Evidence for a Role for AtMYB2 in the Induction of the Arabidopsis Alcohol Dehydrogenase Gene (*ADH1*) by Low Oxygen

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ABSTRACT

The transcription factor AtMYB2 binds to two sequence motifs in the promoter of the Arabidopsis *ADH1* gene. The binding to the GT-motif (5′-TGGTTT-3′) is essential for induction of *ADH1* by low oxygen, while binding to the second motif, MBS-2, is not essential for induction. We show that *AtMYB2* is induced by hypoxia with kinetics compatible with a role in the regulation of *ADH1*. Like *ADH1*, *AtMYB2* has root-limited expression. When driven by a constitutive promoter, *AtMYB2* is able to transactivate *ADH1* expression in transient assays in both Arabidopsis and *Nicotiana plumbaginifolia* protoplasts, and in particle bombardment of *Pisum sativum* leaves. Mutation of the GT-motif abolished binding of AtMYB2 and caused loss of activity of the *ADH1* promoter in both transient assays and transgenic Arabidopsis plants. These results are consistent with *AtMYB2* being a key regulatory factor in the induction of the *ADH1* promoter by low oxygen.

PLANTS respond to conditions of low oxygen by switching carbohydrate metabolism in root cells from an oxidative to a fermentative pathway. In maize, where the molecular events initiated during low oxygen stress have been studied in most detail, transfer to conditions of low oxygen represses aerobic protein synthesis and, at the same time, initiates the synthesis of two transition polypeptides, with molecular weights of approximately 33 kD. After approximately 90 min, a group of about 20 polypeptides, the anaerobic polypeptides (ANPs) are synthesized (Sachs *et al.* 1980; Bailey-Serres and Freeling 1990). Most of these ANPs are enzymes involved in ethanolic fermentation (alcohol dehydrogenase, *ADH*; pyruvate decarboxylase, *PDC*), or in glycolysis (*e.g.*, fructose 1,6-bisphosphate aldolase, sucrose synthase, glucose-6-phosphate isomerase, enolase, glyceraldehyde-3-phosphate dehydrogenase; for review, see Sachs *et al.* 1996). A number of different maize seedling tissues (roots, coleoptile, mesocotyl, endosperm, scutellum, and anther wall) synthesize the ANPs (Okimoto *et al.* 1980). Maize leaves, which have emerged from the coleoptile, do not synthesize the ANPs and do not survive even short periods of anaerobiosis (Okimoto *et al.* 1980).

Sequence elements in the promoter of the maize *ADH1* gene, which are critical for anaerobic induction, have been identified (Walker *et al.* 1987; Olive *et al.* 1990, 1991a,b). The Anaerobic Response Element (ARE) lies between −100 and −140 relative to the transcription start and is a bipartite element with two copies of a GT-element (5′-[T/C]GGTTT-3′), and two GC-elements (5′-GCC[G/C]C-3′). The GC-elements bind a GC-Binding Protein (GCBP-1; Olive *et al.* 1991b); both GC-elements are required for expression of *Adh1*. The GT-motifs are also critical for anaerobic induction and expression (Walker *et al.* 1987) and are "footprinted" *in vivo* by dimethyl sulfate (Ferl and Nick 1987; Paul and Ferl 1997), suggesting proteins bind to these motifs. No GT-binding protein has been identified in maize.

Arabidopsis has a similar anaerobic response to maize (Dolferus *et al.* 1985). Arabidopsis *ADH1* is induced by hypoxic conditions and by a number of other environmental stimuli (low temperature, dehydration) and by the phytohormone ABA (Dolferus *et al.* 1994; De Bruxelles *et al.* 1996). The Arabidopsis *ADH1* promoter contains sequences similar to the maize *Adh1* ARE between −160 and −140, with the GT-motif in the opposite orientation relative to the maize GT-motifs (GT-motif: 5′-AAACCAA-3′; GC-motif: 5′-GCCCC-3′). The GT- and GC-motifs are both necessary for low oxygen induction (Dolferus *et al.* 1994).

The Arabidopsis *ADH1* GT-motif contains a potential Myb binding site. Myb transcription factors bind to a consensus sequence with an AAC central motif (5′-T/CAAC[T/G]G-3′; or 5′-CC[T/A]ACC-3′; Lüscher and Eisenman 1990; Grotewold *et al.* 1991). This prompted us to investigate the involvement of Myb-related transcription factors in the low oxygen induction of the Arabidopsis *ADH1* gene. One candidate Myb was AtMYB2, reported by Urao *et al.* (1993) to be induced by dehydration, salt stress, and exogenous abscisic acid (ABA). *AtMYB2* was able to transactivate a promoter

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containing multimers of the Myb binding site consensus sequence (5′-TAACTG-3′; Urao *et al.* 1996). Recently it was demonstrated that AtMYB2 binds to a Myb recognition site in the Arabidopsis dehydration-responsive gene *rd22* (Abe *et al.* 1997).

In this paper we present evidence that *AtMYB2* is rapidly induced by low oxygen conditions, and that it binds to the GT-motif in the *ADH1* promoter. In transient assays AtMYB2 activates expression of an *ADH1*-GUS construct, and this transactivation does not occur when the GT-motif is mutated.

MATERIALS AND METHODS

Plant material, growth conditions, and stress treatments: *Arabidopsis thaliana* seeds, ecotypes C24 or Columbia (Co-0), used in this study were grown on Murashige and Skoog (MS) medium at 22° (16/8 hr light/dark cycle, 200 μE/sec/cm^2). Stress and ABA treatments were carried out hydroponically, in dishes containing 15 ml liquid MS medium as previously described (Dolferus *et al.* 1994; De Bruxelles *et al.* 1996). Low oxygen treatments were carried out by incubating plantlets in a 5% O_2/95% N_2 gas mixture (hypoxic conditions; Howard *et al.* 1987), for up to 24 hr at 22° in the dark. Dehydration treatment was carried out by incubating the plantlets in medium containing 0.6 m mannitol, for up to 24 hr at 22°. For cold stress treatment, plantlets were incubated at 4–5° for up to 24 hr. ABA (±) *cis-trans* isomers, Sigma, St. Louis) was added to the medium at a final concentration of 0.1 mm for 4 hr. For treatments with the protein synthesis inhibitor cycloheximide, plant material was first preincubated in MS medium containing 10 μm cycloheximide for 1 hr. The solution containing cycloheximide was refreshed before the stress treatment.

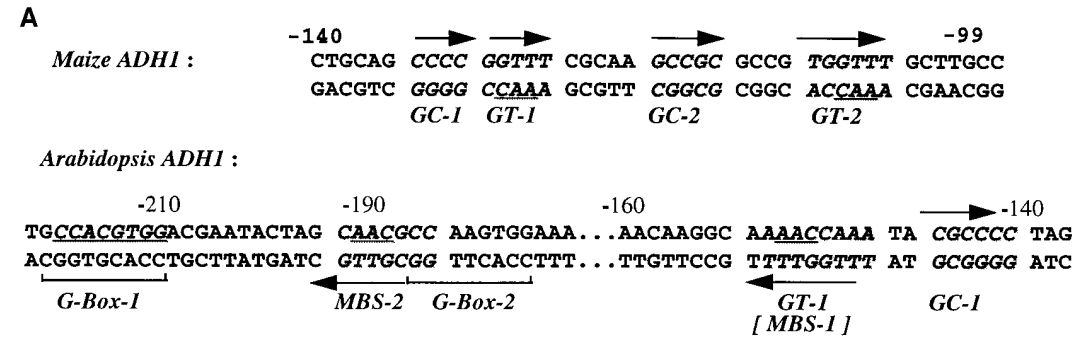
Recombinant DNA techniques: All cloning methods were according to standard procedures (Maniatis *et al.* 1982; Sambrook *et al.* 1989). Plasmid pGEX-RAtmyb 2BE contains the *AtMYB2* cDNA fused to the glutathione-*S*-transferase coding region in plasmid pGEX2T (Urao *et al.* 1993). The plasmid containing the GST-GAMYB fusion protein and the GAMYB probe oligo used in gel retardation were described by Gubler *et al.* (1995). The AtMYB2 fusion protein was purified using the Pharmacia (Piscataway, NJ) GST purification module and used in EMSA (electrophoretic mobility shift assay) experiments. Complementary oligonucleotide probes used in EMSAs (see Figure 1) were annealed, end-labeled using Klenow DNA polymerase, and then gel-purified. Binding reactions (20 μl) contained 1 μl recombinant AtMYB2 (about 50 ng protein), 2 μl 10× EMSA buffer (100 mm Tris-HCl, pH 8.0, 500 mm NaCl, 10 mm EDTA, 0.5% skimmed milk powder, 50% glycerol, and 10 mm DTT), 1 μl DTT (10 mm), 1 μl [poly]dI-dC (1 μg/μl), 15 μl H_2O, and 1 μl labeled target oligonucleotide (0.1 ng; 10,000 cpm). Reactions were incubated at room temperature for 10–15 min. Competition experiments were performed by adding unlabeled competitor oligonucleotide to the reaction prior to the addition of radiolabeled oligonucleotide. Salmon sperm DNA was used as a nonspecific competitor (25 ng per binding reaction; sheared by sonication). Samples were loaded onto a 5% polyacrylamide gel in 0.5× TBE (Maniatis *et al.* 1982).

A full-length *AtMYB2* cDNA, flanked by *Bam*HI sites was obtained using RT-PCR of anaerobically induced root RNA. The resulting cDNA was cloned between the 35S promoter and the 3′ *NOS* terminator sequence of plasmid pART7 (Gleave 1992). The resulting plasmid p35S-*CAtMYB2* was verified by sequencing and used as effector plasmid in all transient assays. Reporter plasmids p*ADH1*-GUS, containing the ADH promoter from position −964 to +53, p35S-GUS, p[ΔGBox-1]ADH-GUS, p[ΔGBox-2]ADH-GUS, p[ΔGT]ADH-GUS, and p[ΔGC]ADH-GUS were described earlier (Dolferus *et al.* 1994; Figure 1B). p[ΔMBS-2]ADH-GUS, containing substitution mutations in the MBS-2 site, was constructed by amplifying fragments overlapping the MBS2 site (5′-TAGCAACGCC-3′) and transforming this site into a *Not*I restriction site (5′-GCGGCCGCAT-3′). The full-length *ADH1* promoter was reconstructed, and the mutated promoter was cloned into plasmid p*ADH1*-GUS to replace the wild-type promoter. The construct was subcloned in binary vector pBIN19 (Bevan 1984) for transformation to Arabidopsis. Binary vectors were mobilized to Agrobacterium strain *AGL1* (Lazo *et al.* 1991) by electroporation (Nagel *et al.* 1990).

RNA extractions, Northern and Southern blot analysis: RNA extraction, gel electrophoresis, Northern blot hybridizations using antisense RNA probes, and filter washing procedures were as described previously (Dolferus *et al.* 1994). Filters were placed on phosphor imager screens (Molecular Dynamics, Sunnyvale, CA) and the hybridization signals quantified. The Arabidopsis ubiquitin gene (Burke *et al.* 1988) was used as a probe to correct for variation in sample loading, by dividing all signal strengths by their respective ubiquitin signal. *AtMYB2* RNA probes were prepared from a clone containing the full-length cDNA. *ADH1* probes were transcribed from a clone containing the entire coding region of the gene. Sucrose synthase RNA probes were prepared from a clone containing the coding region of the Arabidopsis *ASUS1* gene (Martin *et al.* 1993). The *PDC1* probe was prepared from a clone containing the entire coding region of the Arabidopsis *PDC1* gene (Dolferus, Peacock and Dennis, unpublished results). Quantitative RT-PCR was carried out using 1 μg total RNA and the Promega Access RT-PCR system (Madison, WI). Samples were taken during the PCR reaction after 5, 10, 15, and 25 cycles and loaded on agarose gels. Gels were treated for Southern blot hybridization, and filters were hybridized using the *AtMYB2* cDNA. Linearity of signal strength was verified using phosphorimager quantifications. Oligos were used slightly overlapping the 5′ end and 3′ end of the first and second intron positions of the *AtMYB2* gene respectively. These oligos were shown not to amplify genomic DNA as template.

Tissue culture, protoplast transient assays, particle bombardment, and Agrobacterium transformation: Arabidopsis root cultures were established by placing 1-month-old leaf cuttings (ecotype C24) on callus-induction medium (Valvekens *et al.* 1988) for 3 days, prior to infection with *Agrobacterium rhizogenes* (strain A4RS; Vilaine and Casse-Delbart 1987). The leaf disks were cocultivated for 3 days on callus-induction medium, washed in a 200 mg/ml timentin solution (Smithkline Beecham, Dandenong, Australia), and placed on solid MS medium including 100 mg/ml timentin. After 3–4 wk the hairy root explants were transferred to liquid MS medium, and refreshed monthly. Arabidopsis mesophyll protoplasts were prepared from ecotype Co-0, using a modification of previously published procedures (Damm and Willmitzer 1988; Damm *et al.* 1989; Abel and Theologis 1994). Typically, transient assays were carried out using 2×10^{-6} protoplasts and 15–20 μg reporter plasmid DNA, plus or minus the same amount of effector plasmid (p35S-*CAtMYB2*). *Nicotiana plumbaginifolia* suspension cells were maintained and protoplasts were prepared according to Negrutiu *et al.* (1981), using the media described by Kao and Michayluk (1975). Plasmid DNA was introduced using the PEG method for both Arabidopsis and *N. plumbaginifolia* protoplasts (Abel and Theologis 1994).

Particle bombardment of pea leaves was carried out using a homemade helium gun. A total of 25 μl of particles (100



B

OLIGO :	SEQUENCE :
MBS-1	5'– GGGAAAACCAAATCCG –3'
ΔMBS-1/1	5'– GGGAACCCCAAATCCG –3'
ΔMBS-1/2	5'– GGGAAGACCAAATCCG –3'
MBS-2	5'– GGCTAGCAACGCCAAGTGG –3'
ΔMBS-2	5'– GGCTAGCCCCGCCAAGTGG –3'
4 X MBS-1	4 X [5'– GGGAAAACCAAATCCG –3']
ΔGT	5'– GGCCTCTAGACCTAC –3'
ΔGC	5'– TACGAGAATAGT -3'
ΔGBOX1	5'– AAATAACATGTTCACG –3'
ΔGBOX2	5'– GCAACTAACATGTTCCCGAG–3'
GBS	5'–GGCCGATAACAAACTCCGG–3'

Figure 1.—(A) Functional organization of the Arabidopsis *ADH1* promoter compared to the maize *Adh1* promoter. Arrows indicate the orientation of the GT- and GC-motifs in both promoters and the orientation of the arrows shows the orientation of the motifs with respect to the maize *ADH1* promoter. The position of the two potential Myb binding sites in Arabidopsis *ADH1* (MBS-1 and MBS-2) is indicated. (B) Sequences of the MBS-1 and MBS-2 oligos, as well as substitution mutant oligos for both sequences. Nucleotides which are in underlined italics are mutated compared to the wild-type sequence. Double stranded equivalents of these oligos were used in gel retardation experiments. ΔGT, ΔGC, ΔGbox-1 and ΔGbox-2 are the sequences of the substitution mutants used in the original *ADH1* promoter mapping (Dolferus *et al.* 1994). GBS, GAMYB binding site (Gubler *et al.* 1995).

mg/ml in 50% glycerol; 50:50 mixture of tungsten and gold particles) was mixed with 2–6 μg plasmid DNA (1 μg/μl), 25 μl 2.5 m $CaCl_2$, and 10 μl spermidine (0.1 m). The total volume was then adjusted to 40 μl, and 4 μl was used for the bombardment of one leaf. Reporter and effector plasmids were used in a 1:1 ratio. Leaves were incubated on MS plates for 16 hr before GUS staining.

GUS histochemical staining and fluorometric assays: GUS fluorometric assays and *in vivo* histochemical stainings were carried out as previously described (Jefferson 1987; De Block and De Brouwer 1992). The total protein concentration in the extracts was determined using a Bio-Rad (Richmond, CA) protein assay kit (Bradford 1976). Fluorometric and protein assays were carried out in microwell plates, and analyzed using Labsystems (Marlboro, MA) Fluoroskan II and Multiskan Plus readers, in conjunction with Delta Soft II software (Biometallics Inc., Princeton, NJ; Breyne *et al.* 1993). For *in vivo* GUS assays, the staining solution was vacuum infiltrated into the plant tissue. Plant material was fixed in 70% ethanol after GUS staining.

RESULTS

The Arabidopsis *ADH1* promoter has binding sites for *AtMYB2*: The Arabidopsis *ADH1* promoter contains two potential Myb Binding Sites (MBS) in the 200 bp immediately upstream of the start of transcription (Figure 1A). The more distal MBS-2 at −189 to −187 lies in the footprinted region containing G-box-2 (Ferl and Laughner 1989; Dolferus *et al.* 1994). The second motif, MBS-1 at −150 to −148, coincides with the GT-motif (Figure 1A) necessary for mediating the *ADH1* low oxygen response (Dolferus *et al.* 1994; De Bruxelles *et al.* 1996). The MBS-2 motif resembles the classical vertebrate Myb binding sequence (Lüscher and Eisenman 1990; Weston 1992), whereas MBS-1 (within the GT-motif) shows more homology to the maize *P*-Myb binding site (Grotewold *et al.* 1994; Figure 1A). The possibility arises that *AtMYB2* binds to one or both of the two putative Myb binding sequences of the *ADH1* promoter (Figure 1A).

AtMYB2 was expressed in *Escherichia coli* as a glutathione-S-transferase fusion protein (AtMYB2-GST) and affinity purified. The fusion protein was used in EMSAs with oligonucleotides corresponding to the MBS-1 and MBS-2 motifs (Figure 1B). The recombinant AtMYB2 protein retarded both classes of oligonucleotides (Figure 2A). Binding was competed by unlabeled homologues, but not by a nonspecific competitor (salmon sperm DNA; Figure 2A). Binding to the monomers was competed out by 100–200-fold molar excess of either

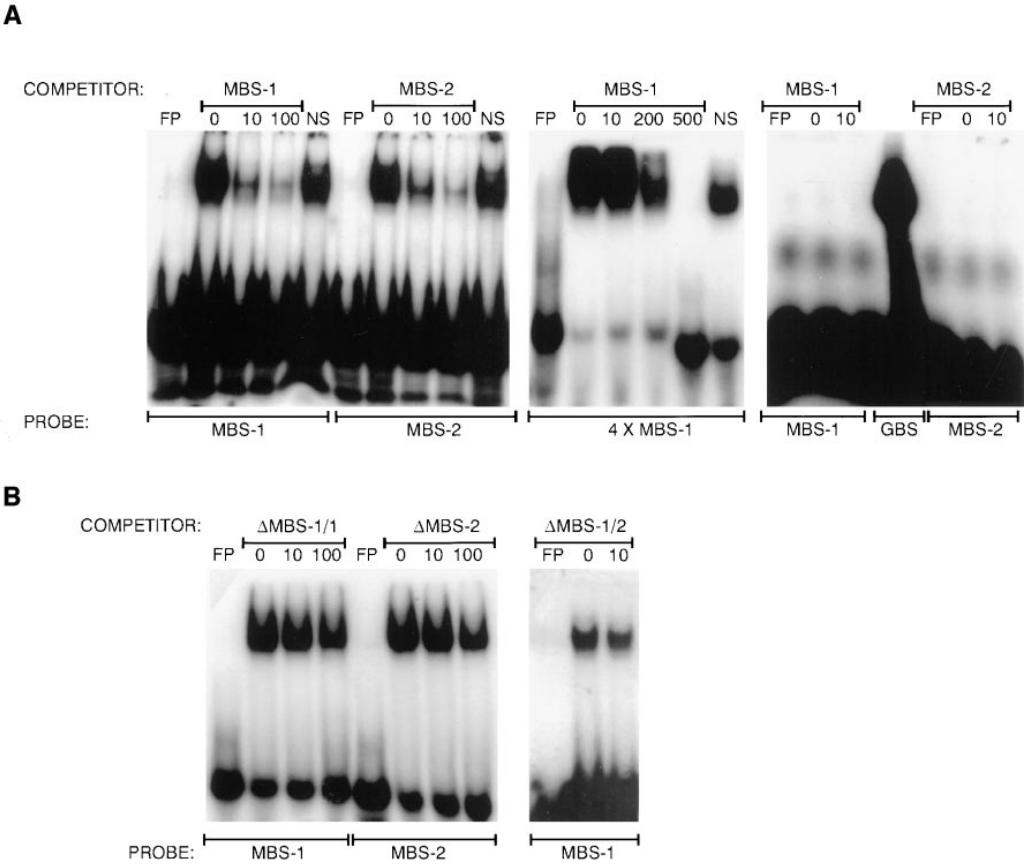


Figure 2.—Gel retardation results, showing binding of purified GST-AtMYB2 fusion protein to ^{32}P-labeled MBS-1 and MBS-2 oligos (see Figure 1B). Numbers on the top of each figure indicate fold molar excess of cold competitor. FP indicates lanes with free probes (no protein added), and NS indicates lanes where nonspecific salmon sperm competitor DNA was used. (A) The left panel shows binding of AtMYB2 to both MBS-1 and MBS-2 sequences. Binding is reduced by cold competitor DNA of the same sequence as the labeled probe, but not by the nonspecific competitor. Middle panel: Binding of AtMYB2 to multimerized (4 × MBS-1) oligos. Higher molar excess of cold competitor is required to eliminate binding. Right panel: GAMYB does not bind to MBS-1 or MBS-2, but shows strong binding to the GAMYB Binding Site (GBS). (B) Binding of AtMYB2 to MBS-1 and MBS-2 can not be competed by competitor oligos with a mutated AAC core sequence (ΔMBS-1/1 and ΔMBS-2 respectively). Mutation of the AAC core to GAC (ΔMBS-1/2 oligo) showed weak competition for binding to labeled wild-type MBS-1 oligo.

of the MBS-1 or MBS-2 oligos. Multimerization of the MBS-1 oligo gave significantly stronger binding than the monomer (Figure 2A), a 500-fold molar excess excluding all binding to the tetramer; at this level, some degree of competition was also observed with the nonspecific competitor (salmon sperm DNA; Figure 2A). AtMYB2-GST did not bind to other motifs of the *ADH1* promoter (G-box-1 or GC-motif sequences; data not shown). Another plant Myb transcription factor, GAMYB (Gubler *et al.* 1995), which binds to a GARE sequence (GA response element; 5′-TAACAAA-3′) of the gibberellic acid inducible α-amylase promoter, did not interact with either MBS-1 or MBS-2 when expressed as a GAMyb-GST fusion protein (Figure 2A).

***AtMYB2* binding to MBS-1 and MBS-2 requires the AAC-core:** The AAC-core sequence of MBS-1 and MBS-2 was mutated to CCC (ΔMBS-1/1 and ΔMBS-2) or GAC (ΔMBS-1/2; Figure 1B). Similar mutations in vertebrate and plant Myb factors abolished binding (Weston 1992; Gubler *et al.* 1995). The CCC core sequence (ΔMBS-1/1 and ΔMBS-2) did not compete for binding to wild-type MBS-1 and MBS-2 probe sequence (Figure 2B); the GAC core (ΔMBS-1/2) had much reduced ability to compete for binding to wild-type MBS-1 (Figure 2B). The EMSA results indicate that AtMYB2 binding requires the AAC-core sequence of both MBS-1 and MBS-2. The fact that GAMYB did not interact with MBS-1 and MBS-2 further suggests that both motifs are specific interaction sites for AtMYB2.

***AtMYB2* expression is induced by low oxygen stress in roots:** We found that *AtMYB2* mRNA levels were increased significantly by low oxygen treatment, with higher induction in roots than leaves (Figure 3A). There was an average of 5.6-fold induction, with root expression levels about seven times higher than in shoots (Figure 3B). Expression peaks within 4 hr, declines by 6–8 hr, and increases again (Figure 3A). The timing of *AtMYB2* induction by hypoxia was compared to that of

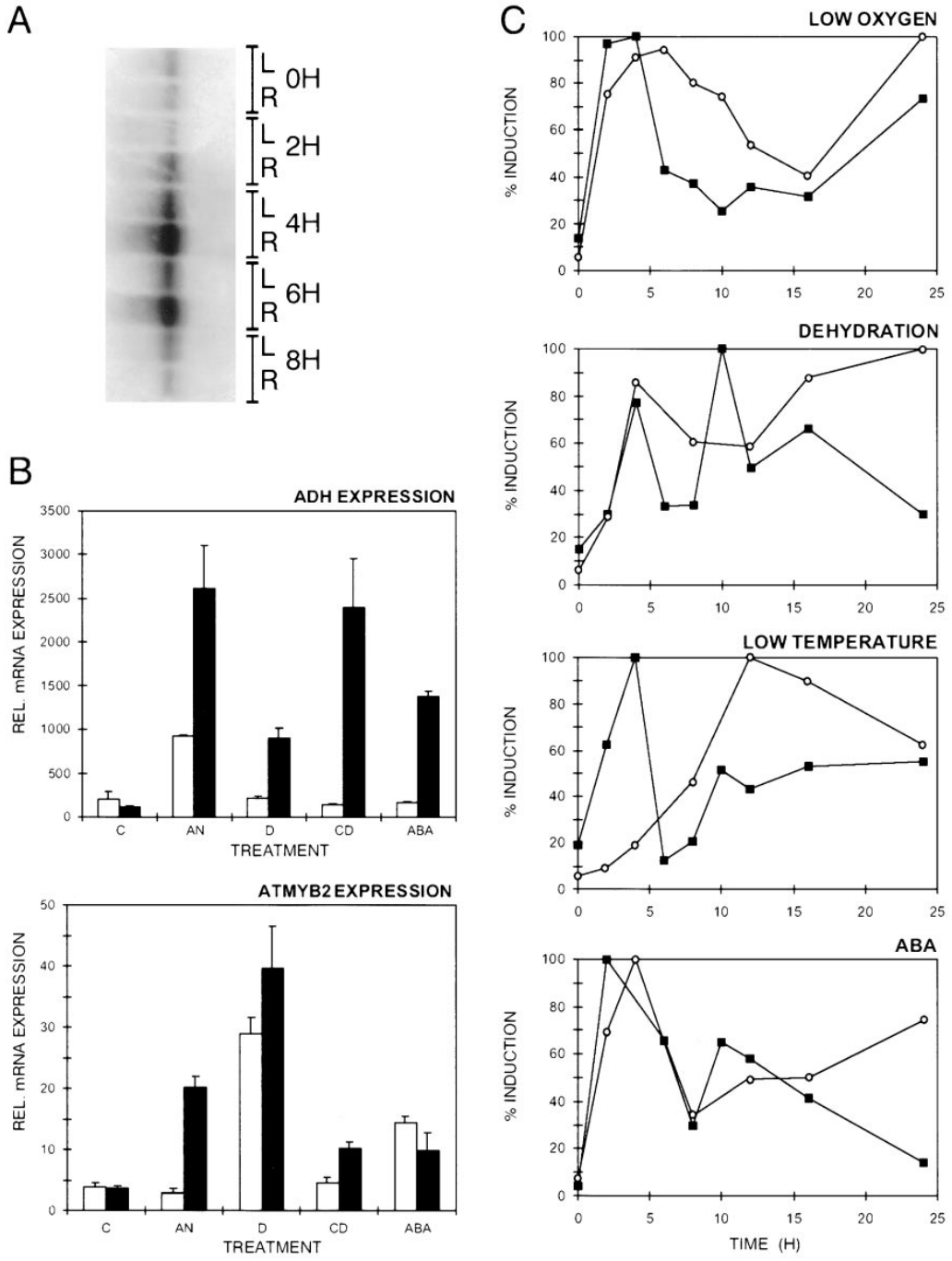


Figure 3.—Northern blot hybridization results showing the *AtMYB2* expression pattern under different imposed stresses as described in materials and methods. Ubiquitin was used as a control to standardize expression levels. (A) Northern blot showing kinetics of *AtMYB2* mRNA accumulation during low oxygen stress treatment. Accumulation of mRNA is preferentially in the roots of the plant (L, leaves; R, roots) over the 8 hours of treatment. (B) Induction of *AtMYB2* and *ADH1* mRNA in shoots and roots by stress treatments. C, control; AN, low oxygen treatment (24 hr); D, dehydration; CD, low temperature; ABA, ABA treatment. *AtMYB2* is induced by all stress treatments, and expression is higher in roots (■) than in shoots (□). Error bars represent standard errors for three repeats. (C) Induction kinetics of *AtMYB2* mRNA (■), compared to *ADH1* mRNA accumulation (○), in Arabidopsis root cultures. Results are expressed as a percentage of maximum mRNA induction obtained for each treatment (24 hr).

ADH1, using RNA extracted from Arabidopsis root cultures (Figure 3C). Induction of *ADH1* mRNA is tightly coupled to the first rise (2–4 hr) in *AtMYB2* mRNA (Figure 3C). Peak *ADH1* levels were obtained between 4 and 6 hr, followed by a decline and a second increase reaching maximal expression after 24 hr, mirroring a second rise in *AtMYB2* mRNA levels.

The induction of *AtMYB2* is also coordinated with the

TABLE 1

Presence of the GT-motif in the promoter of anaerobically induced genes of different plant species

Gene	Sequence	Position	Comments	Reference
Arabidopsis *ADH1*	5′-CAAAACCAAA-3′	−162 to −148	Functionally important	Dolferus *et al.* (1994)
Maize *Adh1*	5′-GCAAACCACG-3′	−100 to −114[a]	Functionally important	Walker *et al.* (1987)
	5′-GAAACCGGG-3′	−125 to −136[a]		Olive *et al.* (1991)
Maize Aldolase	5′-AGAAACCAGC-3′	−56 to −72[a]	Located in functionally important region	Dennis *et al.* (1988)
Pea *Adh1*	5′-ACAAACCAAA-3′	−110 to −96	Located in functionally important region	Llewellyn *et al.* (1987)
Maize *GapC4*	5′-CGAAACCAGC-3′	−300 to −285	Located in functionally important region	Köhler *et al.* (1995)
	5′-CGAAACCATC-3′	−240 to −227		
Consensus	**5′-AAACCA-3′**			
Arabidopsis *PDC1*	5′-AAAAACCACC-3′	−186 to −172[b]	Homology	Dolferus, Peacock and Dennis (unpublished results)
	5′-AGAAACCAAA-3′	−205 to −191[b]		
Arabidopsis *LDH1*	5′-AAAAACCAAA-3′	−143 to −127[b]	Homology	Dolferus, Peacock and Dennis (unpublished results)
Arabidopsis *ASUS1*	5′-TAAAACCTTG-3′	−157 to −142	Homology	
	5′-CAAAACCAAA-3′	−220 to −233[a]	Homology	Martin *et al.* (1993)
	5′-TTTAACCAAA-3′	−342 to −329		
Maize *Adh2*-N	5′-AGAAACCAGG-3′	−131 to −145[a]	Homology	Dennis *et al.* (1985)
Maize Sucrose synthase	5′-CAAAACCAGA-3′	−161 to −174[a]	Homology	Werr *et al.* (1985)
Wheat *Adh1*-A	5′-CGAAACCGGG-3′	−494 to −506[a,b]	Homology	Mitchell *et al.* (1989)
	5′-GCAAACCAAC-3′	−511 to −525[a,b]		
Cotton *Adh2*	5′-CAAAACCACG-3′	−246 to −234[b]	Homology	Millar and Dennis (1996)
	5′-TAAAACCAGA-3′	−213 to −201[b]		
Rice *Adh2*	5′-GGAAAACCACC-3′	−228 to −213	Homology	Xie and Wu (1990)
	5′-GAAAACCGGC-3′	−209 to −193		
Strawberry *Adh1*	5′-CAAAACCAAAACCAA AACCAAAAACCACT-3′	−276 to −310[a,b]	Homology 4 Tandem Repeats	Wolyn and Jelenkovic (1990)
Barley *Adh2*	5′-CACAACCAAA-3′	−155 to −171[b]	Homology	Trick *et al.* (1988)
Petunia *Adh1*	5′-CGAAACCATT-3′	−176 to −161	Homology	Gregerson *et al.* (1991)
Rice *Pdc1*	5′-CCAAACCTTG-3′	−278 to −264[a,b]	Homology	Hossain *et al.* (1996)
	5′-CCAAACCACG-3′	−277 to −264[b]		

	Position:	−3	−2	−1	A	A	C	C	+1	+2	+3
Overall Consensus Sequence	%A:	27	52	94					83	35	39
	%C:	46	14	3					0	24	25
	%G:	15	31	0					10	28	29
	%T:	12	3	3					7	14	7
		−3	−2	−1					+1	+2	+3
	Consensus:	A A	G A C	A	A	A	C	C	G A	G A	G A C
	rd22: (Abe *et al.* 1997)	T	C	T	A	A	C	C	A	C	T

The GT-motif was shown to be functionally important in the maize and Arabidopsis *ADH* genes, and is part of a functionally important region in the maize adolase and glyceraldehyde dehydrogenase genes (*GapC4*) and in the pea *Adh1* gene. These sequences share the common consensus sequence 5′-AAACCA-3′. This sequence was used to identify homologous sequences in the promoter of other anaerobically induced genes and to determine a more accurate consensus sequence. *LDH*, lactate dehydrogenase; *ASUS1*, sucrose synthase; *PDC*, pyruvate dehydrogenase.

[a] Sequence occurring in the reverse orientation.

[b] Positions starting from the ATG.

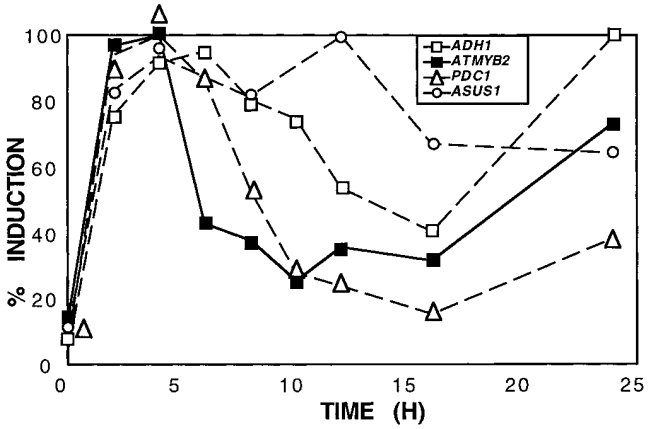


Figure 4.—Induction kinetics of *AtMYB2* mRNA under low oxygen conditions over 24 hr, compared to the kinetics of induction of *ADH1* and two other anaerobically induced genes: *PDC1*, Arabidopsis pyruvate decarboxylase; *ASUS1*, Arabidopsis sucrose synthase. The expression of these genes is root-specific (data not shown). Results are expressed as a percentage of the maximum induction of mRNA obtained for each treatment over 24 hr.

induction of other anaerobically induced Arabidopsis genes, such as the pyruvate decarboxylase (*PDC1*; Dolferus, Peacock and Dennis, unpublished results) and sucrose synthase genes (*ASUS1*; Martin *et al.* 1993). All these genes contain GT-motifs which are potential binding sites for AtMYB2 (Table 1), and they display similar induction kinetics following low oxygen treatment, with peak expression levels found immediately after *AtMYB2* mRNA levels have reached a maximum (Figure 4). These experiments indicate that the timing of *AtMYB2* mRNA accumulation is tightly coupled to expression of anaerobically induced genes, supporting a role for *AtMYB2* in the induction of anaerobic proteins.

Induction of *AtMYB2* by other stresses correlates with *ADH1* induction: Maximal induction of *ADH1* occurs after 8–10 hr of dehydration stress, 20–24 hr of low temperature, and 4 hr of ABA treatment (Dolferus *et al.* 1994; De Bruxelles *et al.* 1996). *AtMYB2* is induced by all these treatments (Urao *et al.* 1993; Figure 3B). Low temperature stress, like hypoxic stress, shows root-limited *AtMYB2* induction. Dehydration and ABA treatment induce AtMYB2 in both leaves and roots, even though *ADH1* is induced predominantly in roots by these treatments (Figure 3B).

AtMYB2 mRNA accumulates following dehydration with kinetics similar to those of *ADH1* mRNA, with two peaks (4 and 10 hr) in both (Figure 3C). We also found two peaks of ABA induction of *AtMYB2* (2 and 10 hr; 24- and 15-fold induction respectively), and of *ADH1* (peaks at 4 and 24 hr; Figure 3C). Low temperature treatment resulted in transient *AtMYB2* mRNA accumulation between 2 and 6 hr (5-fold induction), with induction of *ADH1* mRNA reaching a peak level between 12 and 24 hr. These data suggest that *AtMYB2* expression is correlated both temporally and spatially with *ADH1* expression. The Arabidopsis *rab18* gene is strongly induced by dehydration and ABA in both leaves and roots (Lång and Palva 1992), but not by low oxygen stress (data not shown). *rab18* has a G-box-like element but no GT-motif, and induction kinetics following dehydration and especially ABA treatment are considerably slower than *AtMYB2* and *ADH1* (data not shown), suggesting

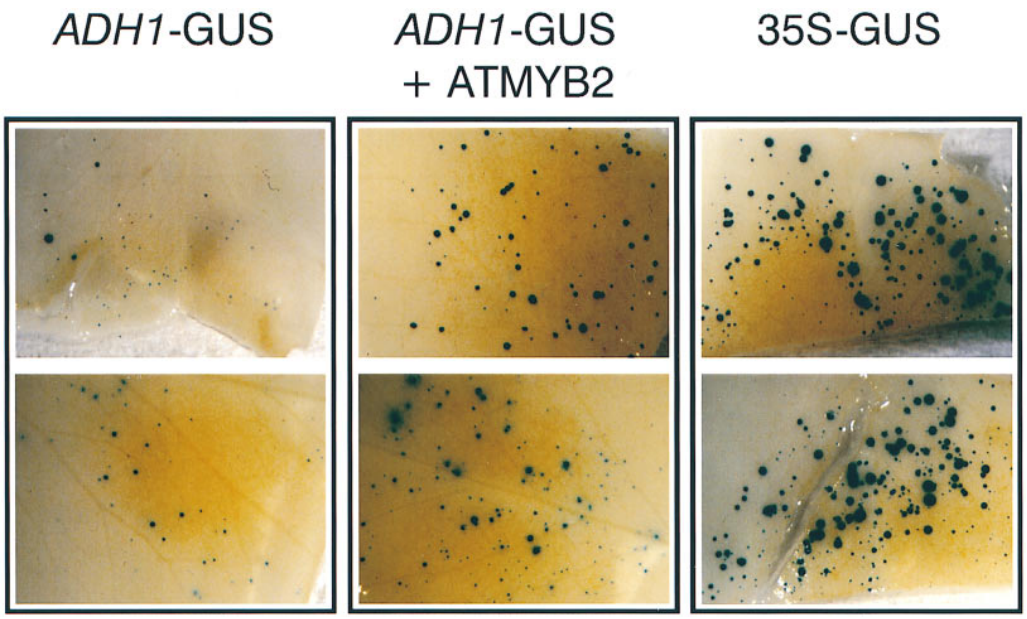


Figure 5.—Particle bombardment using 35S-GUS, *ADH1*-GUS, and 35S-AtMYB2 plasmids and pea leaves. Samples which were cobombarded on three separate occasions by *ADH1*-GUS and 35S-AtMYB2 show larger spot size than samples which were bombarded with the *ADH1*-GUS construct only. Representative leaves are shown for each experiment and construct used.

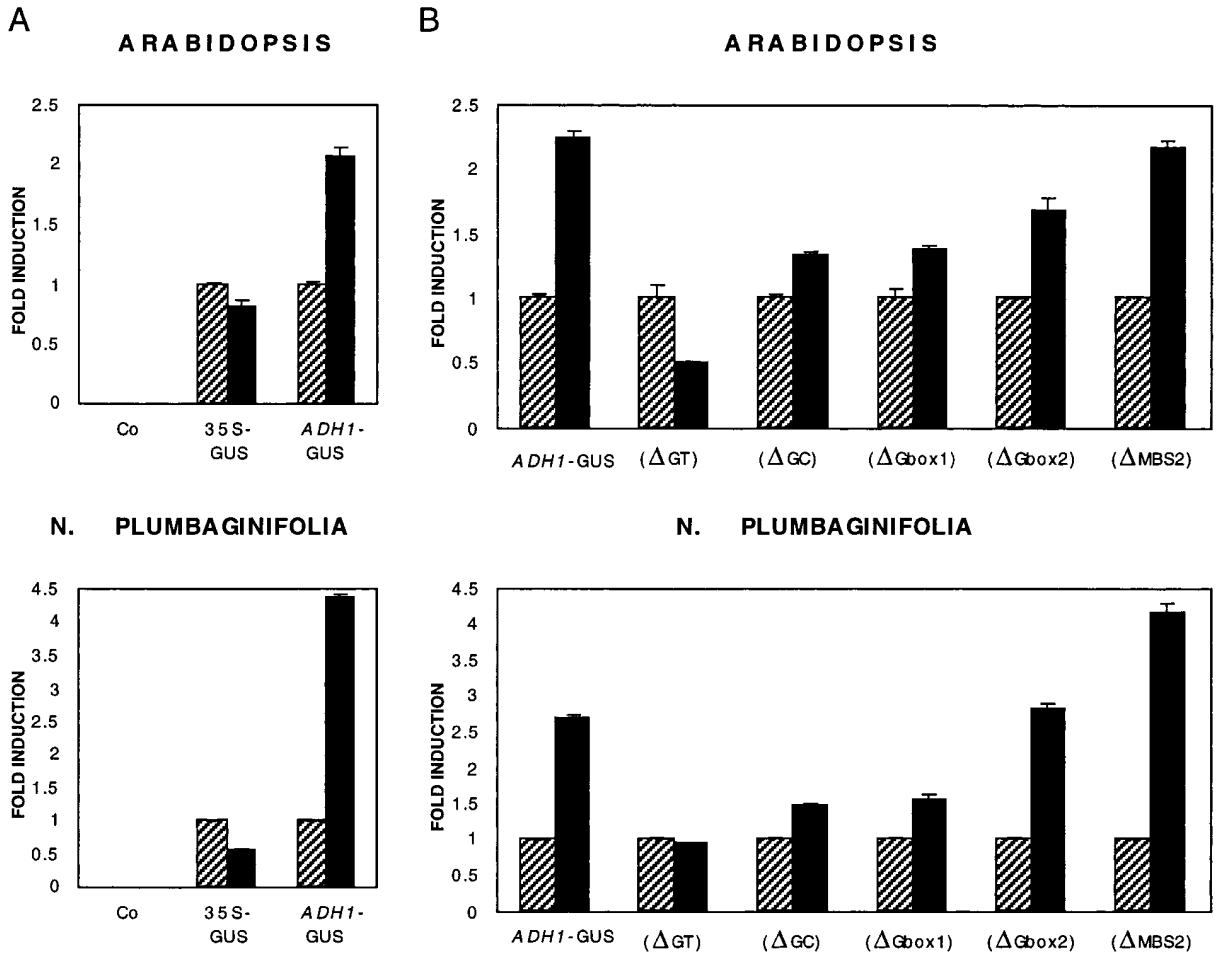


Figure 6.—Transient assays showing AtMYB2 transactivation of an *ADH1*-GUS construct in Arabidopsis mesophyll and *N. plumbaginifolia* suspension protoplasts. ▨, −35S-*ATMYB2*; ■, +35S-*AtMYB2*. All transient assays were repeated at least three times, each assay containing a repetition of each transformation. Although expression levels varied between different protoplast isolations, fold-induction values were very reproducible. Data shown are average fold-inductions over six repeats (standard errors shown as error bars). (A) Transient assays using Arabidopsis mesophyll protoplasts indicate that AtMYB2 is able to transactivate *ADH1*-promoter-driven GUS expression by a factor 2–2.5. This was confirmed also by using protoplasts of *N. plumbaginifolia* suspension cells, where higher transactivations of about 2.5–4.5-fold were consistently observed. (B) Transient assays using the substitution mutant constructs used to map the *ADH1* promoter elements (Dolferus *et al.* 1994; Figure 1B). AtMYB2 is not able to transactivate *ADH1*-GUS expression when the GT-motif (MBS-1) is mutated, but transactivation is unaffected when MBS-2 (p[ΔMBS-2]ADH-GUS) is mutated and is even increased in *N. plumbaginifolia*. Transactivation potential is reduced for the GC-motif, the G-box-1, and G-box-2 mutants.

this gene is regulated by a different set of factors (data not shown).

AtMYB2 does transactivate *ADH1*: To investigate whether *AtMYB2* could transactivate *ADH1* in the absence of hypoxia, an *ADH1*-promoter-GUS reporter construct (*ADH1*-GUS; Dolferus *et al.* 1994) was cointroduced with a 35S-promoter-*AtMYB2* construct as effector plasmid (p35S-CAtMYB2). We first used biolistics, with pea leaves as target tissue, because of the availability of this system. The *ADH1*-GUS reporter plasmid showed increased intensity and size of the blue spots only when the effector construct was present (Figure 5).

For quantitative data, we carried out transient assays in Arabidopsis mesophyll protoplasts. *AtMYB2* transactivated *ADH1* promoter activity, increasing expression by a factor of 2–2.5-fold (Figure 6A). A greater stimulation (2.5–4.5-fold) was observed in *N. plumbaginifolia* suspension cell protoplasts (Figure 6A). Transactivation was low when lower amounts of effector plasmid compared to the reporter plasmid were used (data not shown).

AtMYB2 transactivates the *ADH1* promoter via the GT-motif (MBS-1): The presence of a second potential AtMYB2 binding site, MBS-2, in the *ADH1* promoter suggested the promoter may resemble the maize *Adh1* promoter in having two functionally important GT-motifs (Figure 1). MBS-2 is in an *in vivo* footprinted segment. The area previously mutagenized (G-box-2) did not affect *ADH1* expression (Dolferus *et al.* 1994). We mutagenized the MBS-2 region (5′-TAGCAACGCC-3′), replacing the core AAC with CCG (5′-GCG

GCCGCAT-3′; p(ΔMBS-2)*ADH1*-GUS). Mutation of all the bases of the AAC core eliminates binding to AtMYB2 in EMSA assays (Figure 2B). Mutations of MBS-1 did abolish transactivation in Arabidopsis mesophyll protoplasts (Figure 6B). In contrast, we found that MBS-2 mutations increased transactivation by about 1.5-fold over wild-type levels in *N. plumbaginifolia* protoplasts. This could indicate that different factors interact with the *ADH1* promoter in suspension cells compared to the mesophyll protoplast system, or that different factors interact with the *ADH1* promoter in *N. plumbaginifolia*. Alternatively, mutation of MBS-2 could make more AtMYB2 factor available for binding to MBS-1.

Mutation of the GC-motif (Figure 1B; Dolferus *et al.* 1994) also reduced AtMYB2 transactivation of *ADH1*-GUS expression in both Arabidopsis mesophyll protoplasts and in *N. plumbaginifolia* suspension cell protoplasts (Figure 6B). These results indicate that anaerobic induction of the *ADH1* promoter requires not only AtMYB2 and the GT-motif, but also a factor binding to the GC-motif. Transactivation levels of G-Box-1 mutants (Figure 1B) were reduced in both Arabidopsis mesophyll and *N. plumbaginifolia* suspension protoplasts (Figure 6B), suggesting that the G-box binding factor may play a role in effective binding of AtMYB2. In contrast, G-Box-2 mutants (Figure 1B) in either Arabidopsis and *N. plumbaginifolia* protoplasts did not affect transactivation potential.

In transgenic plants, *ADH1*-GUS expression was decreased dramatically when mutations were introduced into the GT+GC motifs (Dolferus *et al.* 1994). Gbox1 and Gbox2 mutations had expression levels similar to the wild type construct.

Cycloheximide inhibits *ADH1* induction but increases *AtMYB2* expression: If AtMYB2 accumulation is necessary for *ADH1* expression then induction of *ADH1* mRNA would require protein synthesis. Figure 7A shows cycloheximide prevents accumulation of *ADH1* mRNA following inductive conditions, indicating that protein synthesis is required. In contrast, cycloheximide caused a 2–9-fold increase in *AtMYB2* mRNA levels for all treatments (Figure 7B). It is not clear whether this effect is at the transcriptional or post-transcriptional level, but the results do show that *AtMYB2* mRNA can be induced without prior *de novo* protein synthesis.

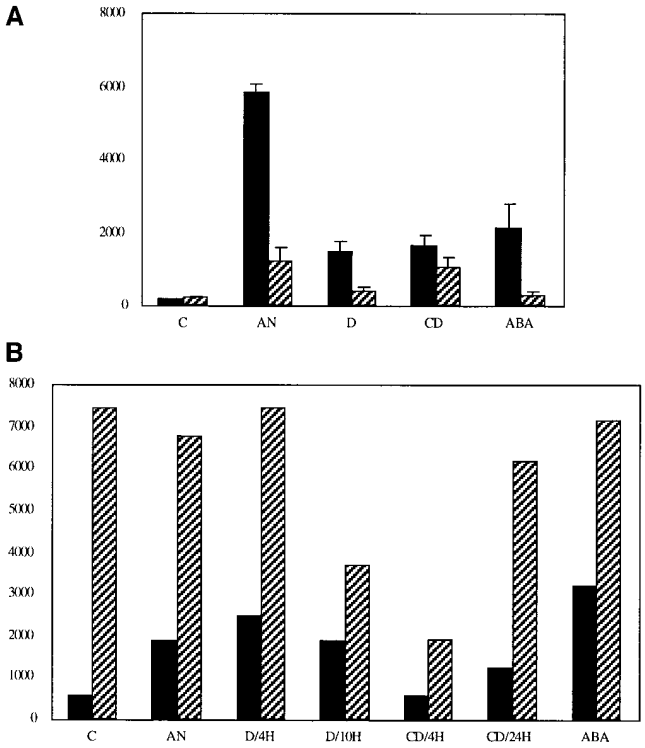


Figure 7.—Effect of cycloheximide on *AtMYB2* and *ADH1* mRNA expression levels. ■, control treatments without cycloheximide; ▨, treatments in the presence of 10 μmol cycloheximide. The scale of the Y-axis is empirical and shows relative mRNA expression levels as measured using the phosphorimager (signal strength divided by signal strength of ubiquitin mRNA expression levels). Ubiquitin mRNA expression levels were not significantly affected by the treatments (data not shown). (A) Northern blot hybridization results showing the inhibition of *ADH1* mRNA accumulation by cycloheximide in four-week-old Arabidopsis plants treated with different stresses. Abbreviations as in Figure 3. (B) RT-PCR combined with Southern blot hybridization was used to study the effect of cycloheximide on *AtMYB2* expression during stress and ABA treatment. RNA from dehydration (D) and low temperature (CD) treated roots was extracted at the two time-points showing maximal induction (see Figure 3C).

DISCUSSION

Our results suggest that AtMYB2 is a key transcription factor in stress-induced *ADH1* gene expression. AtMYB2 binds to two sites in the Arabidopsis *ADH1* promoter, the MBS-1 and MBS-2 motifs. The binding is specific to AtMYB2; neither of two other plant Myb factors, GAMYB (Gubler *et al.* 1995) or AtMYB1 (Urao *et al.* 1993) binds to the motifs. Mutations in MBS-1 eliminate both binding of AtMYB2 and *ADH1* expression, indicating that the binding is critical for *ADH1* expression. Mutation of MBS-2 reduces AtMYB2 binding but does not alter *ADH1* expression (Figure 6), showing that binding of AtMYB2 to MBS-2 does not make any functional contribution to *ADH1* expression.

AtMYB2 has tissue and temporal expression patterns compatible with the proposed role as key regulator of *ADH1* transcription. The tissue-specificity of *AtMYB2*-GUS expression (Urao *et al.* 1993) is similar to the pattern observed for *ADH1*-GUS constructs (Dolferus *et al.* 1994). *AtMYB2* mRNA begins to accumulate soon after the initiation of low oxygen treatment, preceding *ADH1* mRNA accumulation. Other anaerobically induced genes such as *PDC1* and sucrose synthase (*ASus1*) show a similar temporal relationship to *AtMYB2* induction (Figure 4). AtMYB2 may well be a key transcription factor in the regulation of *ADH1* during other environmental stresses, since a similar relationship exists be-

TABLE 2

Comparison of the known recognition sequences of plant Mybs

Name	Recognition sequence	Function	Reference
Vertebrate Myb	[T/C]AACTGG	Proliferation of hematopoietic cells	Lüscher and Eisenman (1990)
Barley *GAMYB*	TAACAAAA	GA-induced gene regulation	Gubler *et al.* (1995)
Maize *P-Myb*	CC[T/A]ACC	Flavanoid biosynthesis	Grotewold *et al.* (1994)
Maize *C1*	TAACTG	Anthocyanin biosynthesis	Roth *et al.* (1991)
Petunia *Myb.Ph3*	AAAC[GC]GTTA TAACTAACT	Flavanoid biosynthesis	Solano *et al.* (1995)
Antirrhinum *Myb305*	CCTACC	Phenylpropanoid biosynthesis	Sablowski *et al.* (1994)
Potato Myb *St1*	TATCC	Function unknown; root-specific expression	Baranowskij *et al.* (1994)
Arabidopsis *CCA1*	AA[A/C]AATCT	Light/phytochrome-induced gene expression	Wang *et al.* (1997)
Arabidopsis *AtMYB2*	AAACCA	Stress-induced gene regulation	This work

tween the induction of *AtMYB2* and *ADH1* mRNA for low temperature stress, dehydration and exogenous application of ABA. This suggestion is consistent with our previous findings that the GT-motif is necessary for all these responses (Dolferus *et al.* 1994; De Bruxelles *et al.* 1996).

The low temperature, dehydration and ABA responses also require the G-box-1 sequence (Dolferus *et al.* 1994; de Bruxelles *et al.* 1996), suggesting that these responses involve a transcription factor which binds to the G-Box-1. AtMYB2 appears to be a transcription factor needed in all stress responses, but interacts with other factors which may differ with the different stress conditions.

Our finding that the induction of transcription and subsequent translation of *ADH1* by low oxygen is sensitive to cycloheximide implies that protein synthesis is required for the operation of this response. On the other hand, cycloheximide does not inhibit *AtMYB2* induction, but actually increases it. We conclude that induction of *AtMYB2* may be the initial response, and that its synthesis is required for the induction of *ADH1*, and probably for the other anaerobic polypeptides. Transition proteins are synthesized before the induction of the anaerobic proteins (Sachs *et al.* 1980), and their molecular weight (33 kD) is similar to the mass of AtMYB2 (27.5 kD). It is possible that AtMYB2 is one of the transition proteins. Independence to cycloheximide may be a feature of transcription factors involved in switching on a coordinate response, and other transcription factors and signal transduction components, such as the maize cold-inducible leucine-zipper transcription factor m*Lip15*, the calcium-dependent protein kinase *ZmCDPK1* (Berberich and Kusano 1997), and *HVA22* (Shen *et al.* 1993) show cycloheximide insensitivity. Neither the GT- nor the GC-motifs are found in the promoter of the *AtMYB2* gene, suggesting that *AtMYB2* expression is not subject to autoregulation, again suggesting that AtMYB2 is the initial step in the response.

The transient expression experiment with cotransfection of 35S-*AtMYB2* and *ADH1*-GUS also showed that AtMYB2 is a key transcription factor for the *ADH1* promoter. While transactivation levels in protoplasts (2–3-fold) were lower than induction levels observed in roots following low oxygen treatment (5–10-fold at protein level; 20–50-fold at mRNA level), they were of the same magnitude as those observed in *N. plumbaginifolia* suspension protoplasts following low oxygen treatment (Llewellyn *et al.* 1987).

In plants, *AtMYB2* expression under low oxygen conditions is confined to the roots. Following dehydration stress or ABA treatment *AtMYB2* mRNA is induced both in leaves and roots, as is *ADH1* (Figure 3B), paralleling the increase in ABA levels in these two tissues (De Bruxelles *et al.* 1996). *ADH1* expression remains root-specific, which suggests that another transcription factor needed for *ADH1* expression is not present in the leaves. In the ABA response of *ADH1* the factor binding to the G-Box-1 may interact with *AtMYB2*. In the barley *HVA22* promoter, the ABA response is dependent on the G-Box and other motifs (Shen *et al.* 1993, 1996; Shen and Ho 1995). Recently, AtMYB2 was proposed to act in conjunction with a Myc-related transcription factor in the drought- and ABA-regulated *rd22* gene (Abe *et al.* 1997). *ADH1* does not have an obvious Myc recognition site, suggesting that in this promoter AtMYB2 must interact with other classes of transcription factors.

Our mutation analysis has shown that AtMYB2 proba-

bly also requires association with the protein binding to the GC-motif (Figure 6; possibly the Arabidopsis homologue of GCBP-1; Olive *et al.* 1991b). In the maize *Adh1* promoter both GT-motifs are closely linked to the GC-motifs. In Arabidopsis the lack of a GC-motif close to the MBS-2 site could explain why this site is not critical for *ADH1* induction. Vertebrate and yeast Myb transcription factors commonly activate transcription in close association with other factors and work in a synergistic manner with these factors (Tice-Baldwin *et al.* 1989; Burk *et al.* 1993). In plants, the maize C1 Myb interacts directly with the B protein (basic helix-loop-helix factor) on the maize *Bronze-1* promoter and activates transcription in a cooperative way (Goff *et al.* 1992).

The GT-motif is present in all anaerobically induced genes (Table 1), and is usually located between positions −300 and −100 relative to the start of transcription. The consensus sequence is 5′-AAACCA-3′. Depending on whether or not a GC-motif is next to the GT-motif, the consensus sequence can be extended to 5′-AAACCAAA-3′ or 5′-AAACCG[G/C][G/C]-3′ respectively (Table 1). The core AtMYB2 recognition sequence in the *rd22* promoter (5′-TAACCA-3′) is similar to the GT-motif (Abe *et al.* 1997; Table 1). The preference for a 5′-[A]AACC[A]-3′ core binding site differentiates AtMYB2 from other known plant Mybs (Table 2). The second AtMYB2 binding site in the *ADH1* promoter (MBS-2; 5′-CAACGCC-3′) is quite different from the GT-motif (MBS-1), and could explain why binding of AtMYB2 to MBS-2 is not functional.

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