme of rice PDC (Rivoal et al., 1990, 1997) whose subunit composition is still unknown. Band 4 (PDC1) had a somewhat similar intensity to other PDC bands in the Western blots in various tissues of rice excluding endosperm and root, and supports a 1:1 stoichiometry (fig. 4.1). (2) However, since PDC activity was increased in the over-expressing 6XARE-PDCI lines (fig. 5.9), it is likely that some PDC1 may form tetrameric holo-PDC with the other residual PDC polypeptides in the transgenic plants. Alternatively, the PDC1 polypeptide alone might form the holo-PDC. But in both cases, the specificity of the active site, coenzyme- and cofactor-binding site in binding substrate (pyruvate), coenzyme (TPP) and cofactor (Mg^{++}), respectively, of these alien/ectopic holo-PDCs might be lower than that of the native holoenzyme. There are many possible combinations for the formation of a PDC holoenzyme. The PDC enzyme from yeast and bacteria is a dimer of dimers but the plant enzyme is an octamer formed by two tetramers arranged side by side (Konig et al., 1998; for reviews see Candy and Duggleby, 1998; Furey et al., 1998; Konig, 1998). PDC tetramers from yeast, maize, and wheat were suggested to be of either \( \alpha_4 \) or \( \alpha_2 \beta_2 \) composition (Ludewig et al., 1974; Ullrich, 1982; Zehender et al., 1987). However, an \( \alpha_4 \) homotetramer was isolated and later purified from bakers yeast by Kuo et al. (1986) and \( \alpha_1 \beta_3, \alpha_2 \beta_2, \alpha_3 \beta_1, \) and \( \beta_4 \) might also exist (Dyda et al., 1990; Furey et al., 1998).

Over-production of the PDC1 polypeptide occurred in shoots, leaf sheaths and leaf blades of three independently transformed lines (PS26, PS29 and PS64) of 6XARE-PDCI. In contrast, no expression of the rice PDCI transgene was observed in roots and endosperm of these transgenic lines. Transgenic cotton plants over-expressing PDC have been generated using the rice PDCI driven by a CaMV 35S-promoter (Ellis et al.,
manuscript in preparation). Over-expression of PDC1 occurred in cotyledons and leaves of three lines of transgenic cotton. However, analysis of the roots of transgenic cotton showed no expression of the rice PDC1. These results suggest that the rice PDC1 is expressed in selected tissues irrespective of the type of promoter used. This result is difficult to explain. The need for special factors for transcription and/or translation can be ruled out since the endogenous PDC1 is expressed in rice roots (fig. 4.3B).

Under greenhouse conditions, plants of the two homozygous families PS64-3 and PS64-4 over-expressing PDC1 were shorter in height only at early stages of growth compared to plants of WT and the azygous family PS64-5. Tadege et al. (1998a) also found this ‘shorter plant’ phenotype in transgenic tobacco plants over-expressing bacterial PDC. However, we could not detect any difference in height in the other two over-expressing PDC1 lines PS26 and PS29. It is likely that this "shorter plant" phenotype might result from consequences of transformation and not from the over-expression of PDC1.

Seeds of the over-expressing PDC1 lines P26, P29 and P64 germinated under anoxia for 5d did not show any retardation or stimulation of germination and/or coleoptile growth. It has also been found that exogenous glucose did not stimulate growth and respiration of Calrose (flooding tolerant rice variety) seedlings germinated in air or under anaerobic conditions for 4d. Endogenous substrates were enough for their growth under such conditions (Atwell and Greenway, 1987). We also found that boosting the ethanol fermentative pathway by over-expressing PDC did not promote rice seed germination under anoxia. In contrast, reduction of EF pathway by lowering the level of ADH activity in rice hampers seed germination under anoxia (Matsumura et al., 1995; section 3.2.2.6.1).

Anoxia tolerance of mature plants (26d old) was tested in two over-expressing PDC1 lines PS26 and PS64. Homozygous families (with transgene) of both lines showed reduced tolerance to anoxia compared to the azygous family (without transgene) of the same line or WT plants. Reduced anoxia tolerance was also observed in roots of tobacco over-expressing PDC and was suggested to be a consequence of the
rapid depletion of the reserve carbohydrate resulting in premature cell death, while replenishment of carbohydrates improved survival under anoxia (Tadege et al., 1998a). We demonstrated that in vitro and in the presence of exogenous glucose, lines PS26 and PS64 produced more ethanol compared to WT and azygous controls. It could be true that plants of these two lines were utilising carbohydrate more rapidly in vivo under anoxia than controls. A positive correlation between carbohydrate content and submergence tolerance is seen in the situation when anaerobic survival is increased by high light intensity and age (Palada and Vergara, 1972; Reddy and Mittra, 1985). We also found that three week old plants of FR13A and IR42, presumably having higher initial carbohydrate content survived during submerged conditions better than two week old plants (data not shown). When rice plants are shaded before submergence (to experimentally reduce carbohydrates), their tolerance of submergence is greatly reduced (Setter et al., 1997a). Likewise, when rice seedlings are submerged in the evenings (when carbohydrate levels are high), their submergence tolerance is higher than when submergence is imposed in the mornings, when carbohydrates are low (Singh et al., 1997). The beneficial effect of slow growth during submergence was clearly demonstrated by Setter and Laureles, (1996) using the GA biosynthesis inhibitor paclobutrazol. This was suggested to be a means of maintaining high carbohydrate supply and hence prolonging energy supply in rice (Setter et al., 1994).

Acetaldehyde is reportedly the cause of ethanol induced injury in carrot cells and exerts its toxic effects by forming acetaldehyde-protein adducts (Perata and Alpi, 1991; Perata et al., 1992; Braun et al., 1995). An enhanced membrane leakage caused by elevated levels of ethanol and acetaldehyde was also suggested to be responsible for the reduced tolerance of roots of transgenic tobacco over-expressing PDC (Drew, 1997; Tadege et al., 1998a). In the presence of exogenous glucose, leaf sheaths of lines PS26 and PS64 produced more acetaldehyde than WT plants. Mature plants of lines PS26 and PS64 might die more quickly than controls as was evident from the fact that the ability of plants of PS26 and PS64 to produce ethanol decreased with time compared to WT plants.
Seeds of these over-expressing \textit{PDC} lines (PS26, PS29 and PS64) did not show any retardation of coleoptile emergence during 5d anoxic germination. This might be because rice seeds are full of starch and do not have substrate scarcity. Moreover, rice seeds also express a high level of ADH (fig. 3.1) which might help to scavenge any excess acetaldehyde.

From the improved anaerobic survival and reduced level of acetaldehyde content of the hybrid plants ($\Phi$AS8-10 x $\delta$PS26-12) over-expressing both \textit{PDC} and \textit{ADH} compared to plants (lines PS26 and PS64) over-expressing \textit{PDC} alone, we suggest that acetaldehyde plays a major role in reducing anoxia tolerance. The improved survival of plants of the $\Phi$AS8-10 x $\delta$PS26-12 cross compared to plants of PS26-12 may arise from the rapid removal of acetaldehyde by the higher level of ADH. Alternatively, plants of $\Phi$AS8-10 x $\delta$PS26-12 are heterozygous whereas plants of PS26-12 are homozygous with respect to \textit{PDC} transgene, which leads to a reduced rate of acetaldehyde generation in these hybrid plants compared to plants of PS26-12. Estimation of soluble sugars before and after submergence and ethanol and acetaldehyde production in the absence of exogenous glucose should give a clear picture about whether depletion of reserve carbohydrate and/or acetaldehyde toxicity caused the reduced anoxia tolerance in the over-expressing \textit{PDC} lines.

However, despite evidence that endogenous ADH is in excess, we need to ask by how much? We were able to increase PDC activity by only two-fold in shoots and under such conditions, endogenous ADH was not able to cope with the accelerated conversion of pyruvate to acetaldehyde. Over-expression of \textit{ADH} ($\Phi$AS8-10 x $\delta$PS26-12) was essential to ameliorate the adverse effect(s) from \textit{PDC} over-expression. Another possibility of the reduced anoxia tolerance of the over-expressing \textit{PDC} transgenic plants might be that the over-expression of \textit{PDC} occurred in shoots but not in roots. Therefore, there might still be scope to over-produce PDC and ADH levels to a greater degree than in our present transgenic plants to increase submergence tolerance compared to WT.
Chapter 6

General Discussion
6. GENERAL DISCUSSION

6.1 Conclusions

An important feature of a rice plant is its ability to grow under waterlogged or submerged conditions. It is not an aquatic plant, however, and needs molecular oxygen for survival. Compared to maize, wheat and barley, rice is extremely tolerant to anoxia. Different organs in rice show different degrees of resistance: under conditions of complete oxygen deprivation, rice roots survive for some days but do not grow, while coleoptiles elongate for more than 23d (Reggiani et al., 1989; Ricard et al., 1991). Seedlings of the most flooding tolerant variety FR13A can survive total submergence for over two weeks (Mazaredo and Vergara, 1982). However, mature plants of most rice varieties can not survive complete submergence of the shoot and root for an extended period. The rice plant avoids and/or tolerates anoxia by two kinds of adaptation. The morphological adaptation to escape low oxygen stress is comprised of aerenchyma, root extension and stem elongation. The second adaptation consists of biochemical changes when anaerobic polypeptides (ANPs) are synthesised under oxygen limitation and energy metabolism is shifted from oxidative to mainly ethanol fermentation (EF). Two ANPs, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) catalyse EF and have been intensively studied over the last few decades. The research described in this thesis has focused on understanding the role of PDC and ADH in anoxia tolerance in rice. The reduced anoxia tolerance of adh null mutants in maize, rice, barley and Arabidopsis and a possible regulatory role of PDC in EF prompted us to investigate whether PDC and ADH limit the rate of EF and whether accelerating EF by over-producing PDC and ADH can improve survival under oxygen limitations.

6.1.1 Manipulation of ADH:

We generated an antisense ADH1 transgenic line (AA108) which had greatly reduced ADH levels of only 4% of WT. In the antisense ADH1 line AA108, both ADH1
and ADH2 polypeptide levels were reduced. The reduction in ADH level had an impact on ethanol production in line AA108 which produced 36% as much ethanol as WT. We found that there was a negative effect on coleoptile growth during germination of seeds of line AA108 under anoxia and no effect on coleoptile and root growth in air. Mature plants of line AA108 with a reduced level of ADH also exhibited a poor ability to tolerate anaerobic conditions compared to both WT and an azygous family with a similar level of ADH as WT plants. The effect of reducing the level of ADH on anoxia tolerance has also been observed in \textit{adh} null mutants of a variety of species including maize (Schwartz, 1969; Freeling and Bennett, 1985; Johnson \textit{et al.}, 1994), barley (Harberd and Edwards, 1982) and rice (Matsumura \textit{et al.}, 1995, 1998). A reduction in ADH level has at least two consequences; a decreased rate of EF, and an elevated level of build-up of acetaldehyde, a cell toxin. Either one of these factors or both together might cause a reduction in the ability to tolerate anoxia (Drew, 1997; Tadege \textit{et al.}, 1999).

We were able to over-produce ADH activity in line AS8 by 439\% of WT and 337\% of a flooding tolerant variety, FR13A. The ADH level in line AS8 was also significantly higher than WT or FR13A under hypoxia. The effect of ADH over-production on ethanol production was insignificant, in agreement with the hypothesis that ADH activity does not limit the rate of EF (for a review see Drew, 1997). It is not surprising that plants of family AS8-10 which over-produced ADH had a similar level of anoxia tolerance as WT and the azygous family AS8-2. This result indicates that over-expression of ADH does not improve anoxia tolerance in rice plants. FR13A, on the other hand, which had much less ADH activity than homozygous family AS8-10 and a similar ADH level as WT, azygous family AS8-2 and IR42 (a flooding intolerant variety), showed 667\% higher survival on average than AS8-10, WT, AS8-2 and IR42. Thus submergence tolerance in rice does not correlate with an elevated level of ADH activity. However, a minimal level of ADH is indispensable to protect plants from a shortage of energy and the deleterious effects of anaerobic metabolism. One unresolved point exists; why do plants contain such a high level of ADH under normal conditions? Perhaps high levels of ADH activity are of great significance during the aerobic
conversion of ethanol to acetaldehyde into the mainstream metabolism following anaerobiosis (Thomson and Greenway, 1991; Waters et al., 1989).

6.1.2 Manipulation of PDC:

We also developed transgenic rice plants over-expressing the rice PDC1 driven by an aerobically inducible promoter. Three independently transformed lines, PS26, PS29 and PS64 showed high levels of PDC1 polypeptide. These lines had a moderate increase in PDC activity, which led to a moderate increase in ethanol production compared to WT or azygous families of these lines. Unlike the antisense ADH1 line AA108, seeds of the over-producing PDC1 lines PS26, PS29 and PS64 germinated under anoxia for 5d did not show any retardation or stimulation of germination and/or coleoptile growth. In contrast, mature plants of homozygous families of these lines had reduced tolerance to anoxia. Reduced anoxia tolerance in the over-expressing PDC transgenic lines might arise because of carbohydrate depletion, acetaldehyde toxicity, or impaired transport of sucrose due to the membrane damage caused by the high level of ethanol and acetaldehyde. As the soluble sugar content before and after submergence was not measured, the carbohydrate depletion model for reduced tolerance in the over-producing PDC plants was unable to be tested. However, rapid utilisation of reserve carbohydrate was suggested as a cause of reduced anoxia tolerance in roots of tobacco plants over-expressing PDC, and a carbohydrate supplement improved survival under anoxia (Tadege et al., 1998a). Observations that anaerobic survival increased with high light intensity (Palada and Vergara, 1972) and age (Reddy and Mittra, 1985) indicate a positive correlation between carbohydrate content and submergence tolerance. The beneficial effect of slow growth during submergence was elegantly demonstrated by Setter and Laureles (1996) and was suggested as a means of maintaining high carbohydrate supply and hence prolonging energy supply in rice (Setter et al., 1994). No variety or cross-breeds are known to possess both submergence tolerance and good elongation capacity (Setter and Laureles, 1996). Transgenic plants with reduced elongation ability only during submergence are a promising solution, and the development of these transgenic plants has already been started in PI, CSIRO, headed by Dr. Marc Ellis and Dr. Liz Dennis.
Acetaldehyde has been envisaged as a cause for reduced survival under anoxia of carrot cells (Perata and Alpi, 1991) and roots of transgenic tobacco over-expressing PDC (Tadege et al., 1998a). Acetaldehyde content was significantly higher in plants over-producing PDC than plants of WT or azygous families (section 5.2.2.4) and might cause the early senescence which was observed in the over-producing PDC lines. Hybrid plants over-producing both PDC and ADH were generated and mature plants were assayed for anoxia tolerance. Hybrid plants showed a significantly increased level of anoxia tolerance but a decreased level of acetaldehyde content relative to plants over-expressing PDC alone (section 5.2.4). This suggests that acetaldehyde is the prime cause of diminished anoxia tolerance in the transgenic plants.

We failed to generate under-expressing PDC transgenic lines. Analysis of 18 independently transformed lines of antisense ACTIN1-PDC1 showed no under-expression of PDC. This raises the question of whether under-expression of PDC is deleterious to plants during seed germination and/or pollen germination. Tadege et al. (1999) suggested a new function of the PDC other than energy metabolism. PDC might participate in a cytosolic pathway that converts pyruvate to acetyl CoA, which then enters the glyoxylate cycle and is involved in lipid metabolism to meet the demand for intermediates during pollen germination and pollen tube growth (Tadege and Kuhlemeier, 1997; Tadege et al., 1999). In the present study, immunoblotting assays of untransformed Taipei plants revealed that PDCs are most abundant in embryos and pollen compared to other tissues. This indicates that PDC might have a function in seed germination and/or pollen tube formation.

6.2 Future research

When exposed to anoxia, plants undergo major metabolic changes to maintain energy production, despite a shut off of respiratory phosphorylation. They do so by increasing the rate of sucrose and starch mobilisation, by accelerating the rate of glycolysis and diversifying its end products, and by accelerating the ethanol fermentative pathway (for a review see Ricard et al., 1994). However, energy
maintenance is not the sole activity of plants under anaerobiosis, as was evident from superoxide dismutase (SOD) being identified as an ANP (Monk et al., 1987b). A role of SOD in anoxia tolerance is well documented; transgenic tobacco plants over-expressing SOD have an increased tolerance to oxygen limitation (Herouart et al. 1993; Sen Gupta et al., 1993). Obviously, enzyme(s) involved in protection against oxidative damage are induced under oxygen limitation to protect important macromolecules and organellar integrity, which are otherwise damaged by reactive oxygen radicals when plants return to air after anaerobic conditions.

A plant’s response to submergence, therefore, appears very complex and is comprised of cell components involved in energy metabolism, sugar mobilisation and its transport and protection against photo-oxidative damage. Hence, to ameliorate a plant’s ability to tolerate submergence, one needs to boost all of the cell components involved in such protection instead of manipulating a single enzyme or a single pathway. Transcription factor(s) may have the potential to improve anoxia tolerance. The ARE sequence responsible for anaerobic induction of genes was initially identified in the maize ADH1 promoter (Walker et al., 1987) and later the GC and GT motifs of ARE were found to be highly conserved in different genes and different species (Hoeren et al., 1998). The Arabidopsis MYB transcription factor AtMYB2 binds specifically to the GT motif of the ADH1 promoter. When driven by a constitutive promoter, AtMYB2 is able to induce ADH1 in transient assays in both Arabidopsis and tobacco protoplasts, and in particle bombardment of pea leaves (Hoeren et al., 1998). Therefore, over-expression of the AtMYB2 could switch on all of the anaerobic genes. However, one impending difficulty in over-expressing a transcription factor under a constitutive promoter is that it would induce every gene containing the ARE motifs, and this might be associated with cell lethality. Over-expression of AtMYB2 only under low oxygen conditions or in the presence of an inducer could be one possible way to evaluate its role during anaerobiosis. Transgenic approaches are presently under way to over- and under-express AtMYB2 under the control of a dexamethazone-inducible promoter, to investigate whether specific expression of AtMYB2 can induce or repress ADH1 and the other anaerobic genes and whether it has any effect on anoxia tolerance (Dr. Erik J. Klok, Dr. Rudy Dolferus and Dr. Liz Dennis, PI, CSIRO, unpublished).
Search for candidate genes for improving anoxia tolerance by genetic manipulation

FR13A, the most flooding tolerant rice variety known, has a very low flooding tolerance compared to flooding tolerant aquatic plants (e.g., *Acorus calamus* can withstand anoxia for at least 6 months; Bucher and Kuhlemeier, 1993). Therefore, a close study of such anoxia tolerant species along with anoxia intolerant species preferably from the same genus as a control, could be valuable and informative. One way to isolate important genes for anoxia tolerance is to prepare a subtractive cDNA library between an anoxia tolerant and an intolerant species from the same genus. Once important genes have been cloned and characterised, a polygenic construct can be made to introduce all genes at the same time.

The concept that regulated enzymes do not control metabolism is emerging. Rather, it is the unregulated enzymes that are important in controlling metabolic fluxes, whereas the regulated enzymes maintain cellular homeostasis and ensure smooth transitions between one metabolic state and another (for a review see Hill, 1998). Most previous work has focused on the enzymes that are induced under anoxia; little work has been done on the enzymes that are suppressed to varying extents in different species under anaerobiosis. It is important to assay these enzymes, specifically before and after anaerobiosis, and to establish a correlation between the extent of reduction of expression and flooding tolerance. As an example, the activity of cytochrome oxidase, which catalyses the terminal reaction in the electron transport chain, was decreased under hypoxia to 80-92% of the aerated control in *Agropyron pungens* and *Hordeum vulgare*, two flooding intolerant and non-wetland species. In contrast, cytochrome oxidase activity was little affected in flooding tolerant species such as rice and three *Salicornia* spp. (Pearson and Havill, 1988). It was suggested that a relatively high cytochrome oxidase activity in wetland species may help to scavenge any available oxygen within roots and thus help reduce energy loss.

One potential candidate gene for improving tolerance under to oxygen conditions corresponds to the *SUB1* locus which contributes to 69% of tolerance against submergence in rice (Xu and Mackill, 1996). Isolation of this gene is in progress (Xu
and Mackill, unpublished). Altering the levels of such genes which correlate with anoxia tolerance could help in promoting anoxia tolerance.

A reasonable amount of information is available about the metabolic pathways in plants, the roles of the enzymes involved in these pathways and levels of induction or repression of some of these enzymes under different environmental stresses such as oxygen limitation, drought, salt stress, and cold. However, the manipulation of the levels of these enzymes by genetic engineering and the study of the effects of over- and under-expression of these enzymes in transgenic plants are still in their infancy. Transgenic plants have great potential in assessing the role of each enzyme in a genetically uniform background under a particular stress, especially one which invokes a complex and multigene regulation in plant cells. The use of transgenic plants may clarify the physiological roles of anaerobic pathways for the generation of submergence tolerant rice.
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