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Transgenic *AEQUORIN* Reveals Organ-Specific Cytosolic Ca^{2+} Responses to Anoxia in *Arabidopsis thaliana* Seedlings[1]

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Using the transgenic *AEQUORIN* system, we showed that the cotyledons and leaves of *Arabidopsis thaliana* seedlings developed a biphasic luminescence response to anoxia, indicating changes in cytosolic Ca^{2+} levels. A fast and transient luminescence peak occurred within minutes of anoxia, followed by a second, prolonged luminescence response that lasted 1.5 to 4 h. The Ca^{2+} channel blockers Gd^{3+}, La^{3+}, and ruthenium red (RR) partially inhibited the first response and promoted a larger and earlier second response, suggesting different origins for these responses. Both Gd^{3+} and RR also partially inhibited anaerobic induction of alcohol dehydrogenase gene expression. However, although anaerobic alcohol dehydrogenase gene induction occurred in seedlings exposed to water-agar medium and in roots, related luminescence responses were absent. Upon return to normoxia, the luminescence of cotyledons, leaves, and roots dropped quickly, before increasing again in a Gd^{3+}-, La^{3+}-, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid-, and RR-sensitive fashion.

When exposed to anoxia, plants undergo major metabolic changes to maintain energy production despite a shut off of respiratory phosphorylation. They do so by increasing the rate of Suc and starch mobilization, by accelerating the rate of glycolysis and diversifying its end products, and by accelerating the ethanol fermentation pathway (reviewed by Ricard et al., 1994). These metabolic changes are associated with major changes in gene expression involving a decrease in general mRNA translatability and an activation of expression of a set of anoxic genes, most of which code for enzymes involved in starch and Glc mobilization, glycolysis, and ethanol fermentation (Sachs et al., 1980; Sachs, 1993; Peschke and Sachs, 1994). The well-characterized *ADH* gene, which codes for the alcohol dehydrogenase enzyme (EC 1.1.1.1), falls in that category of anoxia-induced genes (Sachs et al., 1980; Dolferus et al., 1985; DeLisle and Ferl, 1990). Additionally, anoxia induces a rapid acidification of the cytoplasm, correlated with a substantial decrease in ATP production (Roberts et al., 1984; Saint-Gès et al., 1991).

The existence of anoxia-induced changes in plant metabolism and physiology implies that plant cells sense anoxic conditions and respond to them very quickly by allowing for the production of enough ATP and the regeneration of enough NAD(P)+ to survive (Ricard et al., 1994). The molecular mechanisms by which a plant cell detects anoxia and responds to it are poorly understood. However, experiments with animal and plant cells indicate that intracellular Ca^{2+} may be involved as a second messenger in transducing the anoxic signal into a physiological response. First, a two-phase change in cytosolic Ca^{2+} level is induced by anoxia in rat hepatocytes and cardiac myocytes (Allshire et al., 1988; Miyata et al., 1992; Gasbarrini et al., 1992a, 1992b). Second, RR, a blocker of organellar Ca^{2+} channels, inhibits anaerobic gene expression and poststress survival in maize seedlings. The RR effects are accompanied by a blockage of Ca^{2+} influx and are prevented by the addition of Ca^{2+} to the medium (Subbaiah et al., 1994a, 1994b). Third, anoxia leads to changes in cytosolic Ca^{2+} levels in corn cell cultures. Anoxia-induced Ca^{2+} changes are inhibited by RR, and RR inhibition is prevented by the addition of exogenous Ca^{2+} to the medium (Subbaiah et al., 1994b). Taken together, these data suggest that changes in cytosolic Ca^{2+} levels may be responsible for transducing the anoxic signal into a physiological response in eukaryotic cells.

A molecular genetic approach could substantially help in the characterization of the molecular processes by which plant cells sense anoxic conditions and respond to them. Indeed, such a strategy would allow the identification and characterization of specific players in the pathway, and their interconnection, through the analysis of mutations affecting the response. *Arabidopsis thaliana* is a model system for molecular genetic studies in plants. Its small stature, short generation time, small genome, amenability to

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Abbreviations: *ADH*, alcohol dehydrogenase gene; CCD, charge-coupled device; dH_2O, distilled water; MS, Murashige and Skoog; RLU, relative light unit; RR, ruthenium red.

laboratory experimentation and genetic selfing and crossing and the fast advancements in genome mapping and characterization contribute to its success (Somerville and Meyerowitz, 1994). *A. thaliana* also responds to anoxia. Indeed, several laboratories have shown that the unique *A. thaliana ADH* gene is activated 10- to 15-fold by anoxia in seedlings during germination, as well as in roots and cotyledons of young Arabidopsis seedlings (Dolferus et al., 1985; Chang and Meyerowitz, 1986; Ferl and Laughner, 1989).

Recent observations suggest that signal transduction via cytosolic Ca^{2+} may directly lead to changes in gene expression (Neuhaus et al., 1993). The objective of our research was to investigate the changes induced by anoxia in cytosolic Ca^{2+} in whole plants and tissues and to test whether such changes could account for increased *ADH* expression. These experiments clearly required a simple method of measuring cytosolic Ca^{2+} at the whole plant or tissue level, and for this we took advantage of a new technology developed by us that involves the transformation of plants with the cDNA for *APOAEQUORIN* (Knight et al., 1991). The combination of APOAEQUORIN with coelenterazine generates AEQUORIN, a Ca^{2+}-sensitive luminescent protein. The luminescence of coelenterazine-treated *APOAEQUORIN*-expressing transgenic plants directly reports changes in cytosolic Ca^{2+} levels, independently of the cytoplasmic pH in the physiological range (Cobbold and Lee, 1991; Knight et al., 1991). Since this technique monitors cytosolic Ca^{2+} in whole seedlings, detection of specific tissue variation and monitoring of the kinetics of Ca^{2+} changes are greatly simplified (Knight et al., 1991).

MATERIALS AND METHODS

Genetic Transformation of Plants

The pMAQ2 construct containing the *APOAEQUORIN* gene fused to the cauliflower mosaic virus 35S promoter and nopaline synthase terminator sequences (Knight et al., 1991) was transferred into *Agrobacterium tumefaciens* (strain LBA4404) and used to transform *Arabidopsis thaliana* (No-O ecotype), as described by Valvekens et al. (1988). Kanamycin-resistant seedlings were recovered and transferred to root-inducing medium (Valvekens et al., 1988) in Phytatray containers (Sigma) and incubated in a Conviron (Asheville, NC) TC16 growth chamber (22°C, 80% RH, 16 h of light at 80 μE m^{-2} s^{-1} from cool-white fluorescent tubes, 8 h of dark), where they were allowed to self-pollinate and set seed. T_2 seeds were harvested and plated onto 0.8% agar medium containing 50 μg/mL kanamycin, one-half-strength MS salts (Murashige and Skoog, 1962), and 1.5% Suc ("complete medium"). Kanamycin-resistant seedlings were recovered and transferred to soil, where they were allowed to self-pollinate. Both tissue and T_3 seed were harvested from the T_2 plants. Thirty T_3 seedlings were tested for kanamycin resistance on 0.8% agar medium (one-half-strength MS salts, 1.5% Suc, 50 μg/mL kanamycin), allowing the identification of T_2 plants homozygous for the T-DNA insert. DNA was extracted from tissue derived from the homozygous T_2 plants, digested with *Eco*RI or *Pst*I, electrophoresed on an agarose gel, and transferred to a Hybond N+ membrane (Amersham), as described by Ausubel et al. (1994). The membrane was probed with the *APOAEQUORIN* gene using standard procedures (Sambrook et al., 1989). A T_2 plant carrying only one T-DNA insert was retained for further study. Five-day-old T_3 progeny seedlings from that plant were soaked in 10 μM coelenterazine (Molecular Probes, Eugene, OR) for 7 h in the dark and transferred to a luminometer chamber. Their luminescence was followed before, during, and after a cold-shock stimulus similar to that described by Knight et al. (1991). Plants showing the highest luminescence response to cold-water stimulation were self-pollinated, and their T_4 seeds were used in the experiments described below.

AEQUORIN Reconstitution and Luminescence Measurements

Seedlings were germinated and grown on the surface of vertically oriented agar plates (one-half-strength MS salts, 1.5% Suc, 1.5% agar) within a Conviron TC16 growth chamber (22°C, 80% RH, 16 h of light at 80 μE m^{-2} s^{-1} from cool-white fluorescent tubes, 8 h of dark) for 5 d unless stated otherwise. Individual seedlings were then transferred to microfuge tubes and submerged in 222 μL of 10 μM coelenterazine (first dissolved in 3.8 μL of ethanol) in dH_2O at room temperature in the dark for 7.5 h, unless stated otherwise. The seedlings were not aerated during this treatment to avoid as much as possible a mechanical perturbation of the seedlings as well as coelenterazine oxidation before AEQUORIN reconstitution (Molecular Probes, 1994). At the end of the treatment, the seedlings' roots were brownish, with the most intense coloration occurring at the transition region between roots and hypocotyls and at occasional damaged sites (e.g. broken root hairs, kinks in the roots, etc.). However, this coloration disappeared after 17 to 24 h, whereas the luminescence responses to anoxia reported here were still detectable at that time (see below). Furthermore, this coelenterazine treatment did not affect the rate of growth or the morphology of treated seedlings (data not shown).

For the luminometer assays, individual coelenterazine-treated seedlings were placed in the bottom of plastic luminometer tubes on the surface of a thin layer of 1.5% agar medium containing one-half-strength MS salts and 1.5% Suc (complete medium), except where stated otherwise. The tubes were left undisturbed in the dark for 0.5 or 24 h, as described in the text, before being assayed in a Monolight 1500 luminometer (Analytical Luminescence Laboratory, San Diego, CA). This incubation period after coelenterazine treatment was incorporated in these assays to allow the seedlings to reequilibrate in fully aerobic conditions and to recover from the mechanical perturbation that accompanies seedling transfer. Luminometer measurements were taken at 1-s intervals (recorded as average RLU/s). The data were stored directly into Microsoft Word files on a Macintosh Centris 610 computer and analyzed using the Microsoft Excel program. Gasses and solutions were injected at constant flow rates into the luminometer

chamber through tubes connected to the injection ports available on the luminometer. The gas used for anoxia treatments was composed of N_2 containing 350 μL L^{-1} CO_2 (purchased from Liquid Carbonic Corporation, Milwaukee, WI) at 1 atm of pressure and was humidified by bubbling through 2 L of dH_2O. That bubbling procedure also assured the maintenance of the gas temperature at constant room levels (data not shown). For normoxia treatments, air was also delivered at 1 atm of pressure after bubbling through 2 L of water. For cold-shock stimuli, 1500 μL of ice-cold water were delivered onto the seedling or plant organ under study, using a 5-mL Hamilton (Reno, NV) syringe connected to the second injection port of the luminometer.

For CCD imaging, individual seedlings were placed on complete agar medium (in Petri dishes) and photographed with a CCD imager (model CH220, Photometrics, Tucson, AZ) equipped with a 55-mm F/2.8 Nikon lens and a 2X Teleconverter. Cold shock was induced by injecting 200 μL of ice-cold water onto the plant with a pipetor. Touch stimulation was administered by lightly touching the seedling five times with a Pasteur capillary pipet. Stimulations were performed in the dark at the beginning of 30-s time exposures. The data were collected into and analyzed with the IP Lab Spectrum program (Signal Analytics, Vienna, VA).

For in vitro luminescence reactions, 5-d-old seedlings grown on the surface of a complete agar medium were soaked in 10 μM coelenterazine solution for 7.5 h and allowed to recover in normoxia on complete agar medium for 30 min, as described in "Results." Shoots and roots were dissected with a scalpel and separated from each other. They were subjected to anoxia or normoxia stimulus, as described above, before being frozen in liquid N_2 and homogenized in 250 μL of the protein extraction buffer described by Knight et al. (1991), without coelenterazine. After a 30-s centrifugation at 10,000*g* to remove cell debris, each cell extract was aliquoted in 50-μL samples and frozen at −70°C. For luminescence determinations, 10-μL aliquots were added to a luminometer cuvette containing 40 μL of extraction buffer and transferred to the luminometer. For some experiments, plant extracts were subjected to anoxia or normoxia for 5 min, as described above, before being injected with 100 μL of a 50 mM $CaCl_2$ solution. The extract's luminescence was recorded over time, and the maximum luminescence was determined. For each extract these measurements were repeated at least three times. Extract protein contents were determined using the Bio-Rad Protein Microassay System, as recommended by the supplier. AEQUORIN activity was defined in RLU per microgram of protein.

RNA Extraction and Northern Blot Analyses

Seedlings were germinated and grown in vertically oriented Petri plates (about 150 plants each) on complete agar medium for 5 d in the Conviron TC16 chamber. For anoxia and normoxia treatments, the plates were tightly sealed with Parafilm (American National Can, Neenah, WI) and injected with a continuous flow of either water-saturated N_2 containing 350 μL L^{-1} of CO_2 (for anoxia) or water-saturated air (for normoxia). During these treatments, the plates were submerged in water to avoid gas exchange with outside air. Immediately after gassing, seedlings were frozen in liquid N_2. For experiments in which RNA from specific tissues was needed (see "Results"), the tissues were dissected from the intact seedlings after gassing and before freezing. For experiments in which *ADH* expression was tested on complete or water-agar medium (see "Results"), seedlings were first soaked in water for 7.5 h and then transferred into Petri dishes on complete or water-agar medium for 0.5 h before anoxia or air treatment (all of which was done in the dark). This protocol was used to mimic the treatment of seedlings used in the luminometer assays.

Total RNA was isolated, electrophoresed in formaldehyde-agarose gels, and blotted onto Hybond N+ membranes by capillary action using standard procedures (Masson et al., 1989; Hilson et al., 1993). Hybridizations were done by probing blots successively with [^{32}P]dCTP-labeled fragments (Amersham Random Hexamer Labeling Kit; Chang and Meyerowitz, 1986) of the *ADH* gene, the *APOAEQUORIN* gene (Knight et al., 1991), or the *rDNA* gene (Polans et al., 1986). Between successive hybridizations, the filters were stripped of the radioactive probe derived from the previous hybridization as described by Ausubel et al. (1994). Probed blots were scanned with a Molecular Dynamics (Sunnyvale, CA) Phosphorimager and digitized. The digitized images were analyzed with the Molecular Dynamics ImageQuaNT program.

RESULTS

Anoxia Induces Complex Changes in Cytosolic Ca^{2+} Levels

To determine the effect of anoxia on cytosolic Ca^{2+} levels, we exposed *AEQUORIN*-expressing *A. thaliana* seedlings to 1 atm of water-saturated oxygen-free gas (N_2 + 350 μL L^{-1} CO_2) and quantified seedling luminescence over time. The data, shown in Figure 1, indicate that *A. thaliana* seedlings responded to anoxia by complex changes in cytosolic Ca^{2+} levels. A first transient luminescence peak (peak I) began within 50 s of anoxia. After reaching a maximum very quickly, luminescence decreased progressively to near-baseline levels. It remained close to baseline briefly, then slowly increased to reach a maximum 0.5 to 2 h after initiation of anoxia. After reaching that peak (peak II), luminescence decreased progressively over a period of 1 to 2 h. Throughout the response, rapid luminescence increases were observed. These spikes occurred randomly in plants under both aerobic and anaerobic conditions, and were not due to luminometer background noise.

Although the biphasic luminescence response to anoxia was found in all plants analyzed, some plant-to-plant variability in intensity and timing of peak luminescence was observed. Therefore, we analyzed the response of seven identically treated 5-d-old seedlings to anoxia and averaged the data points of six of the seven plants over the length of the assay. We also determined the SE for the

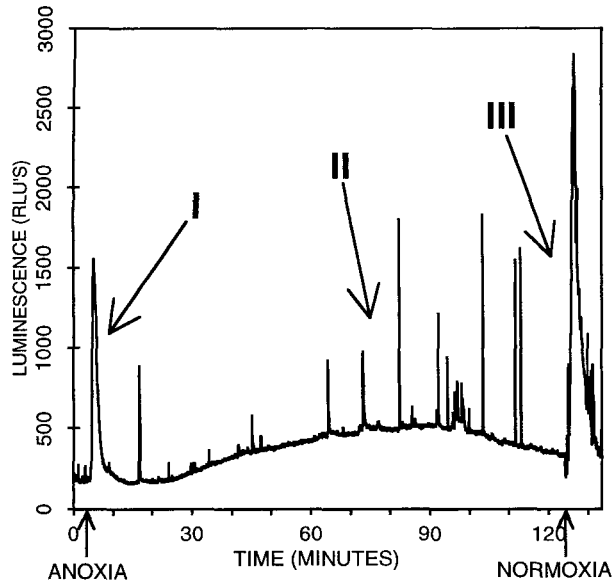


Figure 1. The luminescence response of a typical 5-d-old *AEQUORIN*-expressing Arabidopsis seedling exposed to anoxia. The seedling was placed in a luminometer tube on a thin layer of complete medium and transferred to the luminometer chamber. The level of seedling luminescence was recorded over time. After 4 min of recording, the seedling was subjected to an anoxic challenge (labeled ANOXIA on the *x* axis) that lasted 120 min. At that time, the anoxic treatment was terminated by the introduction of air into the chamber (labeled NORMOXIA on the *x* axis). The peak-I and peak-II luminescence responses to anoxia, as well as the peak-III luminescence response appearing upon return to normoxia, are indicated by arrows.

response at several crucial time points (maxima in peaks I, II, and III). The results, shown in Figure 2A, indicate that the average luminescence response to anoxia is similar to that shown in Figure 1. Data from the seventh plant were not averaged with the others because the peak II luminescence response occurred much earlier than usual (Fig. 2B). About 20% of all tested plants developed an early peak-II response of that type.

To verify that anoxia does not affect the Ca^{2+}-dependent luminescence of reconstituted AEQUORIN, total soluble protein was extracted from coelenterazine-treated 5-d-old seedlings and equal aliquots of that extract were challenged with an excess of $CaCl_2$ after 5 min of normoxia and anoxia. Results indicated no significant changes in Ca^{2+}-dependent luminescence under anoxic and normoxic conditions (642,792 ± 40,730 and 674,286 ± 78,030 RLU, respectively; $n = 3$).

When plants were returned to normoxia, AEQUORIN luminescence immediately decreased and then increased again (Figs. 1, 2A, and 2B). This air-induced luminescence peak (peak III) was transient; its intensity varied from plant to plant.

To determine the effects of repeated anoxia challenges on cytosolic Ca^{2+} levels, we subjected seedlings to a succession of anoxic and normoxic treatments. Figure 3A shows that the peak-I and peak-III luminescence responses described above occurred in a reproducible manner. The first anoxia challenge generated the largest peak-I luminescence response in most plants analyzed, whereas subsequent treatments gave rise to lower and similar peak-I responses. This result suggested that an exposure to anoxia modifies the ability of the seedling to respond to subsequent treatments by luminescence spikes.

To determine if the 30-min recovery period after coelenterazine treatment is sufficient to eliminate the hypoxia effects accompanying that treatment, we subjected seedlings to a succession of two 45-min anoxia treatments, one after 30 min and the other after 24 h, and recorded the luminescence response to both treatments. The results shown in Figure 3, B and C, demonstrated that seedlings respond very similarly to the same anoxic shock applied 30 min or 24 h after the coelenterazine treatment. A very similar average peak-I luminescence response was observed during both treatments, whereas peak II appeared a little later in seedlings allowed to recover for 24 h. The average peak-III luminescence response to the return to normoxia also appeared slightly larger and earlier after a 24-h recovery. All seedlings developed a typical luminescence response with three response peaks. However, the intensity of the peak-I and peak-III responses varied considerably from seedling to seedling, which explains the large error bars shown in Figure 3, B and C. Taken together, these data indicate that a normoxia recovery period of 30 min is sufficient to eliminate any side effect of the

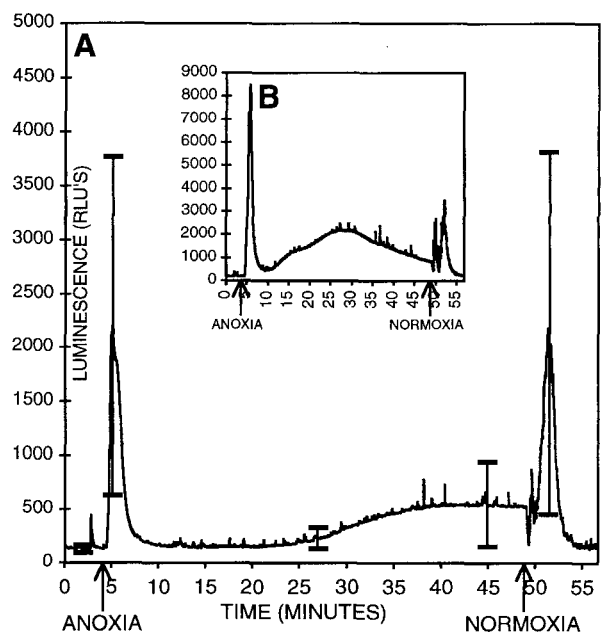


Figure 2. Average luminescence response to anoxia of 5-d-old seedlings. Seven 5-d-old seedlings were independently subjected to the experiment described in the legend to Figure 1. Six of them showed a luminescence profile that was very similar to that shown in Figure 1. A, Average luminescence response of these six seedlings for each data point, along with the SE values calculated for specific data points corresponding to the peak-I, peak-II, and peak-III luminescence responses (bars connected by a vertical line). The seventh seedling developed an early peak-II response (see text). Its luminescence profile during this experimental procedure is shown in B.

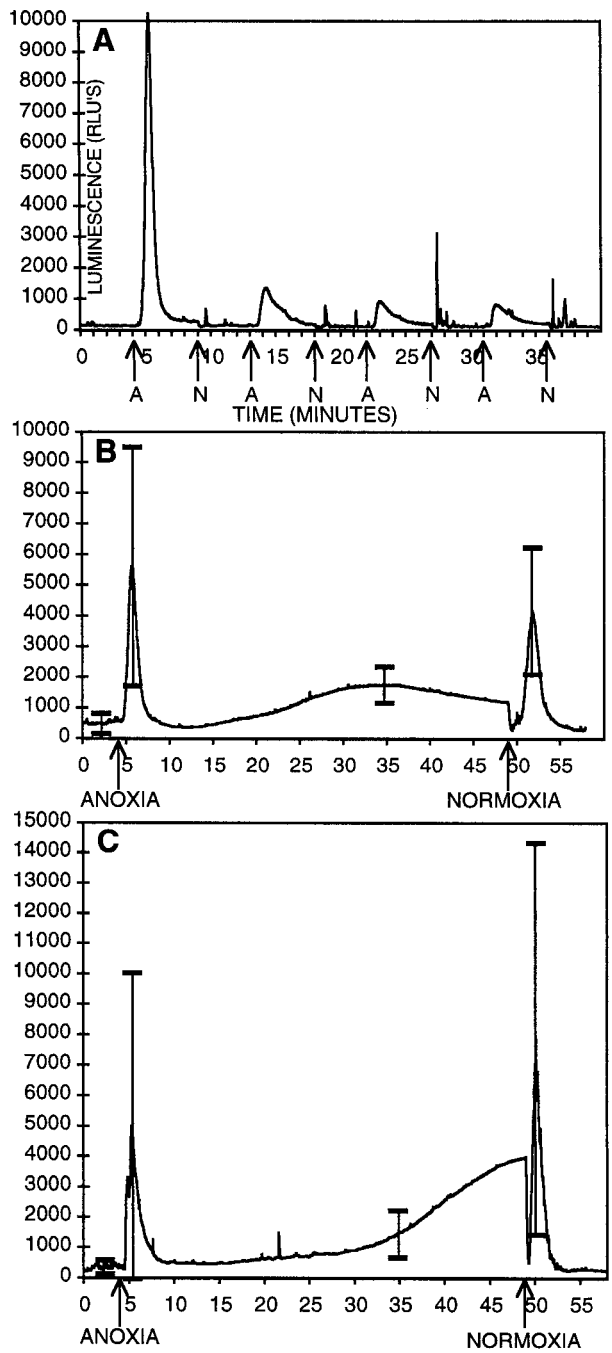


Figure 3. A, The luminescence response of a typical *AEQUORIN*-expressing plant repeatedly stimulated with anoxia (A) followed by normoxia (N). B and C, Average luminescence response of six 5-d-old *A. thaliana* seedlings to a 45-min anoxia treatment initiated 30 min (B) or 24 h (C) after the coelenterazine treatment. The bars indicate the SE values for these data points.

coelenterazine treatment on the peak-I luminescence response to subsequent anoxia.

Ca^{2+} Responses to Anoxia Are Organ Specific

To determine whether different organs respond similarly to anoxia, we measured the luminescence response to anoxia of exposed shoots and roots on half-masked intact seedlings and of dissected cotyledons, hypocotyls, and roots from 5-d-old seedlings grown on agar medium containing one-half-strength MS salts and 1.5% Suc (referred to as the "complete medium" herein). Results indicated that shoots from entire seedlings and dissected cotyledons, but not roots or hypocotyls, respond to anoxia with peak-I and peak-II luminescence responses (Fig. 4, A–E). At times, roots produced small and rapid transient luminescence increases after anoxia initiation, but these peaks looked indistinguishable from background luminescence increases that occurred randomly during both anoxia and normoxia treatments (Fig. 4B). Peak-II-related luminescence increases were never seen.

To verify if the Ca^{2+} response to anoxia is specific to the cotyledons, we subjected leaves from 15-d-old seedlings and inflorescences from 34-d-old seedlings to anoxia and followed the luminescence emitted by these organs over time. The data shown in Figure 4, F and G, indicate that both organs develop a peak-I luminescence response to anoxia similar to that developed by shoots and dissected cotyledons of 5-d-old seedlings. Additionally, when leaves and inflorescences were exposed to longer periods of anoxia (15 min), a peak-II luminescence response appeared and the peak-III luminescence response upon return to normoxia became more apparent (data not shown).

When shoots, dissected cotyledons, leaves, or inflorescences of Arabidopsis seedlings were returned to normoxia, luminescence decreased immediately, then increased transiently before returning to baseline levels (Fig. 4, A, C, F, and G). Roots and hypocotyls also showed a transient luminescence peak after return to normoxia (Fig. 4, B, D, and E). The root's peak-III luminescence occurred in many sharp, transient peaks, whereas the cotyledon's and leaf's luminescence peaks were smoother in nature (Fig. 4, A–G). Finally, we generally found that the peak-III luminescence response appearing upon return to normoxia increased with increased periods of anoxic treatments, which explains the small size of the peak-III luminescence response shown in Figure 4, F and G. In summary, cotyledons, leaves, and inflorescences developed distinct luminescence responses to anoxic treatments compared to roots and hypocotyls, where anoxia-related luminescence increases were questionable at best. All organs responded with AEQUORIN-derived luminescence after return to normoxia.

To determine if functional reconstituted AEQUORIN was present in all parts of the coelenterazine-treated seedlings, we analyzed the levels of AEQUORIN activity in protein extracts from dissected shoots and roots exposed to normoxia or anoxia for 45 min. The results shown in Table I indicate that extracts from anoxia-treated roots contain more reconstituted AEQUORIN than extracts from anoxia-treated shoots, suggesting that the difference in luminescence response to anoxia of shoots and roots does not derive from differences in quantities of reconstituted AEQUORIN in these tissues. Furthermore, extracts from anoxia-treated roots and shoots contained more reconstituted AEQUORIN than extracts from their normoxia-treated counterparts.

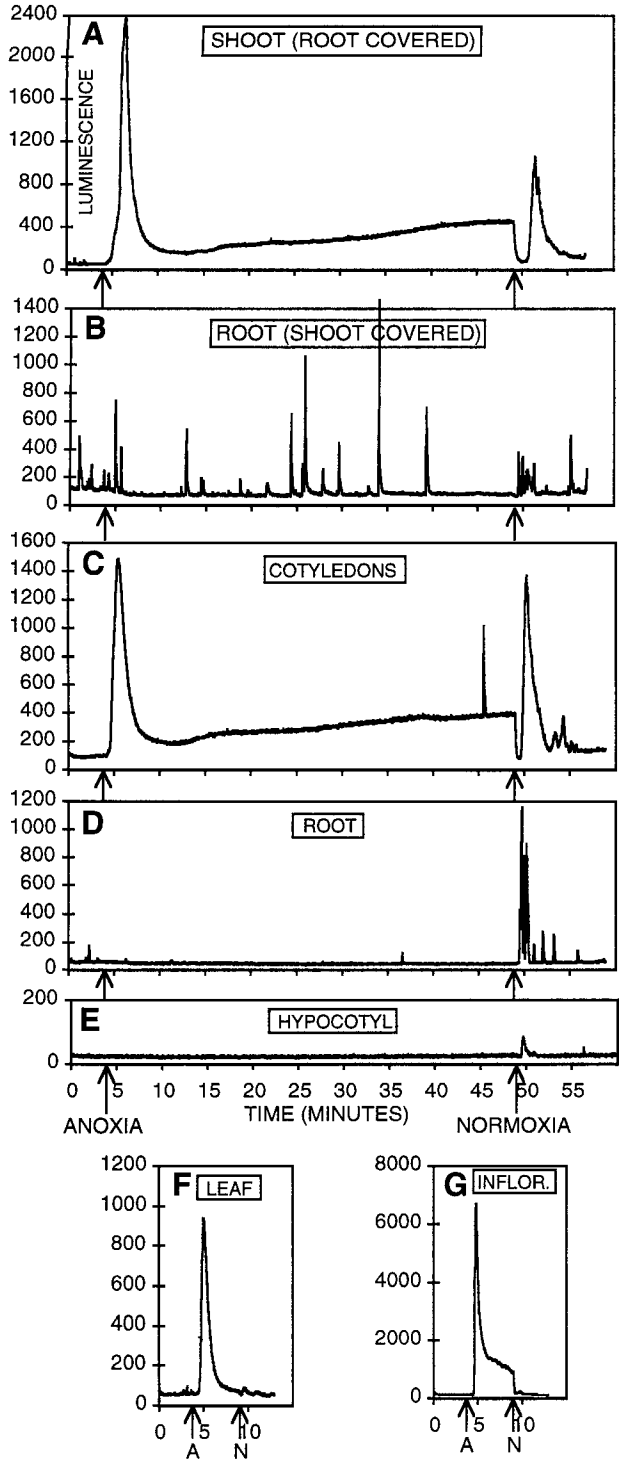


Figure 4. Anoxia-induced luminescence responses of the exposed shoot (A) and root (B) of half-masked *A. thaliana* seedlings, and of dissected cotyledons (C), root (D), hypocotyl (E), leaf (F), and inflorescence (G) of *AEQUORIN*-expressing *A. thaliana* seedlings. Five-day-old seedlings were soaked in 10 μM coelenterazine for 7.5 to 10 h. The root (A) or the shoot (B) of individual seedlings was masked with aluminum foil and placed into a luminometer tube on a thin layer of complete agar medium for 30 min before being assayed (A and B). Alternatively, coelenterazine-pretreated seedlings were dissected into cotyledons, hypocotyls, roots, leaves, and inflorescences (C–G). Each dissected organ was placed into a luminometer tube on a thin layer of complete medium for 0.5 to 2 h before being assayed. The hypocotyl shown in E constitutes an exception to this experimental procedure; it was maintained for 14 h on complete medium after coelenterazine treatment before being assayed. The organs tested in A through E were dissected from 5-d-old seedlings, the leaf shown in F was dissected from a 15-d-old seedling, and the inflorescence shown in G was dissected from a 34-d-old seedling. All plants were grown on complete medium. For F and G, A represents the time of anoxia initiation, and N represents the time of return to normoxia.

Table I. *Amounts of reconstituted AEQUORIN in normoxia- and anoxia-treated organs*

Five-day-old seedlings were treated with coelenterazine for 7.5 h, as described in the text, allowed to recover in normoxia on a thin layer of complete agar medium for 0.5 h, dissected, and exposed to the anoxic/normoxic treatment described below. Extracts were then prepared and analyzed as described in "Materials and Methods."

Organ	Treatment	AEQUORIN Activity
		RLU/μg protein
Shoot	Normoxia (45 min)	22,787
	Anoxia (45 min)	46,123
Root	Normoxia (45 min)	580,652
	Anoxia (45 min)	743,970

To verify that the anoxia treatment did not specifically affect the ability of reconstituted AEQUORIN to report Ca^{2+} changes in response to environmental stimuli in roots, we analyzed the luminescence response of entire seedlings and of dissected shoots and roots to a cold stimulus after 5-, 30-, or 45-min exposures to anoxia or to normoxia. Results shown in Table II indicate that dissected shoots and roots respond similarly to a cold-shock stimulus, whether exposed to anoxia or normoxia. They also indicate that the luminescence response to a cold-shock stimulus is lower for organs exposed to anoxia for 30 to 45 min than for similar organs exposed to normoxia for the same period.

We also imaged the luminescence response to cold-shock and touch stimulation of cotyledons, hypocotyls, and roots of intact seedlings, using the CCD camera system described in "Materials and Methods." Results shown in Figure 5 indicate that both roots and cotyledons respond to a succession of local touch stimuli, as well as to a cold-shock stimulus, by emitting light at the site of stimulation (Fig. 5; data not shown).

We have also attempted to image the luminescence response of seedling tissues to anoxia with the CCD camera system described above. Unfortunately, the amount of light emitted under these conditions was too low and fell below the limit of sensitivity of our camera system (data not shown).

Peak-I Luminescence Is Affected by the Composition of the Medium to Which Seedlings Are Exposed

The anoxia-induced Ca^{2+} increases described above occurred in plants assayed on complete agar medium (Murashige and Skoog, 1962). To determine if medium compo-

Table II. *Luminescence response to a cold shock of entire seedlings and of dissected shoots and roots while exposed to normoxia or to anoxia*

Five-day-old seedlings were treated with coelenterazine for 7.5 h, as described in the text, dissected, and then allowed to recover on a thin layer of complete agar medium for 0.5 h. They were exposed to normoxia or anoxia for the period shown in the second column, and then exposed to a cold-shock stimulus, as described in "Materials and Methods." The third column reports the average peak luminescence response to the cold-shock stimulus of two, three, or five seedlings (fourth column).

Tissue	Postrecovery Treatment	Average Luminescence Response to Cold	No. of Seedlings/ Organs
Dissected roots	Air (30 min)	1,569,948	2
	Anoxia (30 min)	1,023,009	2
Dissected shoots	Air (30 min)	1,567,577	2
	Anoxia (30 min)	823,223	2
Entire seedlings	Air (2 min)	1,955,218	3
	Anoxia (2 min)	1,806,295	3
	Air (45 min)	1,907,817	2
	Anoxia (45 min)	1,217,170	5

sition affects the response, we followed the anoxia-induced luminescence response of individual 5-d-old seedlings placed on agar medium containing either 1.5% Suc and one-half-strength MS salts or nothing ("water-agar medium") for 30 min before assay. Results shown in Figure 6A indicated that seedlings subjected to anoxia while being maintained on water-agar medium developed a normal peak-II luminescence response but showed little evidence of a peak-I response. Seedlings maintained on a water-agar medium for longer periods of time (overnight) occasionally developed a small peak-I response, but that response was always much smaller than the response developed by seedlings maintained on complete agar medium (Fig. 6B). The absence of a peak-I luminescence response in seedlings exposed to water-agar medium could not be attributed to the lack of a specific medium component. Indeed, seedlings exposed to medium containing only one-half-strength MS salts or 1.5% Suc developed anoxia-induced Ca^{2+} responses with kinetics similar to those of seedlings exposed to complete agar medium, even though the amplitude of the peak-I luminescence response was lower (data not shown). When anoxia-treated seedlings maintained on water-agar medium were returned to normoxia, they developed a normal luminescence response, starting with a sharp decrease in light emission followed by a transient peak-III luminescence response (Fig. 6A).

To determine if the seedlings' peak-I luminescence responsiveness to anoxia was a consequence of nutrient uptake before the assay or if it was dependent on continuous contact of the plants with the nutrients during the assay, we transferred 5-d-old coelenterazine-treated seedlings to complete agar medium for 25 min. We then transferred them to either a water-agar medium or to complete agar medium for 5 min and anoxia-stimulated them on that medium. Conversely, coelenterazine-treated seedlings were placed on water-agar medium for 25 min, then transferred to complete or to water-agar medium for 5 min, and anoxia-treated as described above. In all cases, the luminescence response was recorded. Results showed that seedlings transferred from complete agar medium (25 min) onto water-agar medium for 5 min developed a much smaller peak-I luminescence response (average of 733 RLU) than plants transferred from water-agar medium (25 min) onto complete agar medium (average of 2350 RLU). However, that peak-I luminescence response was significantly larger than that of plants exposed to water-agar medium for a total of 30 min (200 RLU, corresponding to baseline levels). The relative intensities of the anoxia-induced peak-I luminescence response of plants exposed first to water-agar medium for 25 min and then to complete agar medium for 5 more min were slightly lower than those of plants exposed to complete agar medium for 30 min before assay (4800 RLU, on average). Taken together, these data suggest that the composition of the agar medium to which plants are exposed during anoxia affects the intensity of the peak-I luminescence more than does the composition of the medium to which plants were exposed before the treatment.

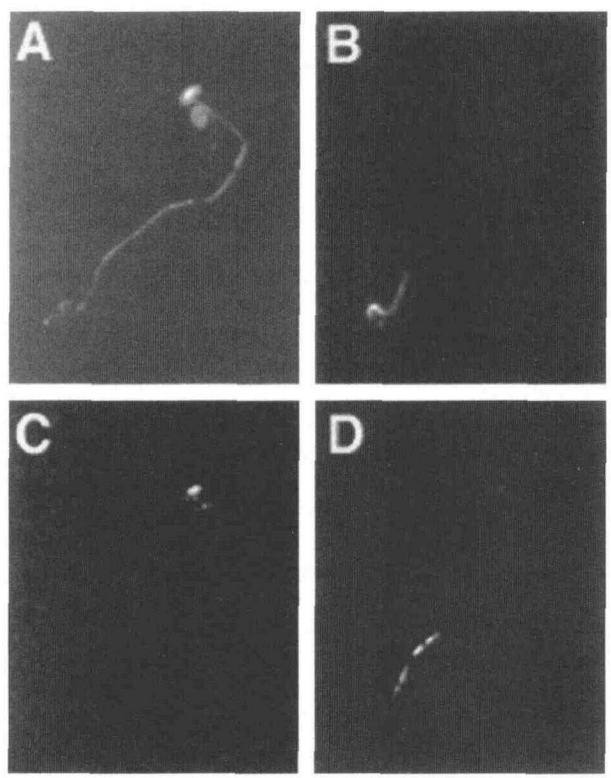


Figure 5. CCD images of the luminescence response of a 5-d-old *AEQUORIN*-expressing *A. thaliana* seedling subjected to cold shock or touch stimulation. A, An image of the plant in room light, showing its position on each frame. B, Luminescence response to a cold-shock stimulus; 200 μL of ice-cold water was injected onto the root, where the luminescence occurred. C, Luminescence response to touch stimulation; the cotyledons were lightly touched five times with the side of a Pasteur pipet. D, Luminescence response to touch stimulation; the root was lightly touched five times with a Pasteur capillary pipet at the sites where the luminescence occurred. Exposure time for the images shown in B through D was 30 s.

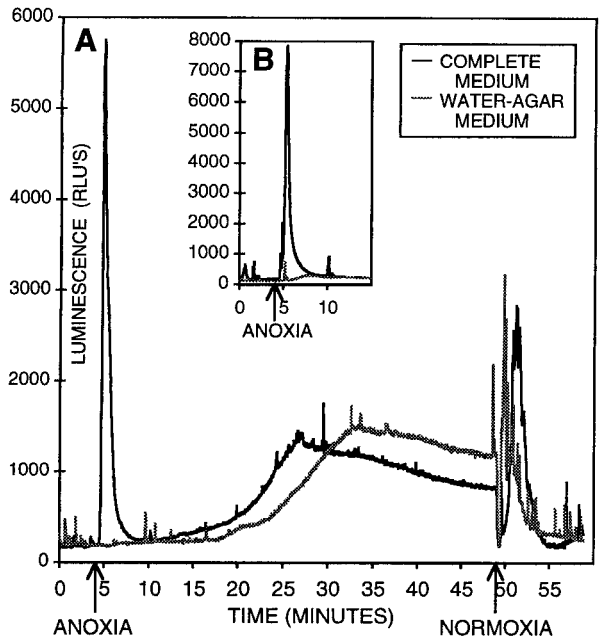


Figure 6. The effect of medium composition on the anoxia-induced luminescence response. Five-day-old seedlings were treated in 10 μM coelenterazine for 7.5 h and then transferred to a luminometer containing complete agar medium (solid curve) or water-agar medium (shaded curve) for 0.5 (A) or 17.5 h (B) before being subjected to the anoxia/normoxia cycle (labeled below the *x* axis).

Various Ca^{2+} Channel Blockers Affect the Luminescence Response Differently

To define whether intracellular Ca^{2+} stores are used as a source of Ca^{2+} for anoxia-induced cytosolic Ca^{2+} increases, we analyzed the luminescence response to anoxia of *A. thaliana* seedlings pretreated with either 10 or 50 μM RR for 2 h (Campbell, 1983; Price et al., 1994). Both RR pretreatments altered the luminescence response very similarly. Figure 7 shows the average response of five seedlings pretreated with 50 μM RR for 2 h. This pretreatment lowered the amplitude of the first luminescence peak (peak I) but did not change its timing. By contrast, RR caused the second luminescence peak (peak II) to appear earlier, with a slightly larger amplitude than that seen in control plants. Additionally, RR pretreatment modified the luminescence response that occurred after return to normoxia (peak III), making it appear later and with a smaller amplitude than in control plants. These RR effects on the luminescence response to anoxia and to the return to normoxia were also found when RR-pretreated seedlings were tested on RR-containing complete agar medium (data not shown).

To determine if plasma membrane Ca^{2+} channels are involved in the response, we pretreated *AEQUORIN*-expressing Arabidopsis seedlings with either 1 or 10 mM Gd^{3+} solutions for 2 h before anoxia exposure (Tester, 1990; Price et al., 1994). Here again, both Gd^{3+} pretreatments very similarly altered the luminescence response to anoxia. Results shown in Figure 7 indicated that Gd^{3+}-pretreated seedlings produced lower responses and slower rates of increase in peak-I luminescence compared to control plants. Additionally, peak-II luminescence responses began very early and reached much larger peak amplitudes than those seen in untreated or RR-pretreated seedlings. On the other hand, the luminescence responses normally observed after normoxia restoration (peak III) were completely abolished. These Gd^{3+} effects on the luminescence response to anoxia and to a return to normoxia were also observed when Gd^{3+}-pretreated seedlings were tested on water-agar medium, with the exception of peak I, which does not exist on such a medium, as described above. However, they could not be tested on Gd^{3+}-containing agar medium because an unidentified precipitate appeared in the medium under these conditions (data not shown). Finally, La^{3+}, another inhibitor of plasma membrane channels (Tester, 1990), had the same effects on the luminescence response to anoxia and return to normoxia as Gd^{3+} (data not shown).

Exogenously Added EGTA Affects the Luminescence Response

To determine if extracellular Ca^{2+} is required for portions of the response to anoxia, as suggested by the Gd^{3+} and La^{3+} experiments described above, the anoxia-induced luminescence response was tested on 5-d-old *A. thaliana* seedlings subjected to 2-h pretreatments with 5 mM EGTA. The results shown in Figure 8 indicated that EGTA par-

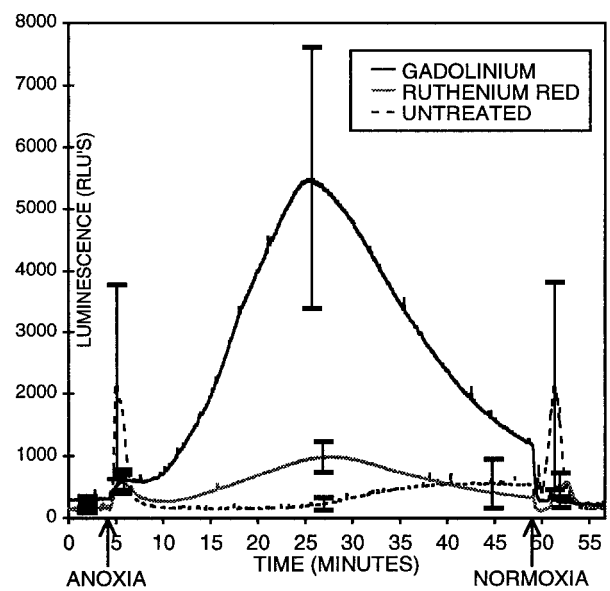


Figure 7. Effect of Ca^{2+} channel blockers on the luminescence response to anoxia and return to normoxia. Seedlings were submerged in 10 μM coelenterazine for 6 h. Then, $GdCl_3$ (solid curve) or RR (shaded curve) solutions were added to final concentrations of 10 mM and 50 μM, respectively, and seedlings were maintained in these solutions for an additional 1.5 h. Similarly, control, untreated seedlings (dotted curve) were maintained for the same period of time in the coelenterazine solution (7.5 h). All plants were blotted and placed individually into luminometer tubes on thin layers of complete agar medium for 0.5 h before being assayed. Graphs represent the averages of data obtained from the responses of four ($GdCl_3$), five (RR), and six seedlings (untreated), along with SE bars.

tially inhibited the amplitude of the peak-I luminescence response. The peak-II luminescence response, however, varied from plant to plant. Some seedlings exhibited brief and small-amplitude peak-II luminescence responses similar to that shown in Figure 8, whereas others produced peak-II responses similar to those seen in untreated control plants. None of the seedlings tested (a total of nine) produced a peak-III luminescence increase upon return to air, even though luminescence immediately dropped to levels identical to the baseline levels of control plants. It is interesting that the EGTA-treated seedlings exhibited elevated baseline luminescence levels (two to five times higher than the baseline levels of untreated seedlings) before being exposed to anoxia (Fig. 8).

To determine if the effects of exogenously added EGTA on the luminescence response were the direct result of Ca^{2+} sequestration, we pretreated 5-d-old *A. thaliana* seedlings with a combination of 5 mM EGTA and 10 mM $CaCl_2$ and recorded their luminescence responses to anoxia. The results shown in Figure 8 revealed a restoration of the anoxia-induced luminescence peak-I and peak-III responses.

Effects of Anoxia on *ADH* mRNA Levels

To determine if anoxia induces *ADH* gene expression in different organs, RNA was extracted from root and shoot tissues of 5-d-old seedlings exposed for 2 h to air or to anaerobiosis and was subjected to northern blot analysis using *ADH*, *APOAEQUORIN*, and *rDNA* sequences as probes (Chang and Meyerowitz, 1986; Knight et al., 1991; Polans et al., 1986). The levels of *ADH* transcript were normalized to the levels of the 25S rRNA found in the same samples. Figure 9 shows that the relative levels of *ADH* transcripts were much higher in both roots and shoots of anoxia-treated seedlings than in air-treated control plants. It also shows that the levels of *APOAEQUORIN* mRNA were higher in shoots than in roots of air-treated seedlings and lower in anoxia-treated roots than in air-treated control roots.

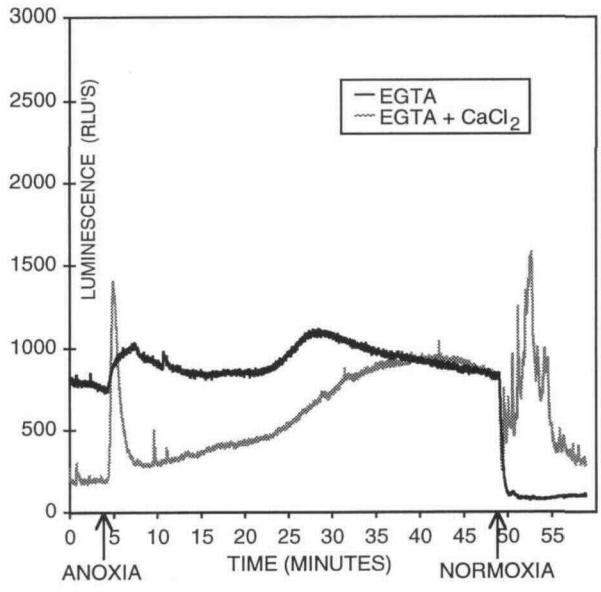
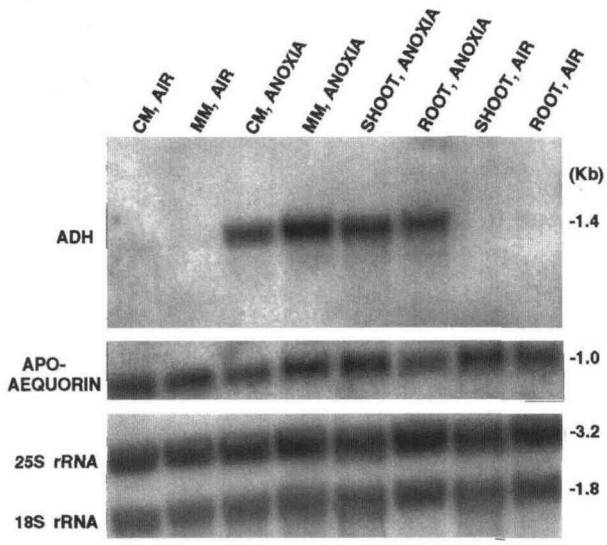


Figure 8. The effect of EGTA on the anoxia response. Five-day-old seedlings were presoaked in 5 mM EGTA (solid curve) or in 5 mM EGTA plus 10 mM $CaCl_2$ (shaded curve) for 1.5 h, then transferred to a 1.5% agar medium containing 1.5% Suc and either 5 mM EGTA (solid curve) or 5 mM EGTA plus 10 mM $CaCl_2$ (shaded curve) for 0.5 h, before being subjected to the anoxia/normoxia cycle of treatment (labeled below the *x* axis). Luminescence was recorded over a period of 60 min.



RELATIVE COUNTS

ADH	2	0	138	284	190	157	4	0
APOAEQ.	31	37	28	42	41	22	38	31
25S rRNA	286	319	383	434	364	495	328	451
ADH/25S	1	0	55	100	80	48	2	0
APO./25S	92	99	63	83	98	38	100	60

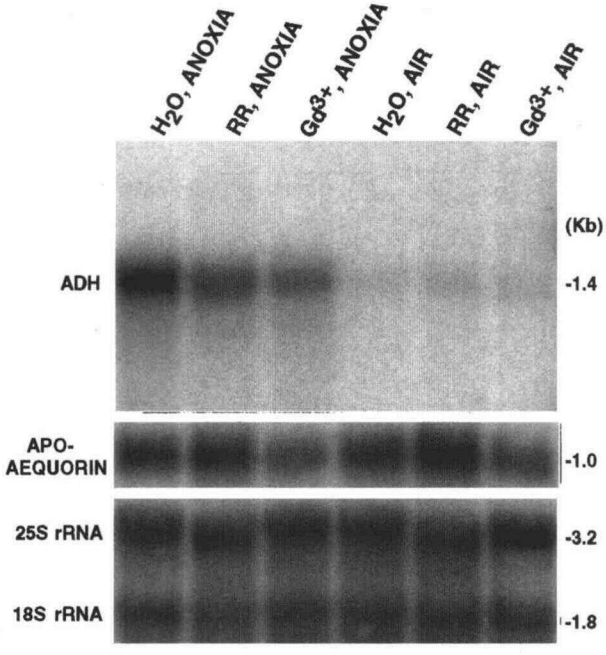
Figure 9. Northern blot analysis of total RNA isolated from plants subjected to either anoxia (N_2 plus 350 μL L^{-1} CO_2) or normoxia (air) for 2 h. RNA was extracted from anoxia- or normoxia-treated seedlings lying on a complete agar medium (CM) or on a water-agar medium (MM) or from roots and shoots dissected from anoxia- or normoxia-treated seedlings and subjected to a northern blot analysis using, successively, the *ADH*, *APOAEQUORIN*, and *rDNA* sequences as probes (see "Materials and Methods"). The origin of each RNA is shown on the top of the figure, the nature of the probe is shown on the left of each panel, and the size of the radioactive band is shown on the right. A quantification of each hybridization band is shown at the bottom of the figure. The total number of radioactive counts was determined with a Phosphorimager and converted into relative counts with the Image QuaNT program. ADH/25S, *ADH radioactive band counts divided by the corresponding 25S rRNA* band counts and normalized to the highest ratio found in that hybridization ("100" value). APO./25S, *APOAEQUORIN* radioactive band counts normalized to the corresponding 25S rRNA band counts, as described above. Background counts were subtracted from all values before normalizations.

As mentioned previously, the anoxia-induced peak-I luminescence response is absent or extremely reduced in seedlings assayed on water-agar medium. To determine how medium composition affects *ADH* transcript levels, we analyzed the levels of *ADH* transcripts in anoxia-treated and air-treated control seedlings contacting complete or water-agar medium, as described above. Figure 9

shows that 2 h of anoxia promote larger increases in *ADH* mRNA levels in plants exposed to water-agar medium than in plants exposed to complete medium. Two other independent experiments gave similar results (data not shown). Additionally, similarly low amounts of *ADH* mRNA were found in air-treated control plants assayed on both medium types (Fig. 9).

Ca^{2+} Channel Blockers Partially Inhibit Anoxia-Induced Increases in *ADH* mRNA Levels

To determine if Gd^{3+} or RR affects anoxia-induced *ADH* expression, we compared the levels of anoxia-induced *ADH* transcripts in 10 mM Gd^{3+}- and 50 μM RR-pretreated *A. thaliana* seedlings to those of water-pretreated control plants. Results shown in Figure 10 indicated that Gd^{3+} and RR inhibited anoxia-mediated induction of *ADH* expression by factors of 5.3 and 2.4, respectively. Similar results were obtained in four independent experiments involving Gd^{3+} and three independent experiments involving RR. Furthermore, Gd^{3+} pretreatment consistently lowered the relative levels of *APOAEQUORIN* mRNA found in air-treated and anoxia-treated seedlings (Fig. 10). The RR pretreatment followed by a 2-h incubation period in normoxia also resulted in a relative increase in *APOAEQUORIN* mRNA level in this experiment (Fig. 10), but that effect was not reproducible (data not shown).



RELATIVE COUNTS

ADH	846	363	183	13	20	19
APOAEQ.	215	230	100	183	298	115
25S rRNA	197	203	220	198	180	253
ADH/25S	100	42	19	2	3	2
APO./25S	66	68	27	56	100	27

Figure 10. Northern blot analysis of total RNA isolated from plants pretreated with Ca^{2+} channel blockers before being exposed to anoxia. Five-day-old seedlings were soaked in either dH_2O (control), 50 μM RR, or 10 mM $GdCl_3$ for 1.5 h, then transferred onto water-agar medium for 0.5 h, before being subjected to anoxic or normoxic treatments for 2 h. The origin of each RNA is shown on the top of the figure, the nature of the probe is shown on the left of each panel, and the size of the radioactive band is shown on the right. A quantification of each hybridization band is shown at the bottom of the figure. Relative counts were calculated as described in the legend to Figure 9.

Table III. *Effect of Ca^{2+} channel antagonists on seedling survivability after anoxic or normoxic treatment*

Five-day-old seedlings were pretreated with the solution described in the first column for 1.5 h and transferred onto complete agar medium for an additional 0.5 h. They were then subjected to the treatment also described in the first column before being allowed to grow for 7 d. The total number of seedlings tested is shown in the second column, and the percentage of green seedlings having resumed growth after 7 d is shown in the third column.

Pretreatment/Treatment	No. of Seedlings	Percentage of Seedlings Alive after 7 d
50 μM RR/2 h air	46	98
50 μM RR/2 h anoxia	47	98
10 mM Gd^{3+}/2 h air	45	58
10 mM Gd^{3+}/2 h anoxia	46	74
dH_2O/2 h air	47	100
dH_2O/2 h anoxia	47	100

Effects of Ca^{2+} Channel Blockers and Anoxia on Seedling Viability

To determine if RR or Gd^{3+} affects the viability of anaerobically or aerobically treated seedlings, 5-d-old seedlings were soaked in either dH_2O, 50 μM RR, or 10 mM $GdCl_3$ for 1.5 h and then placed onto complete agar medium for 0.5 h before being gassed for 2 h with either water-saturated air or water-saturated N_2 containing 350 μL L^{-1} CO_2. The plates containing these plants were then transferred to a growth chamber for 7 d, after which the plants were scored for appearance and survival. Results in Table III show that RR pretreatment does not significantly alter the viability of either anaerobically or aerobically treated seedlings. RR did, however, inhibit root and leaf growth of all plants tested (data not shown). On the other hand, Gd^{3+} pretreatment caused significant but equal mortality of anaerobically and aerobically treated seedlings. Surprisingly, the majority of the Gd^{3+}-pretreated plants surviving such treatments looked healthy and indistinguishable from the dH_2O-pretreated plants after 7 d of growth. Anoxia or air treatment of water-pretreated control seedlings resulted in no fatalities.

For each type of pretreatment (RR, Gd^{3+}, or dH_2O), the anaerobically treated seedlings looked indistinguishable from their aerobically treated counterparts immediately after treatment or after 7 d of growth. However, RR-pretreated seedlings appeared reddish at the end of the treatment, as expected, and a few Gd^{3+}-pretreated seedlings appeared slightly wilted. These two differences between pretreated and control seedlings were the only ones observable immediately after treatment. We found no microscopic evidence of cellular alterations (cellular expansions,

disruptions, or tissue disorganization) on these seedlings immediately after pretreatment.

DISCUSSION

Cytosolic Ca^{2+} changes have been observed in response to a number of environmental stimuli including touch, cold shock, elicitors, and light stimulation (Knight et al., 1991; Poovaiah and Reddy, 1993). Here we show that anoxia induces biphasic cytosolic Ca^{2+} changes in cotyledon and leaf tissues, but not in root and hypocotyl tissues, of *A. thaliana* seedlings. The first anoxia-induced cytosolic Ca^{2+} change (peak I) begins within 1 min of anoxia initiation and lasts less than 10 min. The second phase (peak II) is more progressive in nature, with Ca^{2+} levels increasing and decreasing slowly over a period of 1.5 to 4 h. Qualitatively very similar luminescence responses to anoxia are found for seedlings left to recover in air for 30 min or 24 h after the coelenterazine treatment, suggesting that a 30-min recovery time in normoxia is sufficient to allow a seedling to fully respond to anoxia (Fig. 3, B and C). However, it should also be noted that an anoxic treatment modifies the ability of a seedling to respond to another anoxia challenge applied soon after the first one (within 5 min), as demonstrated in Figure 3A. Furthermore, the peak-II AEQUORIN response to anoxia is delayed and shows different kinetics, whereas the peak-III response to a return to normoxia appears faster after a 24-h recovery period (Fig. 3, B and C). This suggests that some aftereffect of the coelenterazine pretreatment on the response to anoxia remains after 30 min of recovery from that pretreatment.

Even though various strategies have been used to correlate levels of luminescence with cytosolic Ca^{2+} concentrations in living cells (Blinks et al., 1978; Price et al., 1994; Johnson et al., 1995), the Ca^{2+} responses to anoxia described here are difficult to calibrate because they are rather low, falling below the limit of resolution of the ratiometric AEQUORIN response (Knight et al., 1993). An additional complication is that all tissue types do not respond to the stimulus (Fig. 4).

This being said, we have found that the level of luminescence developed by cotyledons in the peak-I phase of the response to anoxia is about 2 orders of magnitude lower than the level of luminescence emitted by such cotyledons in response to a cold-shock stimulus (an average of 5,317 RLU for the peak-I response to anoxia, and an average of 793,262 RLU for the response to cold shock, for a total of six pairs of cotyledons tested). Therefore, if one assumes that all cotyledon cells contribute equally to that response, one can conclude that the peak-I luminescence response to anoxia corresponds to a small increase in cytosolic Ca^{2+} levels in the range of a few hundred nanomolar (Knight et al., 1993). Similar conservative estimates can be reached from the calibration of the peak-II and peak-III luminescence responses.

The intensity of the peak-I luminescence response to anoxia is dependent on the composition of the agar medium used to support the plant at the time of stimulation. Peak I occurs when plants are on agar medium containing Suc and/or one-half-strength MS salts at the time of anoxia exposure, but it is considerably smaller or absent when plants are assayed on a water-agar medium (Fig. 6, A and B). It is interesting that if coelenterazine-treated *A. thaliana* seedlings are exposed to water-agar medium for longer periods of time before anoxia stimulation (e.g. 17.5 h), they regain some ability to develop a biphasic luminescence response to anoxic challenges (Fig. 6B). These data suggest that the peak-I luminescence response to anoxia increases as seedlings exposed to water-agar medium are allowed to recover from the coelenterazine treatment, which subjects them to a long period of hypoxia (at least 7 h). This conclusion would have to be contrasted with the data shown in Figure 3, which indicate that seedlings on complete agar medium have fully recovered 30 min after the coelenterazine treatment. However, we cannot eliminate the possibility that the ethanol present in the medium during coelenterazine treatment contributes, at least partly, to the transient inhibitory effect of that treatment on the luminescence response to anoxia on water-agar medium.

These data also suggest that the magnitude of the peak-I-related cytosolic Ca^{2+} change is affected by the amount of Suc, Ca^{2+}, and/or other components of the medium. It is interesting that previous reports indicated that Suc plays a regulatory role in gene expression (Sheen, 1990; Krapp et al., 1993; Yang et al., 1993), as well as in the circadian regulation of cytosolic Ca^{2+} levels in *A. thaliana* (Johnson et al., 1995). Furthermore, we found that Ca^{2+} added externally to the medium to a concentration of 10 mM causes a larger peak-I luminescence response to anoxia (data not shown).

Although the level of peak-I luminescence response seems to correlate with the presence of Suc in the medium, differentiated chloroplasts—and, hence, active photosynthesis—are not required for anoxia-induced responsiveness. Indeed, etiolated seedlings develop anoxia-induced luminescence responses with the same kinetics as light-grown seedlings, even though the intensity of the luminescence response is lower in etiolated seedlings (data not shown). Differentiated chloroplasts are not sufficient for anoxia responsiveness either, because light-grown seedlings produce no peak-I luminescence response when exposed to anoxia on water-agar medium.

The fact that Gd^{3+}, a putative plasma membrane channel blocker, and RR, a putative inhibitor of channels gating intracellular Ca^{2+} stores, both affect peak-I luminescence suggests that the Ca^{2+} is originating from two sources (Knight et al., 1991; Price et al., 1994). This effect was not reflected by differences in survivability of Gd^{3+}- or RR-treated seedlings to anoxia or normoxia treatments. Indeed, both treatments equally affected the survivability and growth rates of air-treated and anoxia-treated seedlings (Table I). This idea of a two-source Ca^{2+} influx is not without precedent: ABA induces a Ca^{2+} flux into the cytosol of guard cells, apparently through the plasma membrane as well as from intracellular stores (Johannes et al., 1991), and peroxide radicals produce similar Ca^{2+} fluxes from both extracellular and intracellular sources in tobacco (Price et al., 1994). We found that EGTA, which chelates extracellular Ca^{2+}, also partially blocks the peak-I luminescence, further suggesting that part of the Ca^{2+} is derived

from extracellular sources. The effects of EGTA on the peak-I and peak-III luminescence responses were prevented by simultaneously adding 10 mM $CaCl_2$, which suggests that the EGTA-related blockage is due to the chelation of extracellular Ca^{2+} and is not artifactual. It should be cautioned, however, that the sites of action of the Ca^{2+} channel inhibitors used in this study have not been clearly identified in plants. Furthermore, at high concentrations, Gd^{3+} may penetrate into the cytoplasm (Quiquanpoix et al., 1990; Price et al., 1994). Finally, some of the Gd^{3+} pretreatments executed in this study resulted in a slight wilting of a few seedlings immediately after treatment and in some seedling mortality as determined 7 d after treatment (Table I). Therefore, further work is required to demonstrate the origin of the Ca^{2+} entering the cytoplasm upon anoxia stimulation.

Northern blot analysis revealed that Gd^{3+}-treated seedlings exhibit lower levels of *APOAEQUORIN* mRNA than mock-treated plants (Fig. 10). These changes in mRNA levels are probably not responsible for the changes in anoxia-induced luminescence responses. Indeed, the Gd^{3+} treatments were performed at the end of the coelenterazine treatment, when most AEQUORIN had already been reconstituted.

The peak-II luminescence response occurs even when the peak-I luminescence is absent in plants assayed on water-agar medium (Fig. 6A). This suggests that these two Ca^{2+} fluxes are controlled by different mechanisms. This hypothesis is further supported by the results obtained from plants pretreated with Ca^{2+} channel blockers. Both Gd^{3+} and RR inhibit peak-I-related cytosolic Ca^{2+} changes and have an opposite effect on peak II, increasing its amplitude and causing it to occur earlier (Fig. 7). Additionally, as mentioned earlier, longer recovery periods after coelenterazine treatment before anoxia stimulation led to a larger peak-I luminescence response and promoted a delay in the occurrence of the peak-II luminescence response (Fig. 6, A and B; data not shown).

Because the peak-II cytosolic Ca^{2+} response to anoxia is progressive in nature, its appearance may result from slow intracellular changes that affect the cell's ability to maintain cytosolic Ca^{2+} homeostasis. It is interesting that anoxia results in a drop in cytosolic ATP concentration and pH, a rise in cytosolic Na^{+} and Mg^{2+} concentrations, and a buildup of free radicals in the cytosol (Hunter et al., 1983; Roberts et al., 1984; Gasbarinni et al., 1992a, 1992b). These changes could affect the operating efficiencies of Ca^{2+}-ATPases or the gating of Ca^{2+} channels. This model is compatible with the fact that Gd^{3+} and RR increase the amplitude and speed of appearance of the peak-II luminescence response. The effects of these blockers could be an indirect consequence of alterations in the general physiological status of the cells. On the other hand, we have also found an increase in the levels of reconstituted AEQUORIN after long periods of anoxia in both root and shoot tissues (Table I). Such an increase could at least partly contribute to the appearance of the peak-II luminescence response. However, that increase in reconstituted AEQUORIN is not likely to be uniquely responsible for the development of peak II. Indeed, increases in amounts of reconstituted AEQUORIN are found in both root and shoot tissues, yet roots develop no peak-II responses to anoxia and maintain a rather constant luminescence over time. Furthermore, upon return to normoxia, the higher level of luminescence found during peak II in cotyledons and leaves drops abruptly to baseline levels before development of the peak-III response discussed below. After peak III, the luminescence drops back to baseline levels similar to those present before anoxia treatment. Finally, similar responses are found when a seedling is exposed to a succession of two long anoxia treatments (data not shown).

The following explanations for the appearance of peak II under prolonged anoxia are unlikely. For instance, the anoxia-induced cellular changes described above could compromise membrane integrity, leading to leakage of Ca^{2+} into the cytosol or leakage of AEQUORIN into storage spaces containing high Ca^{2+} concentrations. However, the transient and reversible nature of the peak-II cytosolic Ca^{2+}-related luminescence response argues against irreversible membrane damage (Figs. 1–3). Additionally, the anoxia-induced physiological changes described above are not likely to be directly responsible for the luminescence response to anoxia because the AEQUORIN-derived luminescence response to Ca^{2+} rises is insensitive to pH changes in the physiological range and because increased Mg^{2+} concentrations and ionic strength decrease the Ca^{2+} sensitivity of that response (Cobbold and Lee, 1991).

The peak-I and peak-II luminescence responses to anoxia were never observed in roots. Even though less *APOAEQUORIN* mRNA (relative to the amount of 25S rRNA) was found in root tissues than in shoot tissues (Fig. 9), it seems unlikely that a lack of reconstituted AEQUORIN is responsible for their absence, since cold water and touch stimulation can elicit luminescence along the entire length of the root at intensities similar to those elicited on shoot tissues, and many times higher than those seen in anaerobically treated cotyledons (Fig. 5; Table II). Additionally, larger amounts of reconstituted AEQUORIN are found in extracts from anoxia-treated roots than in extracts from normoxia-treated tissues (Table I). Furthermore, these roots develop a large peak-III luminescence response upon return to normoxia (Fig. 4). We have not ruled out, however, the possibility that anoxia causes more internal root cells to luminesce or causes a subset of root cells to luminesce with intensities below the level of resolution of our detection systems. Indeed, the limited amount of light generated by the AEQUORIN complex is not likely to traverse more than one or two layers of cells (Knight et al., 1991).

Researchers have proposed that a sustained rise in cytosolic Ca^{2+} levels is associated with cell damage and death (Farber, 1990; Lemasters et al., 1990; Nicotera et al., 1992). Roots may have evolved to avoid prolonged anoxia-induced cytosolic Ca^{2+} increases and cell damage. Indeed, since roots are buried in soil, they more often experience anoxia during flooding than aerial parts of the plant. It would be interesting to test the relative survivability of Arabidopsis cotyledon and root cells to long periods of anoxia.

Upon return to normoxia, cotyledons, leaves, roots, and hypocotyls develop a transient cytosolic Ca^{2+} rise (peak III). This response may be linked to the accumulation of oxygen free radicals occurring when anoxia-treated cells are returned to normoxia (Hunter et al., 1983; McCord, 1985; Monk et al., 1987; Bowler et al., 1992). Indeed, Price et al. (1994) found that La^{3+}- and RR-sensitive changes in cytosolic Ca^{2+} levels occur in response to challenges with oxygen free radicals in AEQUORIN-expressing tobacco seedlings.

A number of physiological changes occur in response to anoxic stimuli. These changes include an acidification of the cytoplasm (Roberts et al., 1984, 1992), a decrease in protein synthesis (Sachs et al., 1980; Fennoy and Bailey-Serres, 1995), and an increase in expression of various genes, including those coding for the glycolytic and ethanol fermentation enzymes Suc synthase, glyceraldehyde-3-P dehydrogenase, and ADH (Chang and Meyerowitz, 1986; Martin et al., 1993; Yang et al., 1993; Peschke and Sachs, 1994). Because changes in cytosolic Ca^{2+} levels have been shown to result in the activation of numerous signal transduction pathways in plants and animals (Campbell, 1983; Hepler and Wayne, 1985; Neuhaus et al., 1993; Poovaiah and Reddy, 1993), it is likely that the cytosolic Ca^{2+} changes occurring in response to anoxia are involved in the activation of some or all of these physiological responses.

As a preliminary step toward identifying the roles played by the anoxia-induced Ca^{2+} fluxes in the response of plants to anoxia, we tried to determine if anoxia-induced Ca^{2+} fluxes correlate with anoxia-induced *ADH* expression. We found that the first anoxia-induced flux in cytosolic Ca^{2+} level (peak I) occurs earlier than the increase in *ADH* RNA levels. Also, Gd^{3+} and RR both lower the amplitude of the anoxia-induced peak-I luminescence response and inhibit anoxia-induced *ADH* expression (Figs. 7 and 10). However, we were unable to find anoxia-induced cytosolic Ca^{2+} changes in roots even though *ADH* mRNA levels increased in that organ in response to anoxia (Figs. 4 and 9). Additionally, the peak-I luminescence response of plants on water-agar medium was either absent or much smaller than that of plants exposed to complete medium, even though *ADH* mRNA levels increased substantially in plants exposed to these two medium types (Figs. 6 and 9).

Similarly, the anoxia-induced peak-II cytosolic Ca^{2+} rise is probably not responsible for increased *ADH* expression. Indeed, Gd^{3+}- and RR-pretreated seedlings develop enhanced and earlier peak-II luminescence responses but show a lower level of *ADH* induction (Figs. 7 and 10). Taken together, these data indicate that the anoxia-induced changes in cytosolic Ca^{2+} levels do not correlate strictly with the activation of *ADH* expression in anoxic conditions. This implies that different tissues and/or organs use different signal transduction pathways to activate *ADH* expression under anoxia or that Ca^{2+} may not be involved as a second messenger in the transduction pathway, resulting in the activation of *ADH* expression under anoxia. Alternatively, the lack of correlation between *ADH* expression and cytosolic Ca^{2+} responses in plants exposed to water-agar medium and in roots may simply reflect limitations in sensitivity of the system used to detect changes in cytosolic Ca^{2+} levels, as discussed above.

Recently, Subbaiah et al. (1994a, 1994b) showed a correlation between an anoxia-induced cytosolic Ca^{2+} increase and *ADH* and Suc synthase expression in maize suspension-cultured cells. These anoxia-induced cytosolic Ca^{2+} changes were different from those found in *A. thaliana*. The maize cells responded within 2 min of anoxia with a prolonged cytosolic Ca^{2+} increase, apparently lacking a peak-I response. The reasons for these differences are unknown. However, it is possible that the cells used by Subbaiah et al. (1994b) lacked a peak-I response. That hypothesis is compatible with our observation that the anoxia-induced cytosolic Ca^{2+} changes are tissue specific and vary depending on the composition of the culture medium to which the plants are exposed during anoxia (Fig. 6). Alternatively, the unnatural tissue culture environment of maize suspension-cultured cells may alter the anoxia response.

It is also possible that a peak-I luminescence response is present in maize suspension-cultured cells but that its appearance is masked by the precocious appearance of the prolonged Ca^{2+} increase. In that respect, it is interesting to note that about 20% of the maize cells tested also showed two successive Ca^{2+} responses to anoxia (Subbaiah et al., 1994b). Similarly, a succession of short anoxia shocks given to *A. thaliana* seedlings resulted in the precocious appearance of the peak-II luminescence response (Fig. 3), as did treatments with various Ca^{2+} channel blockers (Fig. 7).

At first glance, the large Ca^{2+} increase found in maize cells looks like the peak-II Ca^{2+} response found in Arabidopsis plants (Subbaiah et al., 1994b) (Fig. 2). However, RR completely inhibited that prolonged response in maize cells and increased it and made it appear earlier in *A. thaliana* plants. More work is needed to elucidate the similarities and differences between the maize and *A. thaliana* Ca^{2+} responses to anoxia.

It is interesting to note that the anoxia-induced changes in cytosolic Ca^{2+} levels in *A. thaliana* closely resemble the biphasic cytosolic Ca^{2+} response found in anoxia-treated rat hepatocytes and cardiac myocytes (Allshire et al., 1988; Gasbarrini et al., 1992a; Miyata et al., 1992). Even though we cannot eliminate the possibility that the peak-I and peak-II luminescence responses to anoxia derive from two different cell populations in Arabidopsis seedlings (see above), this observation suggests that some of the mechanisms involved in the cellular responses to anoxia are conserved between animal and plant cells. Although our results question the possible involvement of anoxia-induced cytosolic Ca^{2+} changes in the transduction of anoxic signals into an induction of *ADH* expression (see above), they do not address their involvement in the activation of other physiological processes occurring in response to anoxia. Since *A. thaliana* lends itself to molecular genetic analyses, our system promises to be helpful in elucidating the molecular mechanisms by which eukaryotic cells sense an anoxic stimulus and transduce that information into a physiological response.

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