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Characterization and Expression of Transcripts Induced by Oxygen Deprivation in Maize (*Zea mays* L.)[1]

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Until recently, the only genes described in plants induced by oxygen deprivation (anoxia or hypoxia) encoded enzymes of glucose-phosphate metabolism. In the present study, two flooding-induced maize (*Zea mays* L.) genes that may serve a different function have been identified. These genes, with unique kinetics of mRNA induction under flooding conditions, were not induced by heat, cold, or salt stress or by seedling death. The predicted protein sequence of one gene, *wusl1005*, is similar to that of several other plant genes, including a nasturtium (*Tropaeolum majus* L.) xyloglucan-*endo*-transglycosylase. The predicted protein sequence of the other gene showed no significant homology to genes of known function, indicating that both of these genes may play novel roles in the maize response to oxygen deprivation.

The maize (*Zea mays* L.) anaerobic response is extremely well characterized and serves as a model of coordinate gene regulation in higher plants. In maize seedlings exposed to anaerobic conditions, normal protein synthesis is repressed, and a small group of "transition polypeptides" is immediately produced (Sachs et al., 1980). About 90 min after the onset of anaerobic stress, another set of approximately 20 proteins, the anaerobic polypeptides, is synthesized; this synthesis continues at a steady rate from 5 to 72 h after the onset of anaerobic stress (Sachs et al., 1980). Both transcription and translation are implicated in this gene regulation (Sachs et al., 1980; Bailey-Serres and Freeling, 1990; Russell and Sachs, 1992). The anaerobic polypeptides in maize have been shown to include ADH (Sachs et al., 1980), Suc synthase (Springer et al., 1986), aldolase (Kelley and Freeling, 1984b), Glc-P isomerase (Kelley and Freeling, 1984a), PDC (Laszlo and St. Lawrence, 1983), glyceraldehyde-3-P dehydrogenase (Russell and Sachs, 1989, 1992), enolase, and phosphoglycerate mutase (Bailey-Serres et al., 1988). All of the anaerobic polypeptides so far identified are involved in either glycolysis, Glc metabolism, or fermentation. Such a genetic system presumably has evolved to permit limited ATP production (through glycolysis) and NAD^+ recycling (through fermentation) in the absence of oxygen.

There is evidence that at least some anaerobically induced genes are also induced by other stresses. For example, Russell and Sachs (1989) found heat-shock induction of *adh1* and *gpc3* mRNA levels, although this was not reflected at the level of protein synthesis (Russell and Sachs, 1992). Christie et al. (1991) observed that a 10°C treatment resulted in the induction of ADH1 mRNA and enzyme activity; PDC and aldolase mRNA levels also increased, but enzyme activity was not determined. Also, Irigoyen et al. (1992) detected transient increases in activity of ADH, malate dehydrogenase, and aldolase in drought-stressed alfalfa nodules. Although these gene products clearly fit within the biochemical pathways described above, it is expected that anaerobiosis would also induce genes that are involved in other aspects of adaptation to this stress, such as morphological changes.

In the present study, a number of anaerobically induced RNAs in maize were further characterized for their response to flooding and other stresses. Two previously unidentified clones with novel kinetics of induction were selected for sequence analysis and genomic mapping.

MATERIALS AND METHODS

Clones

A cDNA library was previously constructed from poly(A)$^+$ RNA isolated from roots of Berkeley Fast maize (*Zea mays* L.) inbred seedlings that were treated anaerobically for 6 h (Russell and Sachs, 1989). cDNAs representing two new glyceraldehyde-3-P dehydrogenase genes (one induced by flooding, one not) have been selected from this library and characterized (Russell and Sachs, 1989, 1991). A large number of clones were screened for increasing RNA levels in seedlings over time during oxygen deprivation, and those that showed induction were selected for further study (Sachs, 1991). Some of these were recognized to be well-characterized maize genes (e.g. aldolase and Suc synthase) by cross-hybridization to those sequences and by similar induction kinetics.

Previously identified maize clones used in the present study include ADH1 cDNA clone pZmL793 (Dennis et al., 1984), HSP70 (Rochester et al., 1986), PDC1 cDNA clone

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Abbreviations: ADH, alcohol dehydrogenase; gfu, gene function unknown; ORF, open reading frame; PDC, pyruvate decarboxylase; RFLP, restriction fragment length polymorphism; R_I, recombinant inbred line.

pZMK437pstkpn (Kelley, 1989), and a 26S rRNA clone isolated from the library described above (Russell and Sachs, 1991). Two other clones (previously designated 1042 and 1120) were unidentified when this study was initiated but were subsequently found to represent genes for PDC that are different from each other and from *pdc1* (Kelley, 1989; Peschke and Sachs, 1993). They are now designated *pdc2* and *pdc3*, respectively. Unidentified clones 1005 and 1032 were chosen for study based on their strong induction during flooding.

Plant Materials

B73Ht is a maize ADH^+ line that shows typical survival under hypoxic stress (3–4 d; Lemke-Keyes and Sachs, 1989b). γ25 is a line that is homozygous for the *Adh1-FkFγ25* null allele (Freeling, 1978) and survives less than 24 h of anaerobic treatment; it was included to determine whether induction of any of the clones was a response to secondary effects of anaerobic death rather than anaerobiosis per se. Preemergent seedling shoots of these two lines were used in the stress experiments, as described below. In addition, unstressed 6-week-old B73 leaf tissue and 15-d postpollination B73 endosperm from greenhouse-grown plants were included in some experiments to obtain information concerning the organ specificity of the different clones.

Hypoxic Induction

Seedlings were germinated for 3 to 4 d on moist paper towels in the dark at 28°C and 85% RH. Only seedlings that had not yet extended leaves through their coleoptiles (i.e. preemergent) were used. As many as 15 seedlings were placed in 500-mL jars containing drowning buffer (100 mg L^{-1} of ampicillin, 5 mM Tris-Cl [pH 8]), and as much air as possible was removed from the jars before sealing. Jars were kept in the dark at 28°C for the number of hours indicated. In this report, we are referring to these conditions as "hypoxic," because some oxygen may have been present initially. However, these conditions were nearly anaerobic to begin with and most likely became so by the later times (48–72 h). Previous studies have shown that this treatment and absolute anoxia affect ADH induction in maize seedlings in a very similar manner (Russell and Sachs, 1992).

Other Stresses

B73 seedlings were germinated, grown as above, and then subjected to stress using one of the following treatments:

Control. Seedlings were removed from one moist germination towel to another (to simulate handling experienced by stress treatments) and allowed to grow another 16 h before harvest.

Two-hour heat shock. Seedlings were placed between moist paper towels in a humid chamber and incubated at 40°C for 2 h.

Twenty-hour heat shock. Seedlings were placed between moist paper towels in a humid chamber and incubated at 40°C for 20 h.

Acid. Seedlings were placed on paper towels soaked with 0.05 N HCl (giving a root environment of approximately pH 4) for 4 d. The shoots continued to grow upward and were not in direct contact with the acid.

Salt. Seedlings were placed on paper towels soaked with 5 M NaCl until wilting (approximately 2 d).

Cold. Seedlings were laid on moist paper towels in contact with ice in a Styrofoam box. The box was kept in a cold room (giving a seedling temperature of 0.5–1°C) for an additional 4 d.

RNA Isolation and Northern Hybridization

After the treatments, shoots were excised and frozen in liquid nitrogen. Samples were kept at −80°C until RNA isolation. Total RNA was isolated, and 10-μg samples were fractionated on 1.3% agarose formaldehyde gels as previously described (Russell and Sachs, 1989). RNA was capillary transferred to Nytran nylon membrane (Schleicher & Schuell) using 10× SSC (1.5 M NaCl, 0.15 M trisodium citrate) for 8 to 12 h. Blots were air dried for approximately 20 min before UV cross-linking on a transilluminator for 4 min (Church and Gilbert, 1984). Probes (generally cDNA insert isolated from the vector) were labeled with [α-^{32}P]dCTP using the "random hexamer primer" method (Feinberg and Vogelstein, 1984). Prehybridization, hybridization, and washing were done using the modifications of Church and Gilbert (1984) described by Russell and Sachs (1989). Prehybridization was in 1% BSA, 1 mM EDTA, 0.5 M sodium phosphate (pH 7.2), 5% SDS, and 100 μg mL^{-1} of sheared salmon sperm DNA for 3 h. Hybridization was in fresh solution, with probe added, for 16 to 24 h. Blots were washed twice for 10 min at 65°C in 40 mM sodium phosphate (pH 7.2), 0.5% BSA, 1 mM EDTA, and 5% SDS, followed by two washes at 65°C for 10 min in 40 mM sodium phosphate (pH 7.2), 1 mM EDTA (pH 8.0), 1% SDS. Blots were exposed to XAR-5 film (Kodak) using an intensifying screen at −80°C. When needed, blots were regenerated by soaking in 0.1× SSC, 0.5% SDS, for 30 min at 90°C, followed by overnight exposure to test for probe removal.

DNA Sequencing

Clones 1005 and 1032 were subcloned into M13 mp18 and mp19 vectors (Yanisch-Perron and Messing, 1985). Single-stranded DNA sequencing was done using the Sequenase 2.0 kit (United States Biochemical) according to the manufacturer's recommendations, except that the labeling reaction was done on ice. The sequence was verified by sequencing both strands as well as by use of dITP to resolve the sequence in ambiguous regions, except as indicated in Figure 4A. Comparisons of both nucleotide and predicted amino acid sequences to previously reported sequences were made using Genetics Computer Group (Devereux et al., 1984) and BLAST (Altschul et al., 1990) software. The Genetics Computer Group programs MOTIFS and PROFILESCAN were used to search predicted amino acid sequences for common protein motifs and patterns, and the program ISOELECTRIC was used to determine the isoelectric points (Devereux et al., 1984).

Because no poly(A) tail was present in clone 1032, the

direction of transcription was determined using strand-specific probes to analyze duplicate northern blots. The probes were generated from M13 clones of each strand using reagents described by Hu and Messing (1982). The M13 universal sequencing primer was used, which directed single-stranded probe synthesis downstream into the cDNA insert (similar to the method reported by Sachs et al., 1986).

Southern Hybridization

Maize seedling DNA was isolated (Saghai-Maroof et al., 1984), digested with a 3-fold excess of enzyme under the manufacturer's conditions (New England Biolabs) plus 4 mM spermidine (Dellaporta et al., 1983) and fractionated on 0.8% agarose gels in 45 mM Tris-acetate, 1 mM EDTA (pH 8). Southern blotting, prehybridization, hybridization, and washing were as described by Peschke and Sachs (1993). Blots were exposed to XAR-5 film (Kodak), using an intensifying screen, at −80°C.

RFLP Mapping

RFLP mapping of the gene represented by clone 1032 [*wusl1032(gfu)* = *umc217(gfu)*] was done using both the R_I lines developed by Burr et al. (1988) and Burr and Burr (1991) and the "immortal F_2" population developed at the University of Missouri, Columbia (Gardiner et al., 1993). Because no polymorphism using clone 1005 was found in the immortal F_2 population, its corresponding gene [*wusl1005(gfu)*] was mapped using only the R_I lines. Briefly, both methods involve determining which parental RFLP allele is detected by the probe of interest in each of a number of progeny lines and then comparing that data to a data base produced using a large number of previously mapped probes hybridized to those same lines.

Both 1005 and 1032 hybridized to a number of genomic fragments when the complete cDNA inserts were used to probe Southern blots (data not shown). To simplify the hybridization pattern and facilitate mapping of only the gene of interest, probes corresponding to the 3′ end of each of the RNAs were generated by cleaving the clones with appropriate enzymes. For clone 1005, cleavage was done using an internal *Sst*I site (Fig. 4A) and a *Hin*dIII site in the vector. For clone 1032, two internal *Sst*II sites were used (Fig. 2). In each case, these 3′ subclones detected single restriction fragments that could be easily scored in the mapping experiments (Fig. 3).

RESULTS

Hypoxic and Stress Induction

Figure 1 (left) shows the patterns of mRNA induction in B73 seedling shoots subjected to 0 to 72 h of hypoxic stress; these patterns have been consistent throughout multiple experiments (Sachs, 1991; Peschke and Sachs, 1993). Whereas many hypoxically induced mRNA levels plateau or decrease by 72 h (e.g. *adh1* and the three *pdc* genes), levels of mRNA hybridizing to clone 1005 begin to increase within 6 h and increase until 72 h or later. In contrast, the induction of mRNA hybridizing to clone 1032 occurs much later than for other mRNAs, between 24 and 48 h of hypoxia.

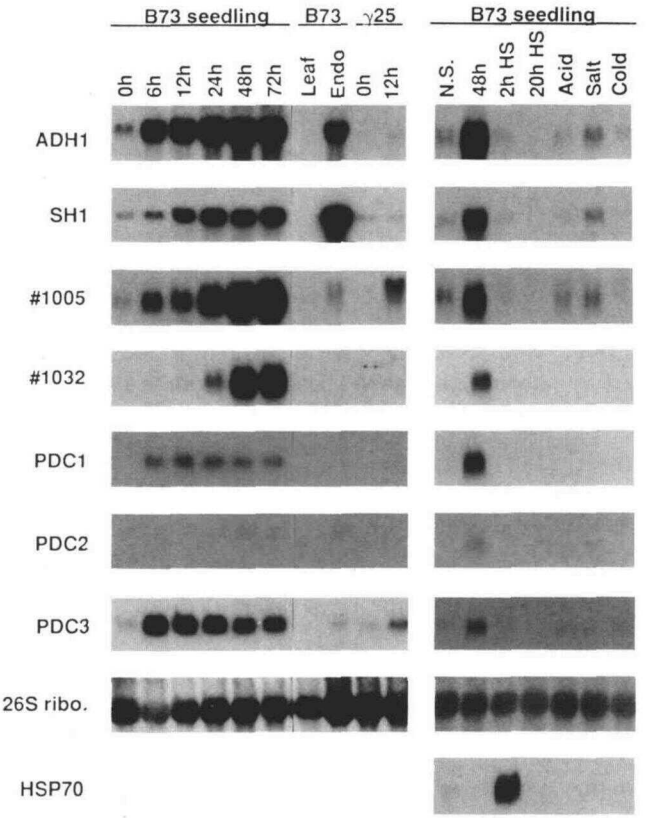

Figure 1. Comparison of hypoxic and stress induction of anaerobically induced RNAs. Left, Lanes 1 to 6, B73 seedling shoots drowned for the number of hours indicated; lane 7, B73 leaf; lane 8, B73 endosperm, 15 d postpollination; lanes 9 and 10, γ25 seedling shoots drowned for 0 or 12 h, respectively. The same blot was used for each probe. Right, B73 seedlings stressed as indicated. Multiple blots were used for probing; hybridization with the maize 26S rRNA (26S ribo.) clone was used to verify equivalent loading of all blots (not shown). 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 to 1°C for 4 d.

The unusually late induction of RNA hybridizing to clone 1032 led to the speculation that its induction may be in response to cell damage or seedling death resulting from hypoxia rather than as a mechanism of tolerance. To address this, a maize line homozygous for the *Adh1-FkFγ25* null mutation (γ25) was included in the analysis, because it dies within 24 h of hypoxia. A 12-h time point was selected for the time of sampling for this line, because tissue death would be expected at that time. RNA hybridizing to clone 1032 could not be detected in the ADH1-null line at either 0 or 12 h, indicating that this transcript is not produced simply as a result of tissue damage. RNAs hybridizing to clones 1005 and PDC3 were induced by hypoxia in this line, although only 1005 RNA could be detected at a level comparable to that seen in B73 12-h tissue. It appears that the RNA detected in the ADH1-null line by the 1005 probe is larger than that detected in the B73 seedling tissue (1700 versus 1400 bases). The lack of induction of RNA levels corresponding to clones

such as SH1 and PDC1 in γ25 is presumed to be due to early tissue death.

As shown in Figure 1 (right), B73 seedlings were subjected to a number of other sublethal stresses. These treatments were chosen to stress the seedlings at various levels of severity and with differing chemical or physical agents to provide the best chance of detecting induction due to cell damage or being near death. Although slight differences were observed in the responses of the different clones, mRNA levels appeared to be responding specifically to hypoxic stress. The most general response noted was a decrease in the levels of nearly every RNA after heat shock and (to a lesser extent) cold treatment. The mRNA detected by probe 1005 showed an increase in size under cold stress relative to other treatments, which may reflect differences in RNA processing or polyadenylation under these conditions. As expected, HSP70 RNA is highly induced by 2 h of heat shock, although it, too, decreases by 20 h. In this experiment *adh1* mRNA levels showed a slight decrease in shoots under heat shock, whereas close to a 2-fold increase was seen previously in both roots and shoots (Russell and Sachs, 1989).

Organ Specificity

To obtain preliminary information concerning organ specificity, leaf RNA from 6-week-old plants and 15-d postpollination endosperm RNA were included in the analysis (Fig. 1, left). Leaf was included because it is one of the few organs in which *adh1* is not normally expressed (Russell and Sachs, 1991). In contrast, many anaerobically induced genes, including *adh1* and *sh1*, have previously been found in developing maize endosperm (Springer et al., 1986; Russell and Sachs, 1991). In the present study, the only RNA present at detectable levels in green leaves corresponded to the maize 26S ribosomal clone. As expected, RNAs encoded by *adh1* and *sh1* were found at high levels in developing endosperm (Fig. 1). RNAs encoded by *pdc2*, *pdc3*, and *wusl1005(gfu)* were also present in the endosperm but at levels only slightly above those in control shoots. Two bands appeared to be present in the B73 endosperm tissue when probed with 1005; one of these was the same size as that detected in B73 seedlings (approximately 1.4 kb), whereas the other appeared to be the same as that detected in the ADH1-null 12-h seedlings (approximately 1.7 kb). In contrast, no band appeared when the tissue was probed with 1032.

DNA Sequencing

Figure 2 shows the DNA sequence of cDNA 1032, with the direction of transcription as determined by northern hybridization with strand-specific probes. The hybridizing RNA is estimated to be 1250 bases in length; the cDNA clone includes 943 bases. In the direction indicated, there is only one ORF that contains a start codon and would encode a polypeptide of greater than 100 amino acids. This ORF would encode a polypeptide containing 232 amino acids (Fig. 2) with a molecular mass of 24 kD and an isoelectric point of pH 10.98. No significant homologies to this amino acid sequence were found (Devereux et al., 1984; Altschul et al., 1990), nor were any common protein motifs or patterns found

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1    AATATTATTGCTTCGACGCAGAGATGTACTTACACTGCATAAGCTCTTAACACATACAGTTGCACATCTT

71   TATTGCTAGCTAACGCAAGGCTACTTTACCCTACGTTAAGACACACAAGTCACTTCAAGCTAATTAAGG

140  ATG CAC GAC GAT CTT GGT GTT CTT CGT CAT GTC GGT GGT GGC GAG GCC GCC GCC
     met his asp asp leu gly val leu arg his val gly gly gly glu ala ala ala

194  CTT GCC GGC GAG CGG GAA GTA GAA GGG GTA GAT CTC GTA GCG CAC CCT GCA GCT
     leu ala gly glu arg glu val glu gly val asp leu val ala his pro ala ala

248  GCT GTA GTT GAT CTG GCA GCC CTG CTG CGC GCC GCA GTA GTC GCC GAA CCT GGA
     ala val val asp leu ala ala leu leu arg ala ala val val ala glu pro gly

302  CAG CGC CGT GGA CAG GCA CTG CGC GCA GGT CAG CGG CGC GAG GTC CCG CGT GCA
     gln arg arg gly gln ala leu arg ala gly gln arg arg glu val pro arg ala

356  CTG CGC CAG CCC GTA GAT GGA CAC GAA CGG CGT GTA CTG CTC CTT GTC CCG CCC
     leu arg gln pro val asp gly his glu arg arg val leu leu leu val pro pro

410  GAG CCC CGC GCT GCC CGC CGC CGA CGC CTG CGC CGT CGC CTT GCC CAT CAC CTT
     glu pro arg ala ala arg arg arg arg leu arg arg arg leu ala his his leu

464  CCC CAC CGC CTT CTC GAA CGC CTT CGG GTT GTC CAT GGC CTG CAC GTT CAC CAG
     pro his arg leu leu glu arg leu arg val val his gly leu his val his gln

518  GAT CAC GCC CGC GTC CGT GTC CAC CTG CCC GAA GAA GTT GGC GTT CTC GTA CCG
     asp his ala arg val arg val his leu pro glu glu val gly val leu val pro

572  CAT GAA GCA GTA GTC GTA CCA TAT CCT CGC GTC GGA ACT GTA GCT GCA GGT ACT
     his glu ala val val val pro tyr pro arg val gly thr val ala ala gly thr

626  GGG GAG CTG CTT GGC GGC GTC GGC AAG GCA GGA CGC GCA GTC GCT GGC GGA GAC
     gly glu leu leu gly gly val gly lys ala gly arg ala val ala gly gly asp

680  GTC GCC GCG GCA TTG CGC GAG GCC GTA GAT GAT GCT GTT GCC CTT GCC GGC GGT
     val ala ala ala leu arg glu ala val asp asp ala val ala leu ala gly gly

734  GGA CGT GGC GTA GCC CCC GGC GGA GGA GGC CGA GGC GAC GAG GTC GGC GAG GAC
     gly arg gly val ala pro gly gly gly gly arg gly asp glu val gly glu asp

788  GGA GTT GAT GTT GGC CAC GGC CTT GCT GCT GCC AGC GTA GCT GCT TCC TGA GCA
     gly val asp val gly his gly leu ala ala ala ser val ala ala ser OPA

842  GTAGCTGCCGATGGAGTCCGCGGCCATGCCCAGCGGGAGCAGCAGAGCCAAGGAAACAACAAGCAGCATG

912  CAGCAGCGCATCCTGCTGGACTCCATGGCAGT
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Figure 2. DNA sequence of clone 1032. The start and stop codons for the single large ORF are underlined. The *Sst*II restriction sites, used to generate a 3′-specific probe, are indicated in bold type. The predicted amino acid sequence of the single large reading frame is given below the DNA sequence. Comparison of this sequence to others in GenBank revealed no significant homologies at the sequence or amino acid level in any reading frame.

(Devereux et al., 1984). Some homologies to a variety of extensins were detected in the predicted amino acid sequence from another ORF that contained a high percentage of Pro. However, these homologies are not thought to be significant because the ORF is very short (105 amino acids), it contains no starting Met, and the Pro's are not contained in the Hyp-rich glycoprotein sequence motifs (e.g., Ser-Pro_4) of either dicots or monocots (Raz et al., 1992).

Although RNA of the opposite polarity was not detected, its sequence (complementary to that shown in Fig. 2) was examined as well. Analysis of the complementary sequence revealed several ORFs corresponding to polypeptides of up to 273 amino acids; again, no significant homologies were detected when the nucleotide or predicted amino acid sequences were compared to those in sequence data bases (Devereux et al., 1984; Altschul et al., 1990).

Southern hybridization to *Eco*RI-digested genomic DNA using the complete 1032 cDNA clone showed hybridization to many bands, whereas a subclone containing the 3′ end of the cDNA hybridized to a single *Eco*RI fragment in each line (Fig. 3A). The 3′ end subclone was used for RFLP mapping because it represented a probe specific to the gene of interest. The northern analysis shown in Figure 1 was produced using the entire cDNA insert as a probe, although using the 3′ fragment as a probe produces an identical pattern of induction (data not shown).

Figure 4A shows the sequence of clone 1005. A second

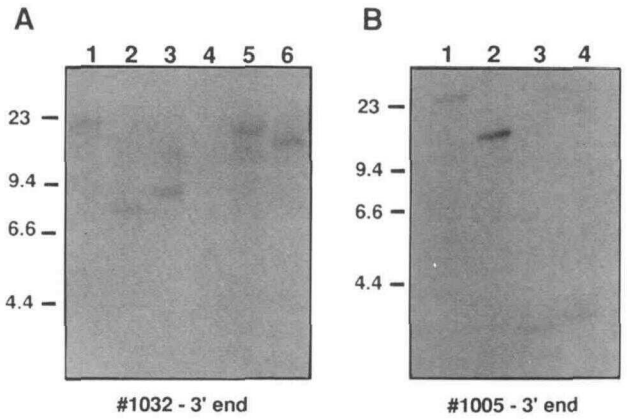

Figure 3. Hybridization of 3′ end subclones of 1032 and 1005 to genomic DNA detects single-copy sequences. A, Probing with 3′ subclone of 1032 (cleavage sites shown in Fig. 2). Lane 1 is maize inbred Mo20, lane 2 is B73, lane 3 is CO159, lane 4 is CM37, lane 5 is Tx303, and lane 6 is T232. All DNA samples were cleaved with *Eco*RI. B, Probing with 3′ subclone of 1005 (cleavage site shown in Fig. 4A). Lane 1 is maize inbred Mo20 cleaved with *Eco*RI, lane 2 is B73 cleaved with *Eco*RI, lane 3 is Mo20 cleaved with *Hin*dIII, and lane 4 is B73 cleaved with *Hin*dIII. Phage λ DNA cleared with *Hin*dIII was used as a size marker and the positions of the higher mol wt fragments are indicated on the left side of each panel.

shorter clone, 1022, that had shown homology to 1005 using plasmid hybridization, was found to be identical with 1005 except that it terminated at a second polyadenylation site 58 bases 3′ to the previously identified one (Fig. 4A). The composite sequence includes 796 bases, about half the length of the transcripts observed (1.4 and 1.7 kb). Neither of the poly(A) sequences are preceded by the canonical AATAAA polyadenylation signal, which is located 10 to 30 bases upstream of the site of polyadenylation in animal cells (Proudfoot and Brownlee, 1976; Wickens and Stephenson, 1984; Birnstiel et al., 1985). Although this signal sequence appears to be necessary for poly(A) formation in animals, it is either absent or found at a different position in more than 50% of the plant genes analyzed (Hunt et al., 1987), including *adh1* (Sachs et al., 1986). Multiple sites of poly(A) addition may explain the various sizes seen in B73 seedlings versus endosperm versus γ25 seedlings; such multiple poly(A) sites were found to cause differences in mRNA size classes encoded by *adh1* (Sachs et al., 1986).

The composite 1005/1022 sequence, which corresponds to approximately the 3′ half of the mRNA, contains three ORFs of 448 bases or more. The predicted product of the first reading frame of this fragment is 50% identical with a soybean seedling gene regulated by brassinosteroids (Zurek and Clouse, 1993; Fig. 4B). The homology is distributed throughout the sequence but is higher in the N-terminal segment of the 1005 sequence, corresponding to the middle of the soybean protein. This homology is also detected in the nucleic acid sequences, which are 60% identical in bases 4 through 321 of 1005 (corresponding to bases 372–689 of the soybean gene sequence). In addition, 1005 showed 74% identity in a 43-amino acid region to an *Arabidopsis* gene that is expressed at high levels in meristems (*meri-5*; Medford et al., 1991). All three of these genes share homology with a recently described xyloglucan-*endo*-transglycosylase from nasturtium, suggested to be involved in cell-wall loosening during elongation (*NXG1*; de Silva et al., 1993). In each case, the amino acid homology appears to be much reduced in the C-terminal half of the predicted protein products. The *Arabidopsis* gene *TCH4*, induced by mechanical stress, also appears to be related in a similar manner to *meri-5* and *NXG1* (Braam and Davis, 1990; J. Braam, personal communication). Clone 1005 was used to

A

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1    GCG AGC GGG GAG CCG TAC ACG GTG CAC ACG AAC GTG TAC AGC CAG GGG AAG GGC
     ala ser gly glu pro tyr thr val his thr asn val tyr ser gln gly lys gly

55   GGG CGG GAG CAG CAG TTC CGG ATG TGG TTC GAC CCC ACG GCG GCC TTC CAC GCC
     gly arg glu gln gln phe arg met trp phe asp pro thr ala ala phe his ala

109  TAC TCC GTG CTG TGG AAC CCC GCC CAC GTC GTC TTC TAC GTG GAC GGC GTC CCC
     tyr ser val leu trp asn pro ala his val val phe tyr val asp gly val pro

163  ATC CGG GAG TTC CGG CGC CGC GGC GAC GGG ACC GTG CCG TTC CCG ACG TCG CAG
     ile arg glu phe arg arg arg gly asp gly thr val pro phe pro thr ser gln

217  CCG ATG CGG GTG TAC GCC AGC GTG TGG GAC GCG GAG GAG TGG GCC ACG CAG GGG
     pro met arg val tyr ala ser val trp asp ala glu glu trp ala thr gln gly

271  GGG CGC GTC CGG ACC GAC TGG TCC AAG GCG CCC TTC GTC GCG TCG TAC CGC GGG
     gly arg val arg thr asp trp ser lys ala pro phe val ala ser tyr arg gly

325  TAC GCC GCC GCC GGG TGC ACC GCG CCG GAC GCC GCC GCC TGC GCG CGC TCC AAC
     tyr ala ala ala gly cys thr ala pro asp ala ala ala cys ala arg ser asn

379  GGC GCA TGG ATG TCG CAG GAG CTC GAC AGC GCC GGC CAG GAG CAG CTC CGC CGG
     gly ala trp met ser gln glu leu asp ser ala gly gln glu gln leu arg arg

433  GCG CAG GCC AGC TAC ATG ATC TAC AAC TAC TGC ACC GAT AAG TAC CGG TTC CCG
     ala gln ala ser tyr met ile tyr asn tyr cys thr asp lys tyr arg phe pro

487  CAG GGC CCG CCG CCC GAG TGC TCG TCG CCG GCC AAG TAG TAG ATGAGTAGAATTGAT
     gln gly pro pro pro glu cys ser ser pro ala lys AMB AMB

544  CGGATACAGAGAGCAATCAATTAATTAAATCGACCCGTCGCTTGGTTTTTGGTTTACACATTGTACTACG

614  GCATAAGTTCGGCGGATATATGTATACGTAGACGGCTAGACGCCATGCATGTGTCTTGGCATGTATCGTA
                                                    •#1005 poly-A start

684  TTCGTATATatcgtaattcgtatgtacctcagtatacctgcatcgtacatttacatgcatatatatctcc
                                          •#1022 poly-A start

754  ctatatataaatatatatggttttattaaaaaaaaaaaaaaaa
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B

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soy     115   L··D··IL···IFT····N·····YL·····RN··T··II
#1005     1   ASGEPYTVHTNVYSQGKGGREQQFRMWFDPTAAFHAYSVL
meri-5  107   M··D···L·····T····DK····HL·····N··T··I·

soy     155   ·K·Q·II·L··NT···V·KNAEPLG····KN····I·S·L
#1005    41   WNPAHVVFYVDGVPIREFRRRGDGTVPFPTSQPMRVYASV
meri-5  147   ···QRIILT··TH·LESLKTMSLSVSC·QRTK···MVRQF

soy     195   ·N·D D···R··L·K·····T·Y··NFK·IEFSSK
#1005    81   WDAE EWATQGGRVRTDWSKAPFVASYRGYAAAGCTAPDA
meri-5  187   MERQSD···KRRSWLKLIGLKLHSWLLTETLRLTRNQTPI

soy     232   SSISN·GAEYEAN···AYSRRR··WV·KYF·····S·LK
#1005   120   AACARSNGAWMSQELDSAGQEQLRRAQASYMIYNYCTDKY
meri-5  227   GTLKKWIQQAKLDSNGFRRIT

soy     272   ·····L·A··KR
#1005   160   RFPQGPPPECSSPAK
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Figure 4. A, DNA sequence of clone 1005, including 58 additional bases at the 3′ end revealed by sequencing an overlapping clone, 1022. An *Sst*I restriction site within this clone, used to generate a 3′-specific probe, is indicated in bold type; the other cleavage site was in the vector. Bases in lowercase were determined by sequencing one strand only using both dGTP and dITP; all other sequence was determined using both strands. The predicted amino acid sequence in the reading frame that showed homology to *Arabidopsis* gene *meri-5* (Medford et al., 1991) is given. B, Comparison between predicted amino acid sequence of 1005, a brassinosteroid-regulated soybean gene (Zurek and Clouse, 1993; GenBank accession No. L22162) and *Arabidopsis* gene *meri-5* (Medford et al., 1991; GenBank accession No. M63166). Sequence homologies were detected using the BLAST server (Altschul et al., 1990).

hybrid select RNA that was translated in vitro to produce a polypeptide of approximately 32 kD (D. Russell, personal communication).

Southern hybridization to genomic DNA using the complete 1005 cDNA insert or a subclone of the 5′ end produced a number of bands (data not shown), indicating the possible existence of multiple genes containing this coding region. As with clone 1032, probing with only the 3′ end revealed a single band (Fig. 3B); therefore, this probe was used for mapping and in northern hybridization. Under low stringency, about 15 copies of *meri-5*-hybridizing sequences can be detected in *Arabidopsis* (J. Medford, personal communication).

RFLP Mapping

The map locations of clones 1005 and 1032 did not correspond to those of any known mutant phenotype or enzyme that might provide additional clues to their function. The 3′ end subclone of 1032 detects a gene [designated *wusl1032(gfu)*] that maps only about 3 cM distal to *pdc3* on chromosome 1S (Peschke and Sachs, 1993), although these two genes show no significant sequence homology or expression similarity. The same map position and gene order were obtained in two independent mapping experiments, one using R_I lines (Burr et al., 1988; Burr and Burr, 1991) and the other using immortal F_2 lines (Gardiner et al., 1993). The 3′ end subclone of 1005 detects a gene [designated *wusl1005(gfu)*] that maps to chromosome 5S. No recombinants were detected between *wusl1005(gfu)* and a previously mapped reference clone, bnl6.22, indicating at a 95% confidence level that these two loci are within about 4 cM of one another (Burr et al., 1988). Table I shows the chromosomal locations of mapped anaerobic genes in maize. Although a number of genes map to chromosomes 1 and 8, they are generally spread out over the chromosomes. The close linkage of *wusl1032(gfu)* and *pdc3* described above is an exception, although it is not known whether there is any functional or evolutionary significance to this.

DISCUSSION

Not All Hypoxically Induced RNAs Represent Glycolytic Genes

Although nearly every hypoxically induced gene identified to date corresponds to an enzyme of Glc-P metabolism, at least two genes (represented by cDNAs 1005 and 1032) are likely to serve a different function. One other RNA that also appears to correspond to a nonglycolytic gene has recently been described (Vogel and Freeling, 1992; J. Vogel, personal communication) that has significant nucleotide homology to a maize mutator transposable element (*mu1.7*) and to a mutator-related sequence (*mrs*).

The majority of research on anaerobic response in plants has focused on the enzymology of energy production, although many other responses have been observed. One of the most well documented of these is the formation of aerenchyma (Jackson, 1985; Justin and Armstrong, 1987). Aerenchyma have most often been studied in plants with only roots submerged (e.g. wheat [Thomson et al., 1990], waxapple [Lin and Lin, 1992]) but are also seen in the roots of fully submerged maize plants (Grineva et al., 1988). This response may be triggered by increased ethylene production and accumulation (Jackson, 1985; Justin and Armstrong, 1991). The similarity of *wusl1005* to a xyloglucanase indicates a possible role in aerenchyma formation or some other change in tissue structure.

Other physiological changes that have been observed in hypoxic plants include alteration of mitochondrial ultrastructure (Couee et al., 1992) and failure of stomata to open even under high light conditions (Vavasseur et al., 1990). There appears to be some correlation between the ability of a plant

Table I. *Chromosomal locations of anaerobic genes in maize*

Gene Name or Clone No.	Symbol	Location	Nearest Markers[a]		Reference[b]
			Proximal	Distal	
Alcohol dehydrogenase1	*adh1*	1L	*umc107*	*npi225*	
Alcohol dehydrogenase2	*adh2*	4S	*bnl5.46*	*umc87*	
Aldolase1	*ald1*	8L	*umc117*	*npi286*	M.M. Sachs, unpublished data[c]
Enolase1	*eno1*	9S	*wx1*	*umc105*	M.M. Sachs, unpublished data
Glyceraldehyde-3-P dehydrogenase3	*gpc3*	4S	Near *gpc1*		Russell and Sachs, 1991
Glyceraldehyde-3-P dehydrogenase4	*gpc4*	5L	*bnl5.71*	*umc126*	M.M. Sachs, unpublished data
Pyruvate decarboxylase 1	*pdc1*	8L	Near *bnl2.369*		Peschke and Sachs, 1993
Pyruvate decarboxylase 2	*pdc2*	8S	*umc124*	*bnl9.11*	Peschke and Sachs, 1993
Pyruvate decarboxylase 3	*pdc3*	1S	*npi286*	*bnl1.326*	Peschke and Sachs, 1993
Shrunken1	*sh1*	9S	*bz1*	*umc113*	
1005	*wusl1005(gfu)*	5S	Near *bnl6.22*		This report
1032	*wusl1032(gfu)* = *umc217(gfu)*	1S	*npi286*	*bnl1.326*	This report

[a] Indicates nearest surrounding maize RFLP probes (Maize Genet. Coop News Lett., vol 66, 1992). [b] Map position from Maize Genet. Coop News Lett. (vol. 66, 1992) unless otherwise indicated. [c] This gene was previously mapped to chromosome 3L (Agrigenetics; 1992 Maize Genet. Coop News Lett.)

species to tolerate anoxia and the accumulation of putrescine, although the mechanism is unknown (Reggiani et al., 1990). Future analysis of *wusl1005(gfu)* and *wusl1032(gfu)* may clarify their involvement in these or other processes.

One class of proteins produced under anaerobic stress that has received little attention is the transition polypeptides (Sachs et al., 1980). These polypeptides are produced beginning during the 1st h of anaerobiosis, although their synthesis decreases relative to the majority of anaerobic polypeptides by 5 h. The in vitro translated product of RNA hybrid-selected with clone 1005 (D. Russell, personal communication) is close to the size of the transition polypeptides (approximately 33 kD), but genetic mutants or antibodies would be needed to test whether the two are related. The kinetics of induction of 1005 mRNA do not correspond to the pattern of accumulation of the transition polypeptides; however, RNA and protein levels are not necessarily well correlated. For example, whereas *adh1* and *gpc3* mRNA levels increase 10-fold under anaerobic stress, protein synthesis rates increase 30- to 60-fold. In contrast, previous studies found that *gpc3* and *adh1* mRNA levels increase greater than 2-fold in roots and shoots under heat stress, but their protein synthesis rates do not increase (Russell and Sachs, 1989, 1992).

Most Hypoxically Induced RNAs Respond Specifically to Hypoxic Stress

It was expected that this study might reveal hypoxically induced clones that represented RNAs induced generally by seedling stress or death, because the late induction of 1032 mRNA suggested that it was not functioning as a mechanism of tolerance, and levels of 1005 mRNA continued to increase even when seedling death was expected to occur. Although subtle differences between the mRNA levels detected by the different clones could be observed under various stresses, in each case hypoxic stress produced the only significant induction seen. In several studies documenting the response of "anaerobic" genes to other stresses, it appears that a secondary effect of the stress being investigated is a reduction in respiration. For example, Christie et al. (1991) postulated that induction of ADH1, PDC, and aldolase at 10°C may be due to disruption of mitochondrial membranes (and therefore respiration) at this temperature. The levels of ADH activity peaked at 10°C and were found to be insignificant at lower temperatures (P. Christie, personal communication). The induction of several glycolytic genes in drought-stressed alfalfa nodules may be due to a change in the nodule surface making it less permeable to oxygen, resulting in lower oxygen levels within the nodule itself (Irigoyen et al., 1992).

Organ-Specific Expression of Hypoxically Induced Genes Varies in the Absence of Flooding Stress

It was previously shown that genes such as *adh1* and *sh1*, although hypoxically inducible in roots and preemergent seedling organs, are expressed constitutively in organs such as the developing endosperm and scutellum and not expressed at all in emerged leaves (Russell and Sachs, 1991). Organ-specific expression of several hypoxically induced genes was examined here to learn whether these genes would all be similarly regulated under nonstress conditions. None of the hypoxically induced RNAs could be detected in unstressed leaf tissue, which is consistent with previous observations (Russell and Sachs, 1989, 1991). Okimoto et al. (1980) reported that no protein synthesis was detected in leaves under anoxic conditions.

When RNA levels in 15-d postpollination endosperm were tested, *adh1* and *sh1* mRNAs were expressed at high levels. At this developmental stage both proteins are present at high levels in endosperm as well (Springer et al., 1986; Russell and Sachs, 1991). Because of this, it was suspected that one or more *pdc* RNAs would also be highly expressed in endosperm, but this appears not to be the case. RNA encoded by *pdc1* is not detected in endosperm; both *pdc2* and *pdc3* RNA were detected but at low levels. Because ADH enzymically follows PDC, this raises questions as to the function of ADH in the developing endosperm. Some PDC must be synthesized in the developing kernel, because mature maize kernels have occasionally been used for its purification (Laszlo, 1981; Lee and Langston-Unkefer, 1985), but this may occur in the embryo or aleurone, and its level and timing of expression relative to that of ADH is unknown. A further puzzle is that ADH activity is not needed for endosperm development; *adh1 adh2* double-null seeds that have no detectable ADH activity (Lemke-Keyes and Sachs, 1989a) are able to develop and germinate under normal oxygen levels. In addition, it has been shown here that two other hypoxically induced genes (represented by clones 1005 and 1032) have little or no transcript accumulation in the developing endosperm, providing further evidence that the observed expression of *adh1* and *sh1* is not simply a response to a hypoxic condition in the endosperm.

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