*Plant Physiology*, October 1999, Vol. 121, pp. 429–436, www.plantphysiol.org © 1999 American Society of Plant Physiologists

# Arabidopsis Alcohol Dehydrogenase Expression in Both Shoots and Roots Is Conditioned by Root Growth Environment[1]

**Hwa-Jee Chung and Robert J. Ferl***

Program in Plant Molecular and Cellular Biology, Department of Horticultural Sciences, University of Florida, Gainesville, Florida 32611

**It is widely accepted that the Arabidopsis *Adh* (alcohol dehydrogenase) gene is constitutively expressed at low levels in the roots of young plants grown on agar media, and that the expression level is greatly induced by anoxic or hypoxic stresses. We questioned whether the agar medium itself created an anaerobic environment for the roots upon their growing into the gel. β-Glucuronidase (GUS) expression driven by the *Adh* promoter was examined by growing transgenic Arabidopsis plants in different growing systems. Whereas roots grown on horizontal-positioned plates showed high Adh/GUS expression levels, roots from vertical-positioned plates had no Adh/GUS expression. Additional results indicate that growth on vertical plates closely mimics the Adh/GUS expression observed for soil-grown seedlings, and that growth on horizontal plates results in induction of high Adh/GUS expression that is consistent with hypoxic or anoxic conditions within the agar of the root zone. Adh/GUS expression in the shoot apex is also highly induced by root penetration of the agar medium. This induction of Adh/GUS in shoot apex and roots is due, at least in part, to mechanisms involving $Ca^{2+}$ signal transduction.**

Plant roots often encounter a number of environmental stresses, including drought and flooding, and respond by changes in cell structure, biochemistry, and gene expression. As a result of flooding, anaerobiosis rapidly represses the synthesis of pre-existing proteins and concomitantly induces the synthesis of new anaerobic proteins (Sachs et al., 1980). Alcohol dehydrogenase (ADH) is one of the anaerobic proteins that catalyzes the reduction of pyruvate to ethanol, resulting in continuous $NAD^+$ regeneration. ADH activity is considered essential for the survival of plants during anaerobic conditions (Johnson et al., 1994). Transcriptional activation of the *Adh* gene has, therefore, become a diagnostic feature of the hypoxic and anoxic responses.

Anaerobic conditions in cells trigger a cascade of biochemical reactions, including changes in cytosolic $Ca^{2+}$ levels with induction of *Adh* mRNAs and an increase in ADH enzyme activity (Subbaiah et al., 1994a, 1994b, 1998; Sedbrook et al., 1996). Calcium is an essential element for cell growth and plays a role as a second messenger in signal transduction pathways (Bush, 1995; Clapham, 1995). Cytosolic $Ca^{2+}$ is implicated in the signaling process of various environmental stresses such as mechanical impedance (Antosiewicz et al., 1995; Legue et al., 1997), light (Im et al., 1996), cold temperature (Monroy and Dhindsa, 1995; Knight et al., 1996; Tahtiharju et al., 1997), drought (Knight et al., 1997), salinity (Knight et al., 1997; Liu and Zhu, 1997, 1998), and hormones such as ABA (Wang et al., 1991; Bustos et al., 1998) and GA (Abo-el-Saad and Wu, 1995; Chen et al., 1997). Pretreatment of maize seedlings with ruthenium red (RR), an inhibitor of intracellular $Ca^{2+}$ flux, dramatically reduced anoxia-induced ADH activity (Subbaiah et al., 1994b). Moreover, transient $Ca^{2+}$ increases in young Arabidopsis seedlings exposed to anoxia were also reduced by treatment with RR and the $Ca^{2+}$ channel blocker gadolinium (Sedbrook et al., 1996). Thus, in both maize and Arabidopsis seedlings, anoxia elevates the cytosolic $Ca^{2+}$ level through efflux from the intracellular $Ca^{2+}$ organelles or influx across the plasma membrane $Ca^{2+}$ channel.

In studying plant response to anaerobic stress, suspension-cultured cells or seedlings grown on horizontal agar medium have been frequently used. Treatment of suspension cells and seedlings with argon (inducing anoxia) or a $N_2/O_2$ gas mixture (inducing hypoxia) was used to mimic the condition plants face during flooding. Dolferus et al. (1994) showed that the Arabidopsis *Adh* gene was constitutively expressed in root tissues, including lateral roots, but expression was not observed in green aerial tissues, when seedlings were grown on horizontal-positioned plates. Anaerobic conditions significantly induced the *Adh* gene in root tissues.

Since roots are very sensitive to anaerobic stress, we have questioned whether the agar medium itself induces hypoxic stress on the plant, resulting in the inappropriate constitutive expression of the *Adh* gene in roots. Therefore, we took advantage of transgenic Arabidopsis plants containing the *Adh* promoter/GUS reporter gene fusion for monitoring *Adh* activity under different growing systems. Arabidopsis has a single *Adh* gene that has been well characterized for its responses to environmental stresses, including hypoxia (Dolferus et al., 1994). Here we report observations of Arabidopsis *Adh*/GUS gene expression patterns in roots and shoot apices when plants were grown under various orientations and conditions of agar medium. In addition, we examine the effects of reagents influencing

[1] This research was supported by the National Aeronautics and Space Agency (grant no. NAG10–0145 to R.J.F.). This manuscript is journal series no. R–06984 of the Florida Agricultural Experiment Station.

* Corresponding author; e-mail robferl@ufl.edu; fax 352–392–4072.

$Ca^{2+}$ concentrations on *Adh*/GUS expression in shoots and roots.

## MATERIALS AND METHODS

### Plant Growth Conditions and Treatments

The plasmid containing the Adh/GUS gene fusion (−846 to +30) (McKendree and Ferl, 1992) was transformed into *Agrobacterium tumefaciens* strain LBA4404 and transferred to Arabidopsis (L.) Heynh., ecotype RLD via root transformation (Valvekens et al., 1988). $F_2$ seeds were germinated in Murashige and Skoog (MS) medium agar plates containing 1% (w/v) Suc and 50 $\mu$g/mL of kanamycin or soil with mixture of peat moss and vermiculite (Vergro transplant mix A). For MS agar plates, $F_2$ seeds were surface sterilized with 70% (v/v) ethanol followed by 50% (v/v) household bleach with approximately 1.5% (v/v) Tween 20, and then washed four times in sterilized water. The agar plates were placed in either a vertical or horizontal position. Phytagel (Sigma, St. Louis) was used as an agar substitute, and concentrations within vertical- or horizontal-positioned plates were 0.25% (v/v) and 0.20%, respectively. For treatment with $Ca^{2+}$ chelators or antagonists, 9-d-old seedlings grown on vertical-positioned plates were transferred to horizontal-positioned plates containing one-quarter-fold diluted MS medium in the presence or absence of $Ca^{2+}$ chelator or antagonists. Because MS medium (Gibco-BRL, Gaithersburg, MD) contains 2 mM $CaCl_2$ as salt components, we reduced the amount of MS salts for $Ca^{2+}$ chelator or antagonist treatments. However, phytagel requires $CaCl_2$ for solidification, and does not solidify in less than one-quarter-fold diluted MS medium. Likewise, the high concentrations of 1 mM $GdCl_3$ or 10 mM EGTA inhibited the solidification in the one-quarter-fold diluted MS medium. Therefore, 5 mM EGTA, 25 $\mu$M RR, and 0.5 mM gadolinium were used in these studies. Plants were grown at 22°C to 24°C under continuous light at 84 $\mu$mol $m^{-2}$ $s^{-1}$. For hypoxic treatment, Arabidopsis seedlings grown on vertical plates were transferred to Petri dishes containing two layers of filter papers soaked with MS liquid solution. The Petri dishes were then put in a 2.5-L gasket jar and continuously sparged (1 L/min) with a 3% $O_2$/97% $N_2$ (v/v) mixture for 24 h in the dark.

### GUS Analysis

For the biochemical GUS assay (Jefferson et al., 1987), sample tissues were homogenized in GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM EDTA, 0.1% [v/v] sarkosyl, 0.1% [v/v] Triton X-100, and 10 mM $\beta$-mercaptoethanol). Samples were centrifuged for 10 min at 4°C, and the supernatant was used for the GUS assay. For fluorimetric reactions, duplicate reactions were carried out by adding 10 mM 4-methylumbelliferyl $\beta$-D-glucuronide (4-MUG) to 1 mM concentration and incubating at 37°C. One reaction was terminated at 5 min as a control, and the second at 65 min with the addition of 0.2 M $Na_2CO_3$. Fluorescence was measured on a fluorometer (excitation wavelength = 365 nm, photoedetector wavelength = 460 nm, Shimadzu, Kyoto) after dilution with 0.2 M $Na_2CO_3$. The protein content of the samples was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's protocol. For histochemical GUS analysis, seedlings were immersed in the GUS reaction buffer (2 mM 5-bromo-4-chloro-3-indolyl-$\beta$-D GlcUA [X-Gluc], 1% [w/v] dimethylformamide, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide, 1 mM EDTA, and 50 mM $NaPO_4$, pH 7.0) followed by brief vacuum infiltration. Tissues were incubated at 37°C for 4 to 16 h. After incubation, seedlings were cleared in 70% (v/v) ethanol to remove chlorophyll for better visualization and photographed with Ektachrome 160 ASA tungsten film (Eastman-Kodak, Rochester, NJ) under dark-field dissecting microscopy.

### Assay of Endogenous ADH Enzyme Activity

The endogenous ADH activity was measured by modification of existing protocols (Russell et al., 1990). Soluble proteins were extracted in cold extraction buffer (50 mM Tris-HCl, pH 8.0, and 15 mM DTT), and centrifuged at 12,000*g* for 15 min at 4°C. The enzyme reaction mixture contained 50 mM Tris-HCl, pH 9.0, 0.867 mM $NAD^+$, and 0.04 volume of extract. The enzyme reaction was initiated by addition of ethanol to 20% (v/v) final concentration of the reaction mixture, and the $A_{340}$ was measured every 15 s for 60 s. Protein concentration of the extract was determined as described above. Activity was calculated as micromoles per minute per milligram of protein.

## RESULTS

### *Adh* Gene Expression in Horizontal Plates, Vertical Plates, and Soil

To investigate whether agar medium induces *Adh* gene expression in root tissues, transgenic Arabidopsis seedlings harboring the *Adh* gene promoter fused to the GUS reporter gene were germinated in soil or on MS medium agar plates oriented in either vertical or horizontal positions. The GUS expression pattern driven by the *Adh* promoter in developing seedlings was monitored by histochemical GUS analysis. As demonstrated in Figure 1, seedlings grown on vertical-positioned plates showed no Adh/GUS expression in root tissues for up to 20 d. In addition, no Adh/GUS activity was observed in 30-d-old plants grown in vertical-positioned plates (data not shown). However, seedlings grown on horizontal-positioned plates initially expressed Adh/GUS activity in the primary root tip approximately 5 d after germination, with Adh/GUS expression gradually increasing throughout the root tissues from 8 to 20 d. The Adh/GUS expression region in root tissues expanded from the root tip, primarily in the root meristem, to the entire root tissue, including the vascular bundles through 20 d (Fig. 1).

Seedlings grown in soil showed a lack of Adh/GUS expression, much like those seedlings grown on vertical-positioned plates, except for a few roots attached to vermiculite particles in soil (Fig. 1). Adh/GUS activity was also quantitatively determined in developing seedlings. The Adh/GUS activities of developing seedlings on vertical-positioned plates remained at basal levels, while seedlings grown on horizontal plates showed significantly

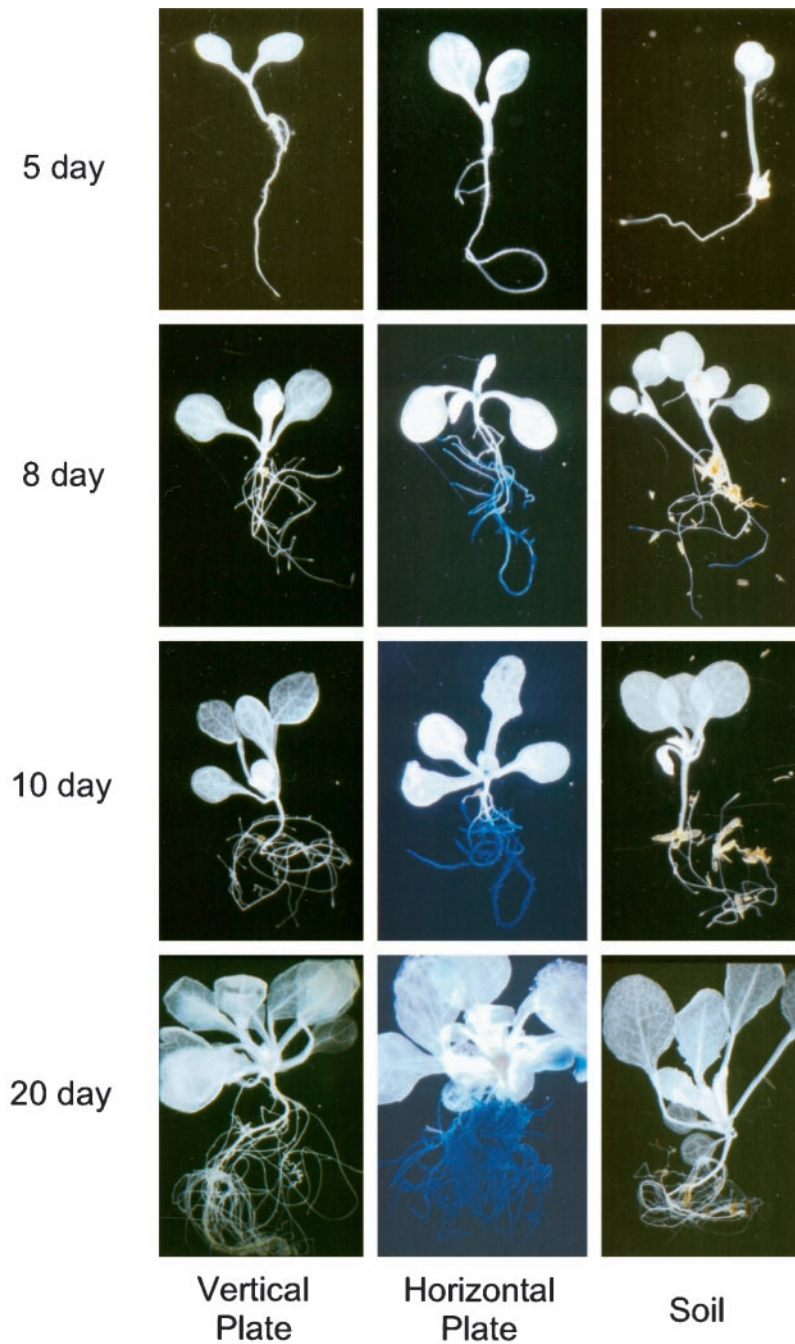



**Figure 1.** The *Adh*/GUS gene expression pattern in plants grown in different growing systems. Seeds were germinated and grown for various periods of time (5, 8, 10, and 20 d) on vertical- and horizontal-positioned MS medium plates, as well as in soil (left, middle, and right columns, respectively). Developing seedlings were stained in 2 mM X-Gluc solution for 16 h at 37°C.

high Adh/GUS activity levels that increased throughout seedling development (Fig. 2A). Increases in Adh/GUS activity of horizontally grown seedlings in the ranges of 3-, 4-, 16-, 30-, and 110-fold were observed in 5, 8, 10, 15, and 20 d of developing seedlings, respectively. These results are consistent with those of the histochemical GUS analysis (Fig. 1). Several different lines of transgenic plants have been tested and shown to have similar Adh/GUS expression patterns and activity (data not shown).

In an effort to establish a physiological significance of the GUS reporter gene activity driven by the *Adh* promoter in both horizontally and vertically grown seedlings, the level of endogenous ADH enzyme activity was determined in developing seedlings. As shown in Figure 2B, ADH activity in seedlings of vertical-positioned plates was low in both the young seedlings and older plants. In contrast, the level of ADH activity in seedlings grown on horizontal-positioned agar plates was greatly increased, and the ADH activity at 20 d was 11-fold greater than for vertically grown seedlings.

### Hypoxic *Adh* Gene Induction by Agar Medium?

Plants subjected to mechanical impedance or touch stimuli have shown similar effects as plants subjected to

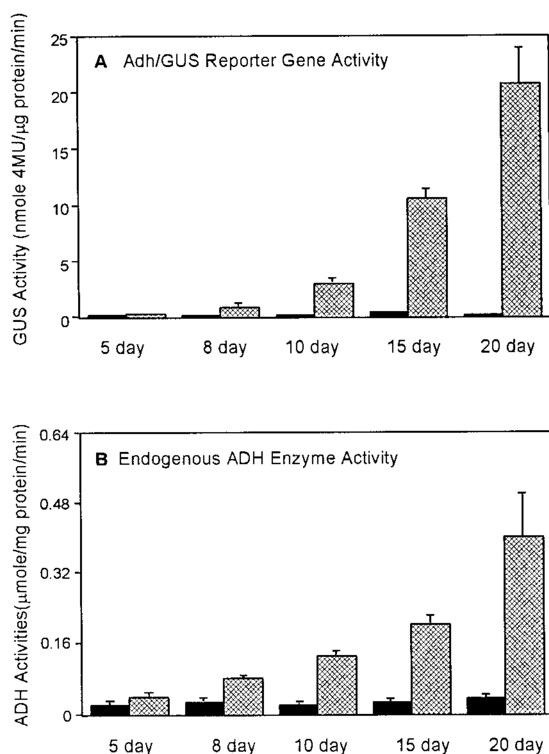



**Figure 2.** Effect of agar medium on *Adh* gene expression. A, The GUS expression level was determined in developing seedlings grown on vertical- (black bars) and horizontal- (checkered bars) positioned plates. GUS activities were the average of triplicate experiments and shown as nanomoles of 4MU per microgram of protein per minute. B, Endogenous ADH enzyme activity of developing seedlings grown on vertical- (black bars) and horizontal- (checkered bars) positioned plates. Enzyme activity is shown as micromoles per minute per milligram of protein.

hypoxia, including increased cytoplasmic $Ca^{2+}$ levels, a stimulated ethylene biosynthesis, and aerenchyma formation in roots (He et al., 1996a, 1996b; Legue et al., 1997). These studies raised the possibility of *Adh* gene induction via mechanical impedance of penetrating the agar rather than hypoxia. Therefore, we questioned how *Adh* gene expression in roots was induced by agar medium. Is it because of the hypoxia created by agar medium? Or, do the root tissues experience mechanical impedance by penetrating or touching the solid agar medium?

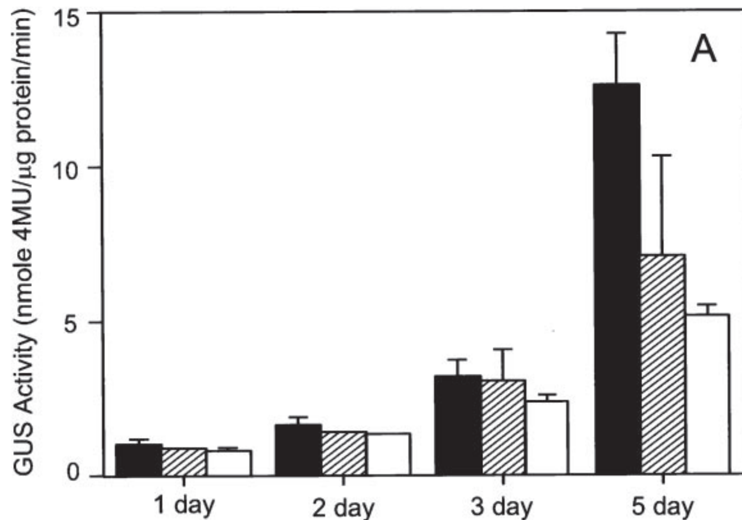
To answer these questions we first transferred 9-d-old seedlings from vertical to horizontal plates containing various concentrations of agar medium. Low phytagel concentration (0.15% [v/v]) resulted in a soft, fragile medium into which most of the root tips penetrated relatively quickly. High concentration (0.30% [v/v]) produced a very hard, solid medium surface that partially inhibited the growth of root tips into the agar medium, requiring more time for most of the root tips to enter the solid medium. The level of Adh/GUS activity in roots of seedlings transferred to the low concentration agar plate was significantly higher than that of roots in the high concentration plates, when assayed at 5 d after transfer (Fig. 3A). Higher agar concentration would present higher mechanical impedance, but did not result in higher Adh/GUS activity. However, lower concentration of agar would present less impedance and therefore quick penetration of root growth through the agar. Figure 3A indicates that faster penetration through low agar concentration did result in higher Adh/GUS activities than did the slower entrance and penetration through the higher impedance agar concentrations.

To confirm that the agar medium causes anaerobic or hypoxic conditions to roots, we altered the growing position of the plates from vertical to horizontal or vice versa. For vertical plates, the plate was reoriented to the horizontal position, and the roots were allowed to penetrate the agar for 7 d. For horizontal plates, the plate was inverted for 5 d until most root tips were coming out from the agar medium and then placed in a vertical position for 2 d to allow root contact of the agar surface. In Figure 3B, most of the root tissue did not show Adh/GUS expression when the plate was placed from a horizontal to a vertical position and the roots grew out of agar medium. On the contrary, placing the plate from a vertical to a horizontal position resulted in high Adh/GUS expression in roots as the roots grew into the agar medium (Fig. 3C). Similar Adh/GUS expression patterns and amounts were observed in the seedlings grown on vertical-positioned plate treated with low oxygen (Fig. 3D).

## Developmental *Adh* Gene Expression and Induction in the Shoot Apex

We examined the Adh/GUS gene expression in the shoot apex of developing seedlings grown on vertical- and horizontal-positioned plates. For 5-d-old seedlings grown on vertical plates, Adh/GUS activity was observed in the shoot apex and hypocotyl (Fig. 4A), consistent with the results of Dolferus et al. (1994). As the seedlings matured through 10 d, Adh/GUS expression in the shoot apex decreased and the stipules began to show expression (Fig. 4B). By 15 d Adh/GUS expression was strictly limited to the stipules (Fig. 4C). Surprisingly, 15-d-old seedlings grown in horizontal plates showed an intense Adh/GUS expression in both the stipules and the shoot apex (Fig. 4D). These results indicate that the shoot apex may receive signals from the roots, and expresses the *Adh*/GUS reporter gene as a result.

It has been shown that treatment with reagents affecting $Ca^{2+}$ influences anoxia-induced *Adh* expression in maize and Arabidopsis seedling roots (Subbaiah et al., 1994a, 1994b; Sedbrook et al., 1996). To determine whether $Ca^{2+}$ signals are involved in *Adh* induction in shoots, we treated seedlings with reagents affecting $Ca^{2+}$ concentration and monitored the change of Adh/GUS expression. Nine-day-old seedlings grown on vertical plates were transferred to horizontal plates containing 25 $\mu$M RR, 0.5 mM gadolinium, or 5 mM EGTA. During the treatment procedure, all seedlings were carefully transferred to the agar medium so that the shoot did not touch the agar medium. Figure 5A shows that transfer to horizontal plates resulted in a large increase in Adh/GUS expression in roots after 3 and 5 d. However, addition of the intracellular $Ca^{2+}$ channel blocker RR to the medium significantly reduced the level of Adh/GUS ex-



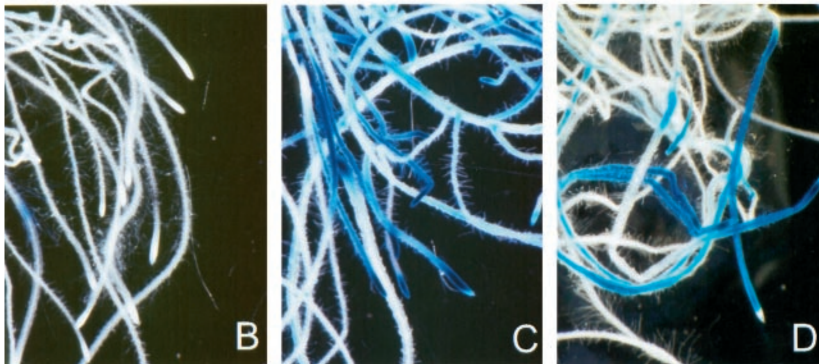




**Figure 3.** Agarose medium creates hypoxia, not mechanical impedance. A, GUS activity in roots growing in 0.15% (black bars), 0.20% (striped bars), and 0.30% (white bars) of agar medium. Nine-day-old seedlings grown on a vertical-positioned plate were transferred to a horizontal-positioned plate containing different concentrations of agar medium. B to D, X-Gluc stained roots of seedlings. At 7 d postgermination, the growing position was changed from horizontal to vertical (B) or from vertical to horizontal (C) and following 7 d of reorientation the roots were stained for GUS activity solution. D, Roots from 2-week-old seedlings that were grown on vertical-positioned plates and treated with hypoxia by placing in 3% $O_2$/97% $N_2$ (v/v).

pression in roots during this period. Likewise, addition of the $Ca^{2+}$ chelator EGTA reduced the induction of Adh/GUS gene expression to levels as low as that of RR-treated seedlings. The most dramatic inhibition of agar-induced Adh/GUS expression was observed in the presence of the plasma membrane $Ca^{2+}$ channel blocker, gadolinium.

Treatment of seedling roots with $Ca^{2+}$ antagonists also affected Adh/GUS expression in shoots (Fig. 5B). In non-treated seedlings no significant increase in Adh/GUS activity was observed during the first 2 d of incubation after transfer from vertical- to horizontal-positioned plates. However, a remarkable increase in Adh/GUS activity was observed by 3 and 5 d after transfer. RR treatment reduced the induction of Adh/GUS activity in the shoots to almost the basal levels seen in vertically grown seedlings. Incubation of seedlings on medium with 5 mM EGTA for 3 d reduced the level of Adh/GUS expression in the shoot, although the effect of EGTA on reduction of Adh/GUS expression was not significant at 5 d. Gadolinium completely blocked induction of the *Adh*/GUS gene in shoots within 24 h.

The Adh/GUS expression pattern of representative seedlings after a 5-d incubation with or without $Ca^{2+}$ antagonists is shown in Figure 6. Seedlings grown on both vertical- and horizontal-positioned plates showed well-developed leaves and roots, including normal-shaped lateral roots (Fig. 6, A, B, F, and G). Plants from horizontal-positioned plates showed uniform and intense Adh/GUS expression in root tissues. Treatment with 25 $\mu$M RR showed no significant effect on leaf development (Fig. 6C). However, RR inhibited the development of lateral roots and confined Adh/GUS expression to the root tip (Fig. 6 h). Addition of 5 mM EGTA had less effect on leaf and root development, with Adh/GUS expression observed in the tip and elongation zone of the root (Fig. 6, D and I). Gadolinium inhibited leaf and lateral root development. The effect of gadolinium on lateral roots was significant, as most lateral roots were arrested during growth (Fig. 6, E and J). In addition to reducing Adh/GUS expression, both RR and gadolinium treatments had an effect on the root tip phenotype, resulting in a ball-shaped or elongated root tip, respectively (Fig. 6, K and L).

## DISCUSSION

Growing plants on horizontal-positioned agar plates is often used in laboratory experiments involving Arabidopsis and other plants. Agar media are also used in specialized applications, such as the growth of plants during space flight (Porterfield et al., 1997). Data presented here indicate that this growing system is not necessarily benign, in that root growth through the agar medium causes con-

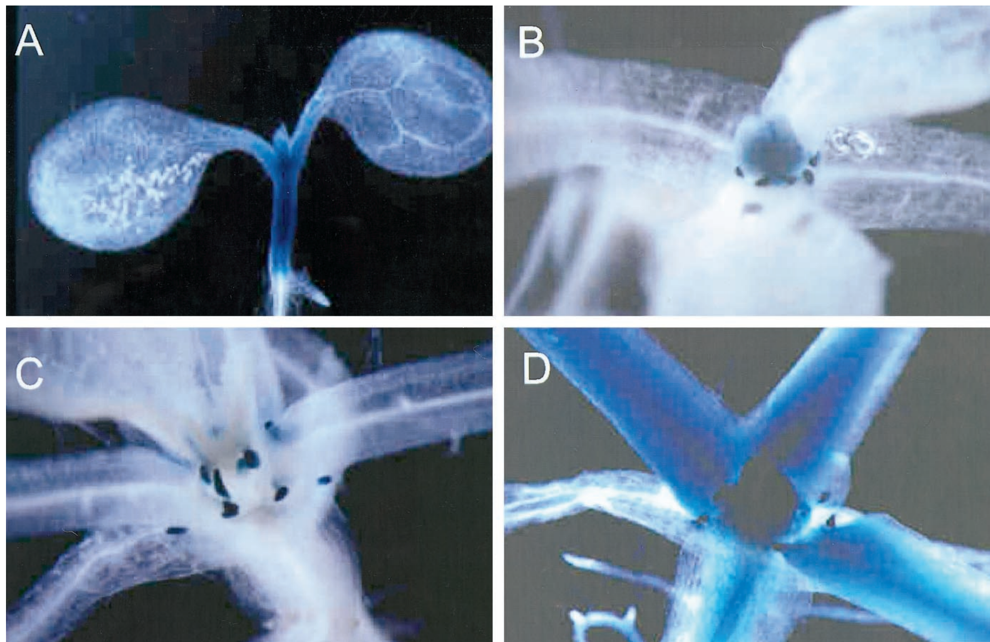



**Figure 4.** The expression of Adh/GUS reporter gene was observed in the shoot apex of developing seedlings. Five-day- (A), 10-d- (B), and 15-d- (C) old seedlings were grown on vertical-positioned plates and 15-d-old seedling grown on horizontal-positioned plates (D). Seedlings were stained in X-Gluc solution to visualize GUS expression in the shoot apex.

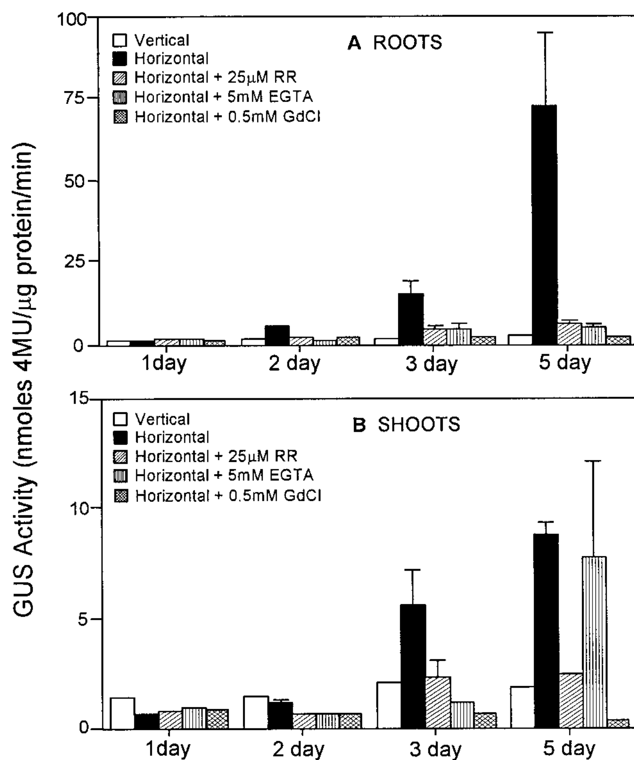



**Figure 5.** Quantitative effects of $Ca^{2+}$ regulators on Adh/GUS expression level in roots (A) and shoots (B). Nine-day-old seedlings grown on vertical-positioned plates (white bars) were transferred to horizontal-positioned plates (black bars) containing 25 $\mu$M RR (diagonal striped bars), 5 mM EGTA (vertical striped bars), and 0.5 mM gadolinium (checkered bars). GUS activities in roots (A) and shoots (B) were measured 1, 2, 3, and 5 d after transfer.

stitutive *Adh* gene expression apparently due to hypoxic stress in the root zone. These data impact conclusions regarding the developmental expression of *Adh* and potentially other genes associated with the response to hypoxia.

These results, using the Adh/GUS reporter as a biological indicator of hypoxic stress perception, fundamentally agree with direct measurements of oxygen concentration in agar. Hojberg and Sorensen (1993) demonstrated that the concentration of oxygen in agar medium surrounding barley roots declined to 9%. If a similar condition exists for Arabidopsis roots grown on horizontal-positioned plates, this decline would be predicted to cause a hypoxic response and increase in Adh/GUS reporter activity.

Hypoxic stress rapidly induces the expression of *Adh* genes in various tissues within 4 to 8 h. Maximal levels of Arabidopsis *Adh* gene expression are attained within 8 h of hypoxic treatment in 4-week-old mature plants (Dolferus et al., 1994). Likewise, maize *Adh*1 and *Adh*2 genes are induced within 4 h by hypoxic treatment of cell suspension cultures (Paul and Ferl, 1991). Surprisingly, *Adh*/GUS expression was not observed in the roots of very young seedlings (3–4 d old) even though their primary roots had entered the agar medium of horizontal plates. However, older seedlings that have fully developed lateral roots showed dramatically high GUS activity in most of their root tissues. This suggests that the hypoxic response of the Arabidopsis *Adh* gene is developmentally regulated, although it is not clear whether developing seedlings respond to hypoxia with different thresholds to various oxygen levels. To test this hypothesis, it would be necessary to determine if various oxygen levels differentially affect *Adh* gene expression in early stages of seedling growth. Another possibility is that a complex relationship exists

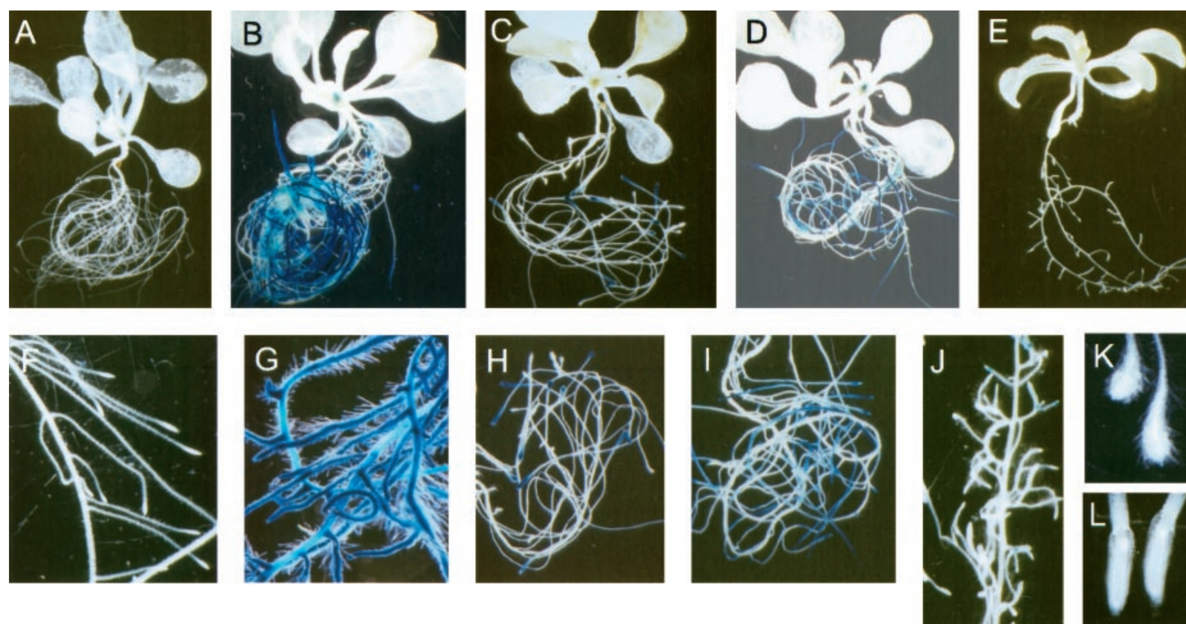



**Figure 6.** Qualitative effects of $Ca^{2+}$ regulators on Adh/GUS expression patterns in seedlings. Five days after transfer to horizontal-positioned plates containing $Ca^{2+}$ antagonists, seedlings were stained in X-Gluc solution for 16 h. Whole seedlings (A–E) and representative roots (F–J) for each treatment condition are shown. A and F, Seedling and root from vertical plates; B and G, horizontal plates without $Ca^{2+}$ antagonists. Seedling and root from horizontal-positioned plates containing: C and H, 5 mM EGTA; D and I, 25 $\mu$M RR; and E and J, 0.5 mM gadolinium. Root tips of seedlings grown on horizontal-positioned plates containing 25 $\mu$M RR (K) and 0.5 mM gadolinium (L) are shown without GUS staining.

among root size, root physiological state, and distance from root tip to the agar surface, such that time of root growth through agar directly influences the generation of hypoxic conditions (Drew, 1997).

There are many reports that root-to-shoot communication occurs when the roots are under stresses such as drought or flooding (Bray, 1997; Jackson, 1997). Both $Ca^{2+}$ and hormones such as ABA and ethylene have been implicated as signaling molecules in this process (Davies et al., 1993; Else et al., 1995; Jackson, 1997). Luminometry of cytosolic aequorin in transgenic plants and fluorescence imaging have been widely used to monitor $Ca^{2+}$ changes upon anaerobic stress (Subbaiah et al., 1994a, 1994b, 1998; Sedbrook et al., 1996). Here we have utilized the *Adh*/GUS reporter gene to investigate whether treatment of the roots with $Ca^{2+}$ antagonists reduced the induction of Arabidopsis Adh expression in shoots. *Adh*/GUS gene expression in the shoot is highly induced by roots growing through agar medium (Fig. 4D). $Ca^{2+}$ antagonists influenced both the shoot and root responses. These results are in agreement with others who observed that RR, gadolinium, and EGTA blocked anoxia-induced increases in $Ca^{2+}$ levels in transgenic aequorin Arabidopsis and partially repressed anoxia-induced Arabidopsis Adh mRNA (Sedbrook et al., 1996).

In the present study, hypoxia-induced *Adh*/GUS gene expression in both roots and shoots was completely blocked by the addition of gadolinium, and significantly inhibited by EGTA and RR treatments (Figs. 5 and 6). In maize suspension cultured cells and seedlings, pretreatment with RR dramatically reduced anoxia-induced ADH activity and *Adh*1 mRNA expression, whereas $Ca^{2+}$ chelator EGTA and plasma membrane $Ca^{2+}$ channel blockers verapamil and bepridil had no affect (Subbaiah et al., 1994a, 1994b). These observations suggest that Arabidopsis may respond to anaerobic stress with increased cytosolic $Ca^{2+}$ released from more than one source in the cell, whereas maize *Adh* levels are regulated by $Ca^{2+}$ from limited cellular sources.

Cytosolic $Ca^{2+}$ is involved in the growth of root hairs and their direction, and the treatment of root hairs with the $Ca^{2+}$ channel blocker verapamil inhibited growth of root hair with a dispersion of cytosolic $Ca^{2+}$ gradient at the tip (Bibikova et al., 1997; Wymer et al., 1997). Similarly, treatment of roots with $Ca^{2+}$ inhibitors altered the root tip phenotype and arrested lateral root development. These changes to root morphology might result from defective mechanisms controlling cell architecture and morphogenesis in roots by blocking $Ca^{2+}$ flux through the cell. Further cytological analyses may be useful in determining how $Ca^{2+}$ affects cell differentiation in root tip.

We have explored the use of the *Adh*/GUS reporter gene system to analyze the stress perception of roots growing through agar medium. Quantitative and qualitative analyses clearly indicate that roots from 1- to 2-week-old Arabidopsis plants perceive hypoxia and mount a stress response as a result of traversing agar growth medium. The Adh/GUS transgenic plant provides a system whereby we can biologically monitor plant perception of stress. These data are in agreement with physical studies that directly monitored oxygen concentrations in zones surrounding growing roots. Taken together, these observations strongly support a model in which the respiratory demands of roots

for oxygen during growth in agar outstrip the ability of diffusion to deliver oxygen to the root surface. The present study also indicates that roots complete the stress perception pathway by transducing a signal to the shoots, resulting in expression of *Adh* in shoot but not in tissues between the shoot and the root. This signal transduction to the shoot is mediated, at least in part, by calcium. Thus, not only does root growth through agar medium result in hypoxia signaling and response in roots, but that signal is propagated to distant parts of the plant. Hence, growth media and conditions influence molecular responses throughout the plant.

**ACKNOWLEDGMENTS**

The authors thank Chris Daugherty for the production of the −846/GUS transgenic Arabidopsis lines. We also thank Maureen Dolan-O'Keefe for critical reading of this manuscript.

Received February 26, 1999; accepted July 4, 1999.

**LITERATURE CITED**

**Abo-el-Saad M, Wu R** (1995) A rice membrane calcium-dependent protein kinase is induced by gibberellin. Plant Physiol **108:** 787–793

**Antosiewicz DM, Polisensky DH, Braam J** (1995) Cellular localization of the $Ca^{2+}$ binding TCH3 protein of *Arabidopsis*. Plant J **8:** 623–636

**Bibikova TN, Zhigilei A, Gilroy S** (1997) Root hair growth in *Arabidopsis thaliana* is directed by calcium and an endogenous polarity. Planta **203:** 495–505

**Bray EA** (1997) Plant responses to water deficit. Trends Plant Sci **2:** 48–54

**Bush DS** (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol **46:** 95–122

**Bustos MM, Iyer M, Gagliardi SJ** (1998) Induction of a β-phaseolin promoter by exogenous abscisic acid in tobacco: developmental regulation and modulation by external sucrose and $Ca^{2+}$ ions. Plant Mol Biol **37:** 265–274

**Chen X, Chang M, Wang B, Wu B** (1997) Cloning of a Ca(2+)-ATPase gene and the role of cytosolic $Ca^{2+}$ in the gibberellin-dependent signaling pathway in aleurone cells. Plant J **11:** 363–371

**Clapham DE** (1995) Calcium signaling. Cell **80:** 259–268

**Davies WJ, Tardieu F, Trejo CL** (1993) Chemical signalling and the adaptation of plants to conditions where water availability is restricted. *In* L Fowden, T Mansfield, J Stoddart, eds, Plant Adaptation to Environmental Stress. Chapman & Hill, London, pp 209–222

**Dolferus R, Jacobs M, Peacock WJ, Dennis ES** (1994) Differential interactions of promoter elements in stress responses of the Arabidopsis Adh gene. Plant Physiol **105:** 1075–1087

**Drew MC** (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annu Rev Plant Physiol Plant Mol Biol **48:** 223–250

**Else MA, Hall EC, Arnold GM, Davies WJ, Jackson MB** (1995) Export of abscisic acid, 1-aminocyclopropane-1-carboxylic acid, phosphate, and nitrate from roots to shoots of flooded tomato plants. Plant Physiol **107:** 377–384

**He C-J, Finlayson SA, Drew MC, Jordan WR, Morgan PW** (1996a) Ethylene biosynthesis during aerenchyma formation in roots of maize subjected to mechanical impedance and hypoxia. Plant Physiol **112:** 1679–1685

**He C-J, Morgan PW, Drew MC** (1996b) Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. Plant Physiol **112:** 463–472

**Hojberg O, Sorensen J** (1993) Microgradients of microbial oxygen consumption in a barley rhizosphere model system. Appl Environ Microbiol **59:** 431–437

**Im CS, Matters GL, Beale SI** (1996) Calcium and calmodulin are involved in blue light induction of the gsa gene for an early chlorophyll biosynthetic step in *Chlamydomonas*. Plant Cell **8:** 2245–2253

**Jackson MB** (1997) Hormones from roots as signals for the shoots of stressed plants. Trends Plant Sci **2:** 22–28

**Jefferson RA, Kavanagh TA, Bevan MW** (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO J **6:** 3901–3907

**Johnson JR, Cobb BG, Drew MC** (1994) Hypoxic induction of anoxia tolerance in roots of *Adh1* null *Zea mays* L. Plant Physiol **105:** 61–67

**Knight H, Trewavas AJ, Knight MR** (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489–503

**Knight H, Trewavas AJ, Knight MR** (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J **12:** 1067–1078

**Legue V, Blancaflor E, Wymer C, Perbal G, Fantin D, Gilroy S** (1997) Cytoplasmic free $Ca^{2+}$ in Arabidopsis roots changes in response to touch but not gravity. Plant Physiol **114:** 789–800

**Liu J, Zhu JK** (1997) An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. Proc Natl Acad Sci USA **94:** 14960–14964

**Liu J, Zhu JK** (1998) A calcium sensor homolog required for plant salt tolerance. Science **280:** 1943–1945

**McKendree WL Jr, Ferl RJ** (1992) Functional elements of the Arabidopsis Adh promoter include the G-box. Plant Mol Biol **19:** 859–862

**Monroy AF, Dhindsa RS** (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25 degrees C. Plant Cell **7:** 321–331

**Paul A-L, Ferl RJ** (1991) *Adh1* and *Adh2* regulation. Maydica **36:** 129–143

**Porterfield DM, Mathews SW, Daugherty CJ, Musgrave ME** (1997) Spaceflight exposure effects on transcription, activity and localization of alcohol dehydrogenase in the roots of *Arabidopsis thaliana*. Plant Physiol **113:** 658–693

**Russell DA, Wong DM-L, Sachs MM** (1990) The anaerobic response of soybean. Plant Physiol **92:** 401–407

**Sachs MM, Freeling M, Okimoto R** (1980) The anaerobic proteins of maize. Cell **20:** 761–767

**Sedbrook JC, Kronebusch PJ, Borisy GG, Trewavas AJ, Masson PH** (1996) Transgenic AEQUORIN reveals organ-specific cytosolic $Ca^{2+}$ responses to anoxia and *Arabidopsis thaliana* seedlings. Plant Physiol **111:** 243–257

**Subbaiah CC, Bush DS, Sachs MM** (1994a) Elevation of cytosolic calcium precedes anoxic gene expression in maize suspension-cultured cells. Plant Cell **6:** 1747–1762

**Subbaiah CC, Bush DS, Sachs MM** (1998) Mitochondrial contribution to the anoxic $Ca^{2+}$ signal in maize suspension-cultured cells. Plant Physiol **118:** 759–771

**Subbaiah CC, Zhang J, Sachs MM** (1994b) Involvement of intracellular calcium in anaerobic gene expression and survival of maize seedlings. Plant Physiol **105:** 369–376

**Tahtiharju S, Sangwan V, Monroy AF, Dhindsa RS, Borg M** (1997) The induction of kin genes in cold-acclimating *Arabidopsis thaliana*: evidence of a role for calcium. Planta **203:** 442–447

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

**Wang M, Van Duijn B, Schram AW** (1991) Abscisic acid induces a cytosolic calcium decrease in barley aleurone protoplasts. FEBS Lett **278:** 69–74

**Wymer CL, Bibikova TN, Gilroy S** (1997) Cytoplasmic free calcium distributions during the development of root hairs of *Arabidopsis thaliana*. Plant J **12:** 427–439