Plant Physiol. (1996) 111: 381–391

Abscisic Acid Induces the Alcohol Dehydrogenase Gene in Arabidopsis

Guy L. de Bruxelles, W. James Peacock, Elizabeth S. Dennis, and Rudy Dolferus*

Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry, G.P.O. Box 1600, Canberra, ACT 2601, Australia (G.L.d.B., W.J.P., E.S.D., R.D.); and Cooperative Research Centre for Plant Science, G.P.O. Box 475, Canberra, ACT 2601, Australia (G.L.d.B., R.D.)

Exogenous abscisic acid (ABA) induced the alcohol dehydrogenase gene (*Adh*) in Arabidopsis roots. Both the G-box-1 element and the GT/GC motifs (anaerobic response element) were required for *Adh* inducibility. Measurement of endogenous ABA levels during stress treatment showed that ABA levels increased during dehydration treatment but not following exposure to either hypoxia or low temperature. Arabidopsis ABA mutants (*aba1* and *abi2*) displayed reduced *Adh* mRNA induction levels following either dehydration treatment or exogenous application of ABA. Low-oxygen response was slightly increased in the *aba1* mutant but was unchanged in *abi2*. Low-temperature response was unaffected in both *aba1* and *abi2* mutants. Our results indicate that, although induction of the *Adh* gene by ABA, dehydration, and low temperature required the same *cis*-acting promoter elements, their regulatory pathways were at least partially separated in a combined dehydration/ABA pathway and an ABA-independent low-temperature pathway. These pathways were in turn independent of a third signal transduction pathway leading to low-oxygen response, which did not involve either ABA or the G-box-1 promoter element.

Plants respond to environmental stresses through a series of adaptive physiological and biochemical changes that enhance their ability to survive the adverse conditions. Many of these adaptive changes involve de novo synthesis of "stress" proteins (see Wray, 1992). The alcohol dehydrogenase gene (*Adh*) from *Arabidopsis thaliana* is induced by a number of environmental stresses, including low oxygen, dehydration, and low temperature (Dolferus et al., 1994), although its biochemical role is apparent only under low-oxygen conditions. Promoter elements involved in the responses to these three environmental stimuli have been mapped (Dolferus et al., 1994), and one element, consisting of a GT motif and a linked GC motif (between positions −160 and −140), was shown to be crucial for expression under all three stress conditions. This GT/GC motif is similar to the maize ARE (Olive et al., 1991a, 1991b), but the dicot ARE equivalent might be structurally different. In Arabidopsis the ARE-like element consists of only one copy of the GT/GC motif, compared to the two present in the maize *Adh1* promoter. In addition, the pea *Adh* and the Arabidopsis *Adh* promoters contain the GT motif in a reverse orientation compared to the maize ARE element (Olive et al., 1991b). The maize *Adh1* ARE element does not function in dicot transgenic tobacco plants without the addition of upstream enhancer sequences (Ellis et al., 1987). In Arabidopsis it is clear that this ARE-like motif also plays a more general role in the response to environmental stresses.

Low-temperature and dehydration induction of *Adh* require the G-box-1 sequence (−216 to −219: 5′-CCACGTGG-3′) in addition to the GT/GC motif, whereas low-oxygen induction requires only the GT/GC motif (Dolferus et al., 1994). This indicates that at least two partially overlapping signal transduction pathways are involved in the induction of the *Adh* promoter by environmental stress. G-box elements contain the core sequence 5′-ACGT-3′, a binding motif for Leu zipper proteins (Schindler et al., 1992a), and are functional in a range of plant gene promoters, primarily those regulated by UV/visible light (Schulze-Lefert et al., 1989) or by ABA, as in the wheat *Em* promoter (Marcotte et al., 1989; Guiltinan et al., 1990) and the rice *rab16* promoter (Mundy et al., 1990). The ABREs from several ABA-responsive genes have been characterized and shown to be G-box-like elements (Vilardell et al., 1991; Michel et al., 1993; Straub et al., 1994). The *Adh* G-box-1 element is identical to the ABRE identified in the *Em* gene. It has previously been observed that, because many non-ABA-responsive promoters also contain G-boxes, the presence of an ABRE or G-box on its own is not enough to confer full ABA responsiveness. Shen and Ho (1995) have recently shown that the promoter of the barley *HVA22* gene contains two ABA response complexes, each containing an ABRE and a novel *cis*-acting element, CE-1, which has a central 5′-CACC-3′ core. In the promoters of most ABA-responsive genes, this central motif is present and located near the ABRE.

ABA has been implicated in responses to environmental stresses such as drought, salinity, cold acclimation, freezing tolerance, flooding, and wounding (Zeevaart and Creelman, 1988; Guy, 1990; Hetherington and Quatrano, 1991; Jackson, 1991; Sánchez-Serrano et al., 1991). This prompted us to investigate whether ABA affects *Adh* gene expression in Arabidopsis and whether it is involved in

* Corresponding author; e-mail rudy@pican.pi.csiro.au; fax 61–6–246–5000.

Abbreviations: ABRE, ABA response elements; *Adh*, alcohol dehydrogenase gene; ARE, anaerobic responsive element; CADH, complete Adh promoter (−964 to +53); *Ler*, Landsberg *erecta*; MS, Murashige and Skoog.

either the low-temperature or dehydration signal transduction pathway(s). Endogenous ABA has been shown to increase in response to dehydration (Zeevaart and Creelman, 1988), suggesting a role for this plant hormone in the dehydration signal transduction pathway. Although the regulation of a number of dehydration-induced genes has been shown to require ABA, ABA does not appear to be the sole mediator of dehydration-induced gene expression, because some dehydration-induced genes are not induced by ABA (Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992; Yamaguchi-Shinozaki and Shinozaki, 1993a, 1993b). Gosti et al. (1995) recently found that endogenous ABA played a role in the regulation of only two of four Arabidopsis clones that were inducible by progressive drought. Similarly, only some cold-induced genes are also inducible by ABA (Lång et al., 1989; Kurkela and Franck, 1990; Gilmour and Thomashow, 1991; Nordin et al., 1991; Cattivelli and Bartels, 1992; Guo et al., 1992; Lång and Palva, 1992; Jarillo et al., 1993), again suggesting that several signal pathways may be operating.

Our results show that in Arabidopsis roots the *Adh* gene is inducible by ABA. The induction of the *Adh* promoter by ABA required the G-box-1 element, which functioned as an ABRE. In addition, the ARE, consisting of the GT/GC motifs, was also essential for ABA induction. We also asked whether ABA is involved in *Adh* induction by environmental stress. Measurement of endogenous ABA levels during environmental stress and analysis of the response in four ABA mutants indicated that *Adh* induction by dehydration was at least in part dependent on the action of ABA, whereas induction by low temperature and low oxygen appeared to be independent of ABA. The *cis*-acting promoter elements essential for ABA induction were the same as those required for response to both dehydration and low-temperature stresses.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Hormone and Stress Treatments

Arabidopsis thaliana ecotype C24 seeds were surface sterilized and sown onto MS medium containing 3% Suc and 0.8% agar. They were then incubated overnight at 4°C, to break seed dormancy before being incubated at 22°C (16-/8-h light/dark cycle, 100 μE s^{-1} m^{-2}) for 4 weeks. During this time the medium was changed once; there were 30 to 40 plantlets per plate. Arabidopsis ABA mutant seeds (*aba1*, *abi1*, *abi2*, and *abi3*) were germinated on MS plates in the presence of 10^{-5} M ABA; after 2 weeks, germinated green seedlings were transferred to fresh MS plates without the hormone and grown for another 2 weeks before being used in experiments. For the experimental treatments, 4-week-old plantlets (30–40) were transferred to Petri dishes containing 12 mL of liquid MS medium 24 h prior to the beginning of the treatment. Environmental stress treatments were as described previously (Dolferus et al., 1994). The low-oxygen treatment was carried out for 24 h in 5% O_2/N_2 in the dark. Low-temperature treatment consisted of plantlets being placed on a shaker at 4°C for 24 h. Dehydration treatment consisted of subjecting plantlets to osmotic stress using liquid MS medium containing 0.6 M mannitol for 24 h at 22°C (16-/8-h light/dark cycle). This treatment is more reliable and reproducible than other dehydration methods, and it resulted in the rapid wilting of the plantlets. ABA [(±)*cis-trans* isomers, Sigma] and GA_3 (Sigma) were dissolved in either double-distilled H_2O or DMSO and added to the MS medium. Higher induction levels were obtained when ABA was applied using DMSO instead of water as a solvent. DMSO alone did not affect *Adh* promoter activity (data not shown). The higher induction was probably due to improved uptake of the hormone into the plant material when dissolved in DMSO. Induction with 0.1 mM ABA (in DMSO) for 4 h was chosen as the standard, except where stated otherwise. Hormone-treated and control plates were incubated on a shaking platform (70–80 rpm) in the growth chamber.

Four ABA mutants obtained from the Nottingham Arabidopsis seed collection (Nottingham, UK) were used in the current study: an ABA-deficient (*aba1*) and three ABA-insensitive (*abi1*, *abi2*, and *abi3*) mutants (Koornneef et al., 1982, 1984). The Arabidopsis ABA-deficient mutant *aba1* has been shown to have significantly lower ABA levels than the wild type (Koornneef et al., 1982; Rock and Zeevaart, 1991). The ABA-insensitive mutants contain normal levels of endogenous ABA, but the sensitivity of the tissues to the hormone is reduced 5 to 20 times (Koornneef et al., 1984; Zeevaart and Creelman, 1988; Finkelstein and Somerville, 1990). These ABA mutants were generated in the ecotype *Ler* background (Koornneef et al., 1982, 1984), whereas the promoter mutants used in the current study were in the C24 background (Dolferus et al., 1994). To validate comparison between the ABA mutants in *Ler* and the *Adh* promoter constructs in C24, we measured endogenous ABA content in both ecotypes and found them to be similar under both unstressed and stressed conditions. The observed response of the *Adh* gene to dehydration and ABA was the same in the *Ler* ecotype and the C24 ecotype (see "Results"). The response of the ABA mutants compared to the *Ler* wild type was analyzed statistically by paired *t* test according to the method described by Mansfield (1986). Results of the *t* test are indicated as *t* values, followed by * (significant to the 5% level) or N.S. (not significant).

RNA Extractions and Northern Blot Procedures

RNA analyses were carried out on segregating populations of T_2 or T_3 plants. RNA extraction from 4-week-old whole plantlets, gel electrophoresis, northern blotting, antisense RNA probe preparation, and filter washing procedures were as described previously (Dolferus et al., 1994). Following hybridization using *Adh*, GUS, and ubiquitin antisense RNA probes, the filters were placed on phosphor screens and the hybridization signals were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Analysis of Northern Blot Hybridization Results

Adh mRNA and *Adh*-promoter-driven GUS mRNA expression levels were analyzed after induction by ABA.

Where possible, five independent transformants of each promoter construct were analyzed. During the initial characterization of the transformants, those with low levels of expression, presumably due to positional effects, were excluded from further analysis (Dolferus et al., 1994). In the current study the effect of each modified promoter construct was analyzed by comparing the expression level of the GUS mRNA with that of the endogenous *Adh* mRNA, which was used as an internal control for the ABA induction conditions. Transformants showing less than wild-type levels of induction of endogenous *Adh* mRNA (<5-fold) were excluded from subsequent analysis.

RNA gels were stained with ethidium bromide, which allowed the uniformity of loading between wells to be visually assessed. In addition, Arabidopsis ubiquitin mRNA expression levels were not affected by ABA treatment and were used to standardize both endogenous *Adh* mRNA and GUS mRNA expression levels. After this correction, relative GUS mRNA expression levels were calculated by dividing GUS mRNA expression levels by their respective endogenous *Adh* mRNA levels.

GUS Histochemical and Fluorometric Assays

GUS fluorometric and histochemical assays were carried out as described by Jefferson (1987). Fluorometric assays and protein assays were carried out in microwell plates and analyzed using Labsystems Fluoroskan II and Multiskan Plus readers in conjunction with Delta Soft II software (Biometallics, Princeton, NJ) as described by Breyne et al. (1993). The total protein concentration in the extracts was determined using a Bio-Rad protein assay kit (Bradford, 1976). BSA was used as a standard.

ABA Assay

(*S*)-(+)-ABA (referred to as ABA) was measured by immunoassay. Following treatment, the plant tissue was snap frozen in liquid nitrogen and stored at −70°C. ABA was then extracted as described by Loveys and van Dijk (1988) and assayed in triplicate by an indirect ELISA method (Walker-Simmons and Sessing, 1987) using a monoclonal antibody obtained from Idetek (San Bruno, CA). Recoveries were calculated from the initial addition of trace amounts of [^{3}H]ABA, and ABA content was expressed as ng/g fresh weight.

RESULTS

Exogenous ABA Induces *Adh* Gene Expression in Arabidopsis Roots

The Arabidopsis *Adh* gene was induced in 4-week-old Arabidopsis plants by exogenous ABA (Fig. 1A). Concentrations of 1, 0.1, 0.01, and 0.001 mM (not shown) ABA all increased *Adh* mRNA levels, with maximum induction occurring with 0.1 mM (Fig. 1B). The maximal steady-state *Adh* mRNA level occurred 4 h after addition of 0.1 mM ABA to the medium. The mRNA level then returned gradually to the uninduced base level by 48 h. The decrease in *Adh* mRNA following the 4-h peak appeared to be due to the

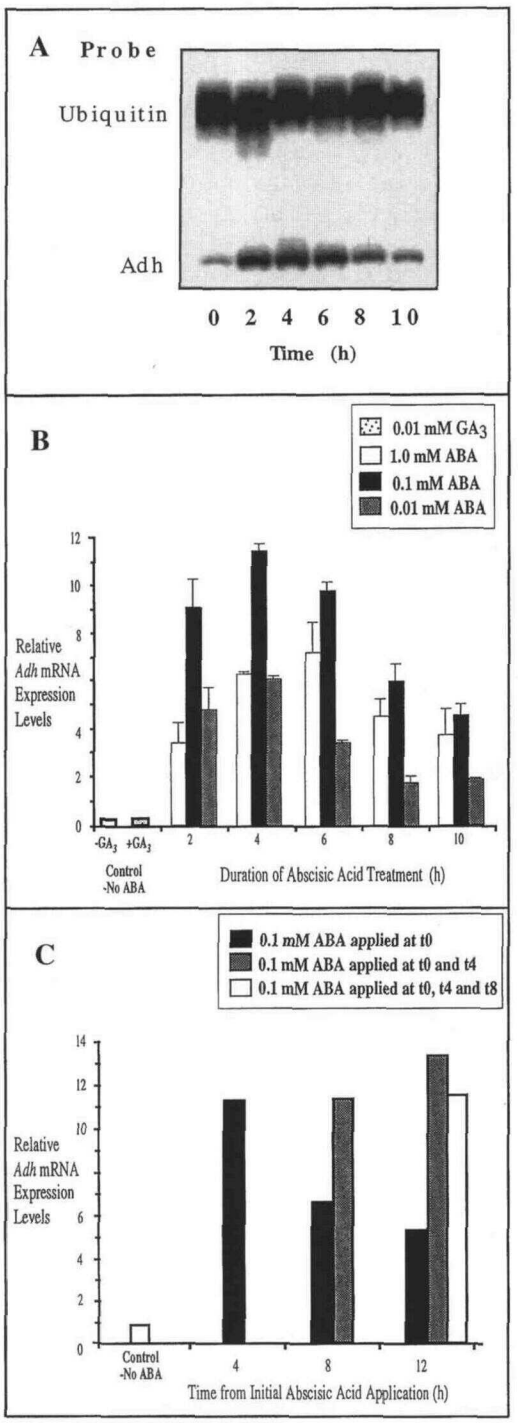

Figure 1. Induction of the Arabidopsis *Adh* promoter by ABA. Four-week-old seedlings were treated with ABA using DMSO as solvent. Total RNA from whole seedlings was extracted and used for northern blot hybridizations with *Adh* and ubiquitin RNA probes. Relative *Adh* mRNA expression levels were determined using ubiquitin as a standard. A, Northern blot hybidization showing the time course of *Adh* mRNA induction by 0.1 mM ABA (in DMSO). B, Induction profiles of the *Adh* mRNA using different concentrations of ABA. GA_3 did not influence *Adh* gene expression, nor did it influence induction by ABA. Mean and SE values of two separate inductions are shown. C, Multiple ABA applications (0.1 mM) restored maximal expression levels of the *Adh* promoter. tx, Time × h.

turnover of the hormone, because the high level of *Adh* expression could be restored by further addition of ABA 4 and 8 h after the start of the treatment (Fig. 1C). However, the expression level could not be increased beyond the maximum observed at 4 h with 0.1 mM ABA, indicating that this level of ABA was sufficient to saturate the response mechanism. The level of mRNA induced by exogenous ABA was comparable to that induced by either dehydration or low temperature but was approximately half that recorded in low-oxygen induction (Dolferus et al., 1994). This suggests that there are two mechanisms for the induction of the *Adh* gene and that ABA falls into the dehydration and low-temperature class.

The effect of ABA is known to be modified by other plant hormones. For example, ABA and GA_3 act antagonistically in the induction of the α-amylase gene in barley aleurone cells (Jacobsen, 1983). We investigated whether GA_3 is involved in the regulation of *Adh* and found that it had no effect (Fig. 1B), nor did GA_3 affect the extent of *Adh* induction by ABA (data not shown).

To determine in which tissues ABA induction of the *Adh* gene occurred, we used Arabidopsis seedlings transformed with a transgene consisting of the *Adh* promoter (−964 to +53) linked to the GUS reporter gene (CADH-GUS). GUS mRNA was induced by ABA in a time similar to that of the endogenous *Adh* gene. ADH enzyme activity increased 3-fold in Arabidopsis seedling roots treated for 8 h with 0.1 mM ABA; this activity began decreasing 12 h after ABA application (data not shown). In roots from CADH-GUS-transformed seedlings, there were similar increases in GUS enzyme activity following ABA application. GUS activity was detectable histochemically only in the roots and not in aerial tissues (Fig. 2, A and B). The root cap, meristem, and terminal stelar regions showed the strongest GUS staining, although low levels of GUS activity were detectable throughout the entire root. This pattern of expression is similar to that of endogenous ADH activity following induction by ABA and shows that the CADH-GUS promoter contains all of the elements required for conferring ABA-induced GUS activity that parallels, spatially, that of endogenous ADH.

Dehydration Treatment Results in Increased Endogenous ABA Levels

We found that the endogenous content of ABA increased 10-fold in wild-type C24 roots following a 24-h dehydration treatment (Fig. 3A). In the same period, a 2- to 5-fold increase in ABA was recorded in the shoots of dehydrated wild-type C24 plants (Fig. 3B). Although dehydration treatment increased endogenous ABA levels in the aerial tissues of the plants as well as in the roots (Fig. 3C), the *Adh* promoter was not activated in shoot tissue. Likewise, 4 h after the application of ABA to the root medium, endogenous ABA content in the shoots increased 20- to 40-fold but did not induce the *Adh* gene in these tissues (data not shown).

The level of endogenous ABA was measured in the roots and shoots of C24 seedlings over a 24-h period following the onset of the dehydration treatment (Fig. 3C). In both roots and shoots there was a steady increase in ABA levels that reached a maximum after 12 h of treatment, although the levels of ABA reached in the roots were higher than those in the shoots.

Our data show that high endogenous ABA levels are correlated with *Adh* activity in Arabidopsis roots, irrespective of whether endogenous ABA is increased by a dehydration treatment or as a result of external application of the hormone. In both experimental approaches, the fact that *Adh* induction occurred only in the roots and not in leaf or stem tissues indicates that other components of the transcription complex and/or signal transduction pathway must differ in the root and aerial tissues.

Induction of the *Adh* Gene by Dehydration Treatment Requires ABA

The leaves of unstressed, 4-week-old seedlings of the ABA-deficient mutant *aba1* contain approximately 10% of the wild-type level of endogenous ABA (Koornneef et al., 1982). Although endogenous ABA levels in the *aba1* mutant increase in response to dehydration treatment, they still remain far lower than those in the dehydrated wild type (Lång et al., 1994). We found that the level of *Adh* mRNA induction was significantly lower in the *aba1* mutant (t = 2.14, *) than in the wild type following 24 h of dehydration treatment (Fig. 4), indicating that ABA is an essential component in the regulation of the *Adh* promoter's response to dehydration. In contrast to this, exogenous ABA resulted in significantly higher levels of *Adh* mRNA in the *aba1* mutants than in the wild type (t = 2.18, *; Fig. 4), suggesting that the *aba1* mutant may have greater sensitivity to ABA than the wild type.

Induction of the *Adh* Gene by ABA Requires the ABI2 Protein

Induction of *Adh* by ABA was further studied in three ABA-insensitive mutants, *abi1*, *abi2*, and *abi3* (Koornneef et al., 1984). The *abi2* mutant showed a significant reduction in the *Adh* response to exogenous ABA compared to wild type (t = 2.13, *), whereas in the *abi1* and especially the *abi3* mutants the response was not significantly different (the respective t values are t = 2.13, N.S.; t = 2.16, N.S.; Fig. 4). Comparable results were obtained for induction by dehydration treatment. *Adh* induction levels in the *abi2* mutant were again significantly reduced compared to wild type (t = 2.08, *), whereas those in the *abi1* and *abi3* mutants were not significantly different (the respective t values are t = 2.08, N.S.; t = 2.1, N.S.; Fig. 4). *Adh* mRNA expression levels in unstressed *abi1*, *abi2*, and *abi3* seedlings were similar to those of the *Ler* wild type (the respective t values are t = 2.08, N.S.; t = 2.06, N.S.; t = 2.1, N.S.; Fig. 4). The fact that the *abi3* mutant displays wild-type response to both exogenous ABA and dehydration treatment is consistent with the observation that the effects of the *abi3* mutation are predominantly seed specific (Finkelstein and Somerville, 1990). The response of the *abi1* mutant to dehydration and ABA treatment was, in contrast to the results reported by Jarillo et

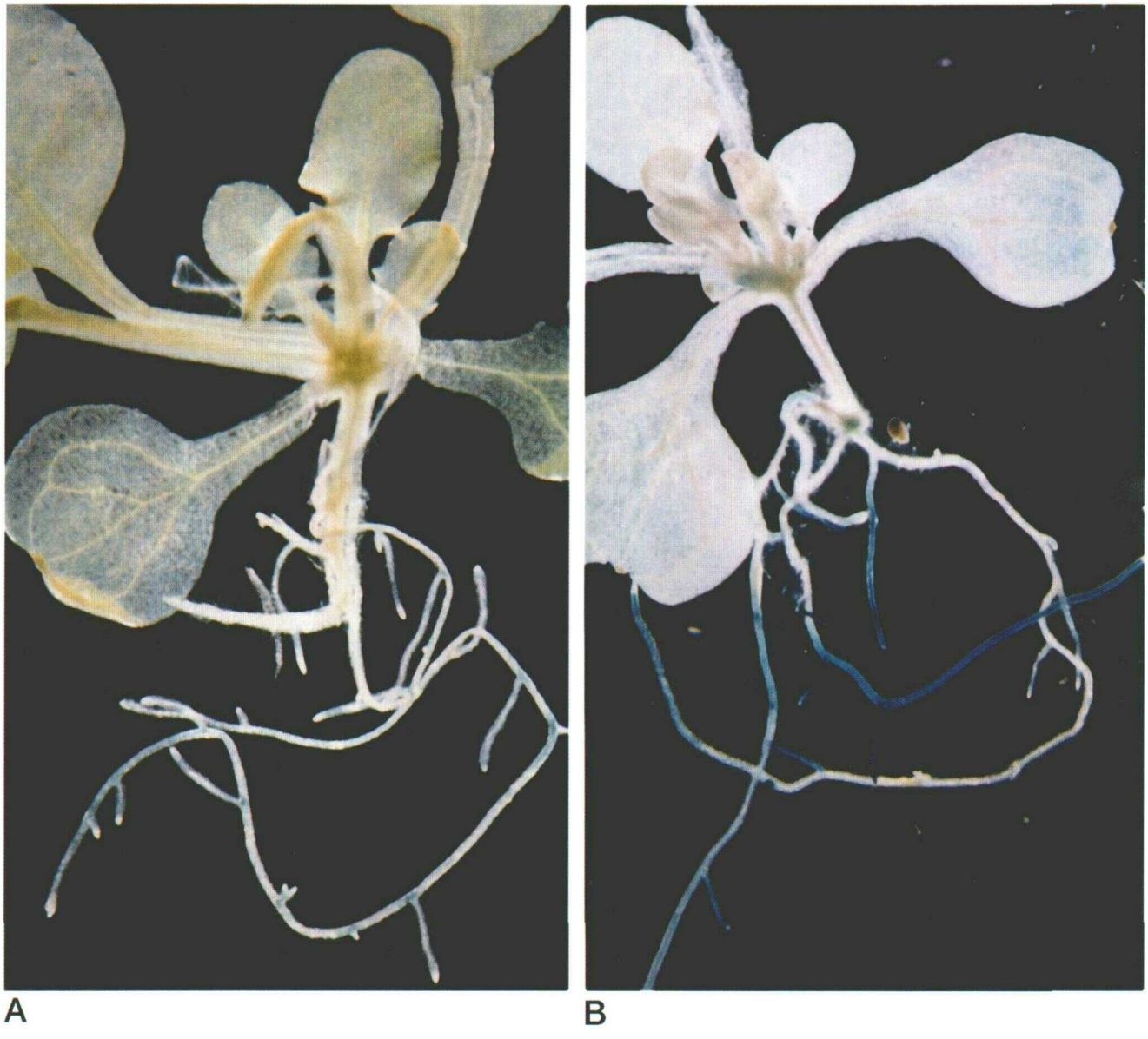

Figure 2. GUS in situ staining pattern of Arabidopsis plants transformed with the CADH-GUS construct (−964 to +53 *Adh*-promoter fragment). A, Four-week-old control uninduced plants. B, Four-week-old plants treated with 0.1 mM ABA for 8 h prior to GUS staining.

al. (1993), only marginally lower and statistically not different from that of the wild type. Our results indicate that the *abi2* protein is involved in the signal transduction pathway regulating the response of the *Adh* gene to increasing ABA concentrations from either endogenous or exogenous sources.

The GT/GC and the G-box-1 Motifs Are Required for ABA Induction

Deletions from the 5′ end and substitution mutants of the *Adh* promoter, linked to the GUS reporter gene (Fig. 5A), identified regions of the *Adh* promoter that were critical for the response to increased ABA content. Deletions from the 5′ end were generated starting from the CADH promoter fragment (−964 to +53), which was shown to confer expression to a reporter gene similar to the endogenous *Adh* gene. The promoter deletion extending to −510 did not change the level of *Adh* induction (Fig. 5B). The subsequent deletions to −384, −309, and −235 showed step-wise reductions in the level of GUS activity in response to exogenous ABA (Fig. 5B), indicating that the region between −510 and −235 contains motifs that contribute to the quantitative level of gene induction. Further deletion to −214 showed a restoration of mRNA induction levels to those of the full-length promoter (Fig. 5B). The simplest explanation for this is that a repressor binding site has been removed. However, this deletion removes two CC bases from the 5′

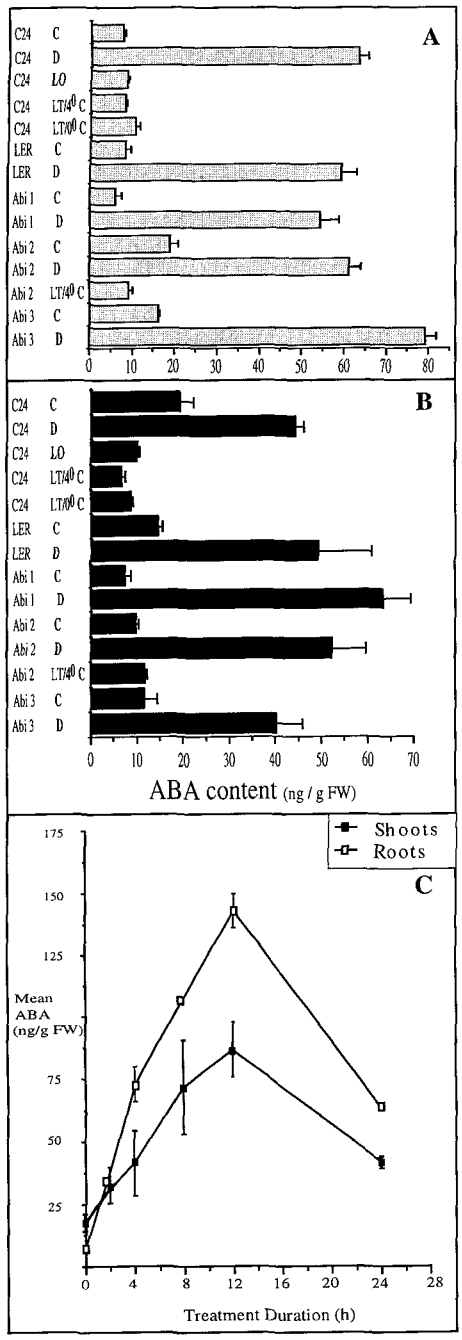

Figure 3. Determination of the endogenous ABA content of roots (A) and shoots (B) of 4-week-old *Ler* and C24 seedlings and the Arabidopsis ABA mutants *abi1*, *abi2*, and *abi3* following exposure to different stress treatments: uninduced control (C), low-oxygen (LO), dehydration (D), and low-temperature treatment at 0°C (LT/0°C) and 4°C (LT/4°C). ABA was assayed in triplicate by an indirect ELISA method as described in "Materials and Methods." Recoveries were calculated from the initial addition of trace amounts of [^{3}H]ABA, and ABA content is expressed as ng/g fresh weight (FW). Data represent the mean and SE values of two replicates per treatment. C, Time course showing the effect of dehydration treatment on the endogenous ABA content of shoots and roots of 4-week-old C24 wild-type plants. ABA content is expressed as ng/g fresh weight (FW). Data represent the mean and SE values of two replicates per treatment. All assays were performed in triplicate.

end of the G-box-1 sequence, replacing them by CG residues from the cloning vector (5′-CCACGTGG-3′ is changed to 5′-CGACGTGG-3′). The two bases flanking each end of the central ACGT core confer binding specificity for different classes of G-box-binding proteins (Schindler et al., 1992a, 1992b; Williams et al., 1992; Izawa et al., 1993). It is possible that this alteration to the G-box-1 motif in deletion −214 changes the behavior or identity of the G-box-binding protein, resulting in enhanced expression.

The importance of the G boxes is made clear in the deletion that extends to −172, removing both the G-box-1 and G-box-2 sequences. *Adh* induction levels drop dramatically in this deletion, and further deletions to −156 and −141 reduce this response level even further. The baseline level of uninduced activity in the set of deleted promoters was not distinguishable from that of the wild-type promoter (Fig. 5B).

The 5′-deletion analysis established that at least two subregions between −575 and −235 contribute to the level of *Adh* response following ABA stimulus. The deletion between −235 and −214 showed a dramatic increase in the level of induction, indicating either that a binding site for a down-modulating protein had been removed or that the deletion had altered the specificity of the family of binding factors capable of binding to the G-box-1 motif. Further deletion past −214 essentially eliminated the *Adh* response to ABA. This region contains the G-box-1, G-box-2, GT, and GC motifs. The reduction in activity was so great that the deletion analysis did not allow us to attribute any significance to any of these motifs. Therefore, a substitution mutation analysis changing each of these motifs in the context of the full promoter was carried out.

Substitution mutations were induced in the CADH promoter background by site-specific mutagenesis at the positions shown in Figure 5A, whereas the rest of the CADH promoter sequence was left intact. Our analysis showed that the GT, GC, and G-box-1 motifs, and to a lesser extent the G-box-2 sequence, are essential for full response to increased ABA (Fig. 5C). Sequence alteration of each motif reduced the level of induction response in roots. The tissue specificity of the gene was not altered in that there was no response visible in the leaves of transgenic plants containing any of the substitution or deletion constructs (data not shown). This suggests that in leaf tissue, one or more of the transcription factors are not present or are not activated by upstream components of the signal transduction pathway.

ABA Is Not Involved in the Low-Temperature or Low-Oxygen Induction of *Adh*

In contrast to dehydration treatment, which elevated the endogenous ABA level, low-temperature and low-oxygen treatments did not result in elevated ABA levels (Fig. 3), yet they significantly induced the *Adh* gene in the root tissue only (Dolferus et al., 1994). Under our experimental conditions, endogenous ABA concentrations (measured daily) remained at unstressed levels for up to 7 d after exposure to low temperature (data not shown). The implication that the low-oxygen and low-temperature stimuli have distinct and ABA-independent signal transduction

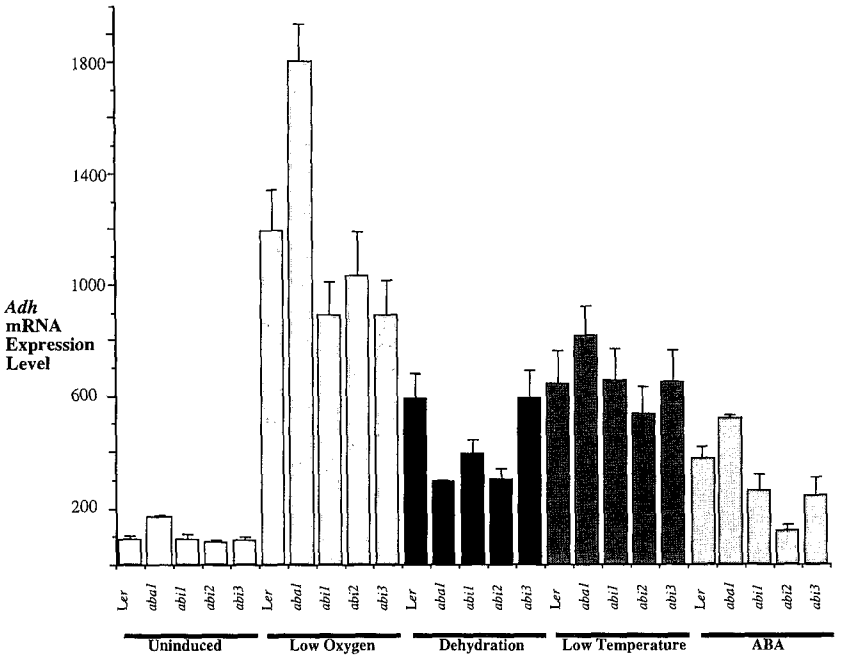

Figure 4. *Adh* mRNA levels under uninduced, low-oxygen, dehydration, low-temperature, and ABA induction conditions for wild-type *Ler*, ABA-deficient mutant *aba1*, and ABA-insensitive mutants *abi1*, *abi2*, and *abi3*. Absolute *Adh* mRNA expression levels were measured using a PhosphorImager. The plotted data were averaged (6-10 repeats) and plotted together with SE bars.

pathways is supported by analysis of the ABA-deficient and ABA-insensitive mutants. In contrast to the significant decrease in *Adh* mRNA induction observed in the *aba1* mutant following dehydration treatment, there was no reduction following either low-temperature or low-oxygen treatment (Fig. 4). The level of *Adh* mRNA induction in *aba1* was higher than that of wild type following low-oxygen treatment (t = 2.13, *) and was not significantly different from that of wild type following low-temperature treatment (t = 2.18, N.S.). None of the ABA-insensitive mutants (*abi1*, *abi2*, or *abi3*) differed significantly from the wild type in their response to either low-oxygen or low-temperature stress (the respective t values for low oxygen are t = 2.07, N.S.; t = 2.06, N.S.; t = 2.10, N.S.; the respective t values for low temperature are t = 2.09, N.S.; t = 2.09, N.S.; t = 2.13, N.S.). Our results indicate that ABA and *abi2* are involved in mediating the response of the *Adh* promoter to dehydration treatment but that neither is involved in response to low oxygen or low temperature (Fig. 4).

DISCUSSION

Our results from substitution mutant analysis show that the G-box-1 and to a lesser extent the G-box-2 elements play a role in ABA-induced *Adh* expression. Our data also show that the GT and GC motifs between positions −160 and −140 are crucial for ABA induction. The requirement for the G-box-1 sequence for response to both dehydration (Dolferus et al., 1994) and ABA indicates that ABA and dehydration stress act through a common promoter element. The G-box-1 sequence is homologous to the ABRE (Guiltinan et al., 1990), and we show in this paper that the Arabidopsis *Adh* gene is indeed inducible by exogenous ABA and that this induction requires the G-box-1 sequence. The GT and GC motifs show similarity to the maize *Adh1* ARE (Olive et al., 1991a, 1991b), and we have previously suggested that both motifs might be part of the same element, which could function as a general stress response element that interacts with a general transcription factor (Dolferus et al., 1994). The G-box-1 binding factor may function by interacting with the factor bound to the GT/GC motif, both regulating transcription (Fig. 6), or it may require the presence of another ABA-responsive element or coupling element to form an ABA-responsive complex, such as the CE-1 element of the barley *HVA22* promoter (Shen and Ho, 1995). In the Arabidopsis *Adh* gene a potential CE-1-like element exists 100 bp upstream of the ABRE, but we have not investigated its role in this study.

The role of the G-box-2 element is less clear. This sequence has neither the central ACGT core sequence required for interaction with Leu-zipper-type transcription factors and G-box-binding factors (Schindler et al., 1992a, 1992b), nor the conserved ABRE CACGTG core sequence (Hetherington and Quatrano, 1991); it is part of a larger in vivo footprint area between positions −195 and −170 (Ferl and Laughner, 1989). The G-box-2 element may overlap with another regulatory element, which acts as an ABA-specific coupling element.

ABA has been previously shown to influence *Adh* gene expression. Maize plantlets pretreated with ABA were shown to have increased tolerance to hypoxia and increased alcohol dehydrogenase activity (Hwang and Van Toai, 1991). ABA was also shown to induce the barley *Adh* gene in aleurone cells (Nolan and Ho, 1988). Jarillo et al. (1993) have demonstrated that both low temperature and ABA induce the *Adh* gene in roots and leaves of etiolated Arabidopsis seedlings. Our results show that under normal lighting conditions, the stress- and ABA-induced *Adh* gene expression is predominantly root specific. We have found that the *Adh* gene is expressed in leaves during callus induction in the dark (Dolferus et al., 1985), in etiolated

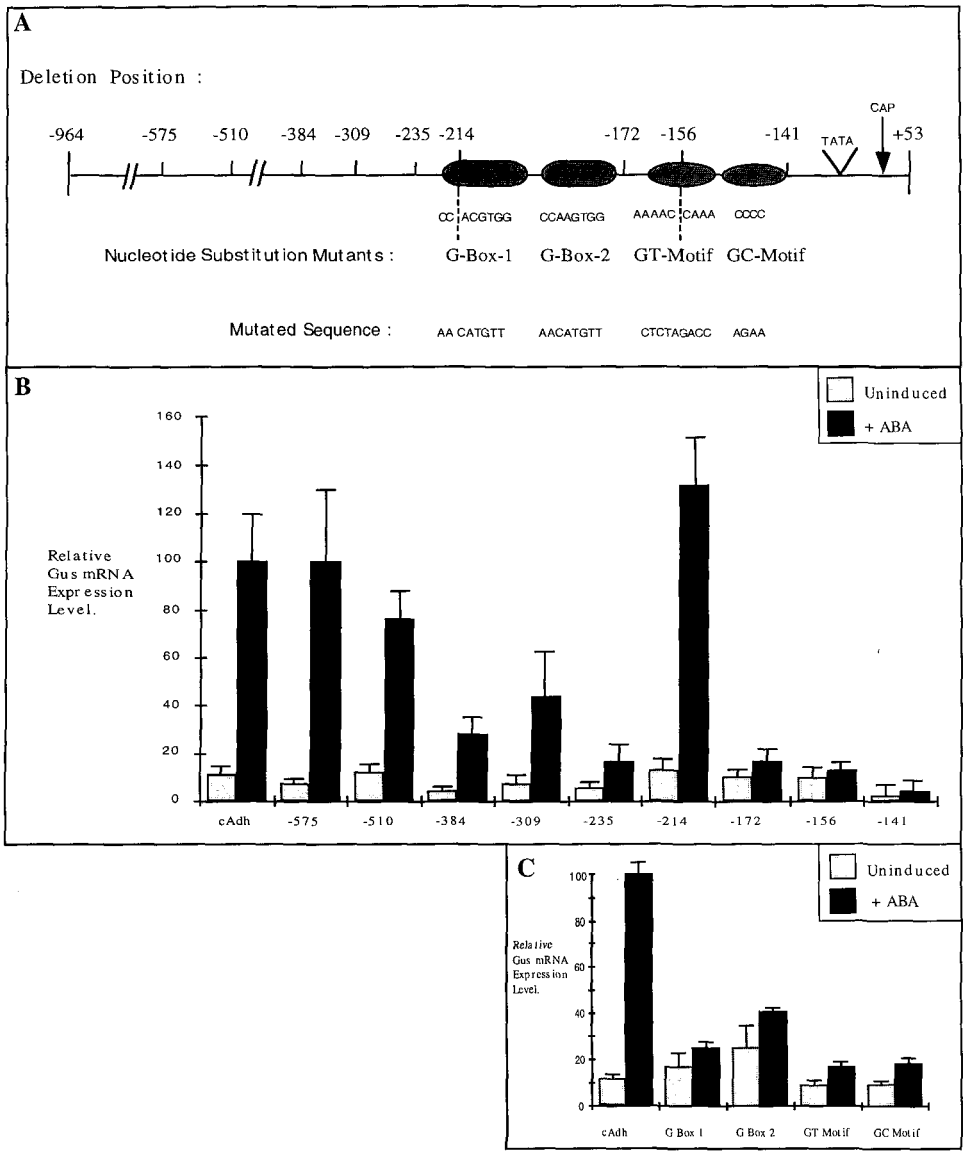

Figure 5. A, Schematic representation of the Arabidopsis *Adh* promoter showing the position of the 5′ deletions and substitution mutants used in this study. The mutated sequences of the G-box-1, G-box-2, GT, and GC motif substitution mutants are shown below. These mutations were introduced in the full-length CADH promoter fragment (−964 to +53). All constructs were transformed to Arabidopsis using a slightly modified root transformation method (Valvekens et al., 1988; Dolferus et al., 1994). B and C, Effect of ABA treatment on GUS mRNA expression for ten 5′ deletion (B) and four substitution mutants (C). Shaded bars show relative *Adh* mRNA expression levels for uninduced seedlings, and solid black bars show the levels for ABA-induced (0.1 mM/4 h) samples. Total RNA (25 μg) from five independent transformants was analyzed by northern blot hybridization, and relative GUS mRNA expression levels were calculated as described in "Materials and Methods." Each column represents the mean and SE values for each promoter construct.

leaves, and after mechanical wounding (G.L. de Bruxelles, R. Dolferus, unpublished data).

We have previously demonstrated that induction of the Arabidopsis *Adh* gene by three environmental stresses (low oxygen, dehydration, and low temperature) involves at least two different signal transduction pathways. Dehydration and low-temperature response both require the G-box-1 element, but response to low-oxygen stress does not depend on the G-box-1 element. Our data do not support the existence of a common regulatory pathway involving ABA for low-oxygen and low-temperature induction as suggested by Jarillo et al. (1993). Several lines of evidence demonstrate that, although dehydration and low-temperature stress response are mediated through the same promoter elements, the signal transduction pathways used by dehydration and low-temperature response can be further separated into ABA-dependent dehydration and ABA-independent, low-temperature signaling pathways (Fig. 6). Measurement of endogenous ABA levels shows that ABA increases during dehydration but not during low-temper-

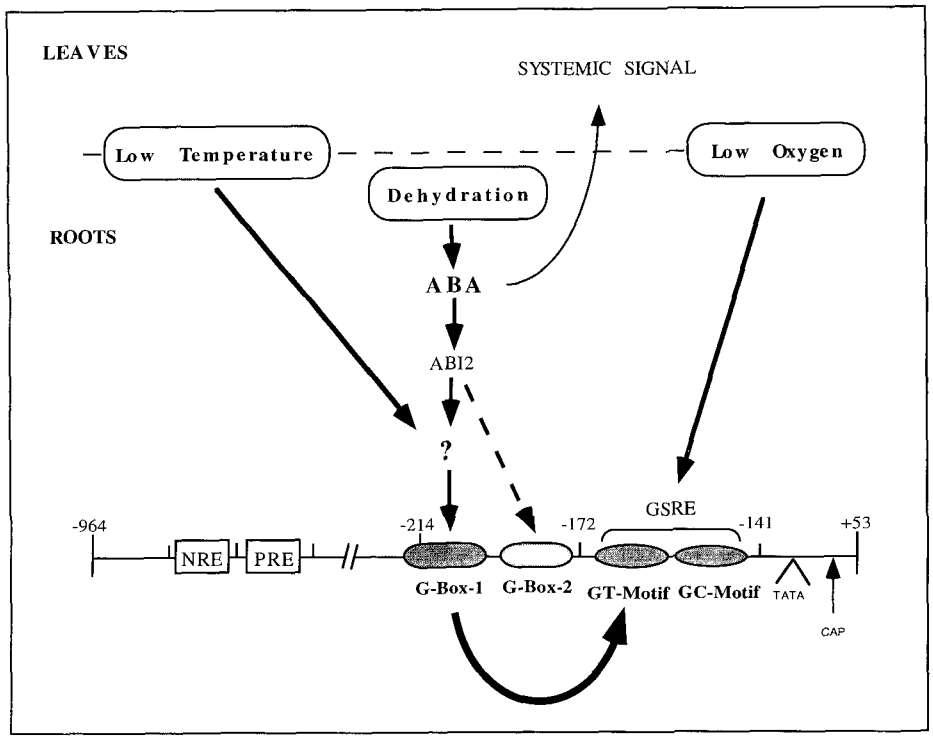

Figure 6. Model for the induction of the Arabidopsis *Adh* gene by environmental stresses and ABA. Both low oxygen and low temperature could be sensed in roots and leaves of the plant; dehydration stress is more likely to be sensed in the roots. Induction of the *Adh* gene by low oxygen, low temperature, dehydration, and ABA requires three signal transduction pathways. Low-oxygen stress is clearly independent from the other stresses and ABA. NRE and PRE represent negative and positive regulatory elements, respectively, and GSRE represents the general stress response element consisting of the GT and GC motifs (Dolferus et al., 1994). Low-temperature stress, dehydration, and ABA require the G-box-1 sequence, but signal transduction pathways are different. Dehydration treatment leads to increased ABA levels in both roots and shoots of the plant, whereas ABA levels remain unchanged after cold treatment. Increased ABA levels could play the role of a stress signal, which results in the amplification of the dehydration stress response in root cells (systemic response). In addition, transport of ABA to the shoot can stimulate other signal transduction pathways, which are involved in processes such as stomatal closure.

ature treatment. The rate of *Adh* induction under dehydration conditions reaches a maximum after 10 to 12 h (Dolferus et al., 1994) and parallels the rate of ABA accumulation in the roots (Fig. 3C). Low-temperature induction is considerably slower, with a maximum after 24 h. The ABA-deficient mutant *aba1* showed a markedly reduced response to dehydration but not to low temperature or exogenous ABA. Additionally, the ABA-insensitive mutant *abi2* was affected only in its response to dehydration and applied ABA but not to low temperature. The *abi3* mutant was not affected in either its response to dehydration or exogenous ABA. Initial characterization of the ABA-insensitive mutants showed *abi1* and *abi2* to be sensitive to wilting (Koornneef et al., 1984), whereas the effect of the *abi3* mutation was predominantly on seed development (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Giraudat et al., 1992). Jarillo et al. (1993) reported that the *abi1* mutation had a more drastic effect on *Adh* gene induction by dehydration and ABA treatment, indicating that the *ABI1* gene product may also play a role in the dehydration/ABA signal transduction pathway. Our results for *abi1* show only a slight but not statistically significant decrease in ABA and dehydration response. The difference in observations between our current study and those of Jarillo et al. (1993) may be due to differences in the experimental conditions used. Jarillo et al. (1993) used dark-grown seedlings dehydrated by air drying, whereas in this study seedlings were dehydrated using a 0.6 M mannitol solution under normal lighting conditions (16 h of light/8 h of dark).

At this stage we do not know whether different transcription factors are mediating dehydration and low-temperature induction, or whether a common G-box-1-binding factor is activated by two different signal transduction pathways. It is also possible that different coupling factors are involved in mediating the response to cold and dehydration and that these as-yet unidentified elements play a role in determining the binding specificity of the G-box transcription factor. Cold induction independent of ABA has been observed for other genes (Lång et al., 1989; Gilmour and Thomashow, 1991; Nordin et al., 1993). Genes induced rapidly by low temperature, such as Arabidopsis *Adh*, appear to be induced by signal transduction pathways independent of ABA. Gilmour and Thomashow (1991) observed increased freezing tolerance in wild-type plants compared to ABA-deficient mutants only when the period

of cold acclimation was longer than 4 d. This may suggest that there are two phases in cold induction, with ABA being involved in gene induction after prolonged cold acclimation. Plants exposed to dehydration treatment or exogenous ABA cold acclimate more efficiently (Cloutier and Siminovitch, 1982; Chen et al., 1983). Under our experimental conditions we were unable to detect increased ABA levels up to 7 d after cold treatment.

Induction of *Adh* by exogenously applied ABA occurs predominantly in the roots and not in the aerial parts of the plant, despite the presence of increased levels of ABA in the shoots following either ABA application or dehydration treatment. This suggests that the presence of ABA is not the only requirement for induction, but that other tissue-specific component(s) are also crucial. The transcription factor binding to the GT/GC motifs might be present only in certain tissues and mediate stress-responsive expression of *Adh* through interactions with upstream regulatory elements such as the G-box-1.

Analysis of the ABA mutants has shown that the *ABI2* gene product, and possibly the *ABI1* gene product (Jarillo et al., 1993), may play a role in the dehydration signal transduction pathway (Fig. 6). We found that the response to dehydration in the *abi2* and especially in the *abi1* mutant is only partially reduced, indicating that the abi1 and abi2 proteins are not complete nulls, and that the existence of both ABA-dependent and ABA-independent dehydration response pathways cannot be excluded (Yamaguchi-Shinozaki and Shinozaki, 1993b, 1994).

In conclusion, we have demonstrated that ABA induces the Arabidopsis *Adh* gene and that induction of the Arabidopsis *Adh* gene by dehydration involves ABA. Induction of *Adh* by low oxygen and low temperature is independent of ABA and occurs through different signal transduction pathways. We intend to dissect the signal transduction pathways leading to these environmental stress responses by selection of mutants blocked in the pathways.

ACKNOWLEDGMENTS

We wish to thank S. Stops for excellent technical assistance and Dr. R. Munns and R. James (Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry) for their assistance with the ABA immunoassays.

Received October 12, 1995; accepted March 11, 1996.

LITERATURE CITED

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Breyne P, De Loose M, Dedonder A, Van Montagu M, Depicker A (1993) Quantitative kinetic analysis of β-glucuronidase activities using a computer-directed microtiter plate reader. Plant Mol Biol Rep **11:** 21–31

Cattivelli L, Bartels D (1992) Biochemistry and molecular biology of cold-inducible enzymes and proteins in higher plants. *In* JL Wray, ed, Inducible Plant Proteins: Their Biochemistry and Molecular Biology. Cambridge University Press, Cambridge, UK, pp 267–288

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol **71:** 362–365

Cloutier Y, Siminovitch D (1982) Correlation between cold- and drought-induced frost hardiness in winter wheat and rye varieties. Plant Physiol **69:** 256–258

Dolferus R, Peacock WJ, Dennis ES (1994) Differential interactions of promoter elements in stress responses of the *Arabidopsis Adh* gene. Plant Physiol **105:** 1075–1078

Ellis JG, Llewellyn DJ, Dennis ES, Peacock WJ (1987) Maize *Adh-1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. EMBO J **6:** 11–16

Ferl RJ, Laughner BH (1989) *In vivo*-detection of regulatory factor binding sites of *Arabidopsis thaliana Adh*. Plant Mol Biol **12:** 357–366

Finkelstein R, Sommerville C (1990) Three classes of abscisic acid (ABA)-insensitive mutations define genes that control overlapping subsets of ABA responses. Plant Physiol **94:** 1172–1179

Gilmour S, Thomashow MF (1991) Cold acclimation and cold regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol Biol **17:** 1233–1240

Giraudat J, Hauge B, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell **4:** 1251–1261

Gosti F, Bertauche N, Vartanian N, Giraudat J (1995) Abscisic acid-dependent and -independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. Mol Gen Genet **246:** 10–18

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol **15:** 11–26

Guiltinan MJ, Marcotte WR Jr, Quatrano RS (1990) A plant leucine zipper protein that recognises an abscisic acid response element. Science **250:** 267–271

Guo W, Ward RW, Thomahow MF (1992) Characterization of a cold-regulated wheat gene related to *Arabidopsis Cor47*. Plant Physiol **100:** 915–922

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223

Hetherington AM, Quatrano RS (1991) Mechanisms of action of abscisic acid at the cellular level. New Phytol **119:** 9–32

Hwang S-Y, Van Toai TT (1991) Abscisic acid induces anaerobiosis tolerance in corn. Plant Physiol **97:** 593–597

Izawa T, Foster R, Chua N-H (1993) Plant b-Zip protein DNA binding specificity. J Mol Biol **230:** 1131–1144

Jackson MB (1991) Regulation of water relationships in flooded plants by ABA from leaves, roots and xylem sap. *In* WJ Davies, HJ Jones, eds, Abscisic Acid: Physiology and Biochemistry. BIOS Scientific Publishers, Oxford, UK, pp 217–226

Jacobsen JV (1983) Regulation of protein synthesis in aleurone cells by gibberellin and abscisic acid. *In* A Crozier, ed, The Biochemistry and Physiology of Gibberellins. Praeger, New York, pp 159–187

Jarillo JA, Leyva A, Salinas J, Martinez-Zapater JM (1993) Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling-tolerant plant. Plant Physiol **101:** 833–837

Jefferson RA (1987) Experimental protocols: assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet **61:** 385–393

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterisation of abscisic-acid-insensitive mutants of *Arabidopsis thaliana*. Physiol Plant **61:** 377–383

Kurkela S, Franck M (1990) Cloning of a cold- and ABA-inducible *Arabidopsis* gene. Plant Mol Biol **15:** 137–144

Lång V, Heino P, Palva ET (1989) Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet **77:** 729–734

Lång V, Mäntylä E, Welin B, Sundberg B, Palva ET (1994) Alterations in water status, endogenous abscisic acid content, and expression of *rab18* gene during the development of freezing tolerance in *Arabidopsis thaliana*. Plant Physiol **104:** 1341–1349

Lång V, Palva ET (1992) The expression of a *rab*-related gene *rab18* is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L) Heynh. Plant Mol Biol **20:** 951–962

Loveys BR, van Dijk HM (1988) Improved extraction of abscisic acid from plant tissue. Aust J Plant Physiol **15:** 421–427

Mansfield E (1986) Basic Statistics with Applications. WW Norton, New York

Marcotte WR Jr, Russell SH, Quatrano RS (1989) Abscisic acid-responsive sequences from the *Em* gene of wheat. Plant Cell **1:** 969–976

Michel D, Salamini F, Bartels D, Dale P, Baga M, Szalay A (1993) Analysis of a desiccation and ABA-responsive promoter isolated from the resurrection plant *Craterostigma plantagineum*. Plant J **4:** 29–40

Mundy J, Yamaguchi-Shinozaki K, Chua N-H (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *rab* gene. Proc Natl Acad Sci USA **87:** 1406–1410

Nolan RC, Ho THD (1988) Hormonal regulation of gene expression in barley aleurone layers: induction and suppression of specific genes. Planta **174:** 551–560

Nordin K, Heino P, Palva ET (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol **16:** 1061–1071

Olive MR, Peacock WJ, Dennis ES (1991a) The anaerobic response element contains two GC-rich sequences essential for binding a nuclear protein and hypoxic activation of the maize *Adh 1* promoter. Nucleic Acids Res **19:** 7053–7060

Olive MR, Walker JC, Singh K, Ellis JG, Llewellyn D, Peacock WJ, Dennis ES (1991b) The anaerobic response element. *In* RG Hermann, B Larkin, eds, Plant Molecular Biology, Vol 2. Plenum Press, New York, pp 673–684

Rock CD, Zeevaart JAD (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid synthesis. Proc Natl Acad Sci USA **88:** 7496–7499

Sánchez-Serrano JJ, Amati S, Ebneth M, Hildmann T, Mertens R, Pena-Cortés H, Prat S, Willmitzer L (1991) The involvement of ABA in wound responses of plants. *In* WJ Davies, HG Jones, eds, Abscisic Acid, Physiology and Biochemistry. Bios Scientific Publishers, Oxford, UK, pp 210–216

Schindler U, Menkens AE, Beckman H, Ecker JR, Cashmore AR (1992a) Heterodimerisation between light-regulated and ubiquitously expressed *Arabidopsis* GBF proteins. EMBO J **11:** 1261–1273

Schindler U, Terzaghi W, Beckman H, Kadesch T, Cashmore AR (1992b) DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor GBF 1. EMBO J **11:** 1275–1289

Schulze-Lefert P, Dangl J, Becker-André M, Hahlbrock K, Schulze W (1989) Inducible *in vivo* DNA fingerprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. EMBO J **8:** 651–656

Shen Q, Ho THD (1995) Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-acting element. Plant Cell **7:** 295–307

Straub PF, Shen Q, Ho THD (1994) Structure and function of an ABA- and stress-regulated barley gene, *HVA1*. Plant Mol Biol **26:** 617–630

Valvekens D, Van Montagu M, Van Lysebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA **85:** 5536–5540

Vilardell J, Mundy J, Stilling B, Leroux B, Pla M, Freyssinet G, Pagès M (1991) Regulation of the maize *rab17* gene promoter in transgenic heterologous systems. Plant Mol Biol **17:** 985–993

Walker-Simmons M, Sessing J (1987) Development of a sensitive immunoassay for abscisic acid in wheat grain utilising a monoclonal antibody. *In* DJ Mares, ed, Fourth International Symposium on Pre-Harvest Sprouting in Cereals. Westview Press, Boulder, CO, pp 591–597

Williams ME, Foster R, Chua NH (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell **4:** 485–496

Wray JL, ed (1992) Inducible Plant Proteins: Their Biochemistry and Molecular Biology. Cambridge University Press, Cambridge, UK

Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterisation of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol **33:** 217–224

Yamaguchi-Shinozaki K, Shinozaki K (1993a) Characterisation of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol Gen Genet **236:** 331–340

Yamaguchi-Shinozaki K, Shinozaki K (1993b) The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of *rd22*, a gene responsive to dehydration stress in *Arabidopsis thaliana*. Mol Gen Genet **238:** 17–25

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an *Arabidopsis* gene involved in responsiveness to drought, low temperature, or high salt stress. Plant Cell **6:** 251–264

Zeevaart JAD, Creelman RA (1988) Metabolism and physiology of abscisic acid. Annu Rev Plant Physiol Plant Mol Biol **39:** 439–473