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Cytosolic Alkalinization Mediated by Abscisic Acid Is Necessary, but Not Sufficient, for Abscisic Acid-Induced Gene Expression in Barley Aleurone Protoplasts[1]

Renske van der Veen, Sjoukje Heimovaara-Dijkstra, and Mei Wang*

Center for Phytotechnology Leiden University/Netherlands Organisation for Applied Scientific Research, Department of Molecular Plant Biotechnology, Wassenaarseweg 64, 2333 AL Leiden, the Netherlands

ABSTRACT

We investigated whether intracellular pH (pH_i) is a causal mediator in abscisic acid (ABA)-induced gene expression. We measured the change in pH_i by a "null-point" method during stimulation of barley (*Hordeum vulgare* cv Himalaya) aleurone protoplasts with ABA and found that ABA induces an increase in pH_i from 7.11 to 7.30 within 45 min after stimulation. This increase is inhibited by plasma membrane H^+-ATPase inhibitors, which induce a decrease in pH_i, both in the presence and absence of ABA. This ABA-induced pH_i increase precedes the expression of *RAB*-16 mRNA, as measured by northern analysis. ABA-induced pH_i changes can be bypassed or clamped by addition of either the weak acids 5,5-dimethyl-2,4-oxazolidinedione and propionic acid, which decrease the pH_i, or the weak bases methylamine and ammonia, which increase the pH_i. Artificial pH_i increases or decreases induced by weak bases or weak acids, respectively, do not induce *RAB*-16 mRNA expression. Clamping of the pH_i at a high value with methylamine or ammonia treatment affected the ABA-induced increase of *RAB*-16 mRNA only slightly. However, inhibition of the ABA-induced pH_i increase with weak acid or proton pump inhibitor treatments strongly inhibited the ABA-induced *RAB*-16 mRNA expression. We conclude that, although the ABA-induced the pH_i increase is correlated with and even precedes the induction of *RAB*-16 mRNA expression and is an essential component of the transduction pathway leading from the hormone to gene expression, it is not sufficient to cause such expression.

The phytohormone ABA influences plant physiology, growth, and development in a variety of ways. ABA, for example, plays a central role in stress responses and enhances adaptation to various stresses, such as desiccation and salt stress (22). ABA is also involved in the regulation of embryogenesis and grain development (7, 26). Most hormone responses are directly or indirectly connected with alteration of gene expression in plant tissue (22). The expression of hormone-regulated genes is controlled by a variety of processes. So far, knowledge about hormone signal transduction in plants and the signal accessibility to the gene level is limited.

Genes that are under ABA control have been isolated from different plant species. These include *RAB*[2] genes (3, 18) and those encoding *BASI* (19) and a 10-kD soluble protein in wheat embryos (Em) (16). The promoters of *RAB* genes are currently being studied to analyze ABA-regulated gene expression. ABA-responsive DNA elements and *trans*-acting hormone-activated regulatory proteins have been identified (20).

Most research concerning the working mechanism of ABA has been focused on the expression of ABA-regulated genes and promoters. However, ABA is able to trigger a series of cellular responses that occur long before ABA-induced gene expression can be observed. It is also known that, in rice cell suspensions, both salt stress and ABA are able to induce a common factor that triggers Em expression (16). Experimental evidence indicates that second messengers such as Ca^{2+} ions mediate ABA actions in response to extracellular signals (17, 25, 29, 30). Although it is difficult to conclude that cytosolic pH (pH_i) is also a second messenger in plant hormone signal transduction, pH_i changes are known to be induced by extracellular stimuli such as light (9) and hormones (10, 12).

The eukaryotic pH_i is strictly regulated. Cells clamp their pH_i at 7.0 to 7.4 by ion transport mechanisms and a high buffering capacity of the cytosol (13). Proton transport across plant plasma membranes has been intensively studied. For example, plasma membrane-located H^+-transporters and H^+-cotransporters (8, 9) have been well studied, and their immediate effects on pH_i have been reported. It has been reported that plant hormones are able to influence H^+-transport as well as pH_i (9). For example, auxin is involved in the activation of a plasma membrane H^+-ATPase (6, 9). In addition, some data show that auxin is able to induce a pH_i decrease by up to 0.2 pH unit (10, 12, 24). It has also been demonstrated that ABA is able to induce an increase of pH_i in *Zea mays* coleoptile and hypocotyl cells (12). However, with regard to the importance of intracellular cytosolic events, we are still far from understanding the role of pH_i. For example, how do plant cells distinguish between pH shifts induced or triggered by a membrane-bound receptor and pH shifts from metabolism or pH shifts induced by extracellular pH changes? In conclusion, it is likely that additional intra-

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[2] Abbreviations: *RAB*, responsive to ABA; BASI, bifunctional α-amylase subtilisin inhibitor; DES, diethylstilbestrol; DMO, 5,5-dimethyl-2,4-oxazolidinedione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pH_e, extracellular pH; pH_i, cytoplasmic pH.

cellular information is necessary for a cell to react to a pH_i shift.

We have assessed the possible role of pH_i in ABA signal transduction leading to specific gene expression by measuring cytosolic pH and pH_i changes in nontreated and ABA-treated barley (*Hordeum vulgare* L. cv Himalaya) aleurone protoplasts. We also investigated the effect of manipulating the pH_i with weak acids, weak bases, or proton pump inhibitors on specific gene expression of ABA-controlled genes.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham (Buckinghamshire, UK). (*R,S*)-ABA (99% chemical purity), DMO, potassium propionate, methylamine, DES, zearalenone, and digitonin were from Sigma (St. Louis, MO). Gene Screen Plus was from DuPont (Boston, MA). Cellulase Onozuka R-10 was obtained from Yakult Honsha (Tokyo, Japan), and Gamborg B5 was from Flow Laboratories (Irvine, UK). PVP K25 was from Fluka Chemie (Buchs, Switzerland), and Pipes was from Janssens Chemical (Tilburg, the Netherlands). All other chemicals were from Merck (Darmstadt, Germany).

Isolation of Protoplasts

Barley (*Hordeum vulgare* L. cv Himalaya, harvest 1985; Department of Agronomy, Washington State University, Pullman, WA) aleurone protoplasts (containing only small vacuoles) were prepared essentially as described by Wang et al. (29). The buffer we used for both washing and incubation of protoplasts was 10 mM Na/K phosphate buffer (0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KH_2PO_4/Na_2HPO_4).

pH_i Measurement by the "Null-Point" Method

Disruption of the plasma membrane of cells in a weakly pH buffered solution will, in principle, lead to a change of the pH_e unless pH_e is equal to the pH_i. The null-point method for determination of pH_i is based on this principle (21). The pH_i can be determined by incubating protoplasts in weakly pH buffered solutions with different pH and subsequently disrupting the plasma membrane (i.e. pH_i is equal to the pH_e, where no pH change could be observed after disruption). The null-point method was used to determine the pH_i in barley aleurone protoplasts. After different treatments, the protoplasts were washed and resuspended in a continuously and gently stirred weakly buffered phosphate buffer (the same phosphate buffer as described above but with 2 mM KH_2PO_4/Na_2HPO_4) with different pH values at a density of 2×10^6 protoplasts/mL. The pH_e was adjusted to the required value with HCl or NaOH, and subsequently digitonin (0.005% w/v) or Triton X-100 (0.02% w/v) was added to permeabilize the plasma membranes of the protoplasts. The resulting pH changes were recorded with a combined pH electrode (Beckman) coupled to a pH monitor (Pharmacia, Uppsala, Sweden) and a pen recorder. The values of the pH_e at which permeabilization induced no apparent shift in pH_e were taken as an estimate of pH_i. In all determinations, correction for the background acidification rate (mainly due to stirring CO_2 into the suspension) was made.

Experiments were performed at room temperature (about 22°C). Mean values ± SD are given (unless specified otherwise), with n = number of independent experiments.

RNA isolation and Northern Analysis

Barley aleurone protoplasts (4×10^5/mL) were incubated in 10 mM phosphate buffer with or without ABA and/or weak acids (DMO at $pH_e = 6.0$; propionic acid at $pH_e = 6.6$) and bases (methylamine, ammonia at $pH_e = 7.4$) in the dark at 25°C for 2 h or otherwise as stated in the text.

Total cellular RNA from 2×10^6 protoplasts was isolated and purified as described by Wang et al. (30). Agarose gel electrophoresis of glyoxylated RNA and transfer to Gene Screen Plus was performed according to the method of Sambrook et al. (23) or the instructions of the manufacturer. Hybridization was performed in 1% SDS, 1 M NaCl, 10% dextran sulfate, and 0.1 mg/mL of sonificated salmon sperm DNA at 65°C, with randomly primed, labeled cDNA hybridization probes (a *RAB*-16 cDNA probe from rice; a *BASI* cDNA probe from barley, and a GAPDH cDNA probe from barley [5]). The amount of ^{32}P-labeled probe hybridizing to the *RAB*-16 mRNA was semiquantitatively determined by measuring the absorbance on autoradiographs with an Ultroscan KL densitometer (LKB).

RESULTS

Measurement of pH_i

The null-point method, first developed to measure pH_i in animal systems (21), was adapted for plant cells. To make the aleurone protoplast plasma membrane selectively permeable, the effects of both digitonin and Triton X-100 were investigated. Triton X-100 and digitonin do not affect the pH_e by themselves (data not shown). The amount of digitonin or Triton X-100 necessary to disrupt the plasma membrane was titrated; as examples, both Triton X-100 and digitonin titration are presented in Figure 1A. As the concentration of either Triton X-100 or digitonin increased, we observed its effect on the disruption of protoplast plasma membrane by changes of the extracellular solution pH (Fig. 1A). After the first change of solution pH, which was induced by either Triton X-100 or digitonin, any further addition of either Triton X-100 or digitonin induced a further change of solution pH (Fig. 1A). When the pH of extracellular solution is lower than the pH_i, disruption of protoplast plasma membranes leads to an increase of solution pH (Fig. 1B). When the pH of the extracellular solution is higher than the pH_i, permeabilization of protoplast plasma membrane causes a decrease in the solution pH (Fig. 1C). However, when the pH of the extracellular solution is equal to pH_i, selective disruption of the plasma membrane will not give any change of pH_e (Fig. 1D).

The basal barley aleurone protoplast pH_i established is 7.11 ± 0.01 (n = 16) in the presence of digitonin and 6.87 ± 0.03 (n = 12) in the presence of Triton X-100. The difference between the values obtained with digitonin and Triton X-100 may indicate that the action of Triton X-100, especially at

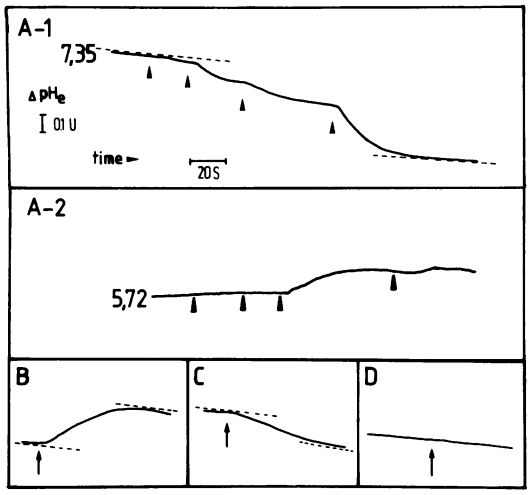

Figure 1. Determination of pH_i by the null-point method. Barley aleurone protoplasts were collected, washed, and suspended in 2 mM phosphate buffer at various pH_e values. Changes in pH_e were induced by addition of Triton X-100 or digitonin to aleurone protoplasts. The arrows indicate the addition of Triton X-100 or digitonin. Time and ΔpH scale are indicated by bars and are identical for all panels. A-1, pH recording of titration curve for Triton X-100. The arrows indicate different amounts of Triton X-100 added to the protoplast suspension (2 mM phosphate buffer, pH_e = 7.35) which are, from left to right, 0.005, 0.01, 0.02, and 0.05% (final concentration, w/v). A-2, pH recording of titration curve of digitonin. The arrows indicate different amounts of digitonin added to the protoplasts suspension (2 mM phosphate buffer, pH_e = 5.72) which are, from left to right, 0.0013, 0.0025, 0.005, and 0.01% (final concentration, w/v). B, pH recording of the change in pH_e induced by digitonin (0.005%, w/v) in protoplasts suspended in 2 mM phosphate buffer, pH_e = 5.70 ($pH_e < pH_i$). C, pH recording of the pH_e change induced by digitonin (0.005%, w/v) in protoplasts suspended in 2 mM phosphate buffer, pH_e = 7.32 ($pH_e > pH_i$). D, pH recording of the pH_e change induced by digitonin (0.005%, w/v) in protoplasts as shown in C, but pH_e = 7.13 ($pH_e = pH_i$).

Table I. *Effect of Proton Pump Inhibitors on ABA-Induced pH_i and Gene Expression*

Barley aleurone protoplasts (4 × 10^5/mL) were incubated in 10 mM phosphate buffer (pH 6.8), to which 100 μM DES or zearalenone and/or 10 μM ABA was added. After 1 h of incubation, samples were taken for pH_i measurement with the digitonin null-point method. The data represent the means ± SD of at least 12 independent determinations. After 2 h of incubation, samples were taken for northern analysis. The level of mRNA was semiquantitatively determined. The gene expression data are presented as ranges. The *RAB*-16 mRNA expression obtained with 10 μM ABA treatment was set to 100%, and the *GAPDH* mRNA expression obtained with buffer treatment was set to 100%.

Conditions	pH_i (*n*)	*RAB* (*n*)	*GAPDH* (*n*)
		%	%
Buffer	7.08 ± 0.02 (15)	9–12 (3)	100 (3)
10 μM ABA	7.21 ± 0.01 (15)	100 (3)	95–105 (3)
100 μM DES	6.88 ± 0.03 (12)	10–15 (2)	100–114 (2)
100 μM zearaleone	6.85 ± 0.04 (12)	9–12 (2)	95–110 (2)
ABA + DES	6.91 ± 0.03 (12)	18–29 (2)	90–113 (2)
ABA + zearaleone	6.89 ± 0.05 (12)	23–39 (2)	97–110 (2)

higher concentrations, is not limited to the plasma membrane only (see "Discussion"); therefore, we mainly used digitonin in the experiments described below. The basal barley aleurone protoplast pH_i values, obtained with the null-point method, are in rather good agreement with values reported by direct pH_i measurement with pH-sensitive microelectrodes (9, 15).

The possibility of measuring pH_i changes with the null-point method had to be tested. In general, inhibition of the plasma membrane proton pump will cause an acidification of the cytoplasm. Therefore, we used the plasma membrane H^+-ATPase inhibitors DES and zearalenone. These inhibitors have been shown to induce a pH_i decrease in other systems (14). Our measurements show that both DES- and zearalenone-induced pH_i changes can be detected with the null-point method (Table I). We conclude that the null-point method applied to barley aleurone protoplasts provides a realistic measurement of pH_i and is able to detect pH_i changes as well.

Are pH_i Changes Correlated with the Induction of *RAB*-16 mRNA Expression?

It has been reported that ABA is able to induce both a pH_i increase (12) and specific gene (*RAB, BASI*) expression (18, 19). We measured whether the induction of *RAB*-16 mRNA expression by ABA is accompanied by sustained changes in pH_i. Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 6.8) with 5 μM ABA. At different

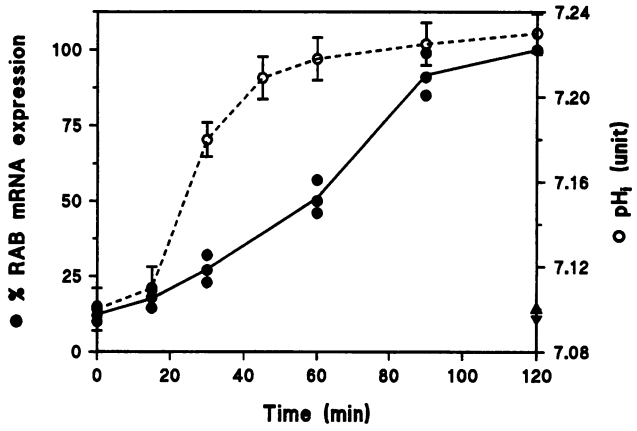

Figure 2. Time course of ABA-induced pH_i and *RAB*-16 mRNA expression. Barley aleurone protoplasts (4 × 10^5/mL) were incubated in 10 mM phosphate buffer (pH 6.8) with or without 5 μM ABA. At different times, the samples were collected for both pH_i measurements and northern analysis. For pH_i measurements (O), the protoplasts were washed twice in 2 mM phosphate buffer (pH 7.0), and the pH_i was measured with the digitonin null-point method (see "Materials and Methods"). The means ± SD of six independent experiments are presented. For northern analysis, the protoplasts were collected, and the total RNA was isolated. The level of *RAB*-16 mRNA expression (●) was semiquantitatively determined, and the mRNA expression obtained after 2 h with ABA was set to 100%. Results of three independent experiments are presented. The triangles (open for pH_i and closed for gene expression) represent control samples incubated without ABA for 2 h.

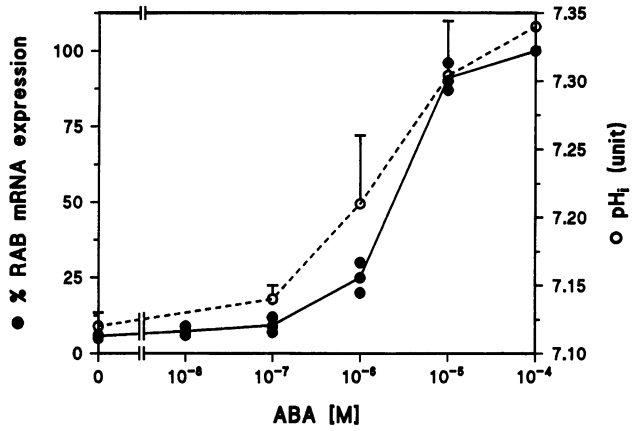

Figure 3. Dose responses of ABA-induced pH_i increase and *RAB*-16 mRNA expression. Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 6.8) with different concentrations of ABA. After 1 h of incubation, the protoplasts were washed twice in 2 mM phosphate buffer (pH 7.0), and the pH_i (O) was measured with the digitonin null-point method (see "Materials and Methods"). The means ± SD of six independent experiments are presented. After 2 h of incubation, the protoplasts were collected for northern analysis (●). The *RAB*-16 mRNA expression at 10^{-4} M ABA was set as 100%. Results of three independent experiments are presented.

incubation times a sample was taken, and the pH_i was measured with the digitonin null-point method. Figure 2 shows that ABA indeed induces a significant increase in pH_i, which reached its maximal level after 45 to 60 min. In the same experiment, the *RAB*-16 mRNA expression was studied by northern analysis. The results of these experiments (Fig. 2) show that ABA induces a time-dependent increase in *RAB*-16 mRNA levels, which reached its maximal level after 90 to 120 min. If we compare ABA-induced pH_i changes with *RAB* mRNA expression (Fig. 2), it is clear that the half-maximal level of ABA-induced pH_i increase was reached at about 30 min, whereas the half-maximal level of ABA-induced *RAB* mRNA synthesis was reached at about 60 min. These differences indicate that the ABA-induced cytoplasmic alkalinization could, at least in theory, be a prerequisite for *RAB*-16 mRNA synthesis. In addition, the ABA-induced pH_i increase encompasses about 0.14 pH units, which is in good agreement with the results obtained by Gehring et al. (12). In the absence of ABA no significant changes in either pH_i or *RAB*-16 mRNA expression were observed (Fig. 2).

The concentration dependency of the ABA-induced pH_i increase and gene expression were studied as well. Protoplasts were incubated in phosphate buffer (pH 6.8) for 1 h with different concentrations of ABA, and subsequently, pH_i was measured by the digitonin method. Figure 3 shows that under these conditions ABA-induced a half-maximal pH_i increase at about 1 μM, whereas at about 2 to 5 μM, ABA was able to induce half-maximal *RAB*-16 mRNA expression (Fig. 3). These results demonstrate that there is no significant difference in the capability of ABA to induce both pH_i changes and gene expression.

In addition, Table I shows that the ABA-induced pH_i increase is absent in protoplasts treated with the plasma membrane H^+-ATPase inhibitors DES and zearalenone. This suggests that the ABA-induced pH_i increase is correlated with an increase in plasma membrane H^+-ATPase activity.

Are pH_i Changes the Cause of Specific Gene Expression?

To test for a possible causal relationship between the ABA-induced gene expression and the ABA-induced increase of pH_i, pH_i was manipulated by adding either weak acids (DMO or potassium propionate) or weak bases (methylamine or ammonia) to the protoplasts. The pH_i was decreased by incubating protoplasts in 10 mM phosphate buffer at pH 6.6 in the presence of DMO or at pH 6.0 in the presence of potassium propionate, and pH_i was increased by adding methylamine or NH_4Cl to protoplasts bathed in 10 mM phosphate buffer, pH 7.4 (28). Figure 4 shows that both weak acids and bases have significant effects on the pH_i. DMO and potassium propionate decrease the pH_i to 6.91 and 6.89, respectively, and prevent the ABA-induced alkalinization. Methylamine and NH_4Cl increase the pH_i to 7.20 and 7.23, thereby mimicking the effect of ABA on pH_i. Simultaneous addition of methylamine or NH_4Cl and ABA has no significant influence on pH_i and demonstrates that both methylamine and NH_4Cl can effectively clamp the pH_i at a high value.

We used weak acids and bases to manipulate the pH_i. Because only the noncharged form of weak acids or bases are able to pass the protoplast membrane, it is essential to apply the weak acids or bases at different pH_e values according to their pK_a values. Therefore, we used the weak acids potassium propionate and DMO at pH_e 6.0 and 6.6, respectively, and the weak bases methylamine and NH_4Cl at pH_e 7.4. Figure 4 shows that the necessary changes in the pH_e have only a small effect on the pH_i. The effects of weak acids

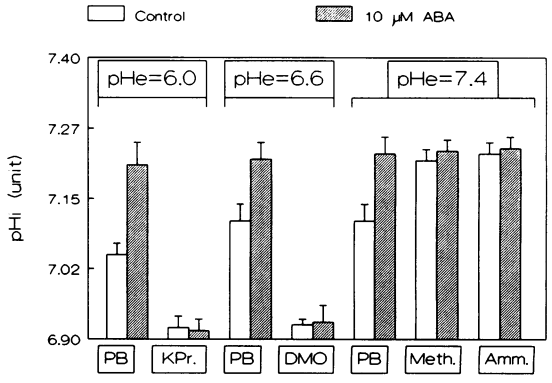

Figure 4. Effects of weak acids and bases on ABA-induced pH_i changes. Barley aleurone protoplasts were incubated in phosphate buffer (PB) with either weak acids (5 mM potassium propionate [KPr] or 7.5 mM DMO) or weak bases (7.5 mM methylamine [Meth.] or 7.5 mM NH_4Cl [Amm.]) in the presence or absence of ABA (10 μM). During incubation, different pH_e values required for loading weak acids or bases were used (see top of figure). After about 45 min of incubation, protoplasts were collected and washed twice in 2 mM phosphate buffer (pH 7.0), and the pH_i was subsequently determined as described in "Materials and Methods" with the digitonin null-point method. The data represent the means ± SD of six independent determinations.

Table II. *Effects of pH_e on ABA-Induced Gene Expression*

Barley aleurone protoplasts (4×10^5/mL) were incubated in 10 mM phosphate buffer at different pH_e in the presence of 10 μM ABA. After 2 h of incubation, samples were taken for northern analysis. The level of *RAB*-16 mRNA expression was semiquantitatively determined. The gene expression data are presented as ranges. The mRNA expression obtained with pH_e 5.0 was set to 100%.

Buffer pH	*RAB* (*n*)
	%
5.0	100 (2)
6.0	95–113 (2)
6.6	92–114 (2)
7.0	89–105 (2)
7.4	85–93 (2)
8.0	65–81 (2)

or bases on pH_i are much more pronounced (Fig. 4). This indicates that the strong effects of weak acids or bases on pH_i are not due to changing pH_e. We investigated whether changes of pH_e would influence ABA-induced gene expression as well. Table II shows that in the pH_e range from 6 to 7.4 ABA-induced *RAB*-16 mRNA expression was not dramatically different.

To investigate the effects of manipulations of pH_i on gene expression, we examined whether the addition of weak acids or bases influences the ABA-induced *RAB*-16 mRNA expression. Aleurone protoplasts were incubated in the presence and absence of ABA with different concentrations of the weak acid DMO. DMO is able to inhibit ABA-induced *RAB*

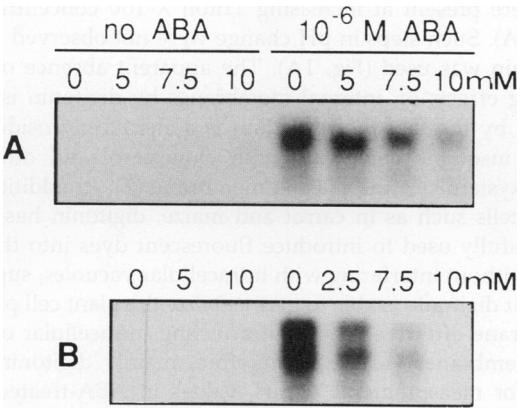

Figure 5. Effect of weak acids on *RAB* gene expression. A, Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 6.6) in the presence of 0, 5, 7.5, or 10 mM DMO with or without ABA (5 μM). After 2 h of incubation, the protoplasts were collected, and total RNA was isolated. The northern blot was probed with ^{32}P-labeled rice *RAB*-16 cDNA. B, As in A, but potassium propionate (0, 2.5, 7.5, or 10 mM) was used as the weak acid. The incubation buffer is 10 mM phosphate buffer (pH 6.0). At least four independent experiments were done, and all gave similar results. One typical example is presented (the two bands that can be observed by hybridization with the rice *RAB*-16 cDNA probe may represent barley mRNAs from the same *RAB* family, which are currently being investigated).

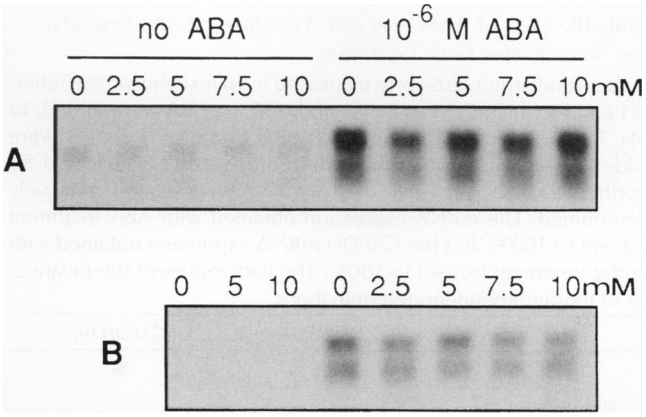

Figure 6. Effect of weak bases on *RAB* gene expression. A, Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 7.4) in the presence of 0, 2.5, 5, 7.5, or 10 mM methylamine with or without ABA (5 μM). After 2 h of incubation, the protoplasts were collected, and total RNA was isolated. The northern blot was probed with ^{32}P-labeled rice *RAB*-16 cDNA. B, As in A, but NH_4Cl (0, 2.5, 5, 7.5, or 10 mM) was used as the weak base. At least four independent experiments were done, and all gave similar results. One typical example is presented (the two bands that can be observed by hybridization with rice *RAB*-16 cDNA probe may represent barley mRNAs from the same *RAB* family, which are currently being investigated).

mRNA expression, and this inhibition is DMO concentration dependent (Fig. 5A). Addition of DMO alone in the absence of ABA did not induce any *RAB*-16 mRNA expression (Fig. 5A). We observed similar results with potassium propionate (Fig. 5B). The inhibition of ABA-induced gene expression by weak acids is likely not due to the effect of pH_e, because the pH_e has only a very small effect on ABA-induced gene expression (Table II). In addition, both DES and zearalenone are able to prevent the ABA-induced pH_i increase as well as the ABA-induced gene expression (Table I).

Addition of methylamine to aleurone protoplasts, which clamps the pH_i at a high value in the presence or absence of ABA, resulted in no substantial effect on ABA-induced *RAB* gene expression (Fig. 6A). Effects of NH_4Cl, the second weak base used (Fig. 6B), support the methylamine data. However, neither methylamine nor NH_4Cl was able to affect the *RAB*-16 mRNA level in the absence of ABA (Fig. 6), indicating that an artificially induced pH_i increase is not able to trigger *RAB*-16 mRNA expression.

We investigated whether another ABA-regulated gene might show the same type of effect as the *RAB*-16 gene when pH_i is altered by a weak acid or weak base. For this purpose, the expression of *BASI* mRNA was studied (Table III). An artificially induced decrease in pH_i by weak acids produced an inhibition of ABA-induced *BASI* mRNA expression, whereas an artificial increase of pH_i by weak bases produced no effect on ABA-induced *BASI* mRNA expression (Table III). These results demonstrate that the effects of pH_i changes on *BASI* gene expression are about the same as for *RAB*-16 gene expression.

To ensure that the inhibition of ABA-induced gene expression by the above mentioned compounds (DMO, potassium

Table III. *Effect of Weak Acid and Weak Base on ABA-Regulated and Nonregulated Gene Expression*

Aleurone protoplasts were incubated in 10 mM phosphate buffer, pH 6.6, to which 7.5 mM DMO and/or 5 μM ABA were added, or pH 7.4, to which 10 mM methylamine and/or 5 μM ABA were added. After 2 h of incubation, the protoplasts were collected for northern analysis. The levels of *BASI* mRNA were semiquantitatively determined. The mRNA expression obtained with ABA treatment was set to 100%, and the *GAPDH* mRNA expression obtained with buffer treatment was set to 100%. The data represent the means ± SD of four independent determinations.

	BASI (*n*)	*GAPDH* (*n*)
	%	%
pH_e 6.6		
Buffer	9.7 ± 3.1 (4)	100.0 (4)
7.5 mM DMO	5.6 ± 2.8 (4)	109.1 ± 11.7 (4)
5 μM ABA	100.0 (4)	106.5 ± 10.6 (4)
ABA + DMO	16.6 ± 5.6 (4)	108.7 ± 7.3 (4)
pH_e 7.4		
Buffer	3.7 ± 0.5 (4)	100.0 (4)
10 mM methylamine	3.4 ± 0.4 (4)	104.8 ± 8.0 (4)
5 μM ABA	100.0 (4)	102.0 ± 12.0 (4)
ABA + methylamine	96.0 ± 7.0 (4)	108.9 ± 9.8 (4)

propionate, and H^+-ATPase inhibitors) is a specific inhibition instead of overall inhibition, the expression of a non-ABA-regulated gene was studied. We rehybridized our northern blots with a barley *GAPDH* cDNA probe (cDNA clone from Cojecki, ref. 5). The semiquantitative data of the northern blots are presented in Tables II and III. These results indicate that an artificially induced decrease in pH_i by weak acids causes a reduction only in specific ABA-induced mRNA expression and that the overall transcription levels are not affected by the used decrease of pH_i. In unpublished experiments, we have observed that *GAPDH* mRNA can be reduced (within 2 h) after treatment of protoplasts with calcium antagonists or heavy metals (Cd^{2+}, La^{3+}). In addition, we rehybridized our northern blots with radioactive polyadenylated oligonucleotides (23) and showed that there was no overall reduction in mRNA levels in pH_i-manipulated and H^+-ATPase inhibitor-treated protoplasts (data not shown).

DISCUSSION

We used the null-point method to measure the pH_i of barley aleurone protoplasts. Our testing experiments demonstrated that (a) the measured basal pH_i (approximately 7.0) measured with the null-point method is in good agreement with values reported for plant cells when different methods were used (9); (b) weak acids or bases are able to influence the pH_i measured by null-point method; (c) proton pump inhibitors in long-term (more than 45 min) are able to induce a decrease in pH_i that can be detected by the null-point method; and (d) changes of pH_e in the range of 6.0 to 7.4 lead (within 45 min) to small changes in pH_i, which can be observed by the null-point method. This evidence shows that the null-point method is a realistic method for measuring plant protoplasts pH_i. However, this method requires a large amount of protoplasts and is, therefore, especially for barley aleurone protoplasts, time consuming. In addition, this method does not allow a continuous pH_i measurement in time and has a rather poor time resolution. Nevertheless, this method seems to have no source of serious errors and will give relatively reliable measurements of the pH_i (14, 21). It has been applied successfully in animal and cellular slime mold cells (1, 21).

Different fluorescent pH indicators developed for pH_i measurements are now available. Although these fluorescent pH indicators have some clear advantages over other methods (4), there are several potential sources of error as well (14). These errors include leakage of indicator from the cytoplasm to the extracellular medium and compartmentalization of the indicator within the cell. In addition, the calibration method may make it difficult to obtain the absolute pH_i, and the preparation of cell samples and loading of the indicator can be difficult. Furthermore, environmental conditions may disturb the measurements and influence the required excitation and emission wavelengths. Compared with the fluorescent pH indicator methods, the null-point method is less complicated and has fewer sources of potential errors. We choose the null-point method for measurement of pH_i in barley aleurone protoplasts, because it could be applied easily without the problem of introduction of a measuring probe into the cytoplasm.

The null-point method requires a permeabilization of the plasma membrane. For this purpose, we tested both Triton X-100 and digitonin. The difference in basal pH_i values obtained with digitonin and Triton X-100 might be explained by a possible action of Triton X-100, especially at higher concentrations, at internal membranes. This explanation is indirectly supported by the observation that at a pH_e that is higher than the pH_i different steps in the decrease of solution pH were present at increasing Triton X-100 concentrations (Fig. 1A). Such steps in pH change were not observed when digitonin was used (Fig. 1A). The apparent absence of disrupting effects on internal membranes by digitonin is supported by the fact that digitonin is a steroid glycoside that forms insoluble complexes with cholesterol and other β-hydroxysterols in the plasma membrane (2). In addition, in plant cells such as in carrot and maize, digitonin has been successfully used to introduce fluorescent dyes into the cytosol without interfering with intracellular vacuoles, suggesting that digitonin is able to permeabilize the plant cell plasma membrane effectively without affecting intracellular organelle membranes (11, 27). Therefore, mainly digitonin was used for measurements of pH_i values in ABA-treated and pH_i-manipulated protoplasts. In addition, for these reasons, no further attempts were made to establish directly the possible effects of Triton X-100 on internal membranes.

We are able to measure the ABA-induced pH_i increase. This increase is both time and ABA concentration dependent. At 10 μM ABA, a 0.15-unit pH_i increase was observed within 45 min (Fig. 2). This response is equal in magnitude but much slower than that reported for an ABA-induced pH_i increase in maize coleoptiles and hypocotyls (12). However, the ABA concentration (100 μM) used by Gehring et al. (12) was much higher than the concentration we used. Stimulation of barley aleurone protoplasts with 100 μM ABA induced a 0.1-unit pH_i increase within 15 min (data not shown), which is

comparable with the results reported for maize (12).

We manipulated the pH_i by adding weak acids, weak bases, and proton pump inhibitors. According to our measurements, the effects of weak acids or bases on the pH_i are in agreement with results obtained from other systems (1, 28). In addition, we showed that introduction of weak acids and bases into the cytoplasm increases the cytoplasmic buffer capacity, because the ABA-induced alkalinization could be inhibited by weak acids (Fig. 4). We investigated the effects of pH_i modulation by weak acids and bases as well as the effects of proton pump inhibitors on gene expression and found that the weak acids (DMO and potassium propionate) inhibit ABA-induced expression of *RAB* and *BASI* genes. Inhibitions of *RAB* gene expression were half-maximal at 5 mM DMO and at 2.5 mM potassium propionate. This difference in the half-maximal inhibition concentration of DMO and potassium propionate is probably due to the pK_a difference (for DMO the is pK_a 6.13; for potassium propionate the pK_a is 4.87). In addition, it has been reported that in cellular slime mold 5 mM DMO is able to induce the same pH_i decrease as 2 mM potassium propionate (28). At the concentrations we used, neither DMO nor potassium propionate affect *GAPDH* expression or total mRNA level, suggesting that the inhibitory effects of DMO and potassium propionate on *RAB*-16 mRNA expression are not due to a general inhibition of transcription. We cannot yet say whether the inhibitory effect of weak acids is at the transcription level or at the mRNA stability level. Although the weak bases methylamine and ammonia are able to bypass ABA to induce an increase in pH_i, these weak bases neither induce *RAB*-16 mRNA expression nor enhance ABA-induced *RAB*-16 mRNA expression. These results demonstrate that an increase in pH_i alone is not sufficient to trigger gene expression.

We have shown that changes in pH_i are necessary but not sufficient to trigger specific gene expression. Therefore, it is likely that more complex regulatory mechanisms are involved in ABA-induced gene expression. Our future research will be focused on the interrelationship of hormone-induced secondary messengers and their function on regulation of gene expression.

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