The Characterization of Alcohol Dehydrogenase in Cotton and its role in the Anaerobic Response

by

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A thesis submitted for the degree of Doctor of Philosophy of the Australian National University, Canberra

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. Part of the work in Chapter Five was done with T. Setter (IRRI, Manila Philippines) while he was visiting CSIRO in 1991. He amplified the Pdc cDNA and ligated it into pBGS. He also constructed the pJ35ShdA plasmid. He has also analysed transgenic calli as referred to in the text.

To the best of my knowledge, the thesis contains no material previously published, or the result of any work by another person, except where due reference is made in the text.

Anthony Millar
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ABSTRACT

This thesis has examined the expression of Alcohol dehydrogenase (ADH) in cotton, a plant that is intolerant to waterlogging and anaerobic conditions. It was found that ADH enzyme activity is induced when cotton is anaerobically stressed. Isozyme analysis found three major ADH isozymes in cotton, and it was proposed that at least two genes were coding for these three isozymes. During anaerobic stress the isozyme pattern alters, with ADH2 being induced in both roots and shoots of seedlings, and ADH1 being induced in the shoots. Biochemical analysis showed that the ADH enzyme has a dimeric structure, having a native molecular weight of approximately 81 kD and a subunit molecular weight of approximately 42 kD.

Using protein labelling and two dimensional (2D) gel electrophoresis techniques, the effect of anaerobic stress on the pattern of protein synthesis was examined in cotton. During aerobic condition several hundred major polypeptides were resolved, but when exposed to anaerobic conditions a shift in protein synthesis was observed, where only approximately 20 major polypeptides were synthesized. Western analysis on a 2D gel found that three of these polypeptides were ADH.

A cDNA library was constructed from root tips of cotton seedlings that had been anaerobically stressed. Adh cDNA clones were isolated and found to fall into three different classes. The nucleotide and deduced amino acid sequence of the Adh cDNA clones have high similarity to Adh genes from other plant species. Using these cDNA clones as probes three different Adh genes were isolated from a cotton genomic library. The promoter regions of these Adh genes were sequenced and found to contain sequences characteristic of functional eucaryotic genes, including TATA boxes and conserved nucleotide sequences around the putative transcription start site. In addition multiple ARE core elements (5'-AAACCA-3') were present, suggesting that transcriptional activity of these promoters can be induce by anaerobic stress.

Cotton was transformed with genes designed to overexpress ADH and pyruvate decarboxylase (PDC) and to underexpress ADH in an attempt to alter the levels of expression of these enzymes, and examine how this affects tolerance to anaerobic stress.
Initially this experiment was to be done with transgenic calli that were expressing different levels of enzyme activity. However due to difficulties in assaying calli it was decided instead to regenerate and analyse plants, from which the results will have more significance. Plants that are overexpressing ADH have now been regenerated, but yet to be assayed for tolerance to anoxia. Plant overexpressing PDC are still being regenerated.
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<tr>
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<td>Abscisic acid</td>
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<td>Alanine</td>
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<td>Asparagine</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>bp</td>
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<td>C</td>
<td>Cytosine</td>
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<td>c.p.m</td>
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<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
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<td>cv.</td>
<td>cultivar</td>
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<td>Cys</td>
<td>Cysteine</td>
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<td>deoxyadenosine triphosphate</td>
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<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
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<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<tr>
<td>dNTP</td>
<td>dATP, dCTP, dGTP and dNTP</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>G</td>
<td>Guanine</td>
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<td>Gln</td>
<td>Glutamine</td>
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<td>Glu</td>
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<td>GUS</td>
<td>bacterial gene for β-glucuronidase</td>
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<td>His</td>
<td>Histidine</td>
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<td>hr.</td>
<td>hour</td>
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<td>Isoleucine</td>
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IPTG  Iso-propyl-β-D-thio-galactopyranoside
kb    Kilobase
kD    Kilodalton
LDH   Lactate dehydrogenase
Leu   Leucine
Lys   Lysine
Met   Methionine
mins. minutes
NAD+  nicotinamide adenine dinucleotide coenzyme I
OD    optical density
p.f.u. plaque forming units
PAGE  Polyacrylamide gel electrophoresis
PCR   polymerase chain reaction
PDC   Pyruvate decarboxylase
Phe   Phenylalanine
Pro   Proline
r.p.m. revolutions per minute
SDS   sodium dodecyl sulphate
Ser   Serine
T     Thymine
T-DNA Transfer DNA
TBE   Tris-borate EDTA buffer
TCA   Trichloroacetic acid
TEMED N,N,N',N', tetramethylenediamine
Thr   Threonine
Tris  Tris(hydroxymethyl)aminomethane
Trp   Tryptophan
Tyr   Tyrosine
UV    ultra violet
Valine

5-bromo-4-chloro-3-indoyl-β-galactoside
CHAPTER ONE

INTRODUCTION

1.1 Responses of plants to waterlogging.

Growing plants have a requirement both for rapid gaseous exchange with the environment and for sufficient water to satisfy the needs for growth and evapotranspiration (Jackson and Drew 1984). Gas spaces in most well drained soils make up 10-60 per cent of the soil volume so oxygen can freely diffuse in while gases like carbon dioxide and ethylene can freely diffuse out. Gas diffuses through water about 10,000 times more slowly than through air (Armstrong 1979), so when soil becomes waterlogged, roots and microorganisms within the soil will quickly exhaust the supply of oxygen resulting in an anaerobic environment. Hence roots would no longer be able to respire and consequently the levels of ATP would fall. Low ATP levels would be expected to lead to the cessation of growth, reduction in the plant's ability to take up nutrients from the soil and ultimately, cause the death of root tips or whole root systems (Jackson and Drew 1984). To offset a drop in ATP levels some plants have evolved a mechanism of structural changes that makes oxygen available to the affected parts and allows respiration (oxidative phosphorylation) to occur. Another strategy is that plants switch root cell carbohydrate metabolism to fermentative pathways that will result in the generation of ATP in the absence of oxygen.

Roots of some plants undergo anatomical changes which enables them to overcome waterlogging. Elevated levels of ethylene (due to accumulation) within waterlogged roots lead to cell separation or partial breakdown of the cortex, resulting in interconnected gas-filled spaces. These spaces are called aerenchyma and allow movement of oxygen from the shoots to the roots thus avoiding anoxic conditions in the rhizosphere and enabling respiration to occur (Jackson and Drew 1984). Aerenchyma contributes to the survival of many wetland species (Kawase 1981) and allows continued root development to occur under waterlogged conditions (Armstrong 1979).
In some wetland species of plants, such as rice (*Oryza sativa*) and wild rice (*Zizania aquatica*), aerenchyma is present even under well oxygenated conditions, suggesting that its development does not require an anaerobic signal (Jackson and Drew 1984). Another structural change is the formation of horizontal adventitious roots that emerge from the base of the shoots. These roots are often permeated by aerenchyma which not only allow oxygen to diffuse down into the roots but also cause the roots to float, helping them stay near the air-water interface where sufficient dissolved oxygen is present to support growth and functions such as nutrient uptake (Jackson and Drew 1984). These specialized roots are adapted anatomically to survive low oxygen tension within soils and will supplement or replace the original seminal root system that eventually succumbs to waterlogging. Plants including barley, wheat, rice, tomato, sunflower, the amphibious *Ludwigia peploides* and the grass *Holcus lanatus* have all been reported to develop adventitious roots (see Jackson and Drew 1984 for a review) of this type.

Adventitious roots and aerenchyma can allow the plant to survive long-term waterlogging conditions. However, these structural changes take some time to develop. For example, at a sudden onset of waterlogging it takes about 24 to 48 hours for aerenchyma to develop in maize (Konings 1982) and 8 to 48 hours in wheat (Thomson et al. 1990). So before aerenchyma can become effective the plant must rely on anaerobic metabolism to enable it to acclimate and survive the transient period of anoxia. The metabolic adaptation to oxygen deficiency is also essential when the plant (or seedling) is entirely submerged, since aerenchyma is ineffective under these conditions. These biochemical changes that the plant undergoes in response to anaerobic stress are examined in this thesis.

1.2 Cotton is susceptible to waterlogging/anaerobic conditions.

Cotton has been chosen as the plant for study in this thesis because it is relatively intolerant to soils that are poorly aerated (de Bruyn 1982). Much of the commercially
grown cotton in Australia is furrow irrigated. Cotton often encounters waterlogging associated with furrow irrigation, especially when irrigation is followed by rainfall. This problem is compounded by the fact that most Australian cotton is planted on cracking grey clays that are poorly aerated (Hodgson and Chan 1982). This is an important agricultural problem, for waterlogging leads to reductions in growth, fruiting, lint yields and causes yellowing (chlorosis) of leaves (Hodgson and Chan 1982, Hodgson 1986). Intolerance of established cotton plants to low oxygen levels stems partly from the plant's inability to transport oxygen efficiently from the aerial portions of the plant to the roots (Vartapetian et al. 1978). This may be attributable to the absence of root aerenchyma (Spieth 1933). However cotton seedlings are also very sensitive to anaerobic conditions, and soil perfused with nitrogen gas can kill taproots of cotton seedlings within three hours; as little as 30 minutes of treatment can be fatal to a proportion of root tips (Huck 1970). Survival during anaerobic conditions depends solely on metabolic processes, thus it is possible cotton is poorly adapted to anaerobic conditions at the biochemical level. This thesis characterizes the response of cotton seedlings to anaerobic conditions at the biochemical and molecular level.

1.3 The molecular responses of plants to anaerobic conditions.

(a) The anaerobic response.

When plants become anaerobically stressed they respond by dramatically altering protein synthesis. This response has been best characterized in the primary root of maize seedlings, where, during anaerobic stress, aerobic protein synthesis is repressed and a small group (about 20) of polypeptides are preferentially synthesized (Sachs et al. 1980). These proteins are known as the Anaerobic Polypeptides (ANPs) and after five hours of anaerobiosis these proteins account for more than 70% of total protein synthesis. This molecular switch in protein synthesis is known as the anaerobic response and has been characterized in a number of different plant species.
Several of the ANPs have been identified as enzymes which are either involved in
glycolysis or catalyse the reaction providing the substrate for glycolysis. These ANPs
include both the ethanolic fermentative enzymes alcohol dehydrogenase (ADH, Sachs
and Freeling 1978, Ferl et al. 1979) and pyruvate decarboxylase (PDC, Wignarajah and
Greenway 1976, Lazlo and St Lawrence 1983), and glycolytic enzymes such as glucose
phosphate isomerase (Kelley and Freeling 1984a), cytosolic fructose-1,6-biphosphate
aldolase (Kelley and Freeling 1984b), glyceraldehyde-3-phosphate dehydrogenase III
(Russell and Sachs 1989 and 1992) and the hexose mobilizing enzyme, sucrose
synthase I (Springer et al. 1986). Anaerobic glycolysis results in a net gain of 2 mol. of
ATP/ mol. of glucose whereas oxidative phosphorylation produces a theoretical
maximum of 36 mol. of ATP/ mol. of glucose. Therefore the synthesis of these ANPs
appears to be a survival mechanism, for glycolysis will be the plant's major source of
ATP under anaerobic conditions (Davies 1980) so an increase in the carbon flux
through the pathway may be needed to compensate for the decrease in efficiency of
ATP production. Enough ATP may then be provided to enable the plant to tolerate
anaerobic stress. Experiments of Saglio et al. (1988) and Johnson et al. (1989) support
this, where they have concluded high rates of alcoholic fermentation confer high
tolerance to anoxia in maize. The anaerobic response and its significance for the plant
survival under anaerobic conditions will be discussed further in the introductions to
Chapters Three and Five.

(b) Alcohol dehydrogenase (ADH).

The most studied ANP of maize is ADH (for review see Freeling and Bennett 1985). Its
expression during anaerobic stress is essential for the plant's survival. It has been shown
that maize ADH1 null mutant seedlings that do not show any ADH activity, will
succumb to waterlogging much faster than wild-type plants (Schwartz 1969). ADH is
probably essential for a number of reasons. The inhibition of oxidative phosphorylation
under anaerobic stress results in the rapid depletion of NAD+ levels. NAD+ is required
for anaerobic glycolysis, therefore NAD+ must be regenerated from NADH. This can
be achieved by ADH, for when ADH reduces acetaldehyde to ethanol it utilizes NADH as a co-factor, oxidizing it to NAD$. The alternative pathway of lactic acid fermentation catalysed by lactate dehydrogenase also results in the regeneration of NAD$. However, Roberts et al. (1984a) have shown that lactic acid fermentation, unlike ethanolic fermentation results in the acidification of the cytoplasm and that the lower pH is closely associated with cell death (Roberts et al. 1984a & b). Ethanolic fermentation allows maintenance of cytoplasmic pH at a tolerable level. This may be an important reason why ADH is essential for the plant's survival during anaerobic stress. The expression of ADH and its physiological role in plants will be further discussed in the introduction to Chapter Two.

(c) Transcriptional and translational control of the anaerobic response.

The anaerobic response is regulated at the transcriptional level. Anaerobic stress results in the increase in the steady-state mRNA levels of the ANPs (Dennis et al. 1984 & 1985, Hake et al. 1985, Springer et al. 1986, Kelley 1989 and Good and Paetkau 1992). After 5 hours of anaerobiosis there is a 50 fold increase in $Adh1$ mRNA levels in the roots of maize seedlings (Gerlach et al. 1982). This anaerobic induction of $Adh1$ mRNA levels is due mainly to an increase in the rate of transcription, but the stability of the transcript is also increased (Rowland and Strommer 1986).

The coordinate induction of the ANPs in maize suggests that a common trans-acting factor influences the promoter region of the genes encoding the ANPs. A 40 bp region within the maize $Adh1$ promoter, called the anaerobic regulatory element (ARE), was shown to contain the signals necessary for transcription under anaerobic conditions (Walker et al. 1987). A motif within the ARE, 5'-TGGTTT-3', was shown to be critical for expression (Walker et al. 1987) and has been found in other maize genes coding for ANPs, and also in anaerobically induced genes in other plant species (Dennis et al. 1987). This suggests that not only is the anaerobic response present in many different plant species but that the mechanism of regulation is conserved. The ARE and its role in transcriptional regulation will be discussed further in Chapter Four.
The anaerobic response of maize is also regulated at the translational level. Sachs et al. (1980) have shown that during anaerobic stress the mRNA encoding the ANPs are selectively translated while the mRNAs encoding the pre-stressed set of proteins are not, thus resulting in the immediate switch in protein synthesis. This translational control in anaerobically stressed plants will be discussed further in the introduction of Chapter Three.

1.4 Outline of thesis.

ADH expression has been shown to be anaerobically induced in a wide variety of plants including rice (Xie and Wu 1989), pea (Llewellyn et al. 1987), Arabidopsis (Dolferus et al. 1985), tomato (Tanksley and Jones 1981), Petunia (Gregerson et al. 1991) and soybean (Russell et al. 1989) (for a summary see Table 2.1). However the expression and anaerobic induction of ADH in cotton has not been characterized. Since ADH appears to play an important role in the anaerobic response this thesis will commence by characterizing its expression in cotton. ADH expression has been shown to be essential for anaerobic survival in maize seedlings (Schwartz 1969), therefore if ADH is only weakly expressed it may explain why cotton is susceptible to anaerobic stress. Estimates of the level of expression of ADH and the extent of its induction by anaerobiosis will be measured using ADH spectrophotometric assays. Additionally, from the examination of the ADH isozyme pattern a clue to the number of Adh genes in cotton may be obtained. The details of these experiments will be presented in Chapter Two.

Chapter Three of the thesis describes experiments that characterize the anaerobic response of cotton. By using protein labelling and two dimensional gel electrophoresis it was examined; (1) whether there is a dramatic shift in protein synthesis like that observed in maize; (2) how many major polypeptides are being expressed during anaerobic stress; and (3) the possible identities of the cotton ANPs. Soybean and pea are both anoxic-intolerant species and only express 4 to 5 ANPs each during anaerobic conditions (Russell et al. 1989) whereas maize, a more anoxic tolerant plant expresses
20 ANPs (Sachs et al. 1980). Russell et al. (1989) proposed that there may be a positive correlation between the number of ANPs and tolerance to anaerobic stress. Huck (1970) in a direct comparison found cotton was less tolerant to anaerobic conditions than soybean, so it is of interest to determine if the correlation between complexity of the ANP pattern and anoxic tolerance also extends to cotton. Because this thesis is concentrating on ADH, the identity of the ANPs that are ADH polypeptides will also be determine. This will indicate if ADH constitutes a major or minor part of the anaerobic response and will allow further insight to the level of expression of ADH in cotton.

Chapter Four of the thesis describes the isolation of Adh cDNA and Adh genomic clones. The Adh cDNAs will be isolated from a library prepared from mRNA of anaerobically stressed roots. The number of different cDNA classes obtained from the library will reveal how many Adh genes are induced by anaerobic stress in roots of cotton. Also from sequencing the cDNAs, information regarding the structure of the ADH enzyme and how similar it is to ADHs in other plant species will be obtained. The Adh cDNAs were used to screen a genomic library for the isolation of Adh genes possessing anaerobically inducible promoters. These promoters were sequenced to see if cotton possesses the DNA motifs shown to be important for anaerobic expression in anaerobically inducible genes from other plant species. Anaerobically inducible Adh promoters may be of benefit in future experiments where the expression of additional proteins under anaerobic conditions may be required.

Studying the proteins/genes switched on in response to anaerobic stress not only expands our knowledge on gene function and regulation but may also provide clues on how to manipulate the response, to produce varieties more tolerant to anaerobic stress. This will be the theme of the fifth chapter where cotton will be transformed with gene constructs modifying the expression levels of ADH and PDC. Although beyond the scope of this thesis these experiments have been initiated with the ultimate goal of defining the precise physiological roles of ADH and PDC under anaerobic conditions and to examine the possibility of manipulation of the ethanolic fermentative pathway to produce a plant more tolerant to anaerobic stress.
It could be argued that introducing the genes into crop plants that result in the formation of adventitious roots with aerenchyma would be the ultimate manipulation to a plant in allowing it to tolerate waterlogging. This may be relevant for growing crops in wetlands or for crops that are waterlogged for the majority of the season. However this appears unnecessary and costly for crop plants that may be waterlogged for only a few days in the entire growing season. Modification of the anaerobic response to allow crop plants to survive transient periods of waterlogging would be more applicable.

**Alcohol dehydrogenase (ADH):** alcohol: NAD⁺ oxidoreductase. E.C.1.1.1.1) is a cytosolic enzyme which catalyses the reversible oxidation of alcohols to ketones utilizing NAD⁺ as a co-factor (Braude et al. 1975). ADH plays a major physiological role in plants during anaerobic stress when carbohydrate metabolism must switch from an oxidative to a fermentative pathway (Davies 1980). ADH is the terminal enzyme in the ethanol fermentation pathway converting acetaldehyde to ethanol and regenerating NAD⁺ in the process. These are two important functions: the removal of acetaldehyde is important because of its phytotoxic effects (Perata and Alt 1991) and the regeneration of NAD⁺ is essential for the continuation of glycolysis, which is the major source of cellular ATP during periods of anaerobiosis.

ADH has been widely studied among higher plants, with the maize ADH system and its genetics being most extensively known (for review, see Freedman and Bennett 1985). In maize, the ADH enzyme being active only as a dimer (Schwartz and Finday 1966) that requires zinc for its formation (Fischer and Schwartz 1975). There are two allelic ADH loci in maize and the products from the Adh₁ and Adh₂ loci are three isoforms by rainbow-discrimination: ADH₁-ADH₁ and ADH₁-ADH₂ chimeras and an intergenic ADH₁-ADH₂ heterodimer (Freedman 1974). The two genes have been found to be expressed in different developmental and organ-specific patterns, with Adh₁ being strongly expressed in seed (embryo, scutellum, endosperm and aleurone), shoots/root, stems, roots and pollen (Hajley-Serrano et al. 1990). Adh₂ is not expressed in pollen and is only weakly expressed in seed and root, however its level of expression can be induced along with Adh₁, under anaerobic conditions in roots (Freedman 1973). The optimal rate...
CHAPTER TWO

The Characterization of ADH Expression in Cotton and its Induction by Anaerobic Stress.

2.1 INTRODUCTION

Alcohol dehydrogenase (ADH; alcohol: NAD\(^+\) oxidoreductase, E.C.1.1.1.1.) is a cytosolic enzyme which catalyses the reversible oxidation of alcohols to aldehydes utilizing NADH as a co-factor (Branden et al. 1975). ADH plays a major physiological role in plants during anaerobic stress when carbohydrate metabolism must switch from an oxidative to a fermentative pathway (Davies 1980). ADH is the terminal enzyme in the ethanolic fermentation pathway converting acetaldehyde to ethanol and regenerating NAD\(^+\) in the process. These are two important functions; the removal of acetaldehyde is important because of its phyto-toxic effects (Perata and Alpi 1991) and the regeneration of NAD\(^+\) is essential for the continuation of glycolysis, which is the major source of cellular ATP during periods of anaerobiosis.

ADH has been widely studied among higher plants with the maize ADH system and its genetics being most extensively known (for review see Freeling and Bennett 1985). In maize, the ADH enzyme being active only as a dimer (Schwartz and Endo 1966) that requires zinc for its formation (Fischer and Schwartz 1973). There are two unlinked \(Adh\) loci in maize and the products from the \(Adh1\) and \(Adh2\) loci form three isozymes by random dimerization; ADH1-ADH1 and ADH2-ADH2 homodimers and an intergenic ADH1-ADH2 heterodimer (Freeling 1974). The two genes have been found to be expressed in different developmental and organ specific patterns, with \(Adh1\) being strongly expressed in seed (embryo, scutellum, endosperm and aleurone), etiolated leaf, roots and pollen (Bailey-Serres et al. 1988). \(Adh2\) is not expressed in pollen and is only weakly expressed in seed and roots, however its level of expression can be induced, along with \(Adh1\), under anaerobic conditions in roots (Freeling 1973). The optimal rate
of induction of these two genes occurs after eight hours of anaerobic conditions and increases at this zero-order rate until root death occurs after 72 hours of treatment. The two genes are also induced, but to a lesser extent, in seed and etiolated leaves (Bailey-Serres et al. 1988). Neither Adh gene is expressed in photosynthetic tissue under aerobic or anaerobic conditions (Okimoto et al. 1980).

Most other plants have multiple Adh genes (Gottlieb 1982), many having a two gene, three isozyme system similar to that of maize (Tanksley and Jones 1981, Xie and Wu 1989, Gregerson et al. 1991) but some plants have a third Adh gene; e.g. wheat and barley (Hanson and Brown 1984). Usually, the pattern of ADH expression in these plants parallels that of maize ADH, that is, in anthers/pollen, mature dry seed and germinating seedlings with at least one of the genes inducible by anaerobic stress, and most strongly induced in roots. Arabidopsis is the only plant known to have one Adh gene (Dolferus et al. 1984). A summary of the expression patterns of Adh genes in other plants is presented in Table 2.1. Although most plants have two Adh genes that have been assigned Adh1 and Adh2, the patterns of expression of the two genes varies from plant to plant meaning that all Adh1 genes are not necessarily evolutionary homologues. Likewise with the Adh2 genes.

It is thought that ADH is expressed where environmental or structural impediments to the availability of oxygen exists. For instance in stems or seeds where there is only a low surface to volume ratio, oxygen would be available to only the outer most tissues and in addition for seed, no oxygen would be available until the rupture of the testa. Therefore in germinating seeds, especially large seeds, oxygen deficiency would occur due to this low surface to volume ratio and would be compounded by rapid oxygen consumption (Leblova et al. 1974) due to rising metabolic activity as the embryo shifts from an inactive to very active state of metabolism (Bewley and Black 1978).

The physiological significance of ADH expression in pollen/anthers is unknown. It has been hypothesized (Okimoto et al. 1980) that ADH might be expressed in anthers/pollen due to accumulation of water in the leaf whorl of a plant after rain or during guttation. However this may not be the case because it has been shown by functional analysis of the maize Adh1 gene that the promoter elements responsible for
Table 2.1  The expression of ADH in various species of plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>ADH1</th>
<th>ADH2</th>
<th>ADH3</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>seed</td>
<td></td>
<td></td>
<td>Dofferus and Jacobs 1984</td>
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<td></td>
<td>pollen</td>
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<tr>
<td></td>
<td>seedling</td>
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<tr>
<td></td>
<td>An</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>seed +++</td>
<td>seed +</td>
<td>An</td>
<td>Bailey-Serres et al. 1988</td>
</tr>
<tr>
<td></td>
<td>An</td>
<td>An</td>
<td>pollen</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>seed</td>
<td>root</td>
<td>An</td>
<td>Xie and Wu 1989</td>
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<tr>
<td></td>
<td>pollen node</td>
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<tr>
<td></td>
<td>sheath</td>
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</tr>
<tr>
<td>Barley</td>
<td>seed +++</td>
<td>seed +</td>
<td>seed +</td>
<td>Hanson and Brown 1984</td>
</tr>
<tr>
<td></td>
<td>An</td>
<td>An</td>
<td>An</td>
<td></td>
</tr>
<tr>
<td>Pearl Millet</td>
<td>seed</td>
<td>An</td>
<td></td>
<td>Bannett-Bouvvillon and Hague 1979</td>
</tr>
<tr>
<td></td>
<td>An</td>
<td></td>
<td></td>
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<tr>
<td>Tomato</td>
<td>seed</td>
<td>An</td>
<td></td>
<td>Tanksley and Jones 1981</td>
</tr>
<tr>
<td></td>
<td>seedling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petunia</td>
<td>An +</td>
<td>An +++</td>
<td>young anther</td>
<td>Gregerson et al. 1991</td>
</tr>
<tr>
<td></td>
<td>old anther</td>
<td></td>
<td>stem, leaf</td>
<td></td>
</tr>
<tr>
<td></td>
<td>root</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sunflower</td>
<td>seed</td>
<td>seed/embryo</td>
<td>pollen</td>
<td>Torres et al. 1977</td>
</tr>
<tr>
<td></td>
<td>embryo</td>
<td></td>
<td>An</td>
<td></td>
</tr>
<tr>
<td></td>
<td>seedling</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Soybean</td>
<td>seedling</td>
<td>An</td>
<td>An only in root</td>
<td>Newman and VanToai 1992</td>
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<tr>
<td></td>
<td>An</td>
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</tbody>
</table>

An = anaerobically inducible
+ = weakly expressed
+++ = strongly expressed
In barley, pearl millet, tomato and soybean expression of ADH in pollen has not been investigated.
anaerobic induction do not confer expression in pollen (M. Olive, pers. comm.). Therefore *Adh* seems to be expressed in pollen under conditions other than anaerobic stress.

Roots and seedlings would encounter frequent periods of anaerobiosis during waterlogging and the induction of ADH in these tissues by low oxygen concentration has been demonstrated for many different plants. In aerobic roots of maize, ADH is only expressed in the root cap and stele (Vogel et al. 1989). ADH may be expressed in the stele in response to local anaerobic conditions, due to this tissue's high oxygen demand and low porosity (Thomson and Greenway 1991). Under anaerobic conditions not only does ADH activity increase in these tissues but *de novo* ADH activity accumulates in the cortex, meristem, epidermis and some longitudinal elements of the vascular system (Vogel et al. 1989). Although maize *Adhl* has been shown to be a non-essential gene, since plants homozygous for an *Adhl* null mutation grow well, the importance of ADH and its anaerobic induction has been highlighted by the fact that these *Adhl* null mutants have a dramatic reduction in their ability to survive anaerobic stress (Schwartz 1969). In addition to regenerating NAD\(^+\) and removing acetaldehyde, ADH is necessary for tight cytoplasmic pH regulation. Roberts *et al.* (1984a) showed that unlike lactic acid fermentation, ethanolic fermentation does not result in cytoplasmic acidosis. In maize ADH1 null mutants it was found that not only were the plants unable to synthesize ethanol and maintain ATP levels but they were also unable to regulate cytoplasmic pH due to competing lactic acid fermentation (Roberts *et al.* 1984a). Cytoplasmic acidosis has been proposed by Roberts *et al.* (1984b) as a determinant of flooding intolerance. Therefore, for a variety of reasons ADH is essential for the plant's survival during anaerobic conditions.

The thesis initiates the examination of the anaerobic response of cotton and this chapter will present the initial genetic and biochemical analysis of the ADH system in this plant, with emphasis on the anaerobic induction of the enzyme. Although ADH has been thoroughly studied in other plants, there has only been very limited study on cotton ADH (Hancock 1982) and no characterization of its anaerobic induction. Cotton has been shown to have a low tolerance to waterlogging during irrigation (de Bruyn
and seedlings have been shown to be very sensitive to anaerobic conditions (Huck 1970). Low levels of expression of ADH might account for this. Cotton seeds have been found to readily synthesize ethanol (Lehle et al. 1991), indicating the presence of ADH, but whether this is the case in seedlings or established plants is unknown. Therefore a major aim of the work is to obtain some idea of the level of expression of ADH in cotton, under both aerobic and anaerobic conditions.

In addition by examining the ADH isozymes, we hope to determine the number of Adh genes that are present in cotton and how many of these are anaerobically inducible. This data will assist in cloning an anaerobically inducible Adh gene from cotton.

The main emphasis in this study will be on G. hirsutum cv. Siokra which is one of the major commercial cotton cultivars grown in Australia. However other cultivars and species will be examined to determine how conserved the ADH system is among the different cottons, in terms of the level of expression of ADH and the pattern of ADH isozymes. By comparing the isozyme patterns, information may also be obtain on the evolutionary relationships between the various species/cultivars of cotton so a brief description of the different cottons and their relationship to one another will follow.

There are approximately 35 diploid (2n=26) species of Gossypium (Malvaceae) and most have been placed into seven genomic groups, A through to G. There are also six allotetraploid (2n=52) species whose genomes have all been classified as having the same two subgenomes (AD). The classification of these species has been based on information concerning cytology, morphology, genetics, taxonomy and geographical distributions (for review see Endrizzi et al. 1985). The main cultivated species are the New World allotetraploid species G. barbadense and G. hirsutum, the latter dominating world cotton production and has accounted for the world wide spread of cotton cultivation. There is still some cultivation of two Old World A genome diploid species, G. arboreum and G. herbaceum which have been cultivated for well over 5000 years (Fryxell 1979).

From cytogenetic and morphological studies it has been established that the A subgenome of G. hirsutum is most closely related to the G. herbaceum genome (Gerstel
1.53, Menzel and Brown 1954) and the D subgenome of *G. hirsutum* to the D genome of *G. raimondii* (Stephen 1944, Hutchinson et al. 1947). The time and place of origin of the allotetraploids is controversial, one main puzzle being that the A genome species are all found in Africa whilst the allotetraploids are all found in the New World. Some consider the allotetraploid formation to be an ancient event (Skovsted 1934, Valicek 1978), when the two original A and D diploid parents overlapped prior to the rifting of the continents, whereas others consider it very recent (Hutchinson et al. 1947, Johnson 1975), where the A genome was transported by man to the New World and under cultivation it hybridized with the D genome. As another alternative Phillips (1963) and Fryxell (1979) argue that the allotetraploids arose not in very ancient or recent times but most likely in the Pleistocene (2 million to 10,000 years ago) following an Atlantic transfer of the progenitor A genome species to the New World. A very ancient origin seems unlikely because of the high degree of similarity of the A and D subgenomes with the A and D genomes of their respective progenitors, but a very recent origin also seems unlikely since the allotetraploids contain an extensive amount of morphological and physiological variability.

The diploid species examined in this study included the putative D progenitor *G. raimondii* and also *G. arboreum* (A genome), a close relative of the putative A progenitor *G. herbaceum* (Phillips 1963), so an analysis between *G. hirsutum* and these diploids may give a brief insight of how related these species are.

2.2 MATERIALS AND METHODS

(a) Plant material.

Seed of *G. hirsutum* cv. Siokra "Blue tag" was obtained from Cotton Seed Distributors Ltd Pty Aust. *G. barbadense* and other cultivars of *G. hirsutum* were obtained from Peter Lawrence of the Australian Tropical Field Crops Genetic Resource Centre. The
diploid species of *Gossypium* were obtained from Brian Hearn, Cotton research station, Narrabri.

To germinate seedlings, seed was delinted in 98% sulphuric acid, then sterilized for 15 mins. in 10% bleach, and germinated in wet vermiculite in a 27°C controlled growth room in the dark. The seeds of the diploid species had to be scratched in order for them to germinate. For mature plants, seeds were planted into soil and grown in a glasshouse.

**(b) Anaerobic treatment and definition of terms.**

Plants were fully submerged in 1 to 2 L of anaerobic induction buffer (10 mM Tris-HCl pH 8.0, 0.1 mM ZnSO$_4$, 50 µg/mL Chloramphenicol) in a 6 L sealed container, through which 100% (v/v) argon was continuously bubbled (approximately 4 to 5 bubbles per second). No more than 30 seedlings per L of induction buffer were used. The anaerobic treatment was carried out in a 25°C control growth room. Plants at the end of the treatment were all washed thoroughly in water.

Throughout the thesis the term anoxia refers to conditions with zero oxygen and hypoxia to conditions with low but not zero levels of oxygen. Oxygen was not removed from the container prior to flushing with argon, therefore initial conditions would be hypoxic but the environment would eventually become anoxic. Because oxygen concentrations were not monitored it is unknown how long the conditions in the container were hypoxic. The term for the conditions used in these experiments will be loosely called "anaerobic conditions" (anaerobiosis) although it would have been a combination of hypoxic as well as anoxic conditions. It should be noted that a period of hypoxia before anoxia is closer to what plants experience in nature rather than immediate anoxic shock. The period of hypoxia allows the plant to acclimatize to the anoxic conditions that follow (Saglio et al. 1988).
(c) Extraction of proteins.

Tissue was ground on ice in cold extraction buffer (50 mM Tris-HCl pH 8.0, 0.1% β-mercaptoethanol, 5% insoluble polyvinyl-pyrolidone) with a mortar and pestle. A small amount of acid washed sand was added to aid grinding. Slurry was pipetted into a 1.5 mL microfuge tube and centrifuged for 15 mins. at 15,000 r.p.m. at 5°C. The supernatant was saved and spun again, and this supernatant was used for assays.

The concentration of protein in the extracts was determined by using the Bio-Rad protein assay kit with bovine serum albumin as the standard (based on Bradford 1976).

(d) ADH spectrophotometric assays.

The spectrophotometric assay of ADH activity involves following the reduction of NAD⁺ to NADH at 340 nm using ethanol as the substrate. The 1 mL enzyme reaction mixture contained 85.5 mM sodium pyrophosphate pH 9.0, 19.1 mM glycine, 1.8 mM nicotinamide adenine dinucleotide (NAD⁺), 1.0 mM glutathione and 0.01-0.02 mL of protein extract. The mixture was inverted and placed in a Perkin-Elmer double beam spectrophotometer and was allowed to equilibrate to 30°C (5 mins.). Ethanol was then added to a final concentration of 0.6 M and the change in O.D was measured for 10 mins. against a cuvette with the identical solution but without ethanol. Data was checked for linearity over time and then converted to ADH activity units. One unit of activity is that which produces 1.6 µmol NADH/min (or the change in 1 absorbance unit at 340 nm/min.) under the assay conditions. All activities were then standardized based on protein concentration of the extracts.

(e) Electrophoresis on cellulose-acetate activity plates.

Samples of protein extracts (of approximately equal concentrations) were loaded using a wire applicator onto Zip zone cellulose-acetate plates (Helena Laboratories). The
plates had been soaked in electrophoresis buffer (50 mM Tris-glycine pH 8.2) for 20 mins. prior to loading. Proteins were electrophoresed at 150 V for 1 hr at room temperature. Plates were then stained for ADH activity in 5 mL of 25 mM Tris-HCl pH 8.0, 0.8 % (v/v) ethanol, 0.144 mM nitro blue tetrazolium, 0.65 mM phenazine methyl sulfate and 0.24 mM NAD⁺ (adapted from Schwartz and Endo 1966). Staining was carried out in the dark at 37°C. The plates were then rinsed with 6% acetic acid for 30 seconds and finally with water for 30 mins. Starch and native PAGE isozyme gels were also performed, but the cellulose-acetate plates gave the best resolution.

(f) Molecular weight determination of the native ADH enzyme.

The molecular weight of the native enzyme was determined on a 0.8 X 29 cm column of Sephacryl S-200 that had been equilibrated with 50mM Tris-HCl pH 8.0 and 1% β-mercaptoethanol overnight at a flow rate of 0.5 mL/min. Blue dextran (2,000,000 kD) was used to determine the void volume (V₀) of the column. The protein standards (1 mg/0.4 mL) were then chromatographed at a flow rate of 0.65 mL/min. and the elution volumes (Ve) determined by measuring total protein absorption at 280 nm for the following marker proteins: β-lactamase (14.1 kD), carbonic anhydrase (30.0 kD), yeast ADH (75 kD and 150 kD), bovine serum albumin (132 kD), and α-amylase (200 kD). The column volume (Vt) had been previously determined, so the Kav (Ve-V₀/Vt-V₀) for each standard could be calculated and plotted against the log of its molecular weight.

A protein extract was prepared as outlined above from seven day old seedlings that had been exposed to anaerobic conditions for the last 48 hrs. Proteins were then precipitated with 60% ammonium sulfate, resuspended in equilibration buffer and then dialysised overnight against equilibration buffer. Approximately 4 mg of protein was chromatographed and 0.5 mL aliquots collected. The Ve was estimated for the ADH enzyme by assaying for ADH activity in the fractions from the column.
(g) Molecular weight determination of the ADH subunit.

The ADH subunit molecular weight was determined by SDS-PAGE and then immunoblotting to locate the enzyme. SDS-PAGE will destroy any multimeric interaction and the mobility of the subunits will be based primarily on molecular weight.

Protein extract samples were each added to an equal volume of boiling buffer (150 mM Tris-HCl pH 6.8, 3% SDS, 20% glycerol, 0.01% bromophenol blue and 4% β-mercaptoethanol) and heated to 96°C for 3 mins. Samples were then run on a 10% acrylamide in 0.375 M Tris-HCl pH 8.8 and 1% SDS resolving gel with a 3.3% acrylamide in 0.125 M Tris-HCl pH 6.8 and 1% SDS stack (Laemmli 1970) together with a set of molecular weight protein standards (Bio-Rad; Myosin 200 kD, β-galactosidase 116.25 kD, Phosphorylase B 92.5 kD, Bovine serum albumin 66.2 kD and Ovalbumin 45 kD). Gels were electrophoresed in 10 mM Tris-glycine pH 8.4 with 1% SDS for 1.5 hrs at 20 mA in a Mighty Small II (Hoefer Scientific Equipment) gel rig. Proteins were then electro-blotted (30 V, 3 hrs at 4°C) onto a nitrocellulose filter which was then stained with 1% amido black for 1 min. to inspect how the extracts had run and to note the mobility of the molecular weight standards. The filter was incubated in blocking solution (25 mM Tris-HCl pH 8.0, 5% w/v milk powder and 150 mM NaCl) for 1 hr at 37°C and then cross-reacted with a 6:1000 fold dilution of a maize ADH1 antibody (a kind gift from M. Freeling's laboratory) in blocking buffer. The filter was then washed three times for 15 mins. in 1X TBS (50 mM Tris-HCl pH 7.5 and 150 mM NaCl) and then incubated with 1:5000 dilution of an antirabbit alkaline phosphatase conjugate antibody (Sigma) in blocking buffer for 2 hrs at 37°C. After further washing (1X TBS, three 15 mins. washes) the filter was then developed by the method of Leary et al. (1983).
2.3 RESULTS

(a) A high specific activity of ADH is found in seeds and declines during germination.

To initiate the characterization of cotton ADH, the specific activity of the enzyme was measured in seed and germinating seedlings, for in other plants this is where ADH has been shown to be strongly expressed (Table 2.1).

The change in specific activity of ADH over time was determined for aerobically grown etiolated seedlings of Siokra. During the first ten days following germination seedlings were harvested every 24 hrs. For each sample of seedlings, extracts were prepared from roots and shoots separately and the specific activity of ADH determined.

Similar to other plants, high levels of ADH activity were found in dry cottonseeds (0 days, Fig. 2.1 A. and 1 B.). This high specific activity level of ADH declined rapidly during the first three days of germination in developing roots and shoots and then decreased only marginally over the next seven days (Fig. 2.1. A and 1 B). By the tenth day the etiolated seedling tissue had started to deteriorate. During germination the protein concentration in the tissues also decreased rapidly but always remained much higher in the shoots than roots.

(b) The specific activity of ADH increases in anaerobically stressed seedlings.

In order to determine if ADH activity was anaerobically inducible, five day old etiolated seedlings of Siokra were subjected to anaerobiosis as stated in the materials and methods. ADH activity was measured in protein extracts which had been prepared from both roots and shoots separately.

In both roots and shoots, a linear relationship can be seen between the increase in specific activity of ADH and the duration of the stress (Fig. 2.1 A. and 1 B.). In both roots and shoots ADH activity peaked after 48 hrs of anaerobiosis. In roots the activity had increased to eight times the aerobic level. In the shoots the induced levels were
Figure 2.1 The specific activity of ADH in germinating seedlings and the induction of ADH by anaerobic stress.

Seeds of *G. hirsutum* cv. Siokra were germinated as described in the materials and methods. During the first ten days of germination seedlings were harvested every 24 hrs. For each sample of seedlings, extracts were prepared separately for the roots (A.) and shoots (B.) and the specific activity of ADH was determined spectrophotometrically.

Five day old etiolated Siokra seedlings were anaerobically stressed for 72 hrs. The specific activity of ADH was determined separately for roots (A.) and shoots (B.) after 0, 4, 8, 16, 26, 48 and 72 hrs of anaerobic conditions.

Each measurement represents readings of three extracts, each extract being prepared from five seedlings. The error bars represent the standard deviation.

Figure 2.1 A: measurements in roots.
Figure 2.1 B: measurements in shoots.
about three to four times greater than the uninduced levels. The induced specific activity of ADH of both roots and shoots reached a similar activity level, which was comparable to the level measured in dry seed.

The tissue of seedlings induced for 72 hrs had begun to degrade in parts, suggesting that the seedlings were dying, explaining why the ADH activity had started to decline.

The measurements of protein concentration indicated that anaerobic treatments did not significantly change the seedling's protein content, therefore the increase in specific activity is not due to general protein breakdown.

(c) ADH activity is anaerobically inducible in green leaf tissue.

Whole plants that had been grown in the glasshouse for six weeks were transferred into anaerobic induction buffer and subjected to anaerobic conditions. Although no ADH activity could be detected in leaves from aerobically grown plants, ADH activity was induced in the leaves when anaerobically stressed. The stress clearly had an adverse affect on the plant and where visible degradation of leaf tissue had occurred, even after one day, ADH activity in the tissue was very low. But in the parts of the leaf that had appeared to remain healthy, ADH activity increased significantly, and continued to do so for three days (Fig. 2.2). The induced specific activity of ADH was found to be lower in green leaves compared to the activity induced in the roots and shoots. This is also the case in rice (Xie and Wu 1989) and may be due to the higher protein content of leaves, for specific activity is based on protein concentration. By the fourth day the majority of the leaf tissue had become necrotic so no attempt was made to measure activity beyond the third day.

(d) Two Adh genes are expressed in the seeds of G. hirsutum.

To determine how many Adh genes are expressed in cotton seed, protein extracts were prepared from whole seeds of Siokra which were then run on cellulose-acetate plates and stained for ADH isozymes. In addition, extracts were made from 18 other different
Figure 2.2 The specific activity of ADH is induced in anaerobically stressed green leaves of Siokra. Values are the mean of four samples ± standard error.
cultivars of *G. hirsutum* and analysed so as to screen for electrophoretic variants that may exist in the germplasm. The ADH isozyme patterns of all 19 cultivars were identical (nine cultivars are shown in Fig. 2.3). Two major ADH isozyme bands were resolved and in every case the cathodic band was stronger than the anodic band. A minor band, running slightly faster than the major bands could also be resolved. The ADH isozymes of another allotetraploid species, *G. barbadense* also displayed an identical pattern (Fig. 2.3, Lane 9).

A possible gene-isozyme system is shown on the side of Fig. 2.3, where it is proposed that the expression of two genes are forming these three isozymes. One gene is coding for the strong cathodic band (designated ADH1:ADH1) and a second gene for the weak anodic band (ADH2:ADH2) with the intermediate isozyme being the heterodimer of the two genes (ADH1:ADH2). Because both catalytic efficiency and level of expression contribute to the intensity of an isozyme band it is unknown whether the cathodic band is stronger because of the level of expression of the enzyme or because that enzyme has a higher catalytic rate.

When extracts from ten individual seeds of *G. hirsutum* cv. Siokra from the same seed batch were examined on an activity plate (data not shown) no change in the isozyme banding pattern was found, implying that the different isozymes come from different loci, not different alleles. The gene coding for the cathodic isozyme has been designated *Adh1*, for in maize, *Adh1* is the predominant isozyme in the seed and is also the cathodic isozyme. The gene coding for the anodic isozyme will be called *Adh2*.

A faint isozyme band is sometimes seen above the ADH1 homodimer (also see Fig. 2.9, Lanes 1 & 8). This could be the expression of yet another gene or it could be a "ghost band", which is an artefact that occurs when a NAD+ co-factor has bound onto the isozyme altering the surface charge and therefore the isozyme's electrophoretic mobility (Jacobson 1968).
Figure 2.3 The ADH isozyme patterns in seed of the allotetraploid cultivars of *G. hirsutum*. Lane (1) Satu (Uganda), (2) VHG 14-26 (USA), (3) IRMA 5028 (Cameroon), (4) TX Lebo 25-81 (Texas), (5) SP 510 (Argentina), (6) Strumica 105 (Yugoslavia), (7) Siokra (Australia), (8) M93-1224 (USSR) and (10) Coker 310 (Sth Carolina). *G. barbadense* cv. Pima S5 is in lane (9). The propose gene-isozyme system is shown to the side.
(e) Anaerobiosis alters the ADH isozyme pattern in roots and shoots of seedlings.

It has been shown that anaerobic stress results in an increase in the specific activity of ADH. To determine which isozyme/s increase under anaerobic conditions extracts were examined on isozyme plates (Fig. 2.4).

Roots that had not been subjected to anaerobiosis (0 hrs) contained three ADH isozymes (Fig. 2.4, Lane 1) with mobilities that were identical to that of the isozymes found in dry seed (data not shown).

When seedlings were anaerobically stressed the intensity of the most anodic isozyme band becomes much more prominent (ADH2:ADH2). In roots, the longer the period of anaerobiosis the more predominant this newly synthesized band becomes compared to the other two isozymes that do not appear to be induced. This implies that the increase in specific activity of ADH in roots shown in Fig. 2.1 A. is due mainly to the induction of ADH2. Although there are three isozymes, some doubt is cast upon the model because the relative activities of the isozymes change independently from one another. It would be expect that the ADH1:ADH2 heterodimer would increase in intensity along with the ADH2:ADH2 isozyme during anaerobic conditions, but instead the proposed heterodimer appears to decrease in intensity. An increase in the ADH1:ADH2 heterodimer may not be occurring because de novo synthesis and assembly of both subunits may be required for the formation of the heterodimers and because at best, ADH1 is only being weakly induced, the formation of the ADH2 homodimer will predominate.

The shoots have a different isozyme pattern to the roots. In shoots, as well as the isozymes found in the seed and roots, an additional more anodic isozyme is weakly expressed, implicating the expression of a third gene (Adh3). However its expression does not appear to be altered by anaerobic stress. When anaerobically stressed the expression of ADH2 is again strongly induced. However unlike the root isozymes, ADH2:ADH2 does not become the predominant isozyme. The ADH1:ADH1 isozyme appears to have been induced and is still more intense than the ADH2:ADH2 isozyme after 72 hours of anaerobic stress. If the isozymes dimerize randomly as happens in
Figure 2.4 The effect of anaerobiosis on ADH isozymes. Protein extracts were separated on a cellulose-acetate plate and then stained for ADH activity. Lanes 1 to 5 are extracts of roots from five day old seedlings exposed to; (1) 0 hrs, (2) 8 hrs, (3) 24 hrs, (4) 48 hrs and (5) 72 hrs of anaerobiosis. Lanes 6-10 are shoot extracts from the same seedlings exposed to; (6) 0 hrs, (7) 8 hrs, (8) 24 hrs, (9) 48 hrs and (10) 72 hrs of anaerobic stress.
maize, where a 1:2:1 ratio of ADH1:ADH1, ADH1:ADH2, ADH2:ADH2 occurs when both genes are expressed (Freeling 1974), we would expect the activity of the proposed heterodimer to predominate. This is not the case and so doubt is again cast upon the proposed gene-isoenzyme system. One explanation is that the genes might be differentially expressed, with ADH1 and ADH2 being synthesized in different cell types and thus being physically separated from one another and so are unable to dimerize.

The mobility of the shoot isozymes become faster after anaerobic stress (compare the mobilities in lanes 6 and 7) accounting for the differences in mobilities of the shoot isozymes when compared to the corresponding root isozymes. It is unclear why this occurs, but it may be due to some alteration in the protein extract (such as the NAD+/NADH ratio) or some tissue specific modifications of the isozymes. When extracts from anaerobically stressed whole seedlings are run, Fig. 2.10 A., two new anodic isozymes appear, suggesting that the two ADH2 isozymes are resolvable.

The shoot isozyme bands are more intense than those of the roots due to the fact that the protein extracts loaded are more concentrated, therefore ADH3 may be present in roots but not visible because insufficient protein was loaded.

(f) Cotton ADH is a dimeric protein; molecular weight estimation of the native and subunit sizes.

It has been shown that there are three main isozyme bands in Siokra. To determine whether the three isozymes are coded for by two or three genes, it was necessary to determine if the enzyme has a dimeric structure like that of ADH from other plants. No ADH electrophoretic variants were available so this question had to be answered by biochemical analysis.

The molecular weight of the native enzyme was estimated by gel filtration on a Sephacryl S-200 column and the subunit size by SDS-PAGE. This analysis was performed because the activities of the isozymes appear to be changing independently of one another and not forming intergenic heterodimers (Fig. 2.4).
Figure 2.5 Estimation of the molecular weight of cotton ADH by gel filtration on a Sephacryl S-200 column.
A gel filtration standard curve was established, being calibrated by the use of marker proteins of known molecular weight. The data were then subjected to linear regression to determine the line of best fit. An ammonium precipitated cotton extract was then chromatographed and all ADH activity eluted as a single broad peak. Based on the data in Fig. 2.5, the native ADH enzyme has a molecular weight of approximately 81 kD, implying that all the isozymes are indistinguishable in size as estimated by gel filtration. The estimated molecular weight is similar to that of the other plant ADHs such as maize, 80 kD (Freeling and Schwartz 1973), Arabidopsis, 87 kD (Dolferus and Jacobs 1984) and soybean 74 kD (Tihanyi et al. 1989) and animal ADHs like horse liver, 80 kD (Eklund et al. 1976). Unlike soybean ADH, the ammonium sulfate precipitation did not inactivate the enzyme (Tihanyi et al. 1989).

In order to determine the subunit molecular weight a soluble protein extract of cotton seed was electrophoresed alongside extracts from maize, wheat, tobacco and pea on a 10% polyacrylamide gel and then subjected to Western analysis with a maize ADH\textsubscript{1} antibody. Fig. 2.6 demonstrates that all extracts have proteins that cross-react with the antibody in the 40-45 kD range, with slight variations in molecular weight for each plant species. Cross-reaction with all the plant extracts demonstrates that all ADHs tested shared antigenic determinants reflecting strong conservation not only in molecular weight, but also of the amino acid sequence between ADHs of different plants.

Both cotton and tobacco extracts have two bands, the slower bands being less intense, indicating that they could have ADH subunits with different molecular weights. These could be contaminating bands caused by antibodies cross-reacting with other proteins, but if this was the case we would expect to see these extra bands in all the samples. They could also be covalently modified forms of ADH although there is no evidence that these exist in other plants and only one peak of activity eluted from the Sephacryl column.

This result together with the column chromatography, indicates that cotton ADH is active as a 81 kD dimer composed of subunits of similar molecular weight (approximately 40 kD). Also because in the column no activity was found in the 40-45
Figure 2.6 Western analysis of protein extracts from cotton (1), tobacco (2), wheat (3), pea (4) and maize (5). Protein extracts were prepared from cotton, wheat, pea and tobacco seeds and maize scutellum. Equal quantities of protein (50 µg) were loaded into each lane of a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was subjected to Western analysis and cross-reacted with an antibody raised against maize ADH1. The mobility of the molecular weight markers are shown to the right of the Western.

Table 2.2 The specific activity of ADH in seed of the different plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>(Units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>7.94</td>
</tr>
<tr>
<td>Tobacco</td>
<td>1.75</td>
</tr>
<tr>
<td>Wheat</td>
<td>1.85</td>
</tr>
<tr>
<td>Pea</td>
<td>3.20</td>
</tr>
<tr>
<td>Maize</td>
<td>3.88</td>
</tr>
</tbody>
</table>
kD range, the monomer alone is not active and therefore requires dimerization for activity. This dimeric structure for cotton ADH gives support to the proposal that the three isozymes seen in cotton are coded by two genes with the intermediate band being a heterodimer.

Examination of the intensities of bands from the Western analysis suggests that cotton has ADH levels in its seed that are significantly higher than tobacco and pea. Because the intensity of the Western blot bands will also depend on epitope similarity, and wheat ADH would be expected to be more homologous to the maize ADH1 than cotton, it is also likely that cotton has more ADH than wheat in seeds. This proposal is supported by the specific activity levels of ADH measured in each extract (Table 2.2). This suggests that allotetraploid cotton has comparatively high levels of ADH expression in seeds.

(g) Siokra has an anaerobically induced ADH activity level that is similar to that of other allotetraploids.

A comparison was made among several different cultivars and species of *Gossypium* to see what level of variability in ADH activity and induction exists between different varieties of cotton. The anaerobic inducibility of ADH of *G. hirsutum* cv. Siokra was compared to an Indian cultivar of *G. hirsutum*, Sujata, to another allotetraploid species, *G. barbadense* cv. Pima S5 and to a diploid species called *G. arboreum*. Sujata was chosen because being an Indian cultivar it may be exposed to flooding during the monsoon. If it has adapted to flooding this may be reflected by a change in the inducibility of its ADH. *G. arboreum* was chosen because it is the only diploid that displays similarity in terms of seed morphology and seedling growth to the allotetraploid species.

All allotetraploid species had similar levels of ADH activity in roots, both for the induced and uninduced samples (Fig. 2.7). Although *G. arboreum* had a similar aerobic root ADH level, the level of ADH activity could only be anaerobically induced to about half that of the allotetraploids.
Figure 2.7 Anaerobic induction among different varieties of cotton. Five day old seedlings of each variety were placed under anaerobic conditions for 24 hrs. Extracts of roots and shoots of both aerobically grown and anaerobically stressed plants were made and the specific activity of ADH determined. Each measurement is from three extracts, each extract is made from five seedlings each. The error bars represent the standard deviations. Solid bars are the anaerobic measurements.
In shoots the aerobic levels of ADH were similar among the allotetraploids but after anaerobic stress these activities were induced to various levels, although the higher level in Sujata was not statistically different to the level found in Siokra. In contrast, *G. arboreum* had both uninduced and induced ADH activity levels that were much lower than the allotetraploids.

Also comparison of the ADH isozyme patterns showed that both of the other allotetraploids had identical isozyme patterns to that of Siokra before and after anaerobiosis (data not shown). So not only are the isozyme patterns conserved among the allotetraploids but also the levels of ADH activity.

(h) The diploid species of *Gossypium* have lower induced specific activity levels of ADH compared to the allotetraploids.

To explore further the differences between allotetraploid and diploid species, aerobic and anaerobically induced levels of ADH activity were determined and compared between Siokra and six diploid species of *Gossypium*. Because of the small size of the diploid seedlings whole plant extracts were prepared instead of root and shoot extracts separately, therefore the data is biased towards the amount of ADH present in the shoot. Extracts were prepared from seven day old etiolated seedlings that had been subjected to anaerobiosis for 0 and 24 hrs. All diploid species had specific ADH activities that could be induced by anaerobiosis, but the specific activities were all lower than the activity of ADH in Siokra (Fig. 2.8). Of the diploids, *G. raimondii* had the highest induced ADH activity level but it was only about 65% of the Siokra induced level. The rest displayed a variety of levels, with *G. nelsonii* having almost no aerobic ADH level at all.

Because each diploid species would be expected to have at least one anaerobically inducible *Adh* loci, the allotetraploid species would be expected to have at least two anaerobically inducible *Adh* loci, acquiring one from each diploid progenitor.
Figure 2.8 Comparison of the anaerobic induction of ADH between G. hirsutum cv. Siokra and diploid species of Gossypium. ADH activity measurement were made on whole plant extracts of seven day old seedlings after 0 and 24 hrs of anaerobiosis. Each measurement is from only one sample made from five to seven plants. Solid bars are the anaerobic measurements.
(i) The diploid species of *Gossypium* display polymorphic ADH isozyme patterns.

The isozyme patterns of the diploid species were examined to determine how many *Adh* genes each species contain and to determine if their isozyme patterns vary. Samples of protein extracts prepared from seeds of 13 diploid species were analysed on cellulose-acetate activity plates. Fig. 2.9 shows that each diploid species has its own unique ADH isozyme pattern except for the two subspecies of *G. sturtianum*, confirming the close evolutionary relationship between these two lines. The plates also showed that the intensity of the ADH isozyme bands among these diploids varies, with *G. thurberi* and *G. nelsonii* possessing very faint bands, whilst *G. raimondii* and *G. anomalum* have intense bands, consistent with the activity data in Fig. 2.8. Although approximately equal amounts of protein from each sample were loaded, no diploid species had ADH isozyme bands that were of greater intensity than that of Siokra, which is again consistent with the activity data. The diploids *G. arboreum* and *G. raimondii* did not possess any isozyme that had a mobility similar to that of Siokra.

(j) The diploid species also have anaerobically inducible isozymes.

The alteration by anaerobiosis of the diploid's ADH isozyme patterns was examined to determine which of the isozymes are induced by the stress. The number of anaerobically inducible *Adh* genes in each species may be determined. In addition comparison of the isozymes of the putative progenitors with Siokra would reveal any common bands. Four separate plates were run (Fig. 2.10 A., B., C. and D.).

*G. arboreum*, and *G. raimondii* were run alongside *G. hirsutum* (Fig. 2.10 A.). A comparison of the mobilities of these isozymes reveals that the newly induced anodic isozyme band of *G. arboreum* (arrow) is similar to the most anodic isozyme band of Siokra which has been anaerobically induced (Lane 2). The only isozyme band of *G. raimondii* is similar to the other anaerobically induced isozyme band of Siokra,
indicating that there could be commonality between the ADH isozymes of the diploid and the allotetraploid species.

Some similarity also extends to the pattern of isozyme expression. It could be hypothesized that the three isozymes of *G. arboreum* are coded by two genes, that have a similar pattern of expression to those of Siokra. Here, the gene coding for the cathodic isozyme is the most predominant form in the seed and seedlings and is induced by anaerobic conditions (Fig. 2.10 A. Lanes 3&4), similar to the ADH1 isozyme of Siokra. A third more anodic isozyme can be seen only after anaerobic stress, implying the induced expression of a second gene. This second gene must also be expressed in the seed (because of the appearance of the heterodimer) and so this isozyme appears to have a similar expression pattern to the ADH2 isozyme of Siokra.

The other putative progenitor *G. raimondii* has a very simple pattern with just one very strongly expressed isozyme. This could be the product of more than one gene with the isozymes having similar mobilities which are unable to be resolved. However, from these two diploid isozyme patterns it can be concluded that the allotetraploids have at least three loci (two from *G. arboreum* and one from *G. raimondii*) of which at least three are anaerobically inducible (the two from *G. arboreum* are inducible and the one from *G. raimondii* must also be inducible).

*G. davidsonii* (D), *G. anomalum* (B) and *G. stocksii* (E) all have isozyme patterns somewhat similar to *G. arboreum* although the mobilities are different. All have two major isozymes in aerobic roots, the cathodic isozyme being strongest, with a third more anodic band becoming visible only under anaerobic conditions. In addition *G. davidsonii* has a fourth isozyme at the far cathodic end implying that this diploid has three *Adh* genes. This fourth isozyme does not appear to be anaerobically inducible, but it is forming faint heterodimers with the more anodic group of isozymes confirming that this is an ADH isozyme. So *G. davidsonii* appears to be an example of a diploid species that has three *Adh* genes.

Although *G. sturtianum* cv. Nandewarense has two strong isozymes in seed it appears to have little or no ADH activity in aerobic seedlings. In anaerobic seedlings it has again
Figure 2.10 The effect of anaerobiosis on the ADH isozyme patterns of the diploid species. The aerobic and anaerobic isozyme profile of Siokra and ten different diploid plant species were examined. Approximately equal protein amounts of the same whole plant extracts described in Fig. 2.8 plus samples from additional species were electrophoresed and stained for ADH isozymes. Odd numbered lanes are aerobic samples while even numbered lanes are anaerobic samples. Lanes; *G. hirsutum* (1) & (2), *G. arboreum* (3) & (4), *G. raimondii* (5) & (6), *G. davidsonii* (7) & (8), *G. somalense* (9) & (10), *G. longycalse* (11) & (12), *G. anomalum* (13) & (14), *G. australe* (15) & (16), *G. nelsonii* (17) & (18), *G. stocksii* (19) & (20), *G. sturtianum* (21) & (22) and *G. thurberi* (23) & (24).
two isozymes but this time the most anodic isozyme is the dominant form while there is little expression of the cathodic isozyme.

*G. austral*e*(C) has one isozyme that has strong activity in seed, seedlings and is the dominant form under anaerobic conditions. The activity of the other isozymes are much weaker and unusual in the fact that the most anodic band disappears under anaerobic conditions and a more anodic band appears. The anaerobic pattern appears to be a two gene, three isozyme system. Therefore six diploids have a cathodic isozyme with strong activity in seed, analogous to ADH1 of Siokra and an anodic isozyme band which only appears after anaerobic treatment, similar to ADH2 of Siokra. Therefore the cathodic isozymes will be assigned to the ADH1 class and the anodic isozymes to the ADH2 class.

*G. nelsonii* has the lowest expression of ADH in both seed and seedlings and this is reflected in its isozyme pattern where only one faint isozyme becomes visible after anaerobic conditions. *G. longycalyse* and *G. somalense* have two major isozymes bands that have very different electrophoretic mobilities. Careful inspection reveals very faint isozymes that run intermediate to these suggesting the formation of heterodimers, although not as readily as some of the other diploid species.

All the diploid isozyme results are summarized in Table 2.3, and the number of expressed *Adh* genes for each species proposed.

One unusual observation is that in some plant species the electrophoretic mobilities of isozymes are faster in the anaerobically prepared extract than in the aerobic extract (compare Lanes 3 & 4, 9 & 10 and 13 & 14). This implies that anaerobic conditions has altered some property of the extract, may be altering the energy charge or the population of NADH co-factors which alters the enzyme's mobility.
Table 2.3. The ADH isozymes of the diploid species of *Gossypium*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genome</th>
<th>Number of isozymes</th>
<th>The possible N° of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Seed</td>
<td>Seedlings</td>
</tr>
<tr>
<td><em>G. arboreum</em></td>
<td>A</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><em>G. raimondii</em></td>
<td>D</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>G. anomalum</em></td>
<td>B</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><em>G. sturtianum</em></td>
<td>C</td>
<td>2-3</td>
<td>2</td>
</tr>
<tr>
<td><em>G. austral</em></td>
<td>C</td>
<td>2-3</td>
<td>3</td>
</tr>
<tr>
<td><em>G. nelsonii</em></td>
<td>?</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>G. thurberi</em></td>
<td>D</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>G. davidsonii</em></td>
<td>D</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><em>G. somalense</em></td>
<td>E</td>
<td>1</td>
<td>2-3</td>
</tr>
<tr>
<td><em>G. stocksii</em></td>
<td>E</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>G. longycalyse</em></td>
<td>F</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>G. bickii</em></td>
<td>G</td>
<td>1</td>
<td>N/E</td>
</tr>
</tbody>
</table>

N/E = not examined.
2.4 DISCUSSION.

Although the *G. hirsutum* cultivars used in the isozyme survey represent Africa, North and South America, Europe, Central Asia and Australia no different electrophoretic variant of ADH in seed could be found. Kloth (1992) examined 24 cultivars of *G. hirsutum* for isozyme variants for malate dehydrogenase but also could not find any variants. In addition the specific activities of ADH before and after anaerobic conditions are similar in seedlings of *G. barbadense* and two cultivars of *G. hirsutum* (Fig. 2.7), implying that not only is the isozyme pattern conserved but also the level of expression of ADH. This high level of conservation suggests that cultivars of *G. hirsutum* and *G. barbadense* are all closely related supporting the theory that the allotetraploid species have evolved relatively recently (Johnson 1975). In addition, the identical isozyme pattern of *G. hirsutum* and *G. barbadense* make it feasible to postulate that these allotetraploids originated from identical progenitors supporting the theory of a monophyletic origin of the allotetraploid species postulated by Gerstel and Sarvella (1956).

In contrast to the allotetraploids, the diploids have polymorphic isozyme patterns, reflecting that these species diverged from one another a long time ago. There is some commonality between the isozyme patterns of *G. hirsutum* and the two diploid species, *G. raimondii* and *G. arboreum*, although clearly the combination of the two sets of isozymes of the diploids would not result in an isozyme pattern identical to that of Siokra. From our limited isozyme data, it seems unlikely that these two species hybridized to form the allotetraploids in recent times and that the species have been evolving separately for sometime, resulting in isozymes with altered mobilities. Alternatively there may have been polymorphisms within the diploid species, and our diploids are not identical to the progenitors.

However some similarities do exist between the patterns of expression of the diploid and allotetraploid isozymes. In most diploid species the cathodic isozyme had strong activity in the seed and aerobic seedling while an anodic isozyme became visible only
after anaerobic stress, similar to the pattern in the allotetraploids. Therefore, from our experimental data, it appears that the regulation of expression of these genes has been conserved more than the charge structure of the enzymes throughout the *Gossypium* family.

Examination and comparison of the staining intensities of the isozyme plates and also the measurements of the specific activity of ADH in the extracts, reveals that the allotetraploids have higher levels of ADH activity compared to the diploids. Therefore the formation of an allotetraploid appears to have resulted in cotton having increased levels and inducibility of ADH. This may be due to the increased number of genes in the allotetraploid. Roose and Gottlieb (1980) examined the expression of ADH in a recently evolved allotetraploid plant *Tragopogen miscellus* and found that expression was at an intermediate level compared to the levels in its diploid progenitors, demonstrating that homoeologous genes in two diploid plant species that specify different amount of ADH can maintain the same relative level of expression in an allotetraploid derivative. This does not seem to be the case in cotton, further suggesting that the allotetraploids are now distantly related to the diploids. Because of the presence of NADH oxidases that are found in cells, caution must always be made when comparing ADH activities between different plant species, for the spectrophotometric assay may be underestimating the level of activity if these oxidases are reconverting NADH (which is being measured) back into the NAD$^+$ form. Different species may have different levels of these enzymes.

A high level of expression of ADH in the seed that quickly declines during the germination process, as seen in Siokra, seems to be universal among plants (Leblova *et al.* 1971). It has been suggested that the expression of ADH in seed occurs because hypoxic conditions exist within the seed (Bewley and Black 1978) and as the seed germinates the protrusion of the radicle and/or shoot stimulates respiration and therefore anaerobic enzymes such as ADH decline. If ADH is expressed in seed because anoxic conditions exist then the mode of induction of the seed specific ADH1 isozymes must be different to that of the ADH2 isozymes. This is because ADH2 is not induced under
the "natural anaerobiosis" occurring in the seed, but only in the seedling if anaerobically stressed. This suggests a role for other factors controlling the expression of ADH such as plant hormones like abscisic acid which has been shown to induce the *Arabidopsis Adh* gene (G. de Bruxelles unpublished).

Like that found in maize (Freeling 1973), cotton ADH activity increases during anaerobiosis at a zero-order rate in both roots and shoots. Although the shoots of seedlings have an additional *Adh* gene that is anaerobically induced, the specific activity only reaches a level similar to that of roots. ADH activity in anaerobically stressed roots increased to eight times the aerobic level and in shoots the level increased three to four times during anaerobic stress. These increases are of the same magnitude as the increases that are seen in maize (Hageman and Flesher 1960), soybean (Russell *et al.* 1990) and rice (Xie and Wu 1989). The induction of the specific activity of ADH in seedlings peaks after 48 hrs of anaerobiosis. This period of induction is longer than that seen in the dicots Pea, 12 hrs (Llewellyn *et al.* 1987) and tomato, 12-18 hrs, (Tankersley and Jones 1981) but shorter than in the monocots rice 84 hrs (Xie and Wu 1989) and maize 72 hrs (Freeling 1973). The duration and levels of the ADH induction probably reflect the experimental conditions as well as the seedling's tolerance to anaerobic conditions. This is highlighted in maize, where seedlings hypoxically pretreated (5% oxygen) before being exposed to anoxic conditions (0% oxygen) had a high level of ADH activity which lasted up to 96 hrs of anoxia, but when no hypoxic pretreatment was given, only a low level of ADH activity was found (Johnson *et al.* 1989).

All the data, including enzyme activities and Western analysis suggests that cotton ADH is strongly expressed under anaerobic conditions and that cotton's intolerance to waterlogging/anaerobiosis is probably not due to a lack of ADH expression. However the enzyme activity data cannot be taken as conclusive, because the *in vivo* activity may be very different from what has been measured *in vitro* particularly in view of the fact that the *in vitro* assay involves measuring the opposite reaction to that occurring *in vivo.*
The finding that ADH is induced in leaves is in contrast to maize where ADH expression and induction is repressed in green tissues (Okimoto et al. 1980), but is similar to rice ADH where it is expressed in leaves (Xie and Wu 1989). As in rice this may reflect an evolutionary adaptation, because for many cotton species the natural habitat is around dry river beds, and therefore the plants may be subjected to total immersion during flooding. However ADH has been reported to be anaerobically induced in leaves of tomato ( Tanksley and Jones 1981) and potato (Matton et al. 1990) so expression in leaves may not be as uncommon as first thought.

Based on the isozyme data we have proposed how many actively expressed Adh genes the diploid species have. Most diploid species of cotton appear to contain two Adh genes which is in keeping with most diploid plants (Gottlieb 1982), although G. davidsonii appears to have three Adh genes, implying that this diploid species has undergone an additional gene duplication. From analysis of the diploid species it could be concluded that the allotetraploids would have acquired two Adh genes from the A genome of which both are anaerobically inducible and at least one from the D genome which is also anaerobically inducible.

In Siokra, Adh1 is expressed most strongly in the seed although Adh2 is also expressed to some extent. Adh2 is expressed strongest in anaerobic roots, where Adh1 does not appear to be induced, although Adh1 is induced in shoots along with Adh2. In addition shoots have a weakly expressed fourth isozyme band implicating the expression of a third gene, but it does not appear to be anaerobically inducible. When root or shoot extracts are run separately, the mobilities of the corresponding ADH2:ADH2 isozyme bands are different (Fig. 2.4). It is unlikely that the root and shoot ADH2 isozymes are coded for by different genes, but rather the different mobilities arise due to some experimental artefact. This is supported by the observation that the mobilities of the shoot isozymes become faster after anaerobic stress, suggesting that either these isozymes have undergone tissue specific modifications, or that there has been some alteration in the shoot extract (such as salts). The fact that when whole seedling extracts are run, two anodic ADH2:ADH2 isozymes bands are resolved (Fig. 2.10 A.), suggests
that salt concentration is not the reason, but rather some tissue specific modification results in these different mobilities. This might be tested by desalting the extracts before running them on the isozyme plates. Cloning the Adh gene that codes for the ADH2 isozyme, along with Northern analysis would verify that the shoot and root ADH2 is coded by the same gene.

The isozyme model has been proposed on the basis of the isozyme patterns, but unfortunately due to the lack of electrophoretic variants it cannot be tested. It is still uncertain how many genes are coding for each isozyme band; in allotetraploids multiple gene copies exist due to the presence of homoeologous genes in each of the subgenomes, therefore two genes could be encoding each isozyme. For the two gene products of the homoeologous genes to be resolved by isozyme gels a change in one of the charged amino acids of one of the products would be necessary. For example, soybean is an allotetraploid and Newman and VanToai (1991) could only detect three ADH isozymes and proposed a two gene model. But when they obtained Adh cDNAs they were able to isolate three different classes (Newman and VanToai 1992) highlighting the limitations that do exist with isozyme gels. Therefore for more subtle changes, electrophoretic methods with higher resolutions would be necessary such as isoelectric focusing. This will be one of the questions addressed in the next chapter.
CHAPTER THREE

The Anaerobic Response of Cotton.

3.1 INTRODUCTION

Higher plants undergo alterations in gene expression during exposure to an environmental stress (for review see Sachs and Ho 1986). Like animals, plants synthesize heat-shock proteins when stressed with high temperature. But whereas animals will synthesize heat-shock proteins in response to a variety of other stresses (Lindquist 1987), plants have different, specific sets of proteins they synthesize in response to other environmental stresses. Such sets of proteins have been found in response to flooding or anaerobiosis (Sachs et al. 1980), high salt (Ericson and Alfinito 1984), UV light (Chappell and Hahlbrock 1984), water-stress (Close et al. 1989), cold (Guy 1990) as well as high temperature (Key et al. 1981). These responses have probably evolved due to the sedentary nature of plants, where unlike animals, plants are unable to alter their location in the environment. Although the physiological significance of some of these stress-induced proteins is unknown, it is thought that they mediate biochemical and/or structural modifications at the cellular level which assist the plant to tolerate the stress until normal conditions again prevail.

In plants, the molecular responses to anaerobiosis and heat are the most dramatic. The responses appear to share common characteristics although different genes are involved in each response. Both responses involve a switch in gene expression from the normal pre-stress set, to only a small battery of stress responsive genes. These switches are regulated at the level of transcription, where rates of mRNA transcription and steady state levels of mRNA increase for the stress responsive genes and decrease for the pre-stressed set of mRNAs. In addition, both responses result in the rapid repression of normal protein synthesis through translational controls during the stress, indicating a need for urgent changes within the stressed cells. This shut down results in the
translation mainly of the stress responsive set of mRNAs and in both responses this occurs in a biphasic manner, with a first set of proteins being expressed for a transient period of time followed by a second set being produced until cell death. Finally both responses are reversible, with normal protein synthesis being restored if plants are returned to non-stressed conditions before cell death occurs, although in maize seedlings after anaerobic stress the primary root eventually dies and adventitious roots form.

The alteration in gene expression occurring during anaerobiosis has been characterized in several plants, namely maize (Sachs et al. 1980), Arabidopsis (Dolferus et al. 1985) and soybean (Russell et al. 1990). The anaerobic response has also been found in plants such as rice, sorghum, Tragopogon, barley and carrots (Roose, M.L., Chen, C.H. and Okimoto, R., unpublished data) demonstrating that this response is widespread throughout the plant kingdom.

The anaerobic response in the primary root of maize seedlings has been the best characterized (Sachs et al. 1980). During the first hour of anaerobiosis, aerobic protein synthesis is repressed and a small set of 33 kD proteins (approximately four), known as the transition polypeptides (TPs) are synthesized. The function of these polypeptides is unknown, but it has been suggested that they may play a role in the regulation of the response (Sachs et al. 1980). In the second hour of anaerobiosis, the synthesis of a second group of about 20 polypeptides is induced; these are the anaerobic polypeptides (ANPs). The group consists of 10 major and 10 minor polypeptides depending on their level of expression as determined by protein labelling and two dimensional gel electrophoresis. After five hours of anaerobiosis the ANPs account for more than 70% of total protein synthesis. They are synthesized in the same proportion and at the same rate during prolonged anaerobic stress, until root death occurs some three days later (Sachs et al. 1980).
Several of the ANPs have been identified as either ethanolic fermentation enzymes, i.e., 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, Lazlo and St Lawrence 1983) or glycolytic enzymes including glucose phosphate isomerase (Kelley and Freeling 1984a), cytosolic fructose-1,6-biphosphate aldolase (Kelley and Freeling 1984b) and glyceraldehyde-3-phosphate dehydrogenase III (GAPDH3, Russell and Sachs 1989 and 1992). The role of these enzymes in the anaerobic pathway of glycolysis is shown in Fig. 3.1. Also the starch mobilizing enzyme, sucrose synthase 1 isozyme (SS1/Sh, Springer et al. 1986) is an ANP. In addition Bailey-Serres et al. (1988) have measured the enzyme activities of other glycolytic enzymes and found that enolase, phosphoglycerol mutase and phosphoglucomutase all have activities induced by anaerobic stress.

These enzymes are all involved in the conversion of sucrose to ethanol and it has been suggested that the anaerobic response evolved as an adaptation to flooding. It is presumed that during aerobic conditions the glycolytic enzymes are at levels sufficient to provide enough substrate for mitochondrial activity. During prolonged anaerobic conditions the levels of these enzymes increase to allow for a greater glycolytic flux, compensating for the switch from the oxidative (36 mol. of ATP/mol. of glucose) to the less efficient ethanolic fermentative pathway (2 mol. of ATP/mol. of glucose), and hence resulting in a Pasteur effect. In general it is thought that the increased flux will generate enough ATP to allow the plant to acclimate to anaerobic stress (Saglio et al. 1988, Hole et al. 1992). However the level of the two enzymes which are thought to be responsible for regulating the glycolytic pathway, phosphofructokinase and pyruvate kinase do not change significantly during anaerobiosis (Bailey-Serres et al. 1988). These and the other stem glycolytic enzymes do not show the same level of inducibility as ADH and this is due to the fact that these enzymes are required at relatively high levels even under aerobic conditions because of their role in aerobic carbohydrate metabolism.
Fig. 3.1 Anaerobic glycolysis in plants. Sucrose and starch are broken down into pyruvate, from which ATP is generated but NAD\(^+\) becomes reduced. For glycolysis to continue NAD\(^+\) must be regenerated, and this can be achieved through ethanolic or lactic acid fermentation. Enzymes that have been identified as maize anaerobic polypeptides (i.e. have been shown to correspond to a spot on a 2D gel) have been written in bold typing. Other enzymes which have not been correlated with a spot but have anaerobically inducible enzyme activities are written in smaller typing. The physiological significance of these pathways will be discussed in Chapter Five.
Several of the ANPs are not involved in the sucrose to ethanol pathway. Lactate dehydrogenase (Hoffman et al. 1986) and alanine aminotransferase (Good and Crosby 1989) in barley have been shown to be anaerobically inducible and are probably anaerobic polypeptides in maize. As shown in Fig. 3.1, these enzymes provide alternatives to ethanol production where lactic acid and alanine have been shown to be major metabolic products of anaerobic metabolism in plants (Smith and ap Rees 1979, Rivoal and Hanson 1993). Their significance will be discussed further in Chapter Five.

In addition Vogel and Freeling (1991) have reported on the cDNA sequence analysis of the maize ANP27, and showed that this ANP has no homology with any known glycolytic enzymes but does have homology (60%) to the maize Mul.7 transposable element. The cDNA encodes a protein having a high content of arginine and proline so it may have a DNA binding/regulatory function.

In maize almost all aerobic mRNAs fail to be translated during anaerobiosis. This does not initially result from mRNA degradation of the aerobic messages but appears to be due to selective translation of the anaerobic messages. Aerobic mRNAs remain translatable in an in vitro system for at least five hours after anaerobic treatment is initiated (Sachs et al. 1980). But after 24 hours of anaerobiosis, the in vitro translation profile is similar to the in vivo profile implying that the aerobic mRNAs have degraded by this time (Sachs et al. 1980). Russell and Sachs (1992) measured the magnitude of the translation control effect by estimating the extent to which selective translation enhances expression. They estimated the relative expression of proteins in an in vitro system and compared it to the levels expressed in vivo. They found that ADH synthesis was enhanced approximately three times, and GAPDH synthesis was enhanced six times in vivo compared to the in vitro estimations. This selective translation was found not to apply for an aerobically expressed gene (β-glucosidase) whose mRNA levels were found to be constant during anoxia but the in vivo synthesis of its product decreases.

This selective synthesis has been highlighted in examination of potato tubers (Vayda and Schaeffer 1988, Butler et al. 1990). When wounded tubers are transferred from
aerobic to hypoxic conditions, the expression of wound responsive proteins is repressed, although the wound mRNAs persist for many hours after the transfer. In addition wounded tubers under hypoxic stress do not synthesis the mRNAs that are induced in response to wounding in an aerobic environment. Presumably this suppression of expression is a partial explanation of why tubers are more vulnerable to pathogenic attack while waterlogged. Thus the anaerobic response appears to have precedence over the wounding response emphasizing just how drastic anaerobic conditions are.

Hypoxically induced alterations in the translational machinery have been observed in soybean (Lin and Key 1967) where it was found that polyribosomes rapidly dissociate under anaerobiosis. It was demonstrated that the dissociation was due to the ribosomes completing their readout and then being unable to reattach to mRNAs to initiate new peptide chains. The translational control therefore appears to be exerted at the level of ribosome recognition of mRNA species. During heat-shock in *Drosophila* the selective translation of heat-shock genes has been shown to be mediated by specific sequences in the 5' untranslated leader regions of the transcripts (McGarry and Lindquist 1985, Hultmark et al. 1986). Translational regulation may be similar in the anaerobic response of plants but to date no sequences in the anaerobic mRNAs have been identified that perform this function.

A rapid increase in monoribosome and ribosomal subunit levels in the roots of hypoxically stressed maize seedlings has been observed (Bailey-Serres and Freeling 1990). In addition, chemical and physical modifications of the protein components of ribosomes were found to be induced by hypoxic stress. Hypoxic stress also altered the quantity and electrophoretic mobility of a number of ribosomal proteins including changes in phosphorylation of these proteins. Furthermore, specific ribosomal modifications were found when *in vivo* labelling revealed that newly synthesized ribosomal proteins were incorporated into the polyribosome structure under stress conditions. How these modifications relate to selective translation of the anaerobic mRNAs is unknown, however this does show that structural modifications to ribosomes
could be involved in the regulatory process. An increase in monoribosomes and a
decrease in polyribosomes levels has also been observed in response to heat-shock (Key
et al. 1981) and drought stress (Manson et al. 1988) in soybean and to heat-shock in
*Drosophila* (Lindquist 1987). This mechanisms is indicative of the translational
regulation occurring in response to these stresses.

The patterns of protein synthesis has been compared in the different organs of maize
(Okimoto et al. 1980) and while aerobic patterns varied greatly, the patterns of protein
synthesis during anaerobiosis in the root, endosperm, scutellum, anther wall, mesocotyl
and coleoptile were essentially the same, indicating that this is a basic cellular response
to anaerobiosis. In contrast, in the leaves of maize during anaerobiosis there was no
detectable synthesis of ANPs. Leaves died much sooner than the other organs,
suggesting that the synthesis of these proteins is essential for each organ's survival.

Although the different ANPs appear to be induced in similar ratios, Hake et al. (1985)
showed by Northern analysis using cDNAs for five ANPs as probes that the mRNAs
were not induced simultaneously, but were induced to different levels with different
timings. *Adhl* and sucrose synthase I (*Sh*) mRNAs displayed identical induction
kinetics, whereas the other anaerobic mRNAs had different induction kinetics. Also
while some genes encoding ANPs respond to anaerobic stress with large increases in
mRNA levels they showed only slight increases in protein and/or activity levels, such as
*Sh* (McElfresh and Chourey 1988) and aldolase (Kelley and Freeling 1984b, Hake et al.
1985). Interpretation of these results is complicated by the fact that much of the analysis
has been done on whole roots rather than on specific tissues. When Rowland et al.
(1989) examined tissue specific expression via *in situ* hybridizations, they found the
highest expression of *Sh* in the vascular cylinder, pith and epidermis whereas *Adhl* was
found to increase in the cortex and epidermis. In addition the sucrose synthase 2
isozyme, whose overall mRNA and protein levels decrease during anaerobiosis, was
found to have increased protein levels in the root cap (Rowland et al. 1989).
A further level of variation has been shown by Kelley and Freeling (1982) in that intermediate concentrations of oxygen result in different patterns of polypeptide synthesis. The pattern of proteins synthesized under an atmosphere of 2% oxygen showed expression of some polypeptides that are not synthesized under either aerobic or anaerobic conditions. Therefore the regulation of the maize anaerobic response is more complex than originally thought, since the genes affected by anaerobic stress do not all respond to the same degree, in the same tissues, under the same reduced oxygen concentrations.

It should also be noted that the anaerobic response differs between plant species; for example the anaerobic response of soybean was found to be much simpler than that of maize (Russell et al. 1990). Although soybean shares characteristics with maize such as mRNA accumulation and selective protein synthesis, its anaerobic response was much simpler having no transition group of polypeptides and synthesizing only four major ANPs during anoxia (Russell et al. 1990). Although, using Northern analysis, Adh was found to be induced, other genes related to glycolysis such as aldolase and Gapdh were found not to be induced. It is possible that a lack of hybridization with the heterologous probes could be an explanation. The other ANPs of soybean have not been identified but it has been suggested that one in the 90 kD region is sucrose synthase (Russell et al. 1990). The response in Arabidopsis has also been examined (Dolferus et al. 1985) and although ADH was found to be induced, Arabidopsis did not appear to have the dramatic switch in protein synthesis that is observed in maize and soybean.

Pea, a flood intolerant plant, also has a simple anaerobic response (Freeling lab unpublished) similar to that of soybean. Russell et al. (1990) speculated that plants like pea and soybean are flood intolerant because they have a limited anaerobic response whereas a more flood tolerant plant like maize has a complex response. But it must be noted that peas do show high rates of ethanol synthesis under anoxia (Roberts et al. 1984b), although the rates are not as high as in rice (Bertani et al. 1980) or maize (Neal and Girton 1955).
The aim of the work to be described in this chapter is to characterize the anaerobic response of cotton seedlings, which have been reported to have roots that are even more susceptible to flooding than soybean (Huck 1970). The number of major polypeptides that are selectively synthesized under anaerobic conditions is investigated. This addresses the question of whether cotton follows the trend of flood intolerant plants (pea and soybean), in having a simple response, compared to the relatively flood tolerant maize which has a more complex response. Additional aims are to determine if cotton has a dramatic shift in polypeptide synthesis as seen in maize and soybean, and to determine if cotton has a transition polypeptide stage like that of maize.

It has been previously shown (Chapter Two) that ADH activity in cotton increases upon exposure of seedlings to anaerobic conditions. To determine whether this increase in activity is due to an increase in de novo synthesis and accumulation of the ADH enzyme under anaerobic conditions, protein labelling and Western analysis were performed. This analysis provided information on how many different ADH polypeptides are being synthesized giving additional insights on how many Adh genes are being anaerobically induced, and to what extent. This information resolves the question of whether ADH makes up a major or minor part of the anaerobic response in cotton.

3.2 MATERIALS AND METHODS

(a) Anaerobic conditions and in vivo protein labelling.

Roots of intact seedlings were labelled with [S-35] methionine using methods essentially as described by Sachs et al. (1980). Seeds of the Siokra "Blue tag" cultivar of cotton were germinated under dark conditions for four days so that the primary roots were 3-5 cm long. Three seedlings were placed into a 4 mL test tube to which 2.5 mL of anaerobic induction buffer was added so as to submerge the roots. The seedlings were then placed into a sealed container through which argon gas was bubbling continuously (4-5 bubbles/sec.). After a period of anaerobic treatment, 0.5 mL of anaerobic induction
buffer (10 mM Tris-HCl pH 8.0, 0.1 mM ZnSO₄ and 50 µg/mL chloramphenicol) containing 100 µCi of [S-35] methionine was added to each tube. After the seedlings had been subjected to a further period of anaerobiosis, their root tips (5 mm) were excised, rinsed in extraction buffer and protein was extracted from the roots as described in section 2.2 (c). The level of [S-35] methionine incorporation was determined by precipitation of a sample of the extract with 10% TCA and counting in a scintillation counter. Aerobic roots were not submerged in buffer but rather the label was painted directly onto the root. The anaerobic conditions for these experiments [described in section 2.2 (b)] are similar to those used by Sachs and co-workers (Sachs et al. 1980, Russell et al. 1990) in their experiments on maize and soybean and thus will allow more meaningful comparisons to be made between the anaerobic responses of the different plants. To verify that the plants had been affected by anaerobiosis the specific activity of ADH was determined.

(b) Two dimensional gel analysis.

Labelled proteins were examined on isoelectric focusing (IEF)/SDS-PAGE two dimensional (2D) gels (O'Farrell 1975), where proteins are separated on the basis of their isoelectric point in the first dimension and by molecular weight in the second dimension. Samples for 2D gels were prepared by precipitating the protein extracts overnight in 10% TCA, 10mM DTT in acetone at -20°C and then spinning at 10 K.r.p.m.. Pellets were washed twice with 10 mM DTT in acetone, and then solubilized in the loading buffer [9 M urea, 10 mM methylamine.HCl, 100 mM DTT, 2% CHAPS, 0.0025% (v/v) pyronin Y, and 5.25% (v/v) carrier ampholines (Pharmacia)]. Samples where then loaded onto IEF gels that contained 3.8% acrylamide, 8.5 M urea 10 mM methylamine.HCl, 2% CHAPS and 5.25% ampholines, pH range 3.5 to 10. Gels were run at 500 V for 17 hrs and then equilibrated in 0.25 M Tris-HCl pH 8.8, 3% SDS and 5% β-mercaptoethanol for 15 mins. before loading onto a 12.5 to 25% polyacrylamide gradient gel in 0.375 M Tris-HCl pH 8.8 and 1% SDS with a 3 % polyacrylamide stack in 0.125 M Tris-HCl pH 6.8 and 1% SDS (Laemmli 1970). Electrophoresis was
performed for 17 hrs at 20 mA per gel. Included in the second dimension was a set of molecular weight standards. Standards used were a [C-14] methylated protein mixture (Amersham) consisting of lysozyme (14 kD), carbonic anhydrase (30 kD), ovalbumin (46 kD), bovine serum albumin (69 kD), phosphorylase b (92.5 kD) and myosin (200 kD).

(c) **Fluorography.**

Fluorography was done essentially as described by Jen and Thach (1982). After electrophoresis gels were soaked in destainer [25% (v/v) ethanol, 7% (v/v) acetic acid and 68% (v/v) water] for 30 mins. and then in fixer [40% (v/v) ethanol, 25% (v/v) acetic acid and 35% (v/v) water] for 1 hr before soaking for 30 mins. in enhancer [66% (v/v) acetic acid, 28% (v/v) methanol, 5% (w/v) 2-methylnaphthalene and 0.5% (w/v) 2, 5-diphenyloxazole]. Gels were then soaked twice in 500 mL of water, 30 mins. each time before being placed on 3MM Whatmans filter paper, vacuum dried and exposed to X-ray film.

(d) **Western analysis.**

Western blotting was adapted from the procedure of Burnette (1981). After performing electrophoresis, proteins were electro-eluted from the gels for 20 hrs at 250 mA at 4°C onto nitrocellulose. The filter was then stained with amido black, and the positions of the molecular weight standards marked. After a 1 hr incubation with blocking buffer (25 mM Tris-HCl pH 8.0, 5% (w/v) milk powder and 150 mM NaCl) the filter was cross-reacted with a maize ADH1 antibody (a kind gift from the Freeling laboratory) overnight at 37°C. After three 15 mins. washes with 1X TBS (50 mM Tris-HCl pH 8.0 and 150 mM NaCl) the filter was cross-reacted with an anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad) for 1 hr at 37°C. The filter was then rewashed as before and finally treated with nitro blue tetrazolium (150 µg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (75 µg/mL) in immunoblot colour buffer (50 mM Tris-HCl
pH 8.9, 50 mM NaCl and 2.5 mM MgCl₂), which results in the formation of an insoluble blue precipitate (Leary et al. 1983).

(e) **Coomassie blue staining.**

After electrophoresis gels were swirled in 5% (w/v) Coomassie Brilliant Blue-R (Sigma), 10% (v/v) methanol, 5% (v/v) acetic acid and 80% water for at least 1 hr. Gels were then swirled in destainer, with destainer being changed numerous times until background regions of the gels were completely destained.

### 3.3 RESULTS

(a) **The pattern of polypeptide synthesis is altered by anaerobic conditions.**

Four day old cotton seedlings were labelled with [S-35]-methionine under both aerobic and anaerobic conditions. Protein extracts were prepared from the primary roots, and the specific activity of ADH was measured to verify that the seedlings had been anaerobically stressed. The activities of ADH were comparable to those in Fig. 2.2, indicating that the seedlings had been subjected to a similar treatment.

Aerobically labelled seedlings always incorporated significantly more counts than seedlings labelled under anaerobiosis (data not shown), suggesting that protein synthesis is not as great under anaerobic conditions as it is under aerobic conditions, although this difference may be a result of the different labelling methods for aerobic and anaerobic conditions. Comparable observations are seen in other plants such as rice (Bertani et al. 1981).

Extracts containing equal numbers of counts were analysed by SDS-PAGE to observe any relative alterations in the expression of the molecular weight classes of polypeptides (Fig. 3.2). The profile of aerobically labelled roots (Lane 1) contains a broad range of molecular weight sizes with only a few major bands. When seedlings had been labelled
Figure 3.2 A fluorograph of a SDS-PAGE gel containing extracts of cotton roots labelled with [S-35] methionine. Approximately 15,000 count from each extract was loaded.

(1) aerobic: labelled for 5 hrs.
(2) anaerobic: labelled for the first 5 hrs of anaerobiosis.
(3) 20 hrs of anaerobiosis, labelled for the last 5 hrs.

The ADH activity (Units/ mg of protein) of each extract which was measured so that an indication of the degree of anaerobic stress was as follows:

(1) 1.07
(2) 1.41 (1.31 X aerobic)
(3) 6.03 (5.63 X aerobic)

The molecular weight standards are in lane 4.
during the last five hrs of a 20 hr anaerobic treatment (Lane 3) the profile changes dramatically, with the intensity of many bands being reduced and protein synthesis concentrated into approximately seven molecular weight classes. From estimation using the standards in Lane 4, the molecular weight of the classes are, 83 kD, 63 kD, 53 kD, 51 kD, 42 kD, 38 kD and a group below 30 kD that are not resolved on this gel. The 38 kD class is the most predominant class, both in aerobic and anaerobic conditions. Another class, the 42 kD class is not present under aerobic conditions but becomes a major class under anaerobic conditions. Proteins labelled during the first five hrs of anaerobiosis (Lane 2) differ from those in the aerobic profile indicating that the anaerobic treatment has already altered the plant's protein synthesis.

(b) Aerobic polypeptide synthesis.

To investigate further the changes in polypeptide synthesis, the extracts described above were run on high resolution 2D gels. In Fig. 3.3, the fluorographs of IEF/SDS-PAGE 2D gels show the differences in labelled polypeptides patterns under aerobic and anaerobic conditions. Under aerobic conditions, the root tips synthesize a large number of proteins (Fig. 3.3a), with the range of molecular weight classes (seen in Fig. 3.2) being resolved into many major spots on the gel. Over-exposure of this fluorograph reveals thousands of minor spots. There are several low molecular weight polypeptides that are strongly expressed. They are not methionine-rich proteins because they also appear strongly in coomassie stained gels (Fig 3.6c) and in a silver stained 2D gel (data not shown).

(c) Anaerobic polypeptide synthesis.

When root tips were labelled under anaerobic conditions, a shift is seen in the pattern of de novo polypeptide synthesis, similar to that seen in maize and soybean. A fluorograph of a 2D gel (Fig. 3.3b) of the polypeptides synthesized during the last five hrs of a 20 hr
Figure 3.3  Fluorographs of IEF/SDS-PAGE two dimensional gels of cotton roots labelled with [S-35] methionine during:
(A) 5 hrs of aerobic conditions.
(B) 20 hrs of anaerobiosis, labelled for the last 5 hrs.
Positions of molecular weight standards run in the SDS dimension are shown on the right.
period of anaerobiosis resolved only about 17 major spots. The nomenclature of Sachs et al. (1980) has been used to describe these polypeptides, which will be referred to as the anaerobic polypeptides (ANPs); they are listed in Table 3.1 with the number referring to their molecular weight. Possible identities of some of these polypeptides have been listed, based on the similarity of their molecular weight when compared to those of the known anaerobic polypeptides of maize and other plant species. Where multiple ANPs have been placed in a group it is being suggested that one or all could correspond to the proposed enzyme.

Ten ANPs (ANP 93, 54, 52, 51, 42a, b and c, 39, 35.6 and 34.4) have no counterpart spot in comparison with the aerobic pattern implying that the synthesis of these polypeptides is exclusive to anaerobic conditions.

The ANP 38.5 group is expressed under both aerobic and anaerobic conditions. From the analysis I am unable to determine if their expression has been induced, although it can be concluded that their synthesis has not been repressed when compared to the other aerobic polypeptides.

The ANP 22.5 and 17.5 groups are highly expressed aerobic polypeptides. Their expression has been repressed but not abolished by anaerobic conditions and therefore it is doubtful that they have a specific function under anaerobic conditions; maize has no ANPs in this molecular weight range. Therefore these polypeptides do not appear to be true ANPs so it will be concluded that cotton has 14 major ANPs.

When the anaerobic and aerobic profiles are exposed longer another 10 minor polypeptides that are expressed exclusively during anoxia are seen (data not shown).

(d) Protein synthesis at the onset of anaerobiosis.

Roots were labelled during the first five hrs of anaerobiosis to examine if cotton, like maize, has a group of transition polypeptides (TPs) expressed exclusively at the onset of anaerobiosis. The analysis, shown in Fig. 3.4, displays a pattern of polypeptide synthesis that is intermediate between the aerobic and anaerobic patterns.
Identification of the anaerobic polypeptides of cotton.
Table 3.1 The anaerobic polypeptides of cotton and their possible identities.

<table>
<thead>
<tr>
<th>ANP</th>
<th>Possible identity/comment</th>
<th>Reference and Mol. Weight.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP 93</td>
<td>Sucrose synthase?</td>
<td>Springer et al. (1986) (87 kD)</td>
</tr>
<tr>
<td>ANP 54</td>
<td>Glucose phosphate isomerase?</td>
<td>Kelley and Freeling (1984a) (55 kD)</td>
</tr>
<tr>
<td>ANP 51</td>
<td>Alanine aminotransferase?</td>
<td>Good and Muench (1992) (50 kD)</td>
</tr>
<tr>
<td>ANP 52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANP 42a</td>
<td>Alcohol dehydrogenase</td>
<td>This chapter</td>
</tr>
<tr>
<td>ANP 42b</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>ANP 42c</td>
<td>&quot;</td>
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</tr>
<tr>
<td>ANP 39</td>
<td>Lactate dehydrogenase?</td>
<td>Honderd and Hanson (1990) (40 kD)</td>
</tr>
<tr>
<td>ANP 38.5a</td>
<td>Expressed aerobically,</td>
<td>Russell and Sachs (1989) (38 kD)</td>
</tr>
<tr>
<td>ANP 38.5b</td>
<td>Glyceraldehyde-3-phosphate</td>
<td></td>
</tr>
<tr>
<td>ANP 38.5c</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>ANP 38.5d</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>ANP 35.6</td>
<td>Aldolase?</td>
<td>Kelley and Freeling (1984b)</td>
</tr>
<tr>
<td>ANP 34.4</td>
<td>Aldolase?</td>
<td>(35.5 kD and 33 kD)</td>
</tr>
<tr>
<td>ANP 22.5</td>
<td>Residual aerobic</td>
<td></td>
</tr>
<tr>
<td>ANP 17.5a</td>
<td>expression, probably</td>
<td></td>
</tr>
<tr>
<td>ANP 17.5b</td>
<td>not true ANPs.</td>
<td></td>
</tr>
</tbody>
</table>
This intermediate labelling pattern contrasts to maize, where during this initial period of anaerobiosis the TPs are expressed exclusively (Sachs et al. 1980). For cotton, only one minor polypeptide (marked TP?) has no corresponding spot in the aerobic or anaerobic patterns and has a molecular weight in the 33 kD range, which is the molecular weight of maize TPs, suggesting that this polypeptide may be similar to the maize. Clearly the expression of this polypeptide is not as exclusive as are the maize TPs during the first several hours of anaerobiosis. However, it is being labelled as heavily as the ANPs which are starting to appear. ANPs such as ANP 54, 51, 42a, 42b, 42c and 39 are already identifiable and have been marked with arrows (Fig. 3.4).

This intermediate expression pattern may reflect the experimental conditions. Because no effort was made to rid the environment of oxygen, the initial labelling conditions would have been aerobic. But even so, several of the highly expressed aerobic proteins are still being synthesized after 15-20 hrs of anaerobiosis (these being labelled ANP 22.5, 17.5a and 17.5b although they are not true ANPs). Cotton does not appear to have the immediate and complete switch in protein synthesis as found in maize, nor does it appear to have a distinct transition stage. Further analysis with shorter pulses may help to clarify this situation.

(e) Co-ordination of the anaerobic response.

It has been suggested that the induction of all the ANPs does not occur simultaneously, but rather the induction occurs in subsets (Hake et al. 1985) and may depend on different levels of oxygen concentration (Kelley and Freeling 1982). When the anaerobic stress conditions are altered by slowing the argon flow rate from four to five bubbles per second down to one bubble per second the anaerobic pattern is altered. Fig. 3.5 shows the labelling pattern of roots of seedlings that were labelled for the last five hrs of such a 20 hr anaerobic treatment. The ratio of the level of expression between the ANPs has changed. ANP 54 is not fully induced and ANPs 52, 35.6 and 34.4 are absent (circles). This is in contrast to ANP 39 and the 42 group which seem to be fully induced. This demonstrates that the ANPs synthesized in the experiment Fig. 3.3b are
Figure 3.4 Fluorograph of IEF/SDS-PAGE two dimensional gel of an extract prepared from cotton roots labelled with [S-35] methionine during the first 5 hrs of anaerobiosis.
Figure 3.5 Alteration in the anaerobic polypeptide pattern by changing the conditions of anaerobiosis.
not all induced in a completely co-ordinated fashion, and may have different timing and oxygen thresholds. The other major polypeptides present in this gel, and not seen in Fig. 3.3b, correspond to aerobic polypeptides, which suggests that the repression of the synthesis of aerobic polypeptides may also occur in subsets in a sequential order.

(f) Identification of the ADH anaerobic polypeptides.

Because no electrophoretic variants or mutants of cotton ADH are available, methods used for maize (Sachs and Freeling 1978) and *Arabidopsis* (Dolferus et al. 1985) to identify which of the spots were ADH products could not be applied. Instead, Western analysis was performed on 1D and 2D gels to identify how many and which spots were ADH polypeptides. Unlike Sachs and co-workers (Sachs et al. 1980, Russell et al. 1990) who used native gels in the first dimension, we opted for IEF gels. Using isozyme gels (Chapter Two) we were unable to determine how many cotton *Adh* genes were expressed, therefore we considered that a native gel would give us no additional information.

Western analysis on a 12.5 cm 1D SDS-polyacrylamide gel revealed that a single molecular weight class cross-reacted with maize ADH1 antibodies (Fig. 3.6 B). This band was not observable in the aerobic sample, but was present in the sample induced for 24 hrs and peaked after 48 hrs. No cross-reactivity could be found in the sample that had been exposed to 120 hrs of anaerobiosis. This demonstrates that ADH polypeptides accumulated under anaerobic conditions, as found in maize (Sachs and Freeling 1978).

Quantitatively, the levels of band intensities correlate with the accumulation of enzyme activity, where ADH activity peaks after 48 hrs and then declines (Fig. 2.1 A), although we would have expected to see a faint band in the aerobic lane. This latter discrepancy may reflect the insensitivity of the antibody.

The Western band, when compared to the molecular weight standards, had a calculated molecular weight of approximately 42 kD, and co-migrated with a Coomassie band that was induced during anaerobiosis (Fig. 3.6 C). In this gel the 42 kD Coomassie band is the only band that can be observed to be induced by anaerobiosis (arrow), although on
Figure 3.6. Five day old cotton seedlings were subjected to anaerobiosis for varying lengths of time. Protein extracts were then prepared from root tips and electrophoresed on 12.5 cm 12.5-25% gradient SDS-polyacrylamide gels. These gels were then subjected to:

(A) fluorography. Samples were run from extracts that had been labelled with [S-35]-methionine for; (1) 5 hrs of aerobic condition, (2) the first 5 hrs of anaerobic conditions, (3) the last 5 hrs of a 20 hr exposure to anaerobic conditions. Each sample contained about 50,000 counts.

(B) Western analysis. Lanes 1 to 5 are extracts of roots exposed to 0 hrs (Lane 1), 24 hrs (2), 48 hrs (3), 72 hrs (4) and 120 hrs (5) of anaerobiosis. 75 µg of protein was loaded onto each lane. The filter has been cross-reacted with an antibody raised against maize ADH1.

(C) Coomassie staining. The extracts were from seedlings that had been exposed to 0 hr (Lane 1), 24 hr (2) and 48 hr (3) of anaerobiosis. 75 µg of protein was loaded onto each lane. Molecular weight standards are lanes marked S.
other occasions when extracts were analysed from plants that had been exposed to 72 hrs of anaerobiosis a 38 kD Coomassie band can also be seen to be induced (data not shown).

The 42 kD band is resolved into three major polypeptides on a 2D gel (Fig. 3.3b). To determine which of these polypeptides were ADH, 2D Western analysis was performed. The 48 hr sample (the most intense) was subjected to Western analysis (Fig. 3.7) and it was found that all three spots cross-react with the maize ADH1 antibody. Therefore ANPs 42a, b and c are all ADH polypeptides, implying that there are three different *Adh* genes being switched on by anaerobic conditions. All three polypeptides had identical molecular weights as determined by their mobilities on the SDS-dimension, which is in contrast to maize where ADH1 and ADH2 have apparent molecular weights of 40 kD and 42 kD respectively (Ferl et al. 1979). In agreement with the protein labelling, the expression of one ADH polypeptide is stronger than the other two.

From the 2D Western it can be concluded that the appearance of the 42 kD Coomassie band is due to the synthesis of three ADH polypeptides, one of which is the most heavily labelled ANP. Therefore from this data it can be concluded that the polypeptide whose expression responds most to anaerobic conditions is ADH. When seedlings are pulse labelled with [S-35]-methionine (Fig. 3.6 C) the induction of the 42 kD band becomes more apparent when compared to the Coomassie band. The pulse label further demonstrates that the ADH protein accumulation reflects an increased rate of protein synthesis, confirming that one contributing factor to the induction of ADH activity seen in Fig. 2.1 is an increase in *de novo* ADH protein synthesis.
Figure 3.7 Five day old cotton seedlings were placed under anaerobic conditions for 48 hrs. Protein extracts from root tips were prepared, and a sample containing 125 µg of protein was subjected to 2D gel electrophoresis. Western analysis was then performed using the maize ADH1 antibody as outlined in the materials and methods.
3.4 DISCUSSION

As outlined in the introduction to this chapter the molecular response to anaerobic stress in roots has been examined in maize, soybean, pea and *Arabidopsis*. This chapter describes the anaerobic response in the root tips of cotton.

These results show that cotton possesses 14 major ANPs, which in a quantitative sense puts it in the same category as maize, which has 20 ANPs (Sachs et al. 1980), rather than that of soybean and pea with 4-5 ANPs (Russell et al. 1990). However comparisons must be made with caution because different 2D gel systems were used, and Sung (1984) claims that IEF/SDS-PAGE gels (used in this study) resolve more polypeptides than the Native/SDS-PAGE 2D gels Sachs and co-workers used. In addition cotton is an allotetraploid thereby possibly duplicating some enzymes, and so may appear to have a more complex response than is the case. For example three cotton ANPs are ADH polypeptides compared to maize where two ANPs are ADH polypeptides. Although Russell et al. (1990) demonstrated that soybean had a simple anaerobic expression pattern, another study by Tihanyi et al. (1989a) came to the conclusion that soybean's anaerobic response was more complex consisting of 28 ANPs. Although their conclusions were less convincing, for the protein labelling and 2D gel electrophoresis were not as clear as the results from Russell et al. (1990), they did show that the enzyme activities of ADH, PDC, LDH, aldolase and phosphoglucomutase were all induced by anaerobic stress. The difference between the results may have been due to the different experimental procedures, particularly the method of anaerobic treatment. This illustrates that comparisons between different results must be made with caution.

Although in a numerical sense the response in cotton has been found to be similar to that in maize, the level of expression of the cotton ANPs may not be as strong as the expression of the maize ANPs during anaerobic stress. Sachs and Freeling (1978) showed that the major ANP molecular weight classes of maize accumulated after 50 or more hrs of anaerobiosis to become the major Coomassie staining bands. In cotton the
accumulation does not appear to be as great, with only the molecular weight classes corresponding to ADH and the ANP 38.5 group visibly increasing during anaerobic stress. Therefore analysis by Coomassie staining suggests that the maize response in terms of the level of expression and/or accumulation of ANPs, is significantly greater.

In a qualitative sense there are several differences between the response of maize and cotton. Cotton lacks several major ANPs that are present in maize. Firstly, in the 65 kD region, where maize has a major polypeptide encoding pyruvate decarboxylase (Laszlo and St Lawrence 1983) cotton has no major ANP in this region implying that PDC is weakly expressed, if at all, in cotton under anaerobic conditions. Other evidence supporting this conclusion is the finding that only very low levels of PDC enzyme activity were detected even after 20 hrs of hypoxia (Dennis et al. 1992). Like cotton, soybean also lacked a spot in the 65 kD region (Russell et al. 1990), although Tihanyi et al. (1989a) found that PDC activity was induced. PDC is at the major branch point in glycolysis between aerobic and anaerobic metabolism and regulation of ethanolic fermentation is likely to occur at this point (Wignarajah and Greenway 1976).

In addition, cotton does not have a major polypeptide at 87 kD whereas both maize and soybean have major spots in this region. In maize the ANP 87 polypeptide corresponds to sucrose synthase, and the gene that encodes it is known to be precisely co-regulated with Adhl during anaerobic stress and to have the highest transcript level of the anaerobic genes (Hake et al. 1985). In cotton a polypeptide is found in the 93 kD region, but it is only weakly expressed in comparison with the other cotton ANPs. On the other hand cotton does have a number of ANPs with molecular weights that do correspond to other ANPs of maize (Table 3.1) and most of these do not seem to be present in soybean. Further analysis of these cotton ANPs will be required to prove their identity.

Like maize and soybean, cotton switches its protein synthesis to only a few selected polypeptides when exposed to anaerobiosis. This metabolic change is in contrast to the response reported for Arabidopsis (Dolferus et al. 1985) where a new stress protein
pattern was not apparent. This difference may result from variation in experimental procedures, although Dolferus (unpublished) has shown by Northern analysis that several glycolytic enzymes induced in maize are not induced in *Arabidopsis* including PDC.

The appearance of the cotton ANPs within the first five hrs of anaerobic treatment demonstrates that the kinetics of induction of the ANPs is similar to those of maize, where the major maize ANPs were visible when labelling was performed in the fourth to fifth hour of anaerobiosis (Sachs et al. 1980). This shows that the cotton plant is able to respond quickly to low oxygen tension. Although cotton has several possible transition polypeptides it is unlike maize in that it does not seem to have a distinct transition polypeptide stage. Protein synthesis in cotton during the early phase of anaerobiosis is a mixture of aerobic and anaerobic polypeptides where particular polypeptides (ANP 22.5 and 17.5 groups) are being expressed under all conditions examined. It seems unlikely that these "ANPs" have a specific role under anaerobiosis, but rather their presence is a consequence of their very high aerobic expression, which has not been fully repressed under anaerobic conditions. Maize and soybean do not appear to have polypeptides that are expressed strongly both in aerobic and anaerobic conditions so perhaps translational repression is not as strong in cotton as in the other plants, although it must be noted that maize did not have aerobic proteins expressed as strongly as these.

It was shown that when the anaerobic conditions were altered, the ratio of expression of some ANPs, remained the same, while others varied. This demonstrates that all the ANPs are not induced in a strictly co-ordinate fashion, but rather they may be expressed/induced in subsets. Evidence for this in maize was found by Hake et al. (1985), and Kelley and Freeling (1982). Therefore instead of considering the anaerobic response to be an all-on or all-off mechanism it might be regarded as a finely tuned mechanism, only responding to a certain degree under a particular set of conditions.
The 2D Western result (Fig. 3.7) shows that ANPs 42a, b and c are all ADH polypeptides therefore cotton possesses three resolvable Adh gene products. The analysis demonstrates that the expression of one gene is induced more strongly than the other two ADHs and this is apparent both with methionine labelling and the Western blot analysis. In both cases the 42c spot is the strongest but in the Western blot the difference between 42c and the other ADH spots is greater than in the methionine labelling analysis. This difference could be due to the limitations of the two systems. In the labelling experiment the intensity of the spots will be dependent on methionine content of the polypeptides and rate of synthesis, while for the Western blot the intensity will be dependent on the extent of cross-reactivity of the antibody with the polypeptide. In addition the Western blot reflects the accumulation of polypeptides over a 48 hr period, while the labelling only reflects the accumulation over a five hr period, so that rates of turnover of the polypeptides will be more important for the former compared with the latter type of analysis.

The accumulation of ADH polypeptides after 48 hrs of anaerobic stress have been resolved by two methods, Western analysis (Fig. 3.7) and isozyme analysis (Fig. 2.4 Lane 4). From the isozyme analysis it was concluded that two different ADH polypeptides were being expressed in roots, and only one of which, ADH2, was anaerobically inducible. In contrast, analysis by 2D Western analysis demonstrated that there are three ADH polypeptides being induced by anaerobic stress in roots. It seems likely that ADH 42c corresponds to the ADH2 isozyme for both have accumulated to be the predominate form of ADH. It is possible that the more weakly expressed ADH 42a or 42b may correspond to the ADH1 isozyme, however the ADH1 isozyme does not appear to be induced by anaerobic stress in roots. Alternatively, all three ADHs could be co-migrating forming the ADH2 isozyme band. The latter case has been found to occur in soybean where only one ADH isozyme band was found in anaerobically induced roots (Russell et al. 1990) but three different soybean Adh cDNA classes have been isolated that are all expressed in anaerobically stressed roots (Newman and VanToai 1992). It is possible that the activity of some isozymes has been lost during
electrophoresis or that the isozymes are not staining because of having a low catalytic activity under the staining conditions.

Why plants have different responses and how these differences affect the plant's tolerance to anaerobic conditions is unknown. From both the Coomassie staining and methionine labelling analysis it seems that ADH is the most strongly induced ANP in cotton. It seems logical that if ADH is strongly induced in cotton then PDC would also be strongly induced, for PDC provides the substrate for ADH. However, PDC appears to be weakly expressed, so the physiological significance of the evolution of a highly expressed ADH under anaerobic conditions is not clear. One possible explanation is that if cotton is very sensitive to acetaldehyde, a high ADH expression level would prevent its accumulation to significant concentrations.

In maize where PDC corresponds to a prominent ANP, ADH activities have been found to be 6 to 9 times greater than PDC activities, suggesting that PDC limits ethanolic fermentation (Wignarajah and Greenway 1976). So in cotton where PDC appears not to correspond to a detectable ANP it seems likely that it is also limiting the rate of ethanolic fermentation. Saglio et al. (1988) and Hole et al. (1992) have concluded that high rates of ethanolic fermentation confer a high tolerance to anoxia in maize. If cotton is intolerant to anaerobic conditions due to low rates of ethanolic fermentation, extra copies of the $Pdc$ gene may generate higher rates of fermentation and thus greater tolerance to anaerobic conditions. This possibility will be further explored in Chapter Five.

The higher expression of ADH 42c compared with the other ADHs, may be a consequence of a stronger promoter and higher steady state mRNA levels. The isolation of the $Adh$ cDNAs coding for these three ADH polypeptides and the characterization of the promoters driving the synthesis of the cDNAs may give clues to why ADH 42c is more strongly expressed than the other two ADHs. Experiments exploring these questions will be described in the next chapter.
CHAPTER FOUR

The Isolation and Characterization of Cotton Adh cDNA and Genomic Clones.

4.1 INTRODUCTION.

Alcohol dehydrogenases are found in a wide range of organisms and make up two evolutionary groups. One group consists of polypeptide chains of approximately 250 amino acid residues that do not require zinc as a co-factor and the other consists of longer polypeptides of about 380 amino acids that require zinc as a co-factor (Jornvall 1985, Jornvall et al. 1987). ADHs from the former group are found in *Drosophila*, while ADHs from the latter group are found in organisms as diverse as mammals, plants and yeasts. The two groups are so distantly related that the alignment of their amino acid sequences is virtually impossible. However the amino acid sequences of the zinc-containing long chain ADHs are highly conserved.

Plant Adh genes were originally isolated from maize (Gerlach et al. 1982, Dennis et al. 1984 and 1985) and taking advantage of the high homology with maize, Adh genes from *Arabidopsis* (Chang and Meyerowitz 1986), pea (Llewellyn et al. 1987), barley (Trick et al. 1988), wheat (Mitchell et al. 1989), rice (Xie and Wu 1990), pearl millet (Gaut and Clegg 1991) and *Petunia* (Gregerson et al. 1991) were able to be cloned. Adh cDNAs have also been isolated from potato (Matton and Brisson 1990), soybean (Newman and VanToai 1992) *Pinus radiata* (Kinlaw et al. 1990) and white clover (Ellison et al. 1990). As expected the sequence homology between the coding regions of these genes is extensive, with the majority of sequences being 70-90% homologous at both the amino acid and nucleotide level and the most divergent of the sequences, pea Adh and barley Adh2, still being approximately 68% homologous at the nucleotide level (see Xie and Wu 1989 for a summary).
Most plants examined have two or more Adh genes. The two maize Adh genes have been the most extensively studied. They are unlinked, Adh1 being located on Chromosome 1 (Schwartz 1971) and Adh2 on Chromosome 4 (Dlouhy 1980). In addition to the conservation between their coding regions (82% conserved at the nucleotide level and 87% at the amino acid level) each maize gene has nine introns occurring in identical positions although there is no sequence homology between the corresponding introns. Also there is little similarity between the 5' and 3' flanking regions of the genes. The position of introns in Adh genes are highly conserved both in monocotyledonous and dicotyledonous species of plants although several of the genes have lost introns (Chang and Meyerowitz 1986, Mitchell et al. 1989). This high level of conservation suggests that an ancestral Adh gene duplication took place and the fact that most plants have at least two Adh genes implies that the initial gene duplication occurred early, predating the separation of the monocotyledonous and dicotyledonous plants which occurred at least 120 million years ago. The presence of a single Adh gene in Arabidopsis suggests a loss of one Adh gene.

The majority of studies on Adh in plants have focussed on the mechanisms that regulate expression of Adh genes during anaerobiosis. When maize seedlings are anaerobically stressed, the increase in ADH enzyme activity is preceded by an increase in the steady-state concentrations of the Adh1 and Adh2 mRNAs (Gerlach et al. 1982, Dennis et al. 1984, 1985). The concentration of Adh1 mRNA reaches a maximum after five hours of anaerobic treatment and exceeds aerobic levels by 50-fold (Gerlach et al. 1982). New transcripts of Adh1 are detectable within one hour of anaerobic treatment demonstrating that at the level of transcription the response to oxygen shortage is rapid (Rowland and Strommer 1986). When anaerobically stressed roots are re-oxygenated within the first hour of recovery the transcription of Adh1 is repressed, and the steady state levels decline gradually (Rowland and Strommer 1986). It was shown by nuclear run-off experiments that an increased transcription rate was responsible for the higher Adh1 steady-state levels (Rowland and Strommer 1986, Dennis et al. 1988b), along with increased stability of the Adh1 transcript (Rowland and Strommer 1986).
During anaerobic stress the steady-state mRNA levels corresponding to the other anaerobic polypeptides also increase. This includes the mRNAs corresponding to sucrose synthase I (Springer et al. 1986), glyceraldehyde-3-phosphate dehydrogenase III (Russell and Sachs 1989), pyruvate decarboxylase (Kelley 1989) and aldolase (Hake et al. 1985). In barley, lactate dehydrogenase mRNA levels have also been shown to be induced by anaerobic stress (Hondred and Hanson 1990).

The identification of the DNA sequences that are required for the increased rate of transcription of these genes during anaerobiosis was initially done by analysing the function of the maize Adhl promoter in a maize protoplast transient assay system (Howard et al. 1987). By linking the Adhl promoter to the chloramphenicol acetyltransferase (CAT) reporter gene and electroporating the construct into maize protoplasts it was determined that the Adhl promoter conferred anaerobic responsiveness. Walker et al. (1987) then performed 5' and 3' deletion analysis of the Adhl promoter and demonstrated that sequences between -140 and -99 relative to the transcription start were able to direct anaerobic induction of transcription, and this 40 bp sequence was called the anaerobic responsive element (ARE). Further deletion analysis indicated that there were two subregions within this element, ARE I and ARE II (Walker et al. 1987). Analysis using linker-scanning mutations verified this, with mutations within either subregion eliminating anaerobic inducibility. However a mutation between or 3' to the subregions did not destroy anaerobic inducibility of the chimeric gene. In each subregion a core sequence 5'-CfGGTTT-3' was found. The importance of this sequence was confirmed when it was found in a similar position relative to the start of transcription in the 5' flanking sequences among many of the Adh genes that had been isolated suggesting that the mechanism of induction has been conserved among the Adh genes of different plant species. In dicotyledonous species of plants the core ARE element, or GT-motif is also found in the inverse complement, 5'-AAACCA-3'.

In addition to Adh genes, this hexanucleotide sequence is found in the promoter regions of other anaerobically induced genes, again in a similar position relative to the
transcription start site (Dennis et al. 1987). This suggests that a common mechanism regulates the anaerobic induction of transcription of the genes involved in the anaerobic response, although it must be noted that the kinetics of induction of the anaerobic genes are not the same (Hake et al. 1985). Functional analysis has been performed on the promoter regions of two other genes, maize aldolase (Dennis et al. 1988a) and pea Adh (Llewellyn et al. 1987). The importance of the core element was confirmed in both systems when it was found to lie in the regions identified to be necessary for anaerobic induction.

The functional properties of the maize Adh1 ARE were further investigated (Olive et al. 1990), using GUS and CAT reporter genes and a maize protoplast transient assay system. When multiple copies of the ARE are linked together, increased levels of anaerobic induction were obtained, with six copies of the ARE giving a 16-fold induction relative to one copy. The ARE was sufficient for induction and it could function in either orientation, thus having enhancer like properties. Spacing between the two subregions could be increased up to 64 bp without alteration of the expression of the reporter gene.

The maize Adh1 promoter has also been analysed in transgenic plants. In transgenic tobacco, the maize Adh1 promoter, linked to the CAT reporter gene, was insufficient by itself to promote any transcriptional activity (Ellis et al. 1987). However, in a gain of function experiment where enhancer like sequences from the octopine synthase gene or 35S enhancer were placed upstream of the maize Adh1 promoter truncated to -140 and containing the ARE, a chimeric promoter was obtained that was active and whose transcription rate was inducible by anaerobic stress (Ellis et al. 1987). When the core ARE motifs were mutated the chimeric promoter lost its ability to be induced by anaerobic stress (J. Ellis unpublished data). Similar experiments were done with the maize aldolase gene in tobacco (Dennis et al. 1988a) and similar results were obtained, demonstrating that the mechanisms of the response to anaerobic stress must be sufficiently conserved between the distantly related plant species, maize and tobacco, to allow regulated expression of the hybrid gene constructs.
The initiation of transcription is mediated by the binding of trans-acting protein factors to the cis-positioned DNA sequences/motifs. Ferl and Nick (1987) used in vivo dimethyl sulphate (DMS) protection to identify putative protein binding sites in the maize Adhl promoter. They found two "constitutive footprint" sites that lay within the two subregions of the ARE and another two footprint sites that lay outside the ARE and were protected only under anaerobic conditions.

Olive et al. (1991) then used gel retardation assays to examine the proteins that were binding to the ARE. When a maize nuclear extract was incubated with a probe consisting of a multimerised 42 bp ARE, multiple protein-DNA complexes were formed, with nuclear proteins binding specifically with the probe. Mutant ARE probes were synthesized and assayed for their ability to bind these proteins, so as to determine the nucleotides within the ARE to which the proteins were binding. At the same time the mutant probes were linked to a reporter gene and their ability to confer anaerobic expression in protoplasts was examined (Olive et al. 1991). In this way the physiological significance of binding in relation to hypoxic induction of transcription was defined.

The region of the ARE to which the protein factors were binding were G/C rich regions, the minimum consensus, 5'-GC(G/C)CC-3', being called the GC-motif. Mutation of these regions blocked binding of proteins and expression of the promoter, thus identifying another region other than the GT-motif (ARE core sequence) that is important for anaerobic induction of the Adhl promoter (Olive et al. 1991). No proteins were found to bind to the GT-motif, but when it was mutated, expression of the promoter was blocked. The study established that binding of a nuclear factor(s) to the GC-motif was necessary for anaerobic expression, although proteins could still bind when mutation to the GT-motifs had abolished anaerobic expression. This suggests that binding is a prerequisite but is not sufficient for anaerobically induced expression to occur. This established that two regions of each of the ARE subregions are important, the GC-motif and the GT-motif. The GC-motif is similar to the motif onto which the human transcription factor Sp1 binds and it was demonstrated that Sp1 protein could
bind the ARE probe, suggesting that the transcription factors may have similar properties (Olive et al. 1991).

Adh has been found to be induced by a number of stresses other than anaerobiosis including cold (Christie et al. 1991), wilting (Dolferus et al. in press) and plant hormones including 2,4-D (Freeling 1973) and ABA (G. de Bruxelles unpublished). In addition the steady-state Adh mRNA level increases in potatoes when subjected to a wide variety of stresses, including treatment with fatty acid elicitors (salicylic, arachidonic and linoleic acids), heat killed mycelial homogenates, UV light as well as anaerobiosis (Matton et al. 1990). How this diversity of stress treatments can result in the same transcriptional response in the plant is not fully understood but is being actively studied in Arabidopsis (Dolferus et al. in press). Like maize the Arabidopsis Adh promoter contains an ARE core element (GT-motif) and a 4C box (related to the GC-motif) and both have been found to be critical for induction by wilting, cold and anaerobic conditions (Dolferus et al. in press). In addition to the GT- and GC-motifs found in the maize Adh1 promoter, a 5′-CCACGTGG-3′ motif is also found in the Arabidopsis Adh promoter. This dyad is better known as the G-box and is found in the promoter region of other plant genes, including the parsley chalcone synthase (CHS) promoter (Schulze-Lefert et al. 1989) and several genes encoding the small subunit of ribulose-1,5-biphosphate carboxylase-oxygenase (RbcS) (Giuliano et al. 1988). These genes also respond to environmental cues, CHS is UV light responsive (Schulze-Lefert et al. 1989) and RbcS is regulated by light (Giuliano et al. 1988). The G-box has been implicated in the transcriptional regulation of both of these responses, although it is uncertain whether it acts as a regulatory component or it just modulates the quantitative level of expression. Although it has been shown that the G-box of the Arabidopsis Adh gene is not necessary for anaerobic induction, it is necessary for induction by cold and wilting implying that in Arabidopsis there are multiple elements involved in the induction of Adh and that different combinations of motifs are involved in the responses to different environmental conditions (Dolferus et al. in press).
In Chapter Three, cotton was shown to express three different ADH polypeptides when exposed to anaerobic stress. The first aim of the work described in this chapter was to determine how many different classes of Adh cDNA clones are present in a library of anaerobically stressed cotton roots. The next aim was to use these cDNAs as probes to isolate Adh genomic clones so the promoters that are driving the synthesis of the anaerobically expressed mRNAs could be obtained. By isolating and characterizing these anaerobically inducible promoters it will be determined if cotton Adh genes have any of the DNA motifs that have been shown by functional analysis in other plant species to be important in the anaerobic induction of transcription.

A complete cotton Adh coding region is sought for experiments (Chapter Five) designed to overexpress or underexpress ADH in transgenic cotton plants. Furthermore, an anaerobically inducible promoter may be useful in other transformation experiments where genes may be required to be expressed in cotton under anaerobic or waterlogged conditions.

4.2 MATERIALS AND METHODS.

(a) RNA extraction.

Total RNA was isolated from the root tips (5 mm) of two day old seedlings that had been subjected to anaerobic conditions for 20 hrs. All solutions prepared were shaken with 0.1% diethylpyrocarbonate (DEPC) overnight and then autoclaved. All glassware was soaked in a 0.1% DEPC solution before autoclaving. Excised tips were frozen in liquid nitrogen immediately after harvesting and ground to a fine powder with a mortar and pestle. The powder was then extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and NTES (0.1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1% SDS). The supernatant from the phenol extraction was then precipitated with three volumes of ethanol and 0.3 M sodium acetate and then pelleted at 4000g for 10 mins. at 4°C. The pellet was dissolved in
water and then RNA was precipitated by adding lithium chloride to a final concentration of 2 M and chilling at 4°C for three hrs. The RNA was pelleted (4000g, 20 mins. at 4°C) and after dissolving in water a final precipitation with isopropanol was performed. Poly(A) RNA was then isolated by chromatography on an oligo (dT)-cellulose (Pharmacia) column (Sambrook et al. 1989). The size of the poly(A) RNA was examined on 0.8% agarose formaldehyde gels and the concentration of the RNA was determined by measuring the OD at 260 nm. From 2 mg of total RNA approximately 20 µg of poly(A) RNA was isolated.

(b) Construction of cDNA library.

cDNA was prepared from 4 µg of poly(A) RNA using a cDNA synthesis kit (Pharmacia) and the manufacturer’s protocol that was provided. First strand synthesis was performed with Moloney Murine Leukemia Virus reverse transcriptase and oligo (T)12-18 as a primer and was monitored by measuring the incorporation of [α-32P]dCTP. Second strand synthesis used RNase H and DNA polymerase I. The double-stranded cDNA product was treated with the Klenow enzyme to ensure that the cDNA had blunt ends. cDNA was then extracted with phenol/chloroform and then purified on a Sephacryl S-200 spin column (provided in the kit) equilibrated in ligation buffer (Sambrook et al. 1989). EcoR1 adaptors were then ligated onto the cDNA overnight at 12°C and then phosphorylated with Polynucleotide kinase/ATP. After purifying through a Sephacryl spin column, the cDNA was then ligated into the EcoR1 cloning site of the expression vector λgt11 (Huynh et al. 1985) supplied in phosphatased form by Pharmacia. Ligated DNA was then packaged and infected into Y1090R- cells where one fifteenth of the cDNA generated yielded approximately 250,000 clones. Using IPTG and the chromogenic indicator X-gal it was established that 98% of the clones contained inserts.
Screening of the λgt11 library was done essentially as described by Huynh et al. (1985). First, maize ADH1 antibodies (a kind gift from M. Freeling's laboratory) were pre-treated to remove any components that could cross-react with Escherichia coli (E. coli) antigens. A plate lysate was prepared by soaking a confluent lawn of non-recombinant λgt11 infected into E. coli Y1090R- cells with 10 mL of phage storage buffer (Sambrook et al. 1989) and 3-4 drops of chloroform overnight at 4°C. The plate lysate was clarified by centrifugation (4000g) for ten mins. and then incubated with a 82 mm diameter nitrocellulose filter for two hrs at 37°C. The plate lysate was then removed and the filter was incubated with 50 mL blocking solution [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2% (w/v) bovine serum albumin and 0.02% (w/v) NaN₃] for one hr at 37°C and then removed. The antibody was diluted in 10 mL of fresh blocking solution (6:1000) and incubated with the nitrocellulose filter at 37°C for 24 hrs. The antibody was now pre-absorbed, it was removed from the filter and was stored at 4°C. Recombinant λgt11 was plated (1×10⁴ p.f.u.) onto Y1090R- cells and incubated at 42°C for three hrs. Nitrocellulose filters that had been soaked in 10 mM IPTG were overlayed on the plates, which were then incubated at 37°C for four hrs. Filters were removed and washed in 1X TBS (50 mM Tris-HCl pH 7.5 and 150 mM NaCl) containing 0.02% NaN₃ at room temperature for 15 mins. before being incubated with blocking solution (above) for one hr at 37°C. Blocked filters were then shaken overnight with the pre-treated ADH1 anti-bodies at 37°C. Filters were then washed three times in 1X TBS, each wash being 30 mins. in duration.

The second antibody used was an anti-rabbit alkaline phosphatase conjugate (Bio-Rad), and positives were developed using the same protocol as that described for Western analysis [Section 3.2 (d)]. Positive plaques were picked and usually went through four rounds of screening until they were purified.
(d) DNA sequencing of cDNA clones.

cDNA inserts were excised from λgt11 and ligated into the EcoR1 site of the sequencing vector pUC118 (Vieira and Messing 1987). Restriction-enzyme mapping, subcloning and deletions were done using standard molecular biology techniques (Sambrook et al. 1989) in the *E. coli* strain JM101 (Yanisch-Perron et al. 1985). Single stranded templates were generated from the recombinant pUC118 subclones when in the JM101 host using the M13K07 helper phage and by following a protocol from the manufacturer, Applied Biosystems. The single stranded templates were then sequenced using the dideoxynucleotide chain termination sequencing method (Sanger et al. 1980) with dye-primers and Taq polymerase. The sequencing reactions were run on an ABI 370A sequencing machine. Sequence data was analysed using the GCG software running on a VAX computer (Devereux et al. 1984).

(e) DNA extraction.

DNA was isolated from five day old etiolated seedlings by a modification of the method of Hughes and Galau (1988). Seedlings were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Then 4 mL of extraction buffer (200 mM 2-[N-cylohexylamino] ethanesulfonic acid/NaOH pH 9.1, 200 mM NaCl, 100 mM EDTA, 2% SDS, 0.5% sodium deoxycholate, 2% Nonidet P-40 and 1% β-mercaptoethanol) for every one gram of tissue was poured over and mixed into the powder with a spatula. The homogenate was then incubated at 55°C for 20 mins. and then the cell debris was spun out at 5000g for 15 mins. at 4°C. Then 1.3 mL per gram of tissue of 5 M potassium acetate was added and mixed into the supernatant before chilling on ice for 40 mins.. The precipitate was pelleted (5000g, 30 mins. at 4°C) and the supernatant collected. DNA was then precipitated with 0.65 volumes of isopropanol overnight at -20°C. DNA was pelleted (10000g for 20 mins. at 4°C) and redissolved in water. The DNA concentration was determined by measuring the OD at 260 nm and the quality checked by electrophoresis on 0.7% agarose gels.
(f) Genomic cloning.

The cotton insert DNA to be used for cloning was prepared by partially digesting Siokra genomic DNA (150 µg) with Sau3A (5 units) for 30 mins. These conditions optimized the percentage of DNA that fell into the 15-20 kb range. The DNA was then sedimented through a 10-40% glycerol gradient in 100 mM Tris-HCl pH 8.0, 10 mM MgCl₂. The 15-20 kb fraction was collected and precipitated with ethanol and redissolved in TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

The λEMBL4 vector (Frischauf et al. 1983) was digested with BamH1 and Sal1 and then ethanol precipitated. The digested arms were then treated with calf intestinal phosphatase (Boeringer) and extracted twice with phenol and once with chloroform. After ethanol precipitation the DNA was dissolved in TE. The DNA concentrations of the prepared λEMBL4 vector and Sau3A insert were estimated on a 0.8% agarose gel.

After ligation of vector and insert DNAs in a 3:1 concentration ratio, DNA was packaged using freeze-thaw lysate and sonic extract. The library was infected into E. coli K803 cells by standard methods (Sambrook et al. 1989).

(g) Plaque hybridization.

All probes used to screen the genomic library were isolated on 0.8-1.0% agarose gels in TBE buffer then purified by electro-elution (Sambrook et al. 1989). [α-³²P]dCTP radionucleotides were incorporated into DNA fragments by the use of a Hexaprime DNA labelling kit (Bresatec) which is based on the random-priming system described by Feinberg and Vogelstein (1983). Unincorporated nucleotides were separated from the incorporated nucleotides through Sephadex G-50 spin columns (Sambrook et al. 1989). Specific activity of the probes was determined on a MINAXIβ Tri-carb 4000 series Liquid scintillation counter and usually was $10^8$ c.p.m./µg of template. Just prior to use all probes were boiled for two mins. and quenched on ice for five mins.
The genomic library was screened by a plaque hybridization method modified from Benton and Davis (1977). Primary screening of the unamplified genomic library was done at a density at $2 \times 10^4$ plaques per 15 cm plate. Plaques were transferred onto nitrocellulose filters which were treated with denaturing solution (0.5 M NaOH and Tris-HCl pH 7.4) and then neutralizer (1.5 M NaCl and Tris-HCl pH 7.4) before being baked at 60°C in a vacuum oven for one hr. Filters were placed in plastic bags and then prehybridized in 50% formamide, 5XSSC, 5X Denhardt's solution, 0.1% SDS, 50 mM NaPO$_4$ pH 6.5 and 0.1 mg/mL of denatured salmon sperm DNA at 42°C on an orbital shaker for three hrs. Hybridization was performed overnight at 42°C with the hybridization solution being essentially the same mixture except with only 20 mM NaPO$_4$ pH 6.5 and 0.04 mg/mL of salmon sperm DNA, and with 1-5 x10$^6$ c.p.m. of probe added per 10 mL of hybridization solution. After hybridization the filters were washed twice in 2X SSC and 0.1% SDS and then twice in 0.1X SSC and 0.1% SDS. Filters were then dried and exposed to X-ray film.

**(h) Sequencing of genomic clones.**

Sequencing primers were synthesized on a Applied Biosystems DNA synthesizer. The first primer corresponded to the antisense sequence of the first nine codons of the coding region of a cotton Adh cDNA. Sequencing was performed by the chain termination method using a T7 Sequencing Kit (Pharmacia) and [α-$^{32}$P]dCTP following the double stranded sequencing protocol supplied by the manufacturer. Reactions were electrophoresed on 6% polyacrylamide in 23% urea, 1xTBE gels (Sambrook et al. 1989). From the sequence generated additional primers were synthesized for further sequencing.

**(i) Southern analysis.**

Genomic DNA (15 µg) was digested with 50 units of the required restriction endonuclease and electrophoresed on 0.7% agarose gels. The gel was then soaked in 0.1
M HCl for five mins., then with denaturing solution (2x 15 mins.) and finally neutralizer (2x 15 mins.). DNA was then transferred to nitrocellulose (Southern 1975) using neutralizer. After the DNA had been UV cross-linked to the filter, prehybridization and hybridization was performed as in section (f) except that each mixture also contained 10% dextran sulphate. Washing was also done at high stringency as in section (g) but with the last wash done at 65°C.

4.3 RESULTS.

(a) Cloning and characterization of a cotton Adh cDNA.

A cDNA library was prepared from two day old cotton root tips that had been anaerobically stressed for 20 hrs. This is similar material to that examined on the 2D gels in Chapter Three, where ADH was a high proportion of the expressed protein, therefore it was expected that by anaerobically stressing the roots the library would be enriched for Adh cDNAs. A maize ADH1 antibody was used to screen approximately 30-40,000 plaques of unamplified cDNA library and approximately 115 plaques cross-reacted with the antibody. Only one in six clones should be in the correct orientation and reading frame for expression, so in theory this result indicates at least 1.76% of the cDNAs in the library code for an ADH polypeptide, assuming all the spots were true positives.

Two of the strongest cross-reacting plaques were purified and λ DNA isolated. The cDNA insert was cleaved out of the λgt11 vector with EcoR1 and subcloned into the plasmid pUC118. The two cDNA inserts were both approximately 1.5 kb in size, and restriction mapping revealed that they had identical Sph1, Nco1 and Sal1 restriction sites. The restriction map of one of the clones, pGhS31 is shown in Fig. 4.1. A. In this diagram the size of the 13 bp adaptors has been ignored, although the EcoR1 sites have been included.
Figure 4.1 Restriction maps of the isolated Adh cDNA classes.

Table 4.1 The similarities between the Adh cDNA coding regions. Values above the diagonal are comparisons at the nucleotide level; those below, at the amino acid level.
The complete nucleotide sequence of the 1.5 kb cDNA of pGhS31 was determined and is shown in Fig. 4.2. Based on the sequence homology that it shares with the maize Adhl (Dennis et al. 1984), pGhS31 is found to code for a complete ADH polypeptide. The cDNA contained 13 bp of 5' untranslated region followed by 1140 bp of coding region, the exact length of the maize Adhl coding region to which it is 73.6% identical at the nucleotide level. There was 337 bp of 3' untranslated region and however no poly(A) tract was present. This indicates some degradation of the cDNA may have occurred.

The deduced primary amino acid sequence of the pGhS31 Adh clone is very similar to other plant ADH polypeptides (82.9% to maize ADH1, 80.5% to maize ADH2 and 85.5% to pea ADH). The calculated molecular weight of the deduced polypeptide is 41,072 daltons which is in close agreement with 42 kD measured by the mobility on SDS gels (Fig. 3.6b). The three dimensional structure of horse liver ADH has been determined, with the identification of important residues in the catalytic domain and co-enzyme binding domain (Eklund et al. 1976). The cotton ADH polypeptide is approximately 50% identical to the horse liver ADH, with most of the structurally and functionally important residues being conserved, such as the residues that provide ligands for the catalytic (Cys^{47}, His^{69} and Cys^{177}) and non-catalytic (Cys^{99}, Cys^{102}, Cys^{105} and Cys^{113}) zinc atoms, suggesting that this cotton ADH has a similar three dimensional structure.

(b) Isolation of other Adh cDNA classes.

Two dimensional (2D) gel electrophoresis had resolved three distinct ADH polypeptides in root tips exposed to anaerobic conditions (Chapter Three), so other classes of Adh cDNAs were screened for within the library. A further ten positive plaques were purified from the original primary screen. Purified plaques were titred and then spotted out onto λ plates, grown and blotted onto nitrocellulose by the method of Beltz et al. (1983) and then screened as outlined in the Materials and Methods [section
The nucleotide and deduced amino acid sequence of the pGhS31 (Adh) cDNA clone.
4.2 (g). cDNA clones were then classified on the basis of whether they could cross-hybridize with the 3' untranslated region of pGhS31. It was expected that this probe would hybridize only to Adh cDNAs in the pGhS31 class; this is based on the observations by Dennis et al. (1985) that even though coding regions of the maize Adhl and Adh2 genes are highly conserved, the 3' non-coding regions are completely divergent and so are unable to cross-hybridize. The purified clones were probed with the 324 bp Ncol/EcoR1 fragment of pGhS31 which is comprised of sequence only from the 3' un-translated region (Fig. 4.1. A). Seven of the ten newly purified plaques hybridized with the 3' un-translated region (data not shown), implying that a total of nine out of the 12 clones fell into the pGhS31 class.

DNA was isolated from the three clones, λGhS33, λGhS43 and λGhS44 that did not hybridize to the 3' end of pGhS31. The cDNA of each λ clone was cleaved and subcloned into pUC118 and further characterized by restriction enzyme mapping. The restriction map of all three clones differed from that of pGhS31. The restriction patterns of the clones pGhS33 and pGhS43 were identical to one another, but were different to that of pGhS44, suggesting that representatives from another two Adh cDNA classes had been isolated.

An EcoR1 cDNA fragment of pGhS44 was sequenced (Fig. 4.3) and was found to be 746 bp long. Based on similarities to other Adhs, the cDNA has 478 bp of coding region followed by a 3' un-translated region of 267 bp. The coding regions of pGhS44 and pGhS31 were very similar, being 89.3% and 94.3% identical at the DNA and amino acid level respectively (Table 4.1). The only similarity between the two 3' untranslated regions was a conserved stretch of 19 identical nucleotides just 3' to the translation stop codon, which carried a convenient Nco1 site. The significance of this conserved region of nucleotides is unknown. Several putative polyadenylation addition sequences are found 18-27 nucleotides from the end of the cDNA.

Sequencing of pGhS33 (Fig. 4.4) revealed that it was also a truncated Adh cDNA containing 417 bp of coding region plus 168 bp of 3' un-translated region. The coding region had less similarity to the other two cDNAs, being 75.6% and 88.0% identical to pGhS31 at the nucleotide and amino acid level respectively and 76.6% and 89.9%
Figure 4.3 The nucleotide and deduced amino acid sequence of the pGhS44 (Adh) clone. The putative polyadenylation signals are underlined.

Figure 4.4 The nucleotide and deduced amino acid sequence of the pGhS33 (Adh) clone. The putative polyadenylation signals are underlined.
identical at the nucleotide and amino acid level to pGhS44. There was no similarity found between pGhS33 and the other cDNAs in their 3' untranslated regions. Two putative polyadenylation addition sequences were found approximately 140 bp from the end of the cDNA.

(c) Southern analysis.

The EcoRI insert from the pGhS31 cDNA clone was used to probe a Southern blot of Siokra genomic DNA that had been digested with various restriction endonucleases (Fig. 4.5). Complex hybridization patterns were obtained, suggesting that there is an Adh gene family in cotton. The least complex of these patterns was obtained with the BglII restriction enzyme where 7-8 bands were found to hybridize. Bands vary in their intensity which may be a reflection on the homologies between the different Adh genes or the length of the hybridizing fragment, although it can not be ruled out that some may result from partially digested DNA.

Southern analysis using the 3' gene specific probe of pGhS31 (the 324 bp NcoI/EcoRI fragment), was performed since this should reveal how many genes exist for the pGhS31 class of cDNA. The probe hybridized with more than one band in each lane of digested DNA (Fig. 4.6) implying that there is likely to be more than one gene for this class of Adh cDNA. The DNA was prepared from bulk seedlings, so it is possible that the multiple bands could result from polymorphisms that may exist within the seed stock. However, the fact that multiple bands are seen for each digestion suggests that there are two genes for this class of cDNA.

The multiple banding pattern obtained is more complex than expected from the isozymes and 2D gels which suggested only three Adh genes. Cotton is an allotetraploid and so it was expected that two genes exist for each of the progenitors. The Southern suggests that more than four Adh genes exist, although Adh pseudo genes may exist in cotton or that there are polymorphisms in the DNA flanking the Adh genes.
Figure 4.5
Southern analysis of *G. hirsutum* cv. Siokra DNA probed with the *EcoR1* insert of pGhS31. DNA has been digested with (2) *BglII*, (3) *BamHI*, (4) *DraI*, (5) *BclI*, (6) *HindIII* and (7) *EcoR1*. In lane (1) is undigested DNA. The mobility of the molecular weight markers are shown on the right (Kb).
Figure 4.6
Southern analysis of *G. hirsutum* cv. Siokra DNA probed with the 324 bp *NcoI/EcoRI* fragment of pGhS31 (Fig. 4.1 A). DNA has been digested with (2) *DraI*, (3) *Sphi*, (4) *BclI*, (5) *HindIII* and (6) *EcoRI*. Undigested DNA is in lane (1). The mobility of the molecular weight markers are shown on the right (Kb).
(d) Isolation of Adh promoters.

The complete Adh cDNA of pGhS31 was used to screen an unamplified genomic library made from DNA partially digested with Sau3A and size selected in the 15-20 kb size range. The probe has a full length Adh coding region which is able to cross-hybridize with the other cDNA classes (data not shown) using the hybridization conditions stated in the Materials and Methods; therefore it was hoped that genes from each Adh cDNA class would be isolated. From the primary and secondary screening of approximately 200,000 plaques, a total of 15 hybridizing plaques were recovered. A 5' specific probe [the 311 bp EcoR1/Bsm1 fragment of pGhS31 (Fig. 4.1. A.)] hybridized to 12 of the 15 clones in the third round of screening. This probe will select for clones that contain Adh 5' end sequences and hence promoters. The last round of screening was done in triplicate, probing each clone separately with the 3' end gene specific probes, from each of the three cDNA classes [Nco1/EcoR1 324 bp fragment of pGhS31, Nco1/EcoR1 236 bp fragment of pGhS44 and Stu1/EcoR1 229 bp fragment of pGhS33 (Fig. 4.1)]. The screen was done so that the genomic clones could be grouped into the cDNA classes. Five of the 12 clones hybridized with the 3' untranslated region of pGhS44 while the 3' ends of the other two cDNAs failed to hybridize to any of the clones. Three of the clones were very weak hybridizers and so no further analysis of these was pursued. This left four clones, λPro8, λPro15, λPro17 and λPro22 that did not hybridize to any 3' end and so remained candidates for the other two classes. λ DNA was isolated from each of these four clones as well as one clone, λPro11 that hybridized to the 3' end of pGhS44. Restriction mapping and Southern analysis of λ DNA demonstrated that each clone had a unique restriction pattern (data not shown) and therefore appeared likely to be a different gene/allele. Southern blot analysis of λ DNA digested with EcoR1 gave only one hybridizing band of unique size for each genomic clone. The hybridizing EcoR1 fragments for each λ clone was successfully subcloned into pUC118 except for λPro17.
(e) Sequencing of Adh promoters.

Using the sequencing primer that corresponded to the antisense sequence of the first nine codons of pGhS31, the nucleotide sequence 300-400 bp upstream from the translation start has been determined for three Adh clones, λPro8, λPro11 and λPro15. These are the regions where the sequences responsible for the anaerobic induction of transcription have been found in other anaerobically induced genes (Dennis et al. 1987). Sequencing was unsuccessful for clone λPro22, which could be due either to the clone not having a promoter or to there being insufficient similarity between the primer and the gene for effective priming. From the sequence obtained from the first sequencing reaction different primers were synthesized and used to sequence approximately 250 bp downstream of the translation start point.

All the clones contain sequences characteristic of eukaryotic promoters indicating that potentially functional Adh promoters have been isolated. In Fig. 4.7 the nucleotide sequences of the putative promoter regions of λPro11 and λPro8 are given and it can be seen that the two clones are highly similar to one another.

λPro11 was the clone that hybridized to the 3' untranslated region of pGhS44. In its upstream region the sequence 5'-TATAAATA-3' is present, which is a perfect match to the consensus TATA box of plant genes coding for enzymes (Joshi 1987). Also 23 bp downstream of this sequence is 5'-TTCATCA-3', that differs from the consensus sequence of the transcription start for plant genes (5'-CTCATCA-3') by one nucleotide (Joshi 1987). This sequence is similar to the nucleotide sequences around the maize Adhl (CTCATCT) and Adh2 (CTCACCA) transcription start sites (Dennis et al. 1984 and 1985), where transcription is initiated from the middle A nucleotide. Therefore it seems likely that this is where transcription is initiated in the λPro11 Adh promoter (arrow). This would imply that the untranslated leader sequence is 73 nucleotides long and 71.2% AT rich, similar to that found in other plant genes. The homology between λPro11 and the Adh coding region of pGhS31 continues for 34 bp downstream of the translation start point after which all homology ceases. This is presumably where the first intron begins and is the identical position within the gene to where the first introns
Figure 4.7 The nucleotide sequence of the promoter region of the λPro11 clone. Underneath is the sequence of the λPro8 clone with the different nucleotides noted and where (·) indicates an identical base and (*) a missing base. Putative TATA boxes and transcription start sequences are underlined with the likely transcription start point marked (arrows). ARE core motifs are in bold typing. The first exon is in small letters.
are situated in Adh genes from other plant species, thus continuing the high degree of conservation of gene structure between Adh genes of different plant species.

In addition to the putative TATA box and transcription start point, the promoter has four 5'-AAACCA-3' motifs within the first 250 bp upstream of the translation start point suggesting that this promoter is anaerobically inducible. In addition, in the promoter there are several half G-box elements, 5'-CCAC-3' and in the first intron there are three 5'-TGGTTT-3' motifs. Whether these sequences have any role in the anaerobic induction of transcription or whether their presence is co- incidental can only be determined by functional analysis. Although the promoter region and first intron contain a total of seven ARE core sequences, it does not have any fully conserved GC-motifs.

Sequence analysis of λPro8 reveals that its sequence is 96% identical to that of λPro11, its sequence being shown below that of λPro11 in Fig. 4.7. In comparison to λPro11 its TATA box sequence is identical and putative transcription start point is almost identical, 5'-CTCCATCA-3'. It has one less 5'-AAACCA-3' motif, where one motif has change to 5'-AAAACA-3'. The high similarity that exists between these two genes suggests that they are either alleles of one locus, or they could be the genes from the two homoeologous loci of the two genomes of cotton.

Sequence analysis of λPro15 (Fig. 4.8) suggests that this clone represents a different locus to that of λPro11 and λPro8. Again the TATA box is identical to the consensus sequence and the putative transcription start only differs by one nucleotide from the plant gene consensus sequence (Joshi 1987) implying that the leader is 74 nucleotides long and 68.5% AT rich. These characteristics suggest that this promoter also has all the signals required to be functional. The promoter again has multiple ARE core sequences (GT-motifs), four 5'-AAACCA-3' upstream from the TATA box and one 5'-TGGTTT-3' element in the first intron. Olive et al. (1991) and Dolferus et al. (in press) demonstrated that GC rich motifs (GC-box) that are related to the human Sp1 binding site were implicated in the hypoxic induction of transcription of the maize Adhl gene. In λPro15, 182 bp upstream from the putative transcription start site there is the sequence 5'-CCGCCC-3' which is identical to the Sp1 hexanucleotide consensus core binding site,
Figure 4.8 The nucleotide sequence of the promoter region of the λPro15 clone. Underlined regions are the putative TATA box and transcription start sequences with the likely start point marked (arrow). Putative ARE core elements and GC-box are in bold typing. The nucleotides of the first exon are in small letters.

Table 4.2 Similarities between the 5' untranslated regions of the different genomic clones.

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<tr>
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<th>λPro11</th>
<th>λPro15</th>
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<tr>
<td>λPro8</td>
<td>96.3%</td>
<td>77.0%</td>
</tr>
<tr>
<td>λPro11</td>
<td>-</td>
<td>77.5%</td>
</tr>
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and could play an analogous role to the maize GC-box as it is in close proximity to a ARE core element.

A comparison of the sequences of λPro15 and λPro11 (Fig. 4.9) indicates that the promoters are approximately 80% identical. A summary of the similarities of the 5' untranslated regions of these three genomic clones is shown in Table 4.2.

None of the genes have any similarity surrounding the translation start codon when compared to the plant consensus sequence 5'-TAAACAATGGCT-3' (Joshi 1987). However it is certain that this is the translation initiation codon not only from comparisons to other Adh genes but also the fact that there are no other ATG codons in the region downstream from the putative transcription start point that are common between the genes. The first intron of all three cotton Adh genes starts in the identical position in Adh genes from other species of plants, extending the observation that the positions of introns are highly conserved within Adh genes.

4.4 DISCUSSION.

Based on sequence and restriction analysis, three different Adh cDNA classes have been isolated from a library prepared from mRNA of anaerobically stressed roots. The three cDNAs cannot be alleles of the same gene because their nucleotide sequences are so different. Of the three classes of cDNAs, pGhS31 and pGhS44 are the most closely related. Cotton is an allotetraploid therefore pGhS31 and pGhS44 could be homologues, derived from each of the two diploid progenitors. In addition cotton would be expected to have four different Adh loci, for most diploid species have two, therefore the isolation of a representative of a fourth cDNA class, which may be homologous to the pGhS33 class, may have been missed.

The isolation of three classes of Adh cDNA is in agreement with the 2D gel analysis performed in Chapter Three, where three different ADH polypeptides were resolved. In addition there appears to be a correlation between the representation of each of these
Figure 4.9 Comparison of the promoter sequences from the \( \lambda \text{Pro}11 \) and \( \lambda \text{Pro}15 \) clones. The nucleotides of the first exon are in small letters. The TATA box and putative transcription start signals are underlined. The ARE core elements and GC-box are in bold typing.
cDNAs in the library with the level of expression of the three ADH polypeptides, as
determined by protein labelling or Western analysis (Chapter Three). Nine of the 12
clones isolated fell into the pGhS31 class, implying that this cDNA codes for ANP 42c
which is the most abundant polypeptide (Fig. 3.3b). Presumably the other two less
abundant Adh cDNAs, pGhS33 and pGhS44, code for the relatively more weakly
expressed ADHs, ANPs 42a and 42b. Future experiments involving Northern analysis
with gene specific probes should verify whether this is the case. Furthermore it would
be expected that pGhS31, the most abundant anaerobically induced cDNA would code
for the most abundant anaerobically induced isozyme, ADH2, and this is demonstrated
in the next chapter.

The pGhS31 has a full length coding region, therefore this cDNA will be used both to
overexpress ADH, resulting in higher levels of ADH expression or produce
complementary antisense Adh RNA in an attempt to reduce ADH expression levels in
transgenic cotton plants (Chapter Five). It maybe important that this clone is used
because it is the most highly expressed Adh cDNA under anaerobic conditions, so the
ADH polypeptide it encodes may have certain advantageous biochemical properties for
dealing with anaerobic stress, such as a favourable catalytic Km for ethanol production
in an acidic cytoplasm. In addition for antisense experiments to be effective in reducing
the anaerobic induced level of ADH, antisense RNA would be ideally 100%
homologous to the highest anaerobically induced Adh cDNA. Thus the cDNA of
pGhS31 is the most appropriate sequence to use for these experiments.

Three genomic clones of cotton Adh have been characterized and each has been found
to contain a different promoter. All three promoters share sequence similarity and a
similar structure with regards to the position and number of the ARE core elements. In
maize, three alleles of Adhl have been isolated, Adh1-IS (Dennis et al. 1984), Adh1-1F
(Sachs et al. 1986) and Adh1-Cm (Osterman and Dennis 1989). All three alleles are
highly homologous in their 5' flanking regions where the largest difference between
these alleles is 5.6%. In contrast maize Adh2 has almost no similarity in the promoter
region when compared to the maize Adhl alleles (Dennis et al. 1985). Taking this into
consideration, and the fact that the promoter regions of λPro11 and λPro8 are 96% identical, these clones are probably either alleles of the same locus or different genes from the homoeologous loci of the different genomes. The promoter of λPro15 only shares 77.5% identity to λPro11, therefore this gene probably corresponds to a Adh gene from a different locus. Further sequencing and comparison is required to confirm these conclusions.

The nucleotide sequence of what is probably the most important regions within these promoters has been determined i.e. 200-300 bp upstream of the translation start point. This is the region where the sequences responsible for anaerobic induction have been found in other anaerobically induced genes (Dennis et al. 1987). The λPro11 clone hybridized to the 3' untranslated region of the pGhS44 cDNA clone, the cDNA which is likely to correspond to either ANP 42a or 42b (Fig. 3.2), which are major anaerobic polypeptides of cotton. Therefore the promoter of λPro11 must contain the DNA motifs necessary for anaerobic induction of transcription. However no completely conserved GC-motifs were found in this promoter, only ARE core elements, suggesting that GC-motifs are not essential for anaerobic induction, although it is possible that there are other sequences within the promoter that perform the same function as a GC-motif.

There are sequences that differ from a GC-motif by only one bp, but whether a sequence like this can function is unsure, for the sequence requirements of a GC-motif have not been experimentally defined. It is possible that whereas the ARE core elements confer anaerobic inducibility a GC-motif may control the level of anaerobic inducibility; it would be interesting to compare the strength of the λPro15 promoter that contains a GC-motif to that of λPro11 which does not. Multiple 5'-AAACCA-3' ARE core motifs were found in each of the promoters that were sequenced. The significance of three or four core ARE elements in the promoter is uncertain, since no other known anaerobic promoter has so many ARE core sequences. Although half G-box elements (5'-CCAC-3') were found, a four base pair nucleotide sequence on average would be expected to occur every 256 nucleotides, therefore this sequence would be expected to occur within a 500 bp region and so its presence is probably not significant.
Although only 5'-AAACCA-3' elements are usually found in Adh promoters of dicotyledonous plant species, 5'-TGGTTT-3' motifs were found in the first intron of each of the cotton genes sequenced; a hexanucleotide sequence would only be expected to occur approximately every 4096 bp, therefore the presence of these 5'-TGGTTT-3' elements is statistically significant (introns are AT rich so the sequence may be expected to occur more often). Putative regulatory motifs have been found previously in plant introns however their functional significance is unknown. For example, in the first intron of the sucrose synthase I gene from maize there is an ARE core element plus flanking nucleotides which give it a 13 bp match to the subregion two of the ARE of maize Adh1 (Springer et al. 1986). In addition, Petunia Adh has four 5'-TGGTTT-3' motifs in its first intron (and one 5'-AAACCA-3' element in its promoter) but none in its other eight introns (Gregerson et al. 1991). Therefore the distribution of these elements does not appear to be random and they may play some role in expression of the gene, but functional analysis is required to resolve their significance.

One aim of this work was to isolate an Adh gene representing each of the cDNAs classes. From a comparison of the sequence of the first 13 bp of 5' untranslated region and the first exon of the genes to the corresponding nucleotides of pGhS31, it was hoped that any clones corresponding to this class of cDNA would be identified. λPro8 only differs by one nucleotide out of 46 and λPro11 differs by two out of 46 to the cDNA pGhS31 (Fig. 4.10), although it is known that λPro11 falls into the pGhS44 class. It is therefore apparent, that the 5' ends of the different Adh genes are more similar to one another than their 3' ends. λPro15 is more divergent to the pGhS31 cDNA than the other two clones (4/46) and so appears also not to code for the pGhS31 cDNA. But λPro11 and λPro15 do have significant similarity between their 5' untranslated regions whereas the maize Adh1 and Adh2 genes do not share much sequence homology with each other in this region (Dennis et al. 1985). Taking the similarity of λPro11 and λPro15 (Fig. 4.9) into consideration and the fact that the coding regions of pGhS33 and pGhS44 are more divergent than maize Adh1 and Adh2, it seems unlikely that λPro15 corresponds to the pGhS33 class either, but rather is another version of the pGhS44 and
Figure 4.10 Comparison of the 5' untranslated regions and the first exons of each genomic clone to the pGhS31 Adh cDNA. Nucleotides which do not match are in small letters.
pGhS31 class suggesting that there are three different loci in this group. Further sequencing is needed to confirm these conclusions. From this finding and the Southern data it appears that cotton has multiple Adh loci, probably more than four.

5.1 INTRODUCTION

(a) Anaerobic metabolism in plants.

The anaerobic response has been better characterized with respect to its molecular biology than to its physiological significance. This chapter describes the initiation of experiments that attempt to examine how a plant's tolerance to anaeria is affected by modifying the levels of expression of the enzymes in the alcoholic fermentative pathway, namely ADH and PDC.

In comparison to animals, all higher plants would be considered tolerant to anaeria because plants are able to survive anaerobic conditions (aerobic/aerobic atmosphere) for periods of between several hours to several months depending on the species of plant (see Jackson and Drew 1984 for a summary).

During anaerobic conditions sugars (hexose, starch) are broken down into pyruvate which is then converted by pyruvate dehydrogenase (PDH) into acetyl CoA. Then the acetyl CoA is channeled in the direction of aerobic metabolism (Fig. 5.1) into the TCA cycle, the electron transport chain and oxidative phosphorylation. This reaction within the mitochondria and requires oxygen to be present as the terminal electron acceptor. If oxygen levels are depleted anaerobic metabolism becomes imperative and ATP production via oxidative phosphorylation ceases. Low ATP levels within the stressed cells will result in the cessation of growth, reduction of the plant's ability to take up nutrients from the soil and cause the death of root tips or even the whole root system (Jackson and Drew 1984). For plants to survive anaerobic conditions, alternative metabolic pathways must become operative to enable ATP to be generated.
CHAPTER FIVE

The Generation of Transgenic Cotton with Altered Levels of ADH and PDC.

5.1 INTRODUCTION

(a) Anaerobic metabolism in plants.

The anaerobic response has been better characterized with respect to its molecular biology than to its physiological significance. This chapter describes the initiation of experiments that attempts to examine how a plant's tolerance to anoxia is affected by modifying the levels of expression of the enzymes in the ethanolic fermentative pathway, namely ADH and PDC.

In comparison to animals, all higher plants would be considered tolerant to anoxia because plants are able to survive anaerobic conditions (nitrogen/argon atmospheres) for periods of between several hours or several months depending on the species of plant (see Jackson and Drew 1984 for a summary).

During aerobic conditions sugars (sucrose, starch) are broken down into pyruvate which is then converted by pyruvate dehydrogenase (PDH) into acetyl CoA. Then the acetyl CoA is channelled in the direction of aerobic metabolism (Fig. 5.1); into the TCA cycle, the electron transport chain and oxidative phosphorylation that function within the mitochondria and require oxygen to be present as the terminal electron acceptor. If oxygen levels are depleted aerobic metabolism becomes inoperative and ATP production via oxidative phosphorylation declines. Low ATP levels within the stressed cells will result in the cessation of growth, reduction of the plant's ability to take up nutrients from the soil and cause the death of root tips or even the whole root system (Jackson and Drew 1984). For plants to survive anaerobic conditions, alternative catabolic pathways must become operative to enable ATP to be generated.
Figure 5.1 Anaerobic metabolism with some of the alternative end products. Sucrose is broken down to pyruvate where under anaerobic conditions it cannot be converted to acetyl CoA. Instead pyruvate can either be converted to ethanol, lactate or alanine. The disadvantage of alanine production is that it does not regenerate NAD+. The production of malate is also shown which was proposed in Crawfords theory to be advantageous to the plant, but as can be seen, it would result in no net gain of ATP. However it must be noted that there are other pathways to malate production. Also shown here is ADH's aerobic role of oxidizing ethanol to acetaldehyde which is then converted to acetate by aldehyde dehydrogenase (AIDH) where it may then enter the TCA cycle.
Anaerobic metabolism results in the synthesis of many end products, including ethanol, lactate (lactic acid), alanine, succinate, glycerol, malate and shikimate (Davies 1980). Of these, the pathways resulting in the synthesis of alanine, lactate or ethanol, which are all end products of anaerobic glycolysis, are known to produce a net gain in ATP (Jackson and Drew 1984, Fig. 5.1). One requirement for continuation of glycolysis during anaerobic stress is that NAD$^+$ must be regenerated from NADH. The pathways that result in the production of either ethanol via pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) or lactate produced by lactate dehydrogenase (LDH) will result in the regeneration of NAD$^+$ (Fig. 5.1). However the production of alanine from pyruvate does not result in the regeneration of NAD$^+$, although it does accumulate to high levels during anoxia in some plants, where alanine aminotransferase (AlaAT) catalyses the transamination reaction (Fig. 5.1). One advantage of alanine production is its synthesis will help prevent cytoplasmic acidosis, for alanine will cause a smaller fall in pH than lactate (Reggiani et al. 1988).

The first metabolic theory of flooding tolerance was proposed by McManmon and Crawford (1971) who incorrectly thought that ethanol was toxic to plant cells and so reasoned that flood tolerant species had metabolic pathways that resulted in the accumulation of alternative end products such as malate (Fig. 5.1). Although malate production does result in the regeneration of NAD$^+$, it would mean that glycolysis would yield no net gain of ATP, for phosphoenolpyruvate (PEP) is not converted to pyruvate (Davies 1980). The theory of Crawford's has been discarded. Only the pathways leading to ethanol and lactate are known to result in NAD$^+$ regeneration and ATP production during anaerobiosis. Although ethanolic or lactic acid fermentation allows glycolysis to continue, the expected net yield of ATP, starting with hexose as a substrate, is only 2 mol. of ATP/mol. of hexose compared with a theoretical maximum of 36 mol. of ATP/mol. of hexose for aerobic respiration. Therefore if sufficient ATP is to be produced to allow the plant to survive, the carbon flux through the pathways must increase. This is observed in many plant species where lactate and ethanol are synthesized in large quantities; this is known as the Pasteur effect. In most plant species sugars are preferentially catabolized to ethanol rather than
lactate when anoxia lasts for more than a few hours (Smith and ap Rees 1979, Bertani et al. 1980, Davies 1980, ap Rees et al. 1987). This reflects the fact that anaerobic conditions will not only depend on whether sufficient energy can be derived to supply the requirement of cell maintenance, but also on whether the metabolic end products are compatible with cell survival. Ethanol is a more compatible end product than lactate as will be discussed below.

(b) Cytoplasmic acidosis as a determinant of flooding tolerance.

Non-invasive NMR techniques were used to examine the initial changes in catabolism that take place during the switch from aerobic to anaerobic metabolism in the root tips of maize (Roberts et al. 1984a). Within two minutes of anoxia the NADH levels rise to a new steady-state level indicating a rapid inhibition of oxidative phosphorylation. Pyruvate was initially found to be converted to lactic acid whose accumulation results in the cytosolic pH becoming acidic (dropping from pH 7.2-7.4 to pH 6.8). At this lower pH LDH activity is inhibited while PDC is activated, resulting in a switch from predominantly lactic acid synthesis to predominantly ethanol synthesis. After this shift in metabolism the cytoplasmic pH remained stable for up to 10 hours. Thus in maize, the transient lactic acid fermentation provides the signal triggering the switch to ethanol production. In this way energy production proceeds without cytoplasmic acidosis.

In maize plants that are homozygous for a non-functional Adhl locus hardly any ethanol is synthesized during anaerobic conditions (Roberts et al. 1984a). In these plants the cytoplasmic pH does not stabilize but instead continues to fall as lactic acid production continues (Roberts et al. 1984a). The events occurring in these plants are similar to those occurring in animal tissue responding to hypoxia, with a build up in lactic acid and ensuing cell death (Dawson et al. 1978). Thus ADH is essential for survival under prolonged anaerobic conditions because it allows the pH of the cytoplasm to remain at a tolerable level.

However, even in wild-type maize plants the acidification of the cytoplasm eventually occurs through leakage of protons from the vacuole. The vacuole is normally
maintained at pH 5.8, whereas the cytoplasm is usually at pH 7.4 (Roberts et al. 1984b). Proton translocating ATPases in the tonoplast normally maintain the relatively high proton concentration of this compartmentation, but with a diminished energy status their activity is presumably inhibited, allowing protons to leak passively to the cytoplasm. The acidification was found to be closely associated with cell death; for example protons were found to leak more quickly from the vacuoles of peas than from maize and appeared to account for the lower tolerance of peas to anaerobiosis (Roberts et al. 1984b). The precise mechanism of damage to the cytoplasm by low pH is unknown, but hypoxia has been shown to cause changes in the fine structure of meristematic cells especially to mitochondria, endoplasmic reticulum, golgi apparatus and chromatin (Aldrich et al. 1985).

Although it was originally believed that ethanol was toxic to plants, there is no evidence that ethanol accumulates in affected tissues to toxic concentrations. Jackson et al. (1982) showed that applied ethanol displayed little toxicity to roots or even to cell protoplasts at concentrations of up to 0.1 M. In the presence of free water, ethanol produced in fermentation readily diffuses out of the cells to the external solution (Bertani et al. 1980). Alternatively ethanol may be transported to other parts of the plant (e.g. leaves) via the xylem where it may be consumed in aerobic tissues or dissipate into the atmosphere via the transpiration stream (Fulton and Erickson 1964) so that accumulation does not pose a threat.

(c) The importance of ADH and the anaerobic polypeptides for anaerobic survival.

Many of the maize anaerobic proteins (ANPs) have been identified as enzymes in the glycolytic and alcohol fermentation pathways (see Chapter Three Fig. 3.1). However the significance of their expression, or level of expression for the adaptation of the plant for prolonged anaerobic survival has not been elucidated. Nevertheless several lines of evidence show that when the ANPs or an ANP is not expressed, anaerobic survival is diminished.
As discussed above, ADH expression has been shown to be critical in the survival of seedlings exposed to anaerobic conditions. Schwartz (1969) was the first to report that maize ADH1 null plants succumbed to flooding more rapidly than did wild-type plants. This has also been shown to be the case in barley (Harberd and Edwards 1982) and Arabidopsis (Jacobs et al. 1988) where plants that are ADH null are less tolerant to anaerobic conditions. This demonstrates that these plants are unable to effectively utilize alternative metabolic pathways and/or tolerate the accumulation of acetaldehyde during anaerobic stress. Acetaldehyde has been demonstrated to be toxic to cells where it can bind proteins and inactivate enzymes (Mauch et al. 1987). In addition to Adh nulls, maize Shl mutants (with deficiencies in sucrose synthase I) have been reported to germinate poorly under hypoxic conditions (Kloeckener-Gruissem and Freeling 1987).

Another line of evidence that the ANPs are important for anaerobic survival comes from the work of Okimoto et al. (1980) who found that maize leaves, which do not have an anaerobic response (i.e. do not synthesize ANPs), die after only 5 hours of an anaerobic environment.

(d) The significance of the level of ADH expression.

Because ADH appears to play such a central role in the anaerobic response many studies have tried to determine if a correlation between the level of ADH activity and the extent of tolerance to flooding exists. There have been almost as many different conclusions as studies performed. The majority of these studies have been done in maize, where there are large variations in both ADH activities and tolerance to flooding in the maize germplasm (Lemke-Keyes and Sachs 1989). No correlation between the ADH activity and flooding tolerance was found (Lemke-Keyes and Sachs 1989) but this may be due to the large differences in genetic background among the maize varieties contributing unequally to anaerobic fitness. Experiments have been done to try to eliminate differences in genetic background by performing appropriate crosses to generate lines with different levels of ADH activity but with randomized genetic background (Marshall et al. 1973, Roberts et al. 1989). Marshall et al. (1973) analysed
maize seedlings where two \textit{Adh1} alleles that coded for electrophoretic variants with different catalytic activities were segregating within the population. They concluded that the plants that inherited the lower activity isozyme were more flood tolerant than plants that inherited the high activity isozyme. They explained these results by assuming that ADH was rate limiting for ethanol synthesis and that ethanol was toxic to the plant cells subscribing to Crawford's metabolic theory of flooding. Roberts et al. (1989) measured survival, cytoplasmic pH and the rates of ethanolic fermentation in root tips differing in ADH activity. They found that only when ADH was at very low levels ethanol synthesis and anoxia tolerance were effected. When ADH falls to very low levels the plant becomes more susceptible to anoxia. This disagrees with Marshall et al. (1973) who showed that different ADH isozymes can influence the flood tolerance of maize seedling, where lower levels of ADH resulted in a plant that was more flood tolerant.

One reason for the discrepancy in results may be the methods of flooding/hypoxia used were different. As will be discussed in detail below [section (e)], the level of induction of ADH activity is known to reflect the conditions of anaerobic stress, where anoxic shock results in a small induction of ADH and a low survival time. Marshall et al. (1973) observed an induction of ADH to a high activity level. However Roberts et al. (1989) did not, indicating that their seedlings were being tested under more adverse condition ("anoxic shock") compared to that of Marshall et al. (1973). This suggests that in the two studies the seedlings were being examined under different environmental conditions, which will effect survival time as will be discussed below [section (e)].

Another possible reason for the different conclusions is that it has been assumed that the \textit{in vitro} ADH enzyme activity reflects the catalytic activity of the enzyme \textit{in vivo}. ADH activity was measured in the ethanol to acetaldehyde direction in an alkaline pH buffer, whereas in the stressed cell the enzyme is performing the opposite reaction, of acetaldehyde to ethanol in an acidic cytoplasm. These studies have assumed that if one isozyme has a higher \textit{in vitro} activity in the ethanol to acetaldehyde direction than another isozyme it will also have a higher \textit{in vivo} acetaldehyde to ethanol activity. This assumption is probably not valid as highlighted by the properties of the two closely
related ADH enzymes in yeast, that have significant catalytic differences, including the Km values, when catalysing the two reactions (Bennetzen and Hall 1982). In yeast ADH I is responsible for ethanol production from acetaldehyde whereas the oxidation of ethanol to acetaldehyde is carried out by ADH II. Therefore the standard in vitro enzyme activity assay would be biased towards measuring the ADH II isozyme activity when in fact it is the activity of ADH I enzyme that is important if it was desired to measure the acetaldehyde to ethanol flux.

A number of other studies have been carried out in which no attempt has been made to randomize the genetic background. Chow (1984) concluded from a study of growing maize in waterlogged conditions to the silking stage that plants with high ADH activity levels were more tolerant to waterlogging than plants with low levels. Conversely other studies (VanToai et al. 1985 and 1987) concluded that there was no correlation of PDC and ADH activity with flooding tolerance, but ADH was assayed in the ethanol to acetaldehyde direction in both these studies. Chan and Burton (1992) who examined Trifolium repens concluded that ADH activity was positively correlated with the rate of ethanol production and that plants with higher levels of ADH activities during flooding have greater flood tolerance.

Additionally it is possible that the role of ADH in catalysing the oxidation of ethanol to acetaldehyde and thus redirecting carbon back into the TCA cycle via acetate (Fig. 5.1) may also contribute to the tolerance to anaerobic conditions (Cossins 1987). This pathway is only able to operate in aerobic parts of the plant, e.g. the aerial parts of the plant when the roots are waterlogged. Thus the ethanol synthesized in roots could be transported to the shoots, where it is catabolized and thus carbon would be conserved.

(e) Hypoxic pretreatments confer tolerance to anoxia.

In nature the oxygen concentrations in waterlogged soil declines over a period of a few hours to a few days depending on the soil temperature and the respiratory activity of roots and microorganisms (Blackwell 1983). Hence, root cells experience a gradual decline in oxygen concentration i.e. they experience hypoxia before anoxia. Some
studies have clearly used "anoxic shock" rather than a gradual decline in oxygen concentrations (Roberts et al. 1984a & 1989). Experimental procedures have a profound effect on survival time. Recent experiments have focused on hypoxic pretreatments of seedlings before the anoxic stress. In an acclimation process these hypoxic pretreatments (HPT) resulted in changes in metabolism that greatly improved the subsequent viability of plants subjected to anoxic conditions. Saglio et al. (1988) showed that if excised root tips from maize seedlings were given an 18 hr HPT with 2-4 kPa partial pressure of oxygen they could survive more than 22 hours of anoxia. In contrast excised root tips from seedlings with no HPT all died within 9 hours of anoxia. This extended survival time may have been mediated by the larger amounts of ATP, larger ATP/ADP ratios and higher rates of ethanol production observed in the pre-treated plants. Unexpectedly when maize Adh1 null plants were given a HPT, they were found to be as tolerant to anoxia as the non-mutants were. ADH activity levels in the mutants were found to increase with ADH2 being responsible for all the activity and the plants produced ethanol at comparable rates to that of non-mutants (Saglio et al. 1988). So from the ADH activities, both in the wild-type and Adh1 null maize seedlings, hypoxic conditions appears to result in a greater induction of ADH than does anoxic conditions. Wignarajah and Greenway (1976) also demonstrated this where ADH and PDC activities were highest in maize roots which were flushed with 8-13% oxygen. The effect of HPT was also examined in intact seedlings (Johnson et al. 1989). They found if maize seedlings were given a 4% oxygen pretreatment for 18 hours viability could be extended from no more than 24 hours to more than 96 hours, where roots were able to resume extension when re-supplied with oxygen. Improved viability of hypoxically pre-treated roots was associated with maintenance of a high energy metabolism, ATP concentrations, total adenylates and adenylate energy charge (Johnson et al. 1989). HPT induced a high activity of ADH which was retained throughout the period of anoxia in contrast to the lack of ADH activity in the anaerobically shocked roots (Johnson et al. 1989). Hole et al. (1992) showed that acclimation was due to a more rapid rate of fermentation where HPT excised root tips consumed [U-14C] glucose and respired up to five times more rapidly than non HPT
root tips. Consistent with this they found HPT root tips produce ethanol more rapidly than non HPT tissues.

This acclimation process has also been found in wheat seedlings (Waters et al. 1991). Seedlings exposed for 15-30 hours of hypoxia had an increased tolerance to anoxia and the alcoholic fermentation rates were 1.4 to 4 fold faster than non-treated plants. Wheat was found to have a weaker Pasteur effect in comparison to plants such as maize and rice so it was proposed that this could be one reason why wheat is less tolerant to anoxia than rice (Waters et al. 1991).

All these observations suggest that hypoxic pretreatments result in a greater induction of the anaerobic proteins which is associated with a more vigourous glycolysis and ethanolic fermentation leading to improvements in energy metabolism, which results in the apical cells of the roots having an increased tolerance to anoxia. However it is unsure of what other acclimation processes are taking place. Xia and Saglio (1992) have shown that hypoxic pretreatments allows the development of a transport system for lactate, which is removed from cells during a subsequent period of anoxia, slowing the rate of cytoplasmic acidosis.

Hwang and VanToai (1991) have also shown that exogenous abscisic acid (ABA) can induce tolerance to anoxia in maize seedlings. Cyclohexamide inhibits this ABA-induced acclimation to anoxia indicating that the tolerance was mediated by the synthesis of new proteins. The ABA treatment was found to induce ADH activity more than three fold and it was suggested that this could account for the increased tolerance although it is uncertain what other acclimation processes may have taken place. For example the activity levels of the other anaerobic proteins were not measured. Nevertheless like the HPTs, application of ABA led to the plant having high levels of ADH activity prior to the exposure to anoxia. Therefore not only may the levels and distribution of ADH activity be important but also the timing of induction.
(f) Aims of the chapter.

These studies suggest that ADH does play a key role in tolerance to anaerobic stress where not only are the activity levels of ADH important but also the timing of expression. The prior expression of ADH before the environment becomes anoxic may enable the plant to acclimate and become more tolerant to anaerobic stress. The experiments described in this chapter set out to test this hypothesis, by generating transgenic cotton plants that are constitutively expressing ADH at a high level of activity. This will specifically test one facet of the acclimation process only, that is where ADH is at high levels prior to anoxia. Consequently any improvement in tolerance to anoxia can unequivocally be assigned to altered ADH activity. An additional benefit from this methodology is that plant transformation results in the production of a number of individual transformed plants usually with differing levels of expression of the transformed gene; therefore plants with a range of ADH expression levels should be obtained.

The level of ADH may be lower by creating transgenic cotton plants that express antisense Adh RNA. The level of expression of the antisense RNA may vary between individual transformants and thus may result in plants with different levels of ADH activity. This experiment may provide information about how much ADH activity can decrease before anaerobic survival is affected.

In Chapter Three, analysis of the anaerobic proteins failed to resolve an ANP corresponding to pyruvate decarboxylase (PDC). In addition preliminary measurements of PDC activity in cotton showed it to be very low even after 20 hours of hypoxia (T.L. Setter unpublished). It has been suggested that in maize the activity of PDC is more likely to be rate limiting to the anaerobic pathway than is the activity of ADH (Wignarajah and Greenway 1976, Waters et al. 1991). Cotton may be intolerant to anaerobic conditions/waterlogging because of a low alcoholic fermentative rate, being limited by a low PDC activity. So in addition to transforming Adh genes, cotton will be transformed with extra copies of Pdc.
Different lines of cotton will be generated with different levels of ADH and PDC activity whilst their genetic background remains constant, allowing for any difference in tolerance to anoxia seen to be unequivocally assigned to the alteration in an enzyme activity. In this way the physiological significance of the level of ADH and PDC activity in anoxic survival may be more precisely defined. If increases either in PDC or ADH activity correlates with an increase in the rate of ethanol synthesis then the rate limiting step of ethanolic fermentation will have been conclusively identified. If an increase in the rate of ethanol synthesis is correlated with an increase in tolerance to anoxia, then it can be said that an increased rate of ethanolic fermentation is responsible for anaerobic survival. Alternatively, if increased ADH activity levels lead to an increased tolerance to anoxia but not to increased rates of ethanol synthesis, it may be suggested that reducing the accumulation of acetaldehyde is enabling the plant to be more tolerant to anoxia.

The first aim in this chapter will be to construct chimeric genes for the overexpression of ADH and PDC. In the last chapter a clone, pGhS31, containing a complete cotton Adh coding region was obtained, and this cDNA will be used to overexpress ADH and also to create antisense Adh RNA in transgenic cotton. The cotton Pde gene has not been isolated, but the maize Pde cDNA has (Kelley 1989). So using this sequence data PCR primers will be made and used to amplify a coding region from a maize Pde gene. Pde antisense experiments appear unnecessary because Pde is probably being only weakly expressed, as outlined in the previous paragraph. These cDNAs will be overexpressed using the highly expressed constitutive 35S promoter of Cauliflower mosaic virus (CaMV) (Guilley et al. 1982).

Initially it was hoped to generate different lines of transgenic callus with altered levels of ADH and PDC, then to examine whether the rate of ethanol production has changed and to see if there are any correlations with respect to tolerance of anaerobic conditions. However the results will show that analysis in callus is difficult and it was concluded that analysis would be easier in transgenic plants and the results would have more physiological significance. Measurements on the transgenic calli with extra copies of Adh and Pdc indicated that the transgenes were active, therefore it was decided to
regenerate transgenic plants from the calli. The commencement of the regeneration of plants and the analysis of the levels of expression of the transgenes will be described in this chapter.

5.2 MATERIALS AND METHODS

(a) Amplification of a maize Pdc cDNA.

To isolate a maize Pdc coding region, PCR products were amplified from cDNA that had been prepared by M. Olive (CSIRO) from a *Zea mays* cv. Black Mexican Sweet XII-II suspension cell line. Two oligonucleotides, a 28-mer sense strand primer 5'­GGAGATCTTTCACGTGCGTTGCGTGCCC-3' and a 32-mer antisense strand primer 3'-TTGGGGGTCACTACTCGGGCAGCTCTTAAGGG-5' were synthesized using an Applied Biosystems 308A DNA synthesizer. Their design was based on the cDNA sequence of a maize Pdc cDNA clone (Kelley 1989) so that the complete coding region would be amplified. The primers had been designed so that the PCR product will have a *BglII* site at the 5' end and an *EcoR1* site at the 3' end (bold letters). A Perkin-Elmer Cetus PCR kit was used and the reaction consisted of 10 ng of cDNA, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 10% DMSO, 0.8 mM dNTPs (equal ratios), 1 µM for each primer and 5 units of *Taq* polymerase. The reaction was covered with 30 µL of mineral oil and subjected to PCR in a thermal cycler. The thermal profile was 5 mins. at 95°C, 1 min. at 55°C and 3 mins. at 72°C then 30X(1 min. at 95°C, 1 min. at 55°C and 3 mins. 72°C) and 10 mins. at 72°C. The vector pBGS-*BglII* [which is the plasmid constructed from the *PvuII* fragment (532-977) of Bluescript inserted in place of the corresponding *PvuII* fragment of pBGS18⁺ (Spratt et al. 1986), and then cleaved with *SmaI* with a *BglII* linker inserted] was used for subcloning the PCR products.
(b) Construction of binary vectors.

All cloning manipulations and constructions were done using standard methods (Sambrook et al. 1989) in the *Escherichia coli* (*E. coli*) strain JM101 (Yanisch-Perron et al. 1985). The *Adh* cDNA from plasmid pGhS31 which contains a complete coding region (Fig. 4.2) was used to overexpress ADH and produce antisense *Adh* RNA. To express the ADH and PDC coding regions, the expression vector pJ35SN (for construction see Lyon et al. 1989) which contains the CaMV 35S promoter and a 3' termination signal from the nopaline synthase gene was used. For transformation into plants the pJ35SN constructs were ligated into the binary vector pGA470 (An et al. 1985) which contains the bacterial *Tn5* neomycin phosphotransferase II (*npt II*) gene which confers resistance to the antibiotic kanamycin.

(c) Transfer of binary vectors from *E. coli* to *Agrobacterium*.

The binary vectors were transferred from *E. coli* into the *Agrobacterium tumefaciens* strain AGL1 (Chaudhury and Signer 1989) by triparental mating with the helper plasmid pRK2013 of *E. coli* HB101 (Ditta et al. 1980). Transconjugants were selected on solid Luria broth (LB) medium (Sambrook et al. 1989) containing rifampicin (50 µg/mL) and tetracycline (5 µg/mL) and then streaked out to single colonies on the same medium containing in addition carbenicillin (5 µg/mL).

In addition to the three strains constructed, a further *Agrobacterium* strain AGL1(470Gus) (Cousins et al. 1991), which contains the CaMV 35S promoter linked to the *gus* reporter gene, was used in order to generate transgenic cotton callus that did not have altered levels of ADH or PDC.

(d) Transformation and regeneration of cotton.

An *Agrobacterium* mediated gene transfer system with selection on kanamycin-containing media was performed as has been described by Cousins et al. (1991).
Agrobacterium was grown for 16 hrs at 28°C in liquid LB medium and then diluted to a density of $1 \times 10^8$ cells/mL in a 1:5 mix of LB and liquid basal media [MS macro and micro elements (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 100 mg/L myoinositol, 30 g/L glucose and with the pH adjusted to 5.8 with KNO$_3$].

Seeds of *G. hirsutum* cv. Coker 315 were germinated aseptically in a Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 2.0 g/L Gelrite and 0.94 g/L of MgCl$_2$. Cotyledons from seven day old seedlings were harvested, co-cultivated with the *Agrobacterium*, cut into pieces and placed on sterile filter paper on solid co-cultivation medium in sterile petri dishes. Medium was the same as the liquid basal media except with the addition of 0.2 mg/L 2,4-D and 0.1 mg/L kinetin. The pH of the medium was adjusted to 5.8 with KNO$_3$ and solidified as before. After 48 hrs in the growth room cotyledons were transferred, six to eight pieces per plate to selection medium (MST 50KC plates), which is the same as co-cultivation medium except with 0.1 mg/L of 2,4-D instead of 0.2 mg/L and with 50 mg/L of kanamycin sulfate and 250 mg/L Cefotaxime. After five to seven weeks, callus that has grown on cotyledons was transferred to MSPN 25KC plates which is the same media but this time with 25 mg/L of kanamycin sulfate instead of 50 mg/L and the hormones 2IP (6 γ, γ-dimethylallyl-amino)-purine N$^6$-(Δ2-isopentenyl)-adenine at 5 mg/L and naphthalene acetic acid at 0.1 mg/L instead of 2,4-D and kinetin.

Plants are regenerated on media in the absence of any further selection on kanamycin allowing for a much more efficient embryogenic process to take place. After four to six weeks callus that had embryogenic structures (green to creamy colour with a grainy texture) were transferred to 50 mL of liquid basal media (same media as before but with no gelling agents, hormones or antibiotics) in 250 mL conical flasks. After three to six weeks the suspension cultures were sieved with 5, 10 and 60 mesh. Generally, large clumpy material was discarded (5 mesh) while the smaller callus material (10 mesh) was plated out onto solid embryo development media that consist of basal media with 1.9 g/L of KNO$_3$. The very fine callus (60 mesh) was subcultured back into liquid basal media and the sieving was continually repeated after every two to three weeks. Frequent sieving led to suspension cultures that gave high quality embryos.
When a radicle emerged from an embryo it was inserted into deep petri dishes of Stewart and Hsu (1977) media solidified with Gelrite and MgCl₂. If the embryo developed it was transferred to a 8 cm x 6.5 cm (diameter) sterile jar containing the same media. All cultures and calli were kept in a 27°C growth room and maintained under low light conditions (5 µmol m⁻² sec⁻¹).

If a root system and true leaves developed the plantlet was transferred to soil. A plastic container was placed over the plantlet to harden off the plant and it was then placed in a 27°C/22°C day/night phytotron cabinet under high light (650 µmol m⁻² sec⁻¹) conditions and grown to maturity.

(e) NPT II dot blot assays.

The npt II gene expression was assayed in plant material using essentially the dot blot method of McDonnell et al. (1987). Protein was extracted by grinding a sample of leaf tissue in 125 mM Tris-HCl pH 6.8, 20% glycerol and 10% β-mercaptoethanol and centrifuging the slurry at 15,000 r.p.m. at 5°C. The supernatant was centrifuged again, and from this 15 µL of extract was mixed with 15 µL of reaction buffer (McDonnell et al. 1987) containing [γ-³²P]-ATP and incubated at 37°C for 30 mins. After the incubation 20 µL from each reaction was spotted and dried onto Whatmans P81 paper which was then washed in 50 mL of 1% SDS with 5 mg of proteinase K for 30 mins. at 60°C. Following two washes in 10 mM NaHPO₄ pH 6.5 at 80°C the paper was dried and exposed to X-ray film overnight. The positives showed up as dark spots.

(f) Anaerobic treatment, protein extractions, ADH enzyme assays and isozyme plates.

Protein extractions, ADH enzyme assays and isozyme plates were performed as described previously in section 2.2 (c), (d) and (e). Anaerobic conditions were again the same as in section 2.2 (b) except that when callus was anaerobically stressed it was not.
submerged in anaerobic induction buffer, but rather left in the petri dish of tissue culture media in a container that was flushed with argon.

5.3 RESULTS

(a) Construction of plasmids for the overexpression and underexpression of ADH.

The \textit{Adh} cDNA coding for the complete ADH polypeptide from \textit{G. hirsutum} cv. Siokra (a 1490 bp \textit{EcoR1} fragment, Fig. 4.2) was excised from the plasmid pGhS31 using \textit{EcoR1} and ligated into the \textit{EcoR1} site of pJ35SN. The fragment was inserted in both the sense (p35SAdhN) and antisense (p35ShdAN) orientation as confirmed with the appropriate restriction digests (\textit{NcoI}/\textit{HindIII}). Names of plasmids or transgenic material derived from the \textit{Adh} cDNA cloned in the sense orientation will contain the initials Adh and those with \textit{Adh} in the antisense orientation, the initials hdA. The p35SAdhN and p35ShdAN plasmids were cleaved with \textit{HindIII} and cloned into the \textit{HindIII} site of the binary vector, pGA470. The resulting plasmids, 470Adh and 470hdA were checked with \textit{EcoR1}, \textit{PstI}, \textit{HindIII} and \textit{EcoR1}/\textit{PstI} digests and all gave the expected restriction patterns. Diagrams of the constructs are shown in Fig. 5.2 A and B. The binary vectors were then transferred into the \textit{Agrobacterium} AGL1 by triparental mating. DNA was prepared from both transformed strains, AGL1(470Adh) and AGL1(470hdA) and the presence and integrity of the binary vectors was verified by Southern analysis on \textit{EcoR1} and \textit{EcoR1}/\textit{NcoI} digested DNA.

(b) Construction of a plasmid to overexpress PDC.

PCR was performed on cDNA prepared from a maize suspension cell line. A PCR reaction yielded one major product whose size was estimated at 1.8 kb, which was the expected size for a maize \textit{Pdc} cDNA. After digestion with \textit{EcoR1} and \textit{BglII} the product was ligated into pBGS-\textit{BglII} that had been cleaved with \textit{EcoR1} and \textit{BglII}. The insert
Figure 5.2 Maps of plasmids constructed for transforming the Adh (A), antisense hdA (B) and Pdc (C) coding regions into cotton. Diagrams are not to scale.

35S = CaMV 35S promoter
nos = nopaline synthase 3' termination signal
BR = right border
BL = left border
Tet = tetracycline resistance
Amp = β-lactamase (ampicillin resistance)
npt II = neomycin phosphotransferase (kanamycin resistance)

--------- = derived from pGA470
--------- = derived from pJ35SN
was mapped with *BamH*I, *Bgl*II, *Hind*III, *Kpn*I, *Nco*I and *Xho*I and the restriction fragments obtained agreed with a restriction map generated from the sequence of the maize *Pdc* cDNA of Kelley (1989). The insert was then cleaved out with *Bgl*II and *EcoR*I and cloned into pJ35SN that had been cleaved with *EcoR*I/*BamH*I resulting in the plasmid p35SPdcN. p35SPdcN was then cleaved with *Pst*I and inserted into the *Xho*I site of pGA470X (*p*GA470 with a *Xho*I linker inserted into the *Hind*III site), and the construct, 470Pdc was confirmed with appropriate restriction digests (*EcoR*I, *Pst*I, *EcoR*I/*Pst*I, *Nco*I, *Xho*I and *Hind*III) and is diagramed in Fig. 5.2 C. The binary vector was then transferred into AGL1 by triparental mating, from which DNA was then prepared and the construct checked with restriction and Southern analysis.

**(c) The generation and analysis of transformed calli.**

Each of the four strains of *Agrobacterium*, AGL1(470Adh), AGL1(470hdA), AGL1(470Pdc) or AGL1(470Gus) were co-cultivated with approximately 30 cotyledons of *G. hirsutum* cv. Coker. Calli was selected from each of the four strains on kanamycin containing medium and was subsequently maintained on MSPN 25KC media.

(i) ADH is expressed in cotton callus tissue.

The control 470Gus calli was examined to determine the level of ADH expression in cotton calli that had not been transformed with extra copies of *Adh* genes. Using ADH spectrophotometric assays it was determined that ADH is strongly expressed in callus, for the specific activity of ADH in aerobically grown calli is higher than the aerobic levels of shoots or roots of seedlings. This higher level of ADH activity in callus is also seen in *Arabidopsis* where ADH levels in callus can be 10-15 times higher than in plantlets (Dolferus et al. 1985). Also in *Arabidopsis* as the callus became older the ADH activity increased to a high activity level (Dolferus et al. 1985). This may also be the case in cotton where younger callus had less ADH activity than older callus (Table 5.1).
Table 5.1 ADH activity levels in transgenic calli.

<table>
<thead>
<tr>
<th>Calli</th>
<th>Age (weeks)</th>
<th>Specific activity of ADH (Units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>470Gus</td>
<td>7</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>470Gus</td>
<td>10</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>470Adh</td>
<td>12</td>
<td>10.8 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 8.2*</td>
</tr>
<tr>
<td>470hdA</td>
<td>12</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 4.1*</td>
</tr>
</tbody>
</table>

Values are the means ± standard errors. 
* = the standard deviations.
This increase in levels of activity could be due to the fact that as callus becomes older it becomes harder and larger resulting in the formation of anoxic zones and resulting in the induction of ADH. Alternatively expression may result from the hormones in the tissue culture media, for example, 2,4-D has been shown to induce ADH activity (Freeling 1973).

(ii) The specific activity of ADH increases in anaerobically stressed callus.

In order to determine whether the endogenous ADH activity levels in cotton callus can be induced by anaerobic stress, measurements were made under aerobic and anaerobic conditions for three independent calli generated from the control Agrobacterium strain AGL1(470Gus). In addition to investigating the level of induction of ADH, this experiment may elucidate the length of survival of cotton callus under anaerobic conditions. In anaerobically stressed seedlings the specific activity of ADH peaked after two days of anaerobic stress and then declined when the seedlings appeared to die (Fig. 2.1). Therefore by monitoring the ADH activity levels of the cotton callus under anaerobic conditions an estimate of the length of time which callus can survive anaerobic conditions will be obtained.

Ten week old calli growing on MSPN 25KC plates were subjected to an anaerobic environment and the specific activity of ADH was monitored. The specific activity increased for the first three days and then plateaued out at this level for over two weeks (Fig. 5.3). The highest specific activity reading was obtained after nine days of stress, being 17.3 activity units/mg of protein. This level is more than twice that obtained in anaerobically stressed roots (Fig. 2.1). This high specific activity of ADH found in calli may be a reflection of the very low protein content of the calli cells, for specific activity is based on protein concentration.

Although at the end of this 17 day period of anaerobic stress calli had turned brown in parts, when the calli were returned to aerobic conditions they were able to commence growth again. This prolonged induction of ADH and survival of anoxic conditions, suggests that cotton calli unlike seedlings are tolerant to anaerobic conditions. This
Figure 5.3  Induction of the specific activity of ADH in cotton callus under anaerobic stress. Each value represents the mean of six readings ± the standard deviation.

Figure 5.4  Anaerobic stress induces the ADH2 isozyme in callus.
would make it difficult to study the effect of overexpressing ADH on the tolerance of calli to anaerobic conditions. This survival may be a reflection of the nutrients and sugars in the media or of the slow growth rate of the callus compared to young root tips.

(iii) ADH2 is the predominant isozyme in callus tissue.

To determine which ADH isozymes are expressed in callus tissue under aerobic and anaerobic conditions protein extracts of calli from the previous experiment exposed to 0, 6 and 24 hrs of anaerobic conditions were run on a cellulose-acetate plate and stained for ADH activity (Fig. 5.4). In aerobic calli (0 hrs) only one faint isozyme is visible which has the same mobility as the ADH2 isozyme. After 24 hrs of anaerobic conditions the activity of this isozyme has increased significantly although two other isozymes are beginning to appear, but are only faintly visible in this photograph (arrows). So like roots the increase in the specific activity of ADH in anaerobically stressed callus is due mainly to the induction of the ADH2 isozyme.

(iv) Calli generated with AGL1(470Adh) have significantly higher levels of ADH activity than calli generated with AGL1(470hdA).

After selection on MST 50KC plates, 25 and 50 calli from independent transformation events were generated from the AGL1(470Adh) and AGL1(470hdA) Agrobacterium strains respectively. These calli could be maintained on MSPN 25KC. The specific activity of ADH was determined in 40 independently generated aerobically grown calli, 20 generated from the 470Adh strain and 20 from the 470hdA strain. On average, the levels of specific activity of Adh were 2.25 times higher in 470Adh calli compared to the 470hdA calli (Table 5.1). The highest ADH activity levels in 470Adh calli were 25.8 and 24.0 units/mg of protein which are greater than the values obtained in the anaerobically induced 470Gus calli (Fig. 5.3). The highest activity in 470hdA calli was 10.7 units/mg of protein. It must be noted that the ADH activity level of the 470hdA calli is no different from that of the 470Gus calli, however there were six zero
readings whereas only one 470Adh callus had a zero activity level, which suggests that in some calli repression of ADH activity is occurring. These measurements suggest that in a number of cases the transgenes have successfully modified the level of ADH in the transgenic calli both upwards and downwards.

The level of ADH activity varied greatly from callus to callus as reflected in the standard deviations (Table 5.1). This variability may be due to the position of insertion of the transgene in the genome which may influence its level of expression. Another source of variation was the presence of multiple cell types (green, creamy, white, dark, soft, hard etc) which varied from callus to callus. The level of ADH activity in the different cell types within a callus was found to vary greatly. For example, in one 470Adh callus, the specific activity of ADH was 5.7 units/mg of protein in a soft white portion of the callus, while in dark green callus cells the activity was measured at 18.0 units/mg of protein. Therefore if a particular callus had a high ADH activity it would be unclear whether it was due to the cell-type or the expression of the transgene. This variation implies that there are multiple factors influencing the expression of the Adh genes (endogenous or trans) making it difficult to obtain values of ADH expression levels that will be reproducible.

Protein samples of various calli were run on isozyme plates and stained for ADH activity. All calli only had one isozyme whose mobility matched that of the ADH2 isozyme (data not shown). Because this was the same isozyme that was found in the 470Gus callus the activity coded by endogenous Adh gene(s) is unable to be distinguished from that of the Adh transgene(s).

(v) Analysis of calli generated with the AGL1(470Pdc) strain.

40 calli from independent transformation events were generated with the Agrobacterium strain AGL1(470Pdc) after selection on MST 50KC plates. Nine calli were analysed for PDC activity (T.L. Setter unpublished) and four calli were found to have a range of levels significantly higher than the 470Gus calli (data not shown) suggesting that the introduction of the 470Pdc transgene has resulted in higher levels of PDC activity. This
confirms that the PCR product codes for a functional PDC polypeptide. Again PDC activity was found to vary among the different callus cell types, where greater PDC activity was found in the brown portions of the callus than in the white or creamy portions (T.L. Setter unpublished).

(vi) Preliminary analysis of ethanol synthesis in transgenic calli.

(The following work has been done by Adel and Tim Setter of IRRI, Manila, Philippines).

Calli from each of the four groups (470Adh, 470hdA, 470Pdc and 470Gus) were exposed to anoxia for 8 days and the rates of ethanol synthesis (\(R_E\)) were measured. The \(R_E\) were variable between different calli, however the 470Pdc callus that had the highest levels of PDC activity also had the highest \(R_E\) values which were 3-100 times greater than any other calli. This suggests that PDC is limiting the rate of ethanol synthesis and that the enzyme is active in the calli under aerobic conditions. Additionally this callus was the only one to grow more than 10% of its initial weight under anoxic conditions, although it had only very low growth rates under aerobic conditions. This low aerobic growth rate may be due to high aldehyde levels for PDC is being expressed constitutively. This is currently being tested.

(d) Regeneration of transformed plants.

Although analysis of the transformed calli is still on going, it was concluded that analysis of transgenic plants would be far superior to that of callus, where such extreme variability in enzyme assays were encountered. Therefore transgenic plants were regenerated. Enzyme activities and tolerance to anoxia may be more easily assayed in plants and the results will have greater physiological significance.

Calli were transferred to solid basal media for the initiation of embryogenic callus. Embryogenic cells were then subcultured into liquid basal media and 15 embryogenic
suspension cell lines were established from the 470Adh calli, 23 suspension cell lines from the 470hdA calli and 6 suspension cell lines from the 470Pdc calli. To determine whether the suspension lines still contained cells that were transformed, the cells were assayed for NPT II activity. Nine of the 15 suspension 470Adh cell lines and 13 of the 23 suspension 470hdA cell lines contained NPT II activity, and from these plant regeneration was attempted.

Of the nine 470Adh suspension cultures with NPT II activity, plants were regenerated from eight. From the 13 470hdA suspension cultures, plants were regenerated from 11. Regeneration of the 470Pdc plants is 6-9 months behind the plants transformed with 470Adh and 470hdA; the work is still in progress and will not be discussed further in this thesis.

(e) Analysis of transgenic cotton plants.

(i) The 470Adh transgene directs the overexpression of ADH.

To determine if the 470Adh transgene was directing the overexpression of ADH, the specific activity of ADH was determined in protein extracts prepared from leaves of the independent primary transformants (T₀). In non-transformed cotton plants ADH is not expressed in aerobic leaf tissue (Fig. 2.2). However the 35S promoter is active in leaves, therefore any ADH enzyme activity detected will be due solely to the expression of the 470Adh transgene. In addition to determining which plants are expressing ADH, the level of expression of the transgene(s) will also be determined.

In three of the eight lines, TAdh-63, TAdh-62 and TAdh-13, detectable ADH activity was found in aerobic leaves and the specific activity levels are shown in Fig. 5.5. Numerous regenerated plants were tested for the other five lines but none of the plants contained any ADH activity in mature leaves. The lines not expressing ADH were either not transformed, or due to truncation of the introduced T-DNA had not obtained a complete copy of the Adh gene. Alternatively the Adh transgene is not being transcribed due to its position in the genome or some form of gene silencing.
Figure 5.5  ADH activity in the different transgenic lines. One measurement was made for each transgenic plant regenerated. Multiple plants were regenerated for the TAdh-62 and TAdh-13 lines and the standard deviations between these measurements are represented as error bars.

Figure 5.6  The pGhS31 Adh cDNA transformed into cotton codes for the ADH2 isozyme. Lanes; (1) & (2) are seed from untransformed Siokra; (3) & (4) are aerobic TAdh-62 leaves.
(ii) The 470Adh gene derived from pGhS31 codes for the ADH2 isozyme.

The Adh transgene originated from the cDNA pGhS31 (Chapter Four). In Chapter Two, it was shown that there are three main ADH isozymes in cotton. To determine which ADH isozyme this cDNA corresponded to, a protein extract was prepared from leaf material from the line TAdh-62 and run along side extracts from seed of untransformed plants (Fig. 5.6). The mobility of the ADH isozyme from the transgenic leaf is the same as the mobility of the ADH2:ADH2 isozyme from seed protein extracts, implying that the pGhS31 Adh cDNA codes for the ADH2 polypeptide.

(iii) Analysis of the antisense lines.

NPT II dot blot assays were performed on numerous plants from the 11 regenerated 470hdA lines and transgenic plants were obtained from each line. Results are summarized in Table 5.2. Of the 11 regenerated lines, only seven of these set seed, the rest being either sterile or died before flowering. Of these seven fertile lines, three were male sterile so they had to be cross-pollinated with wild-type plants. Seed has been collected and the work remains at this stage.

(iv) Determination of the number of copies of T-DNA in the 470Adh lines.

To determine how many copies of the T-DNA the 470Adh transformants had obtained, the expression and segregation of the 470Adh transgene(s) in the T₁ progeny was examined. The TAdh-63 line had the highest specific activity level of ADH but only two plants were regenerated and both were sterile, so no further analysis with this line could be done.

Three of the 11 plants regenerated for TAdh-62 line were fertile, so seeds obtained by self pollination were sown and grown for six weeks. Leaf samples were taken and the specific activity of ADH determined in 13 different plants. The level from each plant is
Table 5.2 Regeneration of 470hdA plants.

<table>
<thead>
<tr>
<th>470hdA Line</th>
<th>NPT II positives</th>
<th>Fertility</th>
<th>No of seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThdA-12</td>
<td>6/6</td>
<td>Four fertile</td>
<td>&gt;500</td>
</tr>
<tr>
<td>ThdA-71</td>
<td>1/1</td>
<td>Fertile</td>
<td>&gt;300</td>
</tr>
<tr>
<td>ThdA-132</td>
<td>4/4</td>
<td>2/4 semi-fertile</td>
<td>&lt;50</td>
</tr>
<tr>
<td>ThdA-134</td>
<td>2/2</td>
<td>All fertile</td>
<td>&gt;300</td>
</tr>
<tr>
<td>ThdA-138</td>
<td>1/1</td>
<td>Some flowers</td>
<td>&lt;30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fertile</td>
<td></td>
</tr>
<tr>
<td>ThdA-162</td>
<td>4/12</td>
<td>Male sterile</td>
<td>&lt;10</td>
</tr>
<tr>
<td>ThdA-163</td>
<td>2/2</td>
<td>Fertile</td>
<td>&gt;500</td>
</tr>
<tr>
<td>ThdA-31</td>
<td>1/1</td>
<td>No flowers</td>
<td>None</td>
</tr>
<tr>
<td>ThdA-41</td>
<td>1/1</td>
<td>Died before it flowered</td>
<td>None</td>
</tr>
<tr>
<td>ThdA-43</td>
<td>1/2</td>
<td>No flowers</td>
<td>None</td>
</tr>
<tr>
<td>ThdA-142</td>
<td>3/3</td>
<td>Sterile</td>
<td>None</td>
</tr>
</tbody>
</table>
shown in Fig. 5.7 A., where each plant has been designated a number, 1 to 13, and each measurement has been calculated from four readings. Although only a small number of progeny were examined, it appears that the T-DNA is segregating as a single locus, where three groups, based on different levels of leaf ADH activity, were obtained, 0 units, approximately 2.1 units and approximately 4.2 units. It is proposed that the primary transformant is heterozygous for one T-DNA locus which is segregating in Mendelian fashion so that plants with 0 activity units are homozygous wild-type (no copy of the T-DNA), those with 2.1 activity units have one copy like the parent (and a similar leaf ADH activity level) and plants with 4.2 activity units are homozygous at the T-DNA locus having two copies of the gene. This will be tested by analysing the segregation of leaf ADH activity in selfed T$_2$ progeny from the proposed homozygous transformants, TAdh-62(5) and TAdh-62(6).

The levels of specific activity of ADH in the selfed progeny of TAdh-13 do not segregate into three clear groups as it did for the TAdh-62 progeny (Fig. 5.7 B.). However four of the 12 plants analysed had no ADH activity, therefore it would again seem likely that there is only one segregating T-DNA. If two loci of T-DNA were present then only one in 16 would be expected to have no ADH activity. The T$_2$ selfed progeny from the TAdh-13(9) and TAdh-13(7) plants, the most likely candidates to be homozygous at the T-DNA locus (the highest expressors) will be analysed.

From 25 independently transformed calli which gave rise to 15 suspension lines, only two fertile lines of transgenic plants expressing leaf ADH were obtained.

5.4 DISCUSSION

By constructing chimeric genes coding for ADH and PDC and introducing them into cotton tissue it was hoped to obtain different lines of transgenic calli that were expressing varying amounts of these enzymes. These lines were then to be subjected to anoxic conditions, from which any correlations of tolerance to anoxia with levels of enzyme activities were to be investigated.
Figure 5.7 The segregation of the specific activity of ADH in leaf tissue from the T1 progeny (obtained by self pollination) of the lines (A) TAdh-62 and (B) TAdh-13. Each value represents the mean of four readings of different leaf samples ± the standard deviation.
The experiment was initiated by analysing the ADH activity in control callus (470Gus calli), so as to characterize the ADH expression levels encoded by the endogenous Adh gene(s). This data provides a basis on which comparisons can be made to the calli with introduced Adh or hdA (antisense) gene(s). The ADH activity in the control callus was found to be at a high expression level, where the specific activity of ADH under both aerobic and anaerobic conditions was higher in callus than the corresponding levels in aerobically or anaerobically grown seedlings (Fig. 2.1). The endogenous isozyme expressed in cotton callus is the ADH2 isozyme, the same isozyme that is strongly induced in roots and shoots of seedlings during anaerobic stress (Fig. 2.4). This suggests that the high level of ADH being expressed in callus is because anoxic zones exist within the callus, resulting from the small surface to volume ratio and thus decreasing the diffusion of air into the interior. This is supported by the fact that as callus becomes older and larger, the specific activity of ADH increases.

Calli were generated using each of the four strains of Agrobacterium (470Gus, 470Adh, 470hdA and 470Pdc). From the results of the enzyme assays it appears that the introduced constructs did successfully modify the levels of expression as intended, where calli generated using the 470Adh strain of Agrobacterium had significantly higher levels than calli generated using the 470hdA strain. Also PDC activity in the 470Pdc-transformed calli were significantly higher than in 470Gus calli. Preliminary analysis of the rate of ethanol synthesis of these calli have shown one callus which has the highest levels of PDC also had the greatest rate of ethanol synthesis, being 3-100 times greater than that of any other callus. This suggests that PDC activity is limiting the rate of ethanol synthesis in cotton which agrees with the conclusion drawn from the 2D gel analysis in Chapter Three. This would also be in agreement with what has been found in other plants, for it has been proposed that PDC is also the rate limiting enzyme in ethanol synthesis in maize, where ADH activity levels are 6 to 9 times the levels of PDC (Wignarajah and Greenway 1976), and in wheat where ADH activities were 7.5 to 46 times greater than PDC (Waters et al. 1991). Therefore if PDC is the rate limiting enzyme of ethanolic fermentation, to increase the carbon flux through the pathway extra copies of Pdc must be transformed into cotton; this is presently in progress. It is
possible that the greater ethanol synthesis of this 470Pdc callus has enabled it to tolerate anaerobic conditions better and hence allowed greater growth than the other calli under anaerobic conditions. This finding is only preliminary and is being further investigated. Although PDC is being expressed constitutively it was thought that under aerobic conditions the activity of PDC in vivo would be low. The fact that the callus with high PDC activity also had the greatest rate of ethanol synthesis either suggest that calli do have anoxic zones within them, or may be the pH of the tissue culture media (pH 5.8) has resulted in the pH of the cells dropping, and thus resulted in the enzyme becoming active.

However, for the analysis proposed earlier, callus was found to be difficult material to work with for a number of reasons. ADH activity levels within a callus varied greatly, where callus age and variation in its cell-types were influencing the level of expression of ADH. Since most calli are made up of multiple cell-types it was difficult to obtain reproducible values for the levels of ADH activity for one callus that could be directly compared to those of another callus. This was highlighted in one callus where the specific activity of ADH was three times higher in one cell-type than in another cell-type. ADH activity even varied greatly in 470Gus calli, as reflected in the size of the error bars in Fig. 5.3, suggesting that it is the endogenous Adh genes whose expression is varying greatly. Alternatively, the differences in ADH activity may be due to different levels of activators or inhibitors in the extracts. In addition, because the same isozyme (ADH2) is expressed endogenously in callus as is encoded by the introduced gene, ADH activity encoded by the transgene(s) cannot be distinguish from ADH activity encoded by the endogenous Adh gene(s) by isozyme analysis, therefore some other type of analysis would be needed to determine if a callus has an actively expressing Adh transgene. The different cell-types themselves would defeat the purpose of the analysis, for although the different calli would have a homogenous genetic background the phenotypes of the calli may be different which could affect their physiological characteristics. In addition, calli appear to be tolerant to anaerobic conditions, where the 470Gus calli survived over two weeks of anaerobic conditions,
although this may change if calli are transferred to a media that lacks glucose. This tolerance is also probably due to a low metabolic rate, where the calli have a doubling time of approximately 10 days (T.L. Setter unpublished data). This is in contrast to root tips that have high metabolic rates, therefore analysis of calli may lack applicability to plants. All these factors suggest that analysis and assaying of transgenic plants would be easier and more appropriate.

Although outside the original scope of this thesis it was decided to regenerate and analyse transgenic plants. Transformation and regeneration of cotton has only become possible in the last couple of years, with genes for herbicide resistance (Bayley et al. 1992) and insect resistance (Perlack et al. 1990) being successfully transferred into cotton. The initial measurements of enzyme activity in transgenic callus material suggest that the chimeric gene constructs are successfully overexpressing ADH and PDC and underexpressing ADH in the presence of antisense, so making it worthwhile to attempt to regenerate the callus into plants.

To date, transformed plants have been obtained which contain either the 470Adh or 470hdA transgenes. Analysis of the 470Adh plants has begun and it has been shown that the transgene is successfully directing the overexpression of ADH in leaf tissue. Two fertile lines have been obtained, each appearing to have only one copy of the T-DNA which is apparently being inherited in a Mendelian fashion. Also, from the measurements of ADH activity, there appears to be a 0:1:2 gene dosage effect, where the plants that are the putative homozygotes for the transgene have twice as much enzyme activity than the putative heterozygotes. This further supports the suggestion that each line has only one segregating locus. The two different lines obtained are expressing ADH at different levels, where TAdh-62 has levels 3-4 times higher than TAdh-13. The line TAdh-62 is a high expressor of ADH because when the specific activity of ADH in the leaf of the putative homozygote is compared to that of the wild-type plant that has been anaerobically stressed for three days (Fig. 2.2) it has approximately twice as much ADH activity. It is unknown why the other five lines of TAdh plants failed to express the transgene.
The work on transgenic plants has also shown that the pGhS31 cDNA sequence encodes the ADH2 isozyme. This is in agreement with other data that has been obtained, for example, that pGhS31 belongs to the most abundant class of Adh cDNA in the library prepared from anaerobically stressed roots, where 9 of the 12 clones fell into this class. Thus it was expected to code for the ADH isozyme that shows the greatest induction upon anaerobic stress (Fig. 2.4), which is the ADH2 isozyme.

**Future directions.**

The 470hdA lines will be analysed to determine which of these lines have lower levels of ADH activity than wild-type plants. Seedlings of the different lines will be anaerobically stressed and the level of ADH activity measured in their root tips and compared to values of the wild-type plants. In addition to measuring the ADH activity, NPT II activity will be measured in root tips of the transgenic seedlings. This will help determine how many copies of the T-DNA is present in each line; it will also show if the T-DNA(s) are segregating with low levels of ADH activity which would be a strong indication that antisense expression is altering the level of ADH expression. Seedlings will also be grown to the next generation and putative homozygotes selected (by quantitative NPT II assays). Seeds of these homozygotes will be collected and their tolerance to anoxia will be determined. By analysing the homozygotes we will be sure that all the seedling have lower levels of ADH activity. If several different lines are obtained with ADH activity levels repressed to different extents, then correlations may be observed between activity and tolerance to stress.

Plants putatively containing the 470Pdc transgene have recently been transferred to soil. If any are positive not only will they be tested for anaerobic survival but they can be crossed to the 470Adh plants so plants overexpressing both ADH and PDC can be obtained. Plants containing extra copies of both Pdc and Adh may be important because although PDC appears to be the rate limiting step in ethanolic fermentation additional ADH may be needed to remove the extra aldehyde produced. It should be noted that the
callus with the highest levels of PDC activity also had the lowest growth rates during aerobic conditions.

The different lines will be tested for rate of ethanol production under anaerobic conditions; the results should show whether PDC does catalyse the rate limiting step of ethanolic fermentation. The different lines will also be tested for survival under anaerobic conditions, to see if there is a correlation between rate of ethanol production and tolerance to anaerobic conditions. From the analysis the physiological significance of these enzymes with respect to the plant’s ability to tolerate anaerobic stress will be defined and give some clue on how to manipulate the anaerobic response resulting in varieties of cotton that have greater tolerance to anaerobic stress.
CHAPTER SIX

CONCLUSIONS.

This thesis has examined the molecular responses of cotton to anaerobic conditions, focusing on the expression and anaerobic induction of ADH in the cultivar "Siokra", one of the major commercial cottons grown in Australia.

Like other species of plants, in cotton the activity of ADH increases when the plant becomes anaerobically stressed. In seedlings the induction of ADH activity peaks after 48 hrs of anaerobic conditions; at this peak the activity level in roots increases to eight times the aerobic level and in shoots increases to three to four times the aerobic level. ADH was also found to be induced in mature cotton leaves, in contrast to maize where there is no expression of ADH in green leaves even following anaerobic stress (Okimoto et al. 1980). Three main ADH isozymes were resolved in both roots and shoots of cotton seedlings and a gene/isozyme system was proposed. Two genes, Adhl and Adh2, code for three isozymes, two homodimers and a proposed intergenic heterodimer, consistent with models from other species of plants. The isozymes were differentially expressed with respect to anaerobic stress. ADH2 was found to be induced in both roots and shoots of anaerobically stressed seedlings, whereas ADH1 was induced only in the shoots of seedlings. Due to the lack of ADH electrophoretic variants within the germplasm of G. hirsutum this gene/isozyme model was unable to be tested, and the assignment of the isozyme with the intermediate mobility as a heterodimer could not be confirmed. Support for the gene-isozyme proposal came from the determination of the native and subunit molecular weights of the cotton ADH enzyme. The native enzyme was estimated to have a molecular weight of 81 kD, while the subunit molecular weight was 42 kD. Thus ADH in cotton forms and is active as a dimer, meaning that the isozyme with the intermediate mobility was potentially a heterodimer. However the fact that Western analysis on a IEF/SDS-PAGE 2D gel resolved three distinct anaerobically induced ADH polypeptides clearly demonstrates
that the proposed gene isozyme system was too simple and the results from the isozyme plates have underestimated the number of different ADH gene products. The IEF analysis may have resolved polypeptides that were unable to be resolved on the isozyme plates. Alternatively some isozymes may not have been detected because they stained poorly due to a catalytic activity that favours the production of ethanol, not acetaldehyde. Southern analysis also indicated that there was multiple Adh genes in cotton, possibly more than four.

The 2D gel electrophoresis and Western analysis gave a clearer indication of the number of different ADH polypeptides expressed during anaerobic stress. One ADH polypeptide is expressed strongly and two other ADH polypeptides are expressed less strongly. In cotton ADH is the most strongly induced ANP.

The induction of three distinct ADH polypeptides was supported by the isolation of three different Adh cDNA classes from an expression library that was prepared from mRNA of anaerobically stressed roots. The frequency of Adh cDNA in the library suggested that Adh mRNA makes up a large proportion of the population of mRNA after 18 hours of anaerobic stress and that the induction of ADH synthesis during anaerobic stress is mediated by the presence of high steady-state levels of Adh mRNA during anaerobiosis. The nucleotide and deduced amino acid sequence of these cDNAs have high similarity to Adh genes from other plant species. From the 12 Adh cDNA clones that were purified, nine fell into one class (pGhS31), two into another class (pGhS33) and one into the remaining class (pGhS44). The distribution was in agreement with labelling and Western analysis where one ADH polypeptide is predominant. The most abundant class of Adh cDNA, pGhS31, encodes the ADH2 isozyme, the most strongly induced isozyme in anaerobically stressed roots.

Adh genes were isolated using the Adh cDNAs as probes. DNA sequencing of three different genomic clones revealed that three different genes/alleles had been isolated. Two of the promoters were highly homologous and may correspond to alleles of one locus, or could be different genes from homoeologous loci. The other promoter appeared to correspond to a different locus. All promoters contained sequences characteristic of functional eucaryotic genes including TATA boxes and conserved
nucleotide sequences surrounding the putative transcription start site. In addition these promoters were found to contain multiple ARE core motifs (5'-AAACCA-3') within the first 250 bp upstream of the translational start site suggesting that anaerobically inducible promoters had been isolated. Also complementary 5'-TGGTTT-3' elements were found within the first intron of all three genes. One promoter was found to contain a putative GC-motif; these motifs have also been shown to be implicated for anaerobic induction in maize and Arabidopsis. The significance of these elements for anaerobic induction of transcriptional activity will have to be confirmed by functional analysis.

The effect of anaerobic stress on the pattern of protein synthesis was examined in cotton. Like maize and soybean, when cotton was anaerobically stressed it was found to undergo a shift in protein synthesis so that only a select few polypeptides were synthesized under anaerobic conditions. Cotton was shown to have 14 ANPs which in a numerical sense implies its anaerobic response is more like the anaerobic response of maize than of soybean or pea. Unlike maize, cotton does not appear to undergo an early transition stage and some proteins were found to be expressed under all conditions examined. The induction of these 14 ANPs may occur independently or in subsets, for when the conditions of anaerobic treatment were altered, the pattern and ratio of expression of the ANPs changed. Although cotton appeared to have ANPs that corresponded to ANPs of other plant species, it did appear that cotton lacked an ANP corresponding to PDC. This was supported by PDC enzyme activity assays (T. Setter unpublished) which indicated that PDC is only weakly expressed in cotton. The lack of PDC expression shifts the emphasis of future analysis to the study of PDC rather than ADH. Northern and Western analysis should be performed to clarify the level of expression and anaerobic induction of PDC in cotton.

Experiments have been initiated that may clarify the physiological significance of PDC and ADH activity in relation to anoxia tolerance. By using gene transfer technology we have attempted to manipulate the levels of ADH and PDC activity in cotton, and then examine if these alterations in enzyme activity have affected the plant's rate of ethanol
synthesis or tolerance to anoxia. PDC was included in this study for PDC unlike ADH, appears to be weakly induced if at all by anaerobic stress. Therefore if PDC is limiting the rate of ethanolic fermentation, higher levels of PDC activity would be needed to increase the rate of ethanol synthesis. From a preliminary analysis of the rates of ethanol synthesis and survival under anoxic conditions of transgenic calli, it appears that PDC is the rate limiting enzyme confirming the prediction from the 2D gel analysis. Whether this high rate of ethanol synthesis in the transgenic 470Pdc callus has also enabled it to have the highest growth rate under anoxic conditions is unknown, but is actively being investigated. This result points to PDC as possibly being the critical factor in cotton's tolerance to anaerobic stress.

The fact that this callus also had low growth rates under aerobic conditions suggests that aldehydes produced by PDC might be detrimental to growth. This, and the fact that ADH appears to be strongly expressed compared to PDC may indicate that cotton is very sensitive to acetaldehyde. In carrot cell cultures exogenous ethanol causes toxicity to cells only if it could be oxidized to acetaldehyde, which even at very low concentration levels (1 mM) showed toxicity (Perata and Alpi 1991). In contrast ethanol itself at high concentrations (80 mM) showed no sign of toxicity (Perata and Alpi 1991). If the regeneration of transgenic cotton plants constitutively expressing PDC proves to be difficult it could be due to the production of acetaldehyde. To avoid this problem PDC may have to be placed under the control of a tightly regulated anaerobically inducible promoter so expression only occurs under anaerobic conditions, when ADH will be present to maintain acetaldehyde levels at low concentrations. The Adh promoters that have been isolated in this study may be suitable for this task. Further analysis of these promoters would be required to ensure that they are functional and have a suitable level of transcriptional activity. Alternatively a double construct could be made containing both Adh and Pdc.

To increase cotton's tolerance to anaerobic stress, it may be necessary to increase PDC activity to allow for an increased rate of ethanol synthesis, but ADH activity may also have to be increased to keep acetaldehyde levels to a minimum. Obtaining a plant which is overexpressing ADH and PDC may be possible in the near future, where, if a plant
overexpressing PDC is regenerated it may then be crossed to a plant overexpressing ADH. Analysis of all these different lines of transgenic plants, (470Adh/Pdc, Adh, Pdc and hdA) may answer a myriad of questions on the physiological significance of these enzymes in relation to anaerobic stress. Also, the levels of aldehydes and alcohols within the plants can be measured to examine whether there is a correlation between these, enzyme activities and anaerobic survival. Hopefully these experiments will determine how the levels of PDC and ADH affect the plant's tolerance to anaerobic stress and whether these factors can be manipulated to create a plant more tolerant to anaerobic conditions.

Even if plants are obtained that are more tolerant to anaerobic conditions this does not necessarily mean that they will also be more tolerant to waterlogging, for other factors in the field will also be contributing to tolerance that has not been consider in this thesis, such as the production and accumulation of phyto-toxic compounds by microorganisms in the waterlogged soils.
REFERENCES


