Anaerobic gene expression and flooding tolerance in maize

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Abstract

Anaerobic treatment dramatically alters the pattern of gene expression in maize (Zea mays L.) seedlings. During anaerobiosis there is an immediate repression of pre-existing protein synthesis and the simultaneous selective synthesis of four polypeptides with a molecular weight of ~33 kDa (the transition polypeptides). After 90 min, this is followed by specific transcription and mRNA accumulation as well as the selective synthesis of approximately 20 anaerobically-induced proteins. Among these anaerobic proteins (ANPs) are enzymes involved in glycolysis and related processes, such as alcohol dehydrogenase (ADH) aldolase, enolase, glucose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate decarboxylase, and sucrose synthase. However, two genes, inducible by oxygen deprivation, have been found that have different functions. One that has homology to xyloglucan endotransglycosylase may be involved in aerenchyma formation during flooding. The anaerobic response is most likely a plant's natural reaction to flooding. A similar condition has been observed in every plant species analysed to date. The anaerobic response and flooding tolerance in maize has been analysed using a combination of molecular, biochemical and genetic approaches. The studies include the characterization of anaerobically inducible genes, the identification of genes involved in flooding tolerance, and analysis of the signal transduction events that are involved in the perception of oxygen deprivation and lead to the anaerobic response.

Key words: Maize, anoxia, flooding, stress.

Introduction

Oxygen limitation is the primary plant stress in flooded soils. The irregular excess of water due to flooding not only threatens the food supply of human populations, but also affects the natural vegetation in river plains. About 20 million acres of corn and soybean were inundated, leading to heavy economic losses during the 1993 flooding in the Mid-Western United States, as estimated by the United States Department of Agriculture, National Agricultural Statistics Service (Suszkiew, 1994). Anoxia or hypoxia is also a problem during the normal ontogeny of plants, particularly in tissues of high bulk or density with intense metabolic activity, for example, vascular cambium (Kozlowski, 1984).

Anaerobic treatment of maize seedlings drastically alters the profile of total protein synthesis (Plate 1). This is known as the anaerobic response. Under an anaerobic environment, 20 proteins that account for more than 70% of the total translation are selectively synthesized (Sachs et al., 1980). Most of the anaerobic proteins (ANPs) identified were found to be enzymes of glycolysis or sugar-phosphate metabolism such as aldolase (Kelley and Tolan, 1986), enolase (Lal et al., unpublished), glucose-6-phosphate isomerase (Kelley and Freeing, 1984), glyceraldehyde-3-phosphate dehydrogenase (Russell and Sachs, 1991), sucrose synthase (Springer et al., 1986), and alcohol dehydrogenase (Freeing, 1973). In addition, the induction of transcription and enzyme activity of pyruvate decarboxylase (Kelley, 1989; Peschke and Sachs, 1993) in maize, and lactate dehydrogenase (Hoffman et al., 1986) in barley, has been reported during anaerobic stress, indicating that they may represent other ANPs. Additionally, three genes that are not involved in glucose-phosphate metabolism (Vogel and Freeing, 1992; Peschke

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Plate 1. Protein synthesis in a maize primary root during anaerobic treatment. Fluorographs of native-SDS 2-D polyacrylamide gels that were loaded with extracts from maize primary roots after the following treatments. (A) 1 h pulse-labelling with \(^{3}H\)-leucine under aerobic conditions. (B-E) Pulse-labelling with \(^{3}H\)-leucine during the specified times under anaerobic conditions. The arrow labelled 'TPs' indicates the position of the transition polypeptides. The unlabelled arrow indicates the position of alcohol dehydrogenase-1 (ADH1) (from Sachs et al., 1980).

and Sachs, 1994; Saab and Sachs, 1995) have been found to be induced by anoxia, and may also encode ANPs.

Anaerobiosis results in an alteration of gene expression in plants leading to the accumulation of the ANPs. Regulation of both transcription and translation is involved in the anaerobic response (Sachs et al., 1980; Ferl et al., 1980; Rowland and Strommer, 1986; Bailey-Serres et al., 1988; Dennis et al., 1989; Russell and Sachs, 1992). At the translation level, anaerobic treatment of maize seedlings disrupts polysomes (Bailey-Serres and Freeking, 1990) and leads to a redirection of protein synthesis (Plate 1; Sachs et al., 1980; Russell and Sachs, 1992). In the first 5 h of anaerobic treatment (transition period) there is a rapid increase in the synthesis of a class of polypeptides (∼33 kDa, the transition polypeptides). After 90 min of anoxia, the synthesis of ANPs is induced. After 72 h, protein synthesis decreases concurrently with the start of cell death (Sachs et al., 1980). Besides this reprogramming of gene expression in a co-ordinated fashion, metabolic (e.g. switch to fermentative pathway, as reviewed in Davies, 1980; Kennedy et al., 1992) and structural changes (e.g. aerenchyma formation, Drew et al., 1985; also reviewed in Konings and Lambers, 1991) occur during flooding. Advances have also been made in molecular-level analyses of several cDNAs and genes involved in the anaerobic response (cf. Sachs, 1994). Table 1 shows the chromosomal locations for maize genes involved in the anaerobic response, for which DNA clones were available for restriction fragment length polymorphism mapping analysis (Peschke and Sachs, 1994). The anaerobic response has been extensively reviewed recently (Armstrong et al., 1994; Drew, 1992; Perata and Alpi, 1993; Ricard et al., 1994; Sachs, 1993, 1994). This article will concentrate on results that were obtained from our recent studies. The emphasis will be on work, in maize, conducted on the glyceraldehyde-3-phosphate dehydro-
The kinetics of induction were the same as anaerobiosis. While the anaerobically induced cDNA was observed for \textit{adh1}, and not what had been expected for the transition polypeptides. The largest clone from each class was chosen to be characterized further. Sequence analysis showed that the two clones had ~80% homology in the open reading frame, but little or no homology in the 3' non-translated region. When used in a GenBank search, it was found that both clones had homology to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from several organisms. Lindquist confirmed that she subsequently found that yeast HSP35 is GAPDH (Lindquist and Craig, 1988). A paper by Brinkmann \textit{et al.} (1987) had just been published that gave sequence information comparing a cytosolic (involved in glycolysis; encoded by \textit{gpc1}) and a chloroplastic (involved in the Calvin cycle; encoded by \textit{gpa1}) GAPDH cDNAs from maize. Both of our clones showed more homology to the cytosolic GAPDH (EC 1.2.1.12; \textit{GAPC1}; Brinkmann \textit{et al.}, 1987). The constitutive clone (\textit{pGAPC2}) had 97% predicted amino acid sequence homology with \textit{GAPC1} and 95% nucleotide homology in the protein coding region, but little or none in the 3' non-translated region. This shows that, while they are very closely related, these cDNAs appear to represent distinct genes (\textit{gpc1} and \textit{gpc2}; Russell and Sachs, 1989). The anaerobically inducible GAPDH (\textit{pGAPC3}) also has ~80% protein coding region sequence homology to \textit{pGAPC1}, but again, little or none in the 3' untranslated region. In a series of experiments that included genetic studies of a GAPDH isozyme variant, RFLP mapping, hybrid select translation, immune precipitation, and 2-D gel electrophoresis followed by Western analysis, it was shown that there are actually four genes encoding cytosolic GAPDH activity in maize (Russell and Sachs, 1989, 1991, 1992). Two that are constitutive and show no organ-specificity (\textit{gpc1} and \textit{gpc2}) and two others that are anaerobically inducible and do exhibit organ-specificity (\textit{gpc3} and \textit{gpc4}) similar to that found with \textit{adh1}. The fourth cDNA as well as genomic sequences of \textit{gpc2}, 3 and 4 have recently been cloned (Manjunath and Sachs, unpublished). The anaerobically inducible GAPDHs (\textit{GAPC3} and \textit{GAPC4}) were identified as an anaerobic polypeptide (ANP31.5; Sachs \textit{et al.}, 1980; Russell and Sachs, 1992). These appear as one elongated smear on our standard 'native'-SDS 2-D PAGE. However, distinct spots represented by the individual isozyme tetramers can be visualized when a modified native gel system is used as the ‘first dimension’ (Russell and Sachs, 1992).

In order to determine the relative contributions of transcriptional versus translational regulation in \textit{gpc} expression, protein accumulation and protein synthesis were investigated during anaerobic stress and heat shock in maize seedlings. \textit{In vitro} translations were used to estimate the levels of different mRNAs in roots following anaerobiosis, recovery from anaerobiosis, and heat shock. This was compared to the \textit{in vivo} protein synthesis rates.
in roots radiolabelled under identical conditions. The labelling was followed by immune precipitation analysis (Russell and Sachs, 1992). In vivo labelling indicates that cytosolic GAPDH (GAPC) and ADH are not heat shock proteins (even though GAPC3 mRNA levels show about a 5-fold increase during heat treatment, it does not appear to be translated during this stress). While both GAPC3 and ADH1 translatable mRNA levels increase about 10-fold during anaerobiosis, in vivo labelling of these proteins (relative to total protein synthesis) is enhanced to a greater degree. This indicates that GAPC3 and ADH1 are selectively translated during anoxia. In contrast, anoxia causes no change in GAPC1 or GAPC2 translatable mRNA levels or in vivo labelling. \( \beta \)-glucosidase mRNA levels are constant during anoxia, but in vivo synthesis decreases. Taken together, the results indicate that during anoxia transcript accumulation as well as selective translation both play a role in ANP gene expression.

At present, promoter function in the gpc2 and gpc4 genes is being analysed in an attempt to identify regulatory sequence elements that are specifically involved in anaerobic induction.

**Enolase**

Enolase (phosphopyruvate hydratase; EC 4.2.1.11) is a glycolytic enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate. The enzyme has been extensively characterized in vertebrates (Giallongo et al., 1986) and similar work has also been done with yeast (Van der Straeten et al., 1991). Enolase in yeast is encoded by two different genes, ENO1 and ENO2. The expression of these two genes share 95% sequence identity with each other (Brindle et al., 1990). The expression of ENO1 and ENO2 depends on the carbon source in the media. The expression of ENO2 is induced 20-fold when cells are grown in the presence of glucose compared to medium containing a gluconeogenic carbon source (Cohen et al., 1987). The differential regulation of the two genes in response to carbon source is regulated at the transcriptional level (Uemera et al., 1986). Expression of the yeast ENO1 gene and hsp70 is induced during stationary phase of the cell cycle (Cohen et al., 1987). Further, a heat shock protein (hsp48) was identified as an isoprotein of enolase, suggesting that enolase may play a role in normal growth and thermal tolerance of yeast (Iada and Yahara, 1985). In vertebrates, three different isozymes of enolase (alpha, beta and gamma) have been characterized (Giallongo et al., 1986). The lens crystalline structural protein in vertebrates has been found to be alpha enolase (Wistaw et al., 1988).

A cDNA encoding maize enolase (pZM245; pENO1) was cloned (Lal et al., 1991) using functional genetic complementation of an enolase deficient mutant of *Escherichia coli*. The gene encoding this cDNA is designated as enol1. A 5-fold increase in ENO1 transcript levels followed by a 2-fold induction in the specific activity of enolase, after 24 h of anaerobic stress was reported in maize roots (Lal et al., 1991). In addition, enolase protein was purified and partially characterized from maize (B73) seeds and antibodies were raised against this antigen (Lal et al., 1994).

Lal et al. (1994) showed that the 56 kDa protein present in the purest fraction of enolase was further resolved into two isoelectric forms upon 2D-IEF analysis. These results suggest that there may be two or more different enolase genes in maize. Representation of the glycolytic enzymes in maize by small multigene families has been reported for alcohol dehydrogenase (Schwartz, 1969), glyceraldehye-3-phosphate dehydrogenase (Russell and Sachs, 1989) and pyruvate decarboxylase (Peschke and Sachs, 1993).

Recently another cDNA encoding maize enolase, pENO2 (Lal et al., unpublished) has been cloned. The pENO2 nucleotide sequence (shares 75.6% identity with pENO1) its deduced amino acid sequence (shares 89.5% identity with ENO1) and its expression during the anaerobic stress response were compared with the pENO1 enolase clone. It was determined that the eno2 gene expression is constitutive in aerobic maize seedlings tissues and transcript levels remain unchanged during anoxia. Genomic Southern blot and sequence analyses confirm that these two enolase cDNAs are the products of two different genes and by using RFLP analysis (Table 1) eno1 was mapped to the short arm of maize chromosome 9 (Peschke and Sachs, 1994) and eno2 to the short arm of chromosome 1 (Benjamin Burr, personal communication). Two previously described major anaerobic proteins ANP45A and ANP45B (Sachs et al., 1980) have been identified as isozymes of enolase in maize by sequencing the N-termini of these proteins after isolation from 2-D gels (Lal et al., unpublished). The difference in molecular weights observed in the two different studies (56 kDa versus 45 kDa) is due to different molecular weight markers used and measuring on 1-D versus 2-D gels (the actual predicted molecular weight for both ENO1 and ENO2 is \( \sim 48 \) kDa). Currently, work is in progress to determine which polypeptide corresponds to which gene, as well as analysing the promoter regions of *eno1* and *eno2*.

**Glucose-phosphate isomerase**

Glucose-6-phosphate isomerase (GPI; EC 5.3.1.9) is a dimeric glycolytic enzyme that catalyses the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate, a reaction that also immediately precedes sucrose biosynthesis in plants. This enzyme has also been called phosphohexose isomerase and phosphoglucose iso-
Two isozyme forms of GPI, one localized in the cytosol and the other in the plastid, have been reported in other plants and may also exist in maize (Weeden and Gottlieb, 1982; Kelley and Freeling, 1984). In general, plants examined contain a single gene for the cytosolic (GPI-C) form with the exception of Clarkia which has two copies, resulting from gene duplication within that species (Weeden and Gottlieb, 1979). Three forms of GPI have been reported in maize (Salamini et al., 1972). GPI-I encoded by phi1 gene is expressed in both the developing endosperm and embryo. In contrast, GPI-II is expressed only in the developing endosperm, whereas GPI-III is found only in the developing embryo. The enzymatic activity of GPI has been reported to be induced under anaerobic stress in maize (Kelley and Freeling, 1984). Using electrophoretically distinguishable alleles of GPI and antibodies against spinach cytosolic GPI, Kelley and Freeling (1984) identified ANP55 in maize as an isozyme of GPI-C encoded by the phi1 gene.

To analyse the source of the multiple GPI isozymes and to study their anaerobic regulation, we isolated 25 clones from a cDNA library constructed from maize roots treated anaerobically for 6 h (Lal and Sachs, 1995). The GPI clones were isolated using a heterologous cDNA probe encoding GPI-C from Arabidopsis (generously provided by Les Gottlieb; Thomas et al., 1993). The clone with the longest insert (2.1 kb) had an open reading frame encoding a protein of 567 amino acid residues with high sequence identity to GPI sequences from other species. Northern blot analysis using the full length cDNA probe detected a dramatic induction of transcript levels in maize roots upon anaerobic treatment. Using this cDNA as a probe in RFLP mapping showed that its gene is located on maize chromosome 1L, where the phi1 gene is expected to be localized, strongly indicating that the GPI cDNA is encoded by this gene (Ed Coe et al., personal communication; Benjamin Burr, personal communication). The phi1 gene was previously characterized and mapped using allozymes (Goodman et al., 1980).

**Xyloglucan endotransglycosylase**

Until recently, the only genes described in plants that are induced by oxygen deprivation (anoxia or hypoxia) encoded enzymes of glucose-phosphate metabolism (mostly glycolysis and fermentation) and thus function to allow limited energy production in the face of limited oxygen supply (Sachs, 1993, 1994). In fact, the majority of research on molecular aspects of oxygen deprivation responses in plants has focused on the enzymology of energy production that aids in short-term survival of flooding. However, flooding also induces structural modifications, such as the formation of cortical intercellular air spaces (aerenchyma; Drew et al., 1979; Justin and Armstrong, 1987). These modifications can facilitate oxygen diffusion to submerged tissues and as such, are generally proposed to aid in prolonging survival during flooding (Drew et al., 1979).

Recent studies identified two anoxia-induced maize genes [wus1005(gfu) and umc217(gfu)] that are not involved in glucose-phosphate metabolism. These genes, with unique kinetics of mRNA induction under anaerobic conditions compared to other anoxia-induced genes (Plate 2) were not induced by heat, cold, or salt stress, or by seedling death. Thus, their induction appears to be specific to oxygen deprivation. The predicted protein sequence of umc217(gfu) showed no significant homology to any gene of known function listed in GenBank (Peschke and Sachs, 1994). An additional anoxia-induced gene with unknown function was identified by Vogel and Freeling (1992).

At present efforts are being concentrated on wus1005(gfu) an oxygen-deprivation responsive gene that may be involved in structural modifications caused by flooding (Peschke and Sachs, 1994; Saab and Sachs, 1995). This gene is highly homologous to xyloglucan endotransglycosylase (XET) a putative cell wall loosening and degradation enzyme. Experiments are being conducted that will address the potential role of 1005 and test the hypothesis that it is associated with structural modifications induced by flooding. A cDNA encoding the complete open reading frame of 1005 (Saab and Sachs, 1995) has recently been cloned and Fig. 1 shows a complete comparison between the deduced amino acid sequences of 1005 and several XETs. XET is a putative cell wall loosening enzyme that is proposed to play a role in wall metabolism during germination, cell expansion and fruit ripening (see below).

One of the earliest cloned sequences homologous to XET was the meri-5 gene from Arabidopsis which is expressed in shoot apical meristems (Medford et al., 1991). However, its homology to XET was not known at the time. Recently, Okazawa et al. (1993) cloned cDNAs encoding XET sequences from Vigna angularis, Triticum aestivum, Arabidopsis thaliana, Lycopersicon esculentum, and Glycine max. The sequences were cloned based on the amino-terminal amino acid sequence of the purified protein from Vigna angularis (Okazawa et al., 1993; Nishitani and Tominaga, 1992). Another XET cDNA was cloned from nasturtium (Tropaeolum majus) seeds (de Silva et al., 1993). The cDNAs range between 975 and 1320 bp in length and the deduced proteins contain several conserved regions.

The deduced amino acid sequence of 1005 is highly homologous to several XET sequences from monocots and dicots, for example, wheat, tomato, Vigna angularis, and Arabidopsis thaliana (Fig. 1). Stretches of homology are present along the entire length of the predicted proteins, and the homology is particularly high in the middle and carboxy terminal regions. For example, 1005...
Plate 2. Comparison of induction of oxygen deprivation-responsive RNAs in maize. Left panel, lanes 1 to 6, B73 pre-emergent shoots drowned for the number of hours indicated; lane 7, B73 leaf (unstressed 6-week-old plants), lane 8, B73 endosperm, 15 d post-pollination; lanes 9 and 10, y25 (ADH1-null mutant) seedling shoots drowned for 0 or 12 h, respectively. The same blot was used for all probes. Right panel, B73 seedlings stressed as indicated. Hybridization with the maize 26S rRNA (26S ribosomal RNA, cDNA) clone was used to verify equivalent loading. N.S., not stressed, 48 h, drowning for 48 h; 2 h HS, 40°C for 2 h; 20 h HS, 40°C for 20 h; Acid, seedlings watered with 0.05 N HCl for 4 d; Salt seedlings watered with 5 M NaCl until wilting (approximately 2 d); Cold, 0.5–1°C for 4 d. ADH1 = alcohol dehydrogenase, SH1 = sucrose synthase and PDC 1, 2, 3 = pyruvate decarboxylase (modified from Peschke and Sachs, 1994).

shares 67% identities and 81% positives with the XET sequence from wheat over a 90 amino acid stretch (starting at position 80 of 1005). The comparison also revealed several amino acid stretches that were conserved among all the species, including one stretch (underlined in Fig. 1) that is proposed to be the catalytic site of related glucanases from Bacillus subtilis (Borriss et al., 1990). These stretches are also shared by a brassinosteroid-regulated gene from soybean (Fig. 1; Zurek and Clouse, 1994). In addition, the predicted mass and isoelectric point of 1005 (31 and 6.23 kDa, respectively) are within range of those predicted from the XET sequences. These results provide good evidence that 1005 encodes XET or a closely related enzyme in maize.

The involvement of XET in flooding responses has not yet been investigated. However, increased XET activity was associated with ethylene-induced tissue softening (Redgwell and Fry, 1993; see below). In addition, ethylene accumulation under flooding is strongly linked to the formation of intercellular air spaces (aerenchyma, discussed below). Based on the above evidence, it is proposed that 1005 is an oxygen deprivation-responsive gene encoding an XET-like protein that may be involved in cell wall loosening or degradation (Saab and Sachs, 1995). Under oxygen deprivation conditions, wall degradation is required for the development of intercellular air spaces (He et al., 1994). XET or related enzymes may well be involved in this process, in concert with a range of degradative enzymes. The potential role of 1005 in flooding-induced cell structural changes, such as tissue deformation, cell lysis, and aerenchyma formation is currently being explored.

Xyloglucan endotransglycosylase (XET): activity and proposed function

XET is a recently discovered enzyme that is involved in the breakdown of cell wall xyloglucans during germination (Fanutti et al., 1993). XET is also proposed to be active in cell wall metabolism during cell expansion and softening (Fry et al., 1992a; Redgwell and Fry, 1993; Nishitani and Tominaga, 1992). Xyloglucans are composed of β-1,4 linked glucans with xylosyl side-chains and are a component of the cell wall matrix in most species. Xyloglucans can hydrogen bond strongly to cellulose and are proposed to cross-link adjacent cellulose microfibrils,
Fig. 1. Comparison of deduced amino acid sequences of 1005 and XET clones in GenBank. Dark regions include identities among the majority of sequences. Underlined sequence is proposed to be the catalytic site of related glucanases (lichenases) from *Bacillus subtilis* (Borriss et al., 1990). A brassinosteroid-regulated clone (BRASSIN) from soybean is also included in this comparison.

acting as tethers (Fry, 1989). As such, xyloglucan chains are hypothesized to restrain cell expansion in response to the driving force of turgor pressure, contributing to cell wall strength (Passioura and Fry, 1992). XET was recently purified from the extracellular space of *Vigna angularis* epicotyls (Nishitani and Tominaga, 1992) and *Tropaeolum majus* cotyledons (Fanutti et al., 1993). The main activity of XET, which has been detected in extracellular spaces (Hetherington and Fry, 1993, Nishitani and Tominaga, 1992) is that of transglycosylation: the enzyme catalyses the cutting and rejoining of xyloglucans (Nishitani and Tominaga, 1992; Fanutti et al., 1993; Fry et al., 1992b). At lower substrate concentrations, however, XET causes hydrolysis of xyloglucans (Fanutti et al., 1993).

It is generally accepted that cell expansion requires continued breakage and rejoining of xyloglucans to allow volume increase while maintaining integrity of the wall (Albersheim, 1976; Talbot and Ray, 1992; Fry et al., 1992a). Thus, XET has been implicated in cell wall loosening during expansion. Indeed, several studies have demonstrated a link between XET and cell expansion in
Aerenchyma formation was observed in the cortex of tissues of fully submerged maize plants (Grineva et al., 1988). Under these conditions, several anatomical and morphological changes were observed in the roots and shoots after 24 h of submergence which continued through 72 h after submergence, the duration of the experiments. In adventitious roots (which maintained some elongation during the treatment) these changes included fusion of xylem vessels, deformation of endodermis and parenchyma cells, and the formation of intercellular cavities, all of which occurred in the maturation and elongation zones, but not in the meristematic zone (the actual dimensions of the elongation zone were not determined, however). Subjecting the entire plant to flooding conditions caused profound structural modifications in the shoot as well. These included distortions of sclerenchyma cell walls and the formation of intercellular spaces in stem and leaf tissues (Grineva et al., 1988).

There is strong evidence indicating that formation of intercellular air spaces is promoted by accumulation of endogenous ethylene in submerged tissues (Drew et al., 1979; Jackson et al., 1985; Justin and Armstrong, 1991). Formation of aerenchyma can also be induced in well-aerated plants that are starved for nitrogen or phosphorus, and this response is proposed to be triggered by increased sensitivity to endogenous ethylene (He et al., 1992). The formation of aerenchyma was found to be associated with increased activity of cellulase, which presumably contributes to cell lysis (Drew, 1992; Grineva and Bragina, 1993; He et al., 1994). However, additional wall and cytoplasmic degradation enzymes are also likely to be involved in the process (Campbell and Drew, 1983; He et al., 1994).

**Analysis of the signal transduction pathway leading to the anaerobic response**

All organisms including primitive prokaryotes, need to sense environmental signals and encode and transfer these stimuli to a target (e.g. the nucleus) before their metabolism is altered to suit the environmental changes. This is needed not only to avoid and adjust to unfavourable conditions, but also to obtain food and resources for their growth and reproduction. The mechanisms of signal transduction (the process of stimulus–response coupling) are, in general, well understood in bacterial and animal systems. However, plants, with their unique organization, provide further opportunities to understand how information is processed in complex biological systems. It is being investigated how maize seedlings and cultured cells perceive changes in O₂ availability.

The genes encoding the ANPs (e.g. ADH) are rapidly turned on by oxygen deprivation (including mild hypoxia) and rapidly turned off by reoxygenation (Wignarajah and Greenway, 1976; Paul and Ferl, 1991). Such a response implicates a fast and precise O₂-sensing system operating in plant cells. However, very little is known about how...
plants sense the changes in O₂ availability and how this information is translated into an adaptive response. There is some evidence for the involvement of phytohormones such as ethylene (Jackson, 1985; He et al., 1994) and ABA (Zhang and Davies, 1987; Hwang and Van Toai, 1991) as intercellular or long distance signals during anoxia (Drew, 1990). However, the intracellular pathway of O₂ sensing still represents a 'black box'. Haemoglobin was suggested to signal O₂ deprivation intracellularly in plants (Appleby et al., 1988). Although a barley haemoglobin gene was recently shown to be anaerobically inducible, the kinetics of induction do not support this proposal (Taylor et al., 1994).

In prokaryotes such as Escherichia coli (Spiro and Guest, 1991) and Rhizobium meliloti (Gilles-Gonzalez et al., 1991) changes in O₂ concentration are sensed by a two-component regulatory system, which is homologous to other families of bacterial regulators that mediate a range of adaptive responses. The lower eukaryote, Saccharomyces cerevisiae, also responds to anoxia and haem is proposed to be a transducer of the signal (Poyton and Burke, 1992; Zitomer and Lowry, 1992). In vertebrates, hypoxia elevates cytosolic Ca²⁺ and this could lead to gene activation (Aldashev et al., 1991). Recently, Youngson et al. (1993) identified an O₂-sensitive K⁺ channel in the plasma membrane of pulmonary neuroepithelial cells. Except for this fragmentary information, very little is known of how low O₂ signals are transduced even in widely studied animal systems.

**Involvement of calcium ions in the signal transduction pathway leading to the anaerobic response**

Deprivation of O₂ leads to disturbances in ionic balance of plant cells, reflecting energy depletion and membrane depolarization. These transient changes, particularly in Ca²⁺ and H⁺, are immediately recognized, amplified and finally translated into long-lasting biochemical and physiological responses by plant cells. Among the ionic changes induced by anoxia, alterations in cytosolic pH have been studied extensively. Preliminary evidence was presented for the triggering role of pH in metabolic adaptation (Roberts et al., 1984; Fox et al., 1995). Recently, it has been shown that gene expression and physiological changes in response to O₂ deprivation are preceded and signalled by an elevation of cytosolic Ca²⁺ in maize seedlings and cultured cells (Subbaiah et al., 1994a, b).

Transient changes in cytosolic Ca²⁺ have been implicated in many signal transduction pathways in plants, including environmental stresses (reviewed in Hepler and Wayne, 1985; Poovaiah and Reddy, 1993). Studies were initiated to assess the role of calcium as a second messenger in signalling of anoxia in the roots of intact maize seedlings and an assay system has been developed to measure the response in terms of expression of marker genes and whole plant survival (Subbaiah et al., 1994b). Using specific antagonists and analysing intracellular free calcium by photometry and imaging (in collaboration with Douglas Bush of Rutgers University, Newark) it has been demonstrated that, both in suspension-cultured cells and intact seedlings, calcium acts as a transducer of low O₂ signals (Subbaiah et al., 1994a). Calcium-dependent protein phosphorylation changes were also found to occur during the early hours of anaerobic treatment. A polypeptide in the range of 85 kDa was preferentially labelled within 2 h of anoxia. In vitro nucleic acid-binding studies indicate that these post-translational changes may contribute to the differential gene expression (Subbaiah and Sachs, 1993). There is some preliminary evidence for a systemic anoxia stimulus as evidenced by expression of anoxia-inducible genes in distal non-stressed tissues (unpublished).

The effect of various Ca²⁺-antagonists on the anoxic induction of adhl and shl as well as the post-stress recovery of maize seedlings has been studied. Ruthenium red (RR; a known blocker of mitochondrial Ca²⁺ fluxes in animal cells) repressed the activation of the anoxia-specific genes. Further, RR-treated seedlings failed to recover after only 2 h of flooding (anoxia) in contrast to untreated maize (cv. B73) seedlings which are known to survive 72 h of submergence (Subbaiah et al., 1994b). Even ADH null mutants survive ~6 h of submergence (Lemke-Keyes and Sachs, 1989b; see below). This shows that RR acted on a very early and/or critical step in the adaptive response to anoxia. Ca²⁺ influx was not necessary for the adaptive response to anoxia, since Ca²⁺-chelators did not affect the anoxic response or survival. Hence, RR may act on one of the intracellular Ca²⁺ stores and the Ca²⁺ mobilized from this source is a physiological transducer of anoxic stress signals in maize (Subbaiah et al., 1994b).

Fluorescence imaging and photometry of [Ca] in maize suspension-cultured cells (cell line P3377) were used to investigate the above proposal further. Two complementary approaches were taken: (1) real-time analysis of anoxia-induced changes in [Ca], and (2) experimental manipulation of [Ca], levels and then assaying anoxia-specific responses. O₂ depletion caused an immediate increase in [Ca], and this was reversible within a few seconds of reoxygenation. The kinetics of Ca²⁺-rise showed that it occurred much earlier than any detectable changes in gene expression. The occurrence of [Ca]₀-elevation was independent of extracellular Ca²⁺ ([Ca]₀). RR decreased the resting levels of [Ca]₀ and blocked the anoxic Ca²⁺ elevation. The compound also affected post-anoxic cell viability and the induction of marker genes. Ca²⁺-chelators and plasma membrane Ca²⁺-channel blockers failed to block the [Ca]₀ and were equally
ineffective on anoxic gene expression. Caffeine, which induced an elevation of \([\text{Ca}^2+]\) under aerobic conditions, caused an increase in ADH activity under normoxia. These results thus support our proposal that \(\text{Ca}^{2+}\) is a physiological transducer of anoxia signals in plants (Subbaiah et al., 1994a).

Having established the role of cytosolic \(\text{Ca}^{2+}\) as an essential player in the transduction of anoxia signals in plants, it is now possible to answer a few fundamental questions on the \(\text{Ca}^{2+}\)-mediated pathway of \(\text{O}_2\) sensing. A single cell system has been developed which is amenable to address these important questions in intracellular signalling.

Where is \(\text{O}_2\) deprivation sensed in the cell? Identifying the origin of the \(\text{Ca}^{2+}\) signal

It is important to investigate where the \(\text{O}_2\) levels are sensed in the cell and then what leads to \([\text{Ca}^2+]\) elevation. Since oxygen is more diffusive than any potential signal molecule that has to traverse the cellular membranes, anoxia may be first sensed at the mitochondrial electron transport chain, where \(\text{O}_2\) can no longer be available as an electron acceptor. However, the role of plasma membrane redox systems and associated second messengers also need to be examined. It is expected that detecting the origin of the calcium signal may indicate the nature of the primary sensor, since \([\text{Ca}^2+]\) rise appears to be an essential link in the chain of events that lead to the whole plant response to anoxia.

How is the \(\text{Ca}^{2+}\) signal perceived?

The \(\text{Ca}^{2+}\) transients need to be amplified and targeted to an appropriate compartment where the response finally originates. Protein kinases have been shown to participate as sensors or amplifiers in many signalling pathways (Mori et al., 1993). Changes in protein phosphorylation were assayed during the induction of anoxia in maize (cv. B73) roots. Detergent solubilized root extracts of maize seedlings that were incubated aerobically (control) or submerged for 2 h (anoxia) were compared for their endogenous protein phosphorylation activities. The anoxic extracts showed an increased labelling of an ~85 kDa polypeptide. Addition of micromolar levels of calcium to the assay buffer dramatically enhanced the phosphorylation of this protein both in control and anoxic extracts; and this could be abolished by EGTA. Enzyme renaturation in activity gels (containing casein as a substrate) or in membrane blots of root extracts indicated that the 85 kDa polypeptide itself could be a kinase. The \({}^{32}\text{P}\)-labelling of this protein was resistant to heparin, but was affected by the calmodulin inhibitor chlorpromazine (Subbaiah and Sachs, 1993). These studies will be followed up to identify the nature of the phosphoprotein and its role in anoxia signalling.

Among the intracellular responses to \(\text{O}_2\) deprivation, ionic disturbances appear to be the earliest. These transient changes, particularly in \(\text{Ca}^{2+}\) and \(\text{H}^+\), are immediately recognized and relayed to the adaptive machinery through the downstream signalling intermediates. Anoxia-induced alterations in cytosolic pH (\([\text{pH}]_c\) have been studied extensively (Roberts et al., 1984; Fox et al., 1995). Our recent studies showed that changes in cytosolic \(\text{Ca}^{2+}\) are crucial for gene expression and physiological responses induced under \(\text{O}_2\) deprivation in maize (Subbaiah et al., 1994a, b). Presently, we are attempting to uncover how these ionic changes converge to bring about oxidative responses in plant cells. The activation of glutamate decarboxylase (GAD) under anoxia is chosen as the adaptive response and as the focal point of interaction between \(\text{pH}\) and calcium. By its \(\text{pH}\)-dependent activation and proton-consuming catalytic activity, GAD has been shown to be important for cytosolic \(\text{pH}\) regulation (Carroll et al., 1994). At the same time, \(\text{Ca}^{2+}\)/calmodulin has been shown to bind GAD and stimulate its activity (Snedden et al., 1995). How intracellular \(\text{Ca}^{2+}\) and \(\text{pH}\) independently and mutually regulate this enzyme in anoxic maize cells will be analysed. Furthermore, GAD is regulated \textit{in vivo} by reversible phosphorylation in animal cells. A calcium and calmodulin-dependent protein phosphatase, calcineurin, has been shown to be involved in the dephosphorylation-dependent activation of GAD in brain cells (Bao et al., 1995). Plant glutamate decarboxylases do possess potential sites for phosphorylation at their carboxy termini (Arazi et al., 1995). A protein phosphatase with partial homology to calcineurin (CANP) has also been cloned in \textit{Arabidopsis} (Meyer et al., 1994). Thus, analysis of \([\text{pH}]_c\), \([\text{Ca}^2+]\), and their interaction in anoxic cells, using GAD activation as an example, may provide an understanding of how the early events and potential signalling intermediates are interconnected to bring about whole cell responses to anoxia (Fig. 2).

The maize anoxic response is well characterized at the molecular level and hence provides a useful model to further understanding of abiotic stress signalling in plants. Work is in progress to unravel some of the early molecular responses and integrate them with the reprogramming of gene expression and, further, with whole plant adaptation. From the analyses, it is hoped to elucidate the cross-talk among the early events/intermediates that occur downstream to \([\text{Ca}^2+]\) elevation and well before gene activation (Fig. 2). These analyses will also be correlated with the whole plant anoxic tolerance at every step. In the future, the information generated from these studies will be used to develop screening assays for genotypes that are variant in anoxia-tolerance. Such variants/mutants not only serve as tools for further analysis, but are also of agronomic importance. Components of signalling pathway have become targets for chemotherapy in human and animal model systems (Brugge, 1993; Edgington, 1993) and offer...
allows alcohol dehydrogenase-1 (ADH1) null individuals to survive only a few hours of anoxia. The ATN survival trait was found to segregate as a single, recessive locus. *atnl* was found to be assorting independently of *adh1* and *adh2* (Lemke-Keyes and Sachs, 1989a). There is speculation that *atnl* might be null for either lactate dehydrogenase (LDH) or pyruvate decarboxylase (PDC) since ADH nulls might be more affected by cytoplasmic acidosis or accumulation of acetaldehyde, respectively. Recent results indicate that *atnl* may affect the expression of LDH as well as PDC in seedling tissues. However, individuals that are homozygous for *atnl* do have these enzyme activities and it is planned to examine this trait further.

**Conclusions**

Anoxia is one of the most important abiotic stresses encountered by higher organisms. The anaerobic-stress response of maize offers a unique opportunity to characterize the regulatory components of a family of 20 genes that are co-ordinately expressed. The anaerobic-specific proteins appear to be encoded by a set of genes whose expression is stimulated by a deprivation of oxygen, a condition that would occur in nature during flooding. Regulation of protein synthesis under anaerobiosis appears to occur at both the levels of transcription and translation and several genes involved in the anaerobic response have been isolated and characterized. The present goal is to determine which structural features of these genes might explain their selective expression during anaerobiosis. Genes that confer increased flooding and anaerobic tolerance in maize are also being analysed and this trait was found in some exotic maize accessions. Genetic analysis indicates that tolerance is a fairly simple dominant trait. Additionally, a recessive factor that increases anaerobic tolerance in plants that are null for ADH activity has been found and it is intended to isolate and characterize the genes involved in tolerance to anaerobic-stress and to determine how this trait relates to the anaerobic response. Furthermore, a novel gene has been isolated that is likely to be involved in structural modifications that are proposed to prolong flooding survival. Ongoing studies will address the function of this gene and confirm its role in structural modifications.

**Major objectives are to analyse the anaerobic response on several levels with the ultimate goals of understanding the mechanisms involved in the transcription and translation level controls involved in regulating this highly co-ordinate response. Studies will involve genetic and molecular analysis of trans-acting genes and variant or mutant anaerobic specific genes that exhibit altered expression. Physiological and biochemical experiments**

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Cross-talk between potential intermediates in maize anoxia-signalling. Experimental evidence is available for the events marked in solid arrows. The proposed interactions between the events/intermediates (thin broken arrows) will be the subject of our future investigations. (N, nucleus; V, vacuole; mt, mitochondria; ER, endoplasmic reticulum; [Ca**2+**], or [Ca**2+**]~i, intra- or extra-cellular free Ca**2+**; CaM, calmodulin; [pH]; RR, ruthenium red; GAD, glutamate decarboxylase; GABA, y-aminobutyric acid, CSA, cyclosporin A; CANP, calcineurin-like protein phosphatase).
will be carried out to determine the nature of the translation-level repression of pre-existing protein synthesis. Is translation initiation or elongation involved? Is there a competition between 'anaerobic-specific' mRNAs and the pre-existing mRNAs?

Another goal is to understand how maize perceives the changes in external O\textsubscript{2} concentration and adapt its growth and metabolism in short and long time scales. To this end, the initial molecular events in maize roots during anoxia are being examined, together with the nature of endogenous signal(s) and the mechanism of its (their) transduction within the root system and other organs.

It is hoped that an understanding of the anaerobic response will lead to an understanding of flooding tolerance. With the advances in genetic transformation techniques, it is likely that genes encoding stress proteins, or other factors, from a tolerant plant will be introduced into plants that are normally sensitive to the stress condition. This approach is not only important for the molecular analysis of DNA sequences that are responsive to an anaerobic stimulus, but will also pave the way for constructing 'tailor-made' flood-resistant crop plants.

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