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Evidence for Cotransport of Nitrate and Protons in Maize Roots[1]

I. Effects of Nitrate on the Membrane Potential

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ABSTRACT

The electrical response of nitrate-grown maize (*Zea mays* L.) roots to 0.1 millimolar nitrate was comprised of two sequential parts: a rapid and transient depolarization of the membrane potential, followed by a slower, net hyperpolarization to a value more negative than the original resting potential. The magnitude of the response was smaller in roots of seedlings grown in the absence of nitrate, but, within 3 hours of initial exposure to 0.1 millimolar nitrate, increased to that of nitrate-grown roots. Chloride elicited a separate electrical response with a pattern similar to that of the nitrate response. However, the results presented in this study strongly indicate that the electrical response to nitrate reflects the activity of a nitrate-inducible membrane transport system for nitrate which is distinct from that for chloride. Inhibitors of the plasmalemma H^+-ATPase (vanadate, diethylstilbestrol) completely inhibited both parts of the electrical response to nitrate, as did alkaline external pH. The magnitude of the initial nitrate-dependent, membrane potential depolarization was independent of nitrate concentration, but the subsequent nitrate-dependent hyperpolarization showed saturable dependence with an apparent K_m of 0.05 millimolar. These results support a model for nitrate uptake in maize roots which includes a depolarizing NO_3^-/H^+ symport. The model proposes that the nitrate-dependent membrane potential hyperpolarization is due to the plasma membrane proton pump, which is secondarily stimulated by the operation of the NO_3^-/H^+ symport.

The absorption of nitrate by roots of higher plants is generally thought to be thermodynamically active and to require a significant input of energy (2, 11). The mechanism of absorption, however, is a matter of controversy.

Because root nitrate absorption often leads to an alkalinization of the external solution, a popular early hypothesis was that an OH^-/NO_3^- or HCO_3^-/NO_3^- exchange mechanism mediated the process (12). More recently, however, Ullrich, Novacky and coworkers (20, 25, 26) reported that nitrate caused a rapid, pH-dependent depolarization of the cell membrane potential of *Lemna gibba* fronds (*i.e.* the potential became less negative). The transient depolarization was followed by a gradual repolarization of the membrane potential. In nitrogen-starved plants, the degree of depolarization was enhanced by nitrate pretreatment, in correlation with observations of nitrate-induced acceleration of nitrate uptake. These authors explained these results by the operation of a nitrate-inducible, NO_3^-/H^+ symport mechanism (H^+:NO_3^- stoichiometry > 1), in which active nitrate influx was coupled to passive influx of protons across the plasma membrane. The subsequent repolarization was proposed to be due to a stimulation of the H^+-translocating, plasma membrane ATPase caused by changes in either cytoplasmic pH or the membrane potential itself.

Results from other studies of nitrate uptake by plants, however, have led to alternative proposals. In studies of nitrate-starved and nitrate-induced excised maize roots, Thibaud and Grignon (24) reported that nitrate-starved (noninduced) roots excreted protons in the presence of $Ca(NO_3)_2$, while nitrate-induced roots displayed a net H^+ influx in the same solution. (Both types of roots acidified the media in the presence of only $CaSO_4$.) These authors also reported that exposure of induced roots to nitrate caused a small, steady hyperpolarization (about 15 mV) of the membrane potential which depolarized upon nitrate removal, and that DES[3] (an inhibitor of H^+-translocating ATPases) did not alter this electrogenic response. To explain the results, these authors proposed a 2 NO_3^-/1 OH^- antiport mechanism which was not directly coupled to a proton pump (within the time frame of these experiments [<0.5 h]).

In studies of nitrate uptake in *Chara corallina*, Deane-Drummond (4, 5) reported that: (a) OH^- efflux and nitrate uptake were not consistently related, (b) DES inhibited nitrate uptake and H^+ pump activity differentially, (c) the membrane potential of these cells was electrically silent to nitrate, (d) nitrate efflux and estimates of cytoplasmic nitrate concentrations were linearly related, and (e) NH_4^+ inhibited net nitrate uptake by stimulating nitrate efflux. From the first two results above, Deane-Drummond argued that coupling of nitrate transport and proton pump activity is not obligatory. A model

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[3] Abbreviations: DES, diethylstilbestrol; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; FC, fusicoccin; FITC, fluorescein isothiocyanate; PGO, phenylglyoxal; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene.

was proposed (4) in which nitrate influx and nitrate efflux involved discrete carriers, each mediating nitrate/anion exchange; however, no evidence was presented for the identity of the counter anion, and no attempt was made to identify the source of energy for the processes.

It is difficult to provide a coherent explanation for all of the apparent discrepancies among these studies. In the present study, however, we have attempted to explain some of these discrepancies by extending the previous work of Thibaud and Grignon (24), and Ullrich, Novacky and coworkers (20, 25, 26). We have monitored nitrate effects on root membrane potentials of maize seedlings as affected by: (a) nitrate pretreatment (nitrate-induced *versus* noninduced seedlings), (b) external nitrate concentrations and pH, (c) putative inhibitors of anion transport (SITS and PGO), (d) inhibitors (vanadate and DES) and a stimulator (FC) of proton-translocating ATPases, and (e) cations (K^+ and NH_4^+) which cause a depolarization of the resting membrane potential of maize root cells. Because chloride uptake and nitrate uptake share some common regulatory features in higher plants (3, 6, 7, 23), we also have examined the effects of chloride on maize root membrane potentials. In an accompanying paper (19), we report the results of investigations of net nitrate and proton fluxes in maize seedling roots receiving similar treatments.

MATERIALS AND METHODS

Plant Materials

Zea mays L. seeds (3377 Pioneer) were surface-sterilized in 0.5% NaOCl, and then germinated for 2 d in the dark on filter paper saturated with 0.2 mM $CaSO_4$. Subsequently eight germinated seedlings were selected for uniform growth, transferred to polyethylene cups with polyethylene mesh bottoms (two seedlings per cup), and then covered with black polyethylene beads. Four cups (eight seedlings) were then placed into precut holes in the covers of black polyethylene containers containing 2.4 L of aerated solution which consisted of either 0.2 mM $CaSO_4$, or 0.2 mM $CaCl_2$ for noninduced plants, and 0.15 mM $CaSO_4$ or $CaCl_2$ plus 0.05 mM $Ca(NO_3)_2$ for nitrate-induced plants. The seedlings were grown for 3 d at 22°C under low-light conditions. The primary root of intact 5-d-old seedlings was used for all electrical measurements.

Chemicals

All experimental chemicals were purchased from Sigma Chemical Company, St. Louis, MO. For experiments with DES or FC, all solutions (including control solutions) contained 0.25% (v/v) ethanol. For experiments with vanadate, fresh stock solution of 5 mM sodium orthovanadate (Na_3VO_4) was prepared and the concentration determined by the methods of O'Neill and Spanswick (21). For the electrophysiology experiments, the stock solution was diluted to a concentration of 0.2 mM and titrated to pH 6.0 with H_2SO_4.

Membrane Potential Measurements

The microelectrode system was constructed such that either membrane potentials or nitrate and proton fluxes (quantified with ion-selective microelectrodes) could be measured (see ref. 19 for details concerning flux measurements). The maize seedling was secured in a Plexiglas chamber mounted to the stage of an Olympus BH-2 microscope (Spectra Services, Rochester, NY). Cells of the root epidermis and cortex were impaled (2–3 cm from the root apex) using a hydraulically driven Narashige micromanipulator (Model MO-204; Narashige USA, Greenvale, NY) mounted onto the microscope stage. Membrane potentials were measured using a WPI model KS-750 amplifier (World Precision Instruments, Inc., New Haven, CT) and microelectrodes (tip diameter = 0.5 μm) made from single-barreled borosilicate glass tubing and filled with 3 M KCl solution (adjusted to pH 2 to reduce tip potentials). Reference electrodes were made in an identical manner, and placed within the chamber housing the seed to minimize K^+-contamination of the solution bathing the root.

RESULTS

Over 70 measurements of the electrical response of maize roots to 0.1 mM nitrate were made in more than 25 nitrate-grown intact seedlings. Measurements were made in simple calcium-salt solutions in the absence of effectors of either root-cell membrane potential or nitrate uptake. In greater than 90% of these measurements, a qualitatively similar response of the membrane potential was observed as illustrated for two representative nitrate-grown maize roots in Figure 1. Upon introduction of 0.1 mM nitrate to these roots, a rapid and transient depolarization was observed (*i.e.* the potential became less negative). The initial depolarization was followed by a gradual hyperpolarization to fairly constant values which were more negative than the original values. Return of the roots to nitrate-free solutions caused a gradual depolarization to values approximately equal to the original ones. In less than 10% of these measurements, no rapid depolarization was observed; only the more gradual hyperpolarization and

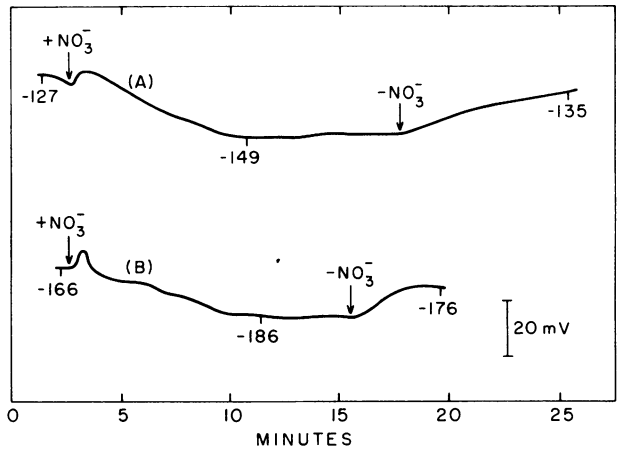

Figure 1. Effect of nitrate on cell membrane potential in two representative nitrate-grown maize roots. Seedlings were grown in (A) 0.15 mM $CaCl_2$ and 0.05 mM $Ca(NO_3)_2$ or (B) 0.15 mM $CaSO_4$ and 0.05 mM $Ca(NO_3)_2$. Impalements were made in (A) 0.2 mM $CaCl_2$ or (B) 0.2 mM $CaSO_4$. Arrows indicate times at which changes were made between nitrate-free solutions and nitrate-containing solutions.

depolarization were observed upon introduction and withdrawal of nitrate, respectively (data not shown).

The electrical response to 0.1 mM nitrate was qualitatively the same in nitrate-induced roots of seedlings grown and impaled in a $CaCl_2$-background (Fig. 1A) as in those grown and impaled in a $CaSO_4$-background (Fig. 1B). Maximum initial depolarizations occurred in both types of root within 0.2 to 0.4 min of introduction of nitrate. The magnitude of this transient depolarization (when observed) ranged from 1 to 12 mV with mean values of 6 mV for both types of roots (Table I). The subsequent gradual hyperpolarization occurred for about 4 to 7 min for both root-types. Mean values (±SEM) for the net change in membrane potential due to nitrate introduction were greater for $CaSO_4$-grown roots (22 ± 8 mV) than for $CaCl_2$-grown roots (16 ± 8 mV), but the significance of the difference was marginal (Table I). The transient depolarization was observed in most measurements of both types of root.

Figure 2 shows representative responses of the membrane potential to varying concentrations of nitrate in roots of nitrate-grown seedlings. Figure 3 depicts the concentration-dependence of the two components of the electrical response. The transient depolarization showed essentially no concentration-dependence between 0.01 and 10 mM nitrate (Figs. 2 and 3A). The net hyperpolarization of the membrane potential, however, was markedly dependent upon nitrate concentration. The dependence displayed saturability with an apparent K_m of 0.05 mM nitrate (Figs. 2 and 3B).

The initial electrical response to 0.1 mM nitrate was smaller in seedlings grown in nitrate-free conditions (noninduced) than the response in seedlings grown with nitrate (*cf.* Tables I and II). When 0.1 mM nitrate was initially introduced to noninduced roots, a transient depolarization of the membrane potential was not detected as clearly as in induced roots. This was especially the case for noninduced roots grown in 0.2 mM $CaCl_2$; small (2 mV) transient depolarizations were seen in only two of nine measurements. In contrast, nitrate-dependent transient depolarizations were more evident in noninduced $CaSO_4$-grown roots (10 of 12 measurements). However, the magnitude of this response was decidedly smaller than that of induced $CaSO_4$-grown roots (6 mV for induced roots *versus* 3 mV for noninduced; Tables I and II, respectively). In both $CaSO_4$- and $CaCl_2$-grown noninduced roots, however, the nitrate-dependent net hyperpolarization was detected more clearly than the depolarization. A net hyperpolarization of the membrane potential was observed in 7 of 9 measurements for noninduced $CaCl_2$-grown roots and in all 12 measurements of noninduced $CaSO_4$-roots (Table II). The magnitude of the nitrate-dependent hyperpolarization, however, like that of the nitrate-dependent depolarization, clearly was smaller in noninduced roots than in induced roots (*cf.* Tables I and II).

Repeated exposures of noninduced roots to 0.1 mM nitrate increased the magnitude of the electrical response to nitrate. Figure 4 shows the response to initial exposures of nitrate in a representative, noninduced, $CaCl_2$-grown root. When 0.1 mM nitrate was first introduced, the membrane potential did not rapidly and transiently depolarize, but slowly hyperpolarized. After several 20 to 30 min exposures to nitrate, which were interspersed with exposures to nitrate-free solutions, the characteristic pattern of nitrate-grown roots began to emerge. When introduced to 0.1 mM nitrate for the third time, approximately 90 min after commencement of the first exposure to nitrate, a transient 2 to 3 mV depolarization was observed, followed by a small hyperpolarization (Fig. 4B). Two to 3 h after initial nitrate exposure, the magnitudes of both components of the response were greater than those in Figure 4B (data not shown), and closely resembled those of the response in nitrate-grown roots.

The electrical response of nitrate-induced and noninduced roots to chloride (0.4 mM) was similar to that of nitrate-grown roots to nitrate (*i.e.* initial transient depolarization followed by net hyperpolarization) (Fig. 5). When a 0.2 mM $CaCl_2$ solution was replaced with a solution of 0.05 mM $Ca(NO_3)_2$ plus 0.15 mM $CaCl_2$, a typical nitrate-response was observed in nitrate-grown roots (Fig. 5A), but was absent in roots previously grown without nitrate (Fig. 5B). These results agree with those presented in Figure 4 indicating that noninduced, $CaCl_2$-grown roots initially show only a small electrical response to 0.1 mM nitrate. The response to chloride was not dependent upon chloride-pretreatment; roots grown either in $CaSO_4$- or $CaCl_2$-backgrounds displayed the same pattern

Table I. *Response of the Membrane Potential to 0.1 mM* Nitrate in Roots of Nitrate-Grown Maize Seedlings Bathed in Simple Calcium-Salt Solutions[a]

Growth Solution	Magnitude of Transient Depolarization[b]	Magnitude of Net Hyperpolarization
	mV	
0.15 mM $CaCl_2$	Mean = 6 ± 3 mV	Mean = 16 ± 8 mV
0.05 mM $Ca(NO_3)_2$[c]	Range = 1–12 mV	Range = 4–30 mV
	No. of observations = 35	No. of observations = 36
0.15 mM $CaSO_4$	Mean = 6 ± 3 mV	Mean = 22 ± 8 mV
0.05 mM $Ca(NO_3)_2$[d]	Range = 1–12 mV	Range = 4–39 mV
	No. of observations = 30	No. of observations = 36

[a] Seedling growth, root impalements, and solution changes as outlined in legend of Figure 1. [b] Compiled from responses in which rapid, transient depolarizations were observed. Mean values are listed with ± SEM. [c] Measurements made with 12 seedlings, average resting membrane potential = −153 ± 14 mV. [d] Measurements made with 15 seedlings, average resting membrane potential = −139 ± 18 mV.

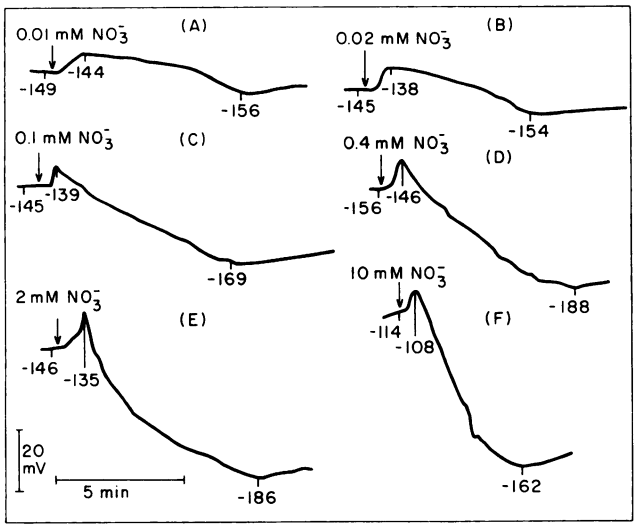

Figure 2. Effect of nitrate concentration on the response of the membrane potential to nitrate in nitrate-grown maize roots. Seedlings were grown in 0.15 mM $CaSO_4$ and 0.05 mM $Ca(NO_3)_2$, and impalements were made in 0.2 mM $CaSO_4$. Arrows for the top four traces (A–D) show time of replacing 0.2 mM $CaSO_4$ with solutions containing 0.01 to 0.4 mM nitrate. Calcium concentration was kept constant by replacing $CaSO_4$ with $Ca(NO_3)_2$. In trace E, arrow indicates replacement of 1 mM $CaSO_4$ with 1 mM $Ca(NO_3)_2$ (2 mM NO_3^-). In trace F, arrow indicates replacement of 5 mM $CaSO_4$ with 5 mM $Ca(NO_3)_2$ (10 mM NO_3^-).

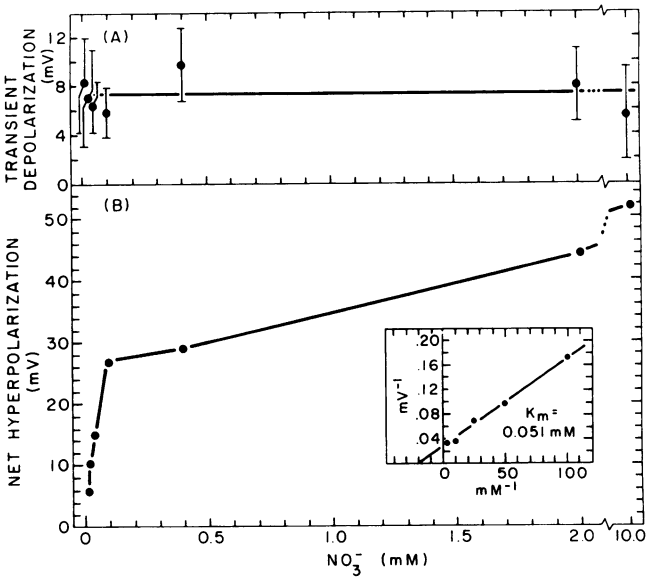

Figure 3. Concentration dependence of transient depolarizations of the membrane potential (panel A) and subsequent net hyperpolarizations (panel B) caused by nitrate. Data were compiled from three separate experiments. Values in either panel represent the mean of at least two replicate measurements from each of two experiments ($n > 4$). In panel A, bars represent ±SEM, and the horizontal line represents the mean value of the transient depolarization averaged over nitrate concentrations between 0.01 and 10 mM. Inset in panel B represents a double-reciprocal plot of measurements made within the nitrate concentration range of 0.01 to 0.4 mM. Seedlings were grown and impaled as described in the legend of Figure 2.

(data not shown). Observations of the response to 0.4 mM chloride were made in five roots. Values for the transient depolarization averaged 13 ± 3 mV, while the net hyperpolarization averaged 24 ± 7 mV; both means are greater than the respective means for nitrate responses contained in Table I. The magnitude of the response to 0.1 mM chloride (transient depolarization = 12 ± 3 mV and net hyperpolarization = 24 ± 6 mV; $n = 3$) was essentially the same as that of the response to 0.4 mM chloride.

The electrical response to 0.1 mM nitrate in nitrate-grown roots displayed sensitivity to variation in external pH (Fig. 6). Between pH 4.4 and 7, the response was essentially unchanged. At pH 8, however, the normal response to nitrate was conspicuously absent; roots were electrically silent upon the addition of nitrate (Fig. 6B). If, after a 20 to 30 min exposure to pH 8 conditions, roots were returned to solutions buffered at pH 6, they remained electrically silent to nitrate for up to 30 minutes (data not shown). The effect of longer recovery periods was not investigated, nor was the effect of external pH on the electrical response to chloride.

The presence of either of two inhibitors of proton-translocating ATPases, vanadate, or DES, dramatically inhibited the electrical response to 0.1 mM nitrate (Fig. 7). Treatment of nitrate-grown roots with 0.20 mM sodium orthovanadate (Na_3VO_4) for approximately 30 min caused a net 60 mV depolarization of the membrane potential (Fig. 7A). After the 30 min treatment with vanadate, the addition or withdrawal of nitrate had no effect on the membrane potential. Treatment with 0.05 mM DES for 30 min also brought about a large net depolarization of the resting membrane potential and a significant inhibition of the nitrate response (Fig. 7B). Complete inhibition by DES of the electrical response to nitrate required a longer exposure period than did inhibition by vanadate.

Two putative inhibitors of anion transport, SITS and PGO, differed in their ability to inhibit the electrical response to 0.1 mM nitrate (Fig. 8). Treatment of nitrate-grown roots with either of these compounds (0.5 mM SITS and PGO at either 0.5 or 1.0 mM) caused large net depolarizations of the membrane potential; depolarization due to SITS, however, was slower than that caused by PGO. Pretreatment of roots with 1 mM PGO (Fig. 8B) or 0.5 mM PGO (data not shown) completely inhibited the nitrate response. In contrast, even after a similar 30-min pretreatment, the presence of 0.5 mM SITS did not alter the response of the membrane potential to nitrate (Fig. 8A). Both compounds, however, completely inhibited the electrical response to chloride (Fig. 8, A and C). These results suggest that (a) both of these compounds may have some direct inhibitory effect on H^+-ATPases as shown previously (10, 15, 16); (b) the inhibitory effect of PGO was greater than that of SITS; and (c) SITS specifically inhibited chloride transport. PGO has been shown previously to inhibit both chloride and nitrate uptake in maize roots (8).

The electrical response of maize roots to 0.1 mM nitrate was qualitatively independent of the magnitude of the resting membrane potential (Fig. 9). Three membrane potential-altering treatments were applied: 0.01 mM FC, 0.1 mM KCl, and 0.2 mM NH_4Cl.

Table II. *Response of the Membrane Potential to Initial Exposures of 0.1 mM Nitrate in Roots of Maize Seedlings Grown Previously in the Absence of Nitrate*

Roots were impaled in respective growth solutions, and membrane potential changes were measured upon introduction of either 0.15 mM $CaCl_2$ plus 0.05 mM $Ca(NO_3)_2$ or 0.15 mM $CaSO_4$ plus 0.05 mM $Ca(NO_3)_2$, for $CaCl_2$- and $CaSO_4$-grown roots, respectively. First exposures to nitrate were 7 to 15 min in duration. Following 15-min subsequent exposures to original growth solutions, second exposures to nitrate were applied, and the response of the membrane potential was measured again. Values of means are listed with ± SEM.

Growth Solution	Magnitude of Transient Depolarization	Magnitude of Net Hyperpolarization
	mV	
0.2 mM $CaCl_2$	Mean = 0.4 ± 1 mV Range = 0–2 mV No. of observations = 9	Mean = 5 ± 4 mV Range = 0–14 mV No. of observations = 9
0.2 mM $CaSO_4$	Mean = 3 ± 1 mV Range = 0–4 mV No. of observations = 12	Mean = 7 ± 4 mV Range = 1–13 mV No. of observations = 12

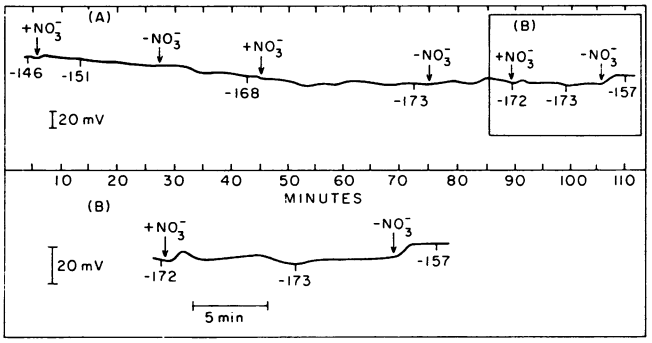

Figure 4. Effect of sequential nitrate exposures on the response of the membrane potential to nitrate in a $CaCl_2$-grown (noninduced) root. Seedling growth and root impalements were both in 0.2 mM $CaCl_2$. Arrows indicate solution changes from 0.2 mM $CaCl_2$ to 0.15 mM $CaCl_2$ and 0.05 mM $Ca(NO_3)_2$, and vice versa. The trace in panel B represents a magnification of the boxed trace in panel A.

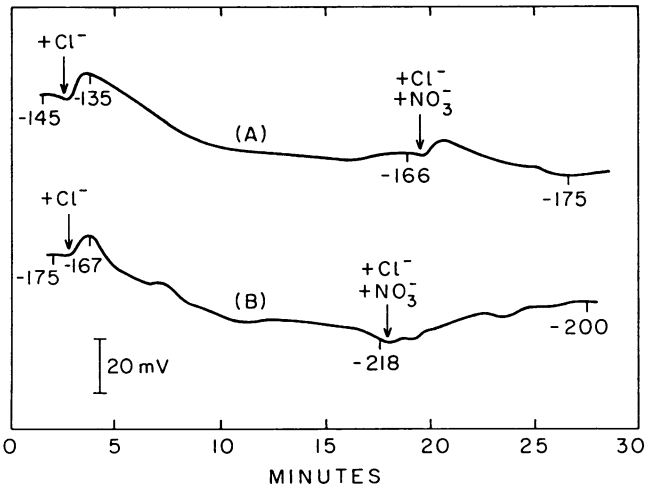

Figure 5. Effect of chloride and nitrate on the membrane potential in maize roots grown on (A) 0.15 mM $CaSO_4$ and 0.05 mM $Ca(NO_3)_2$ or (B) 0.2 mM $CaSO_4$. Impalements were made in 0.2 mM $CaSO_4$. First arrow for each trace indicates the time at which 0.2 mM $CaCl_2$ replaced 0.2 mM $CaSO_4$. The second arrow indicates solution changes from 0.2 mM $CaCl_2$ to 0.15 mM $CaCl_2$ and 0.05 mM $Ca(NO_3)_2$.

Treatment of roots with 0.01 mM FC led to a net hyperpolarization of the resting membrane potential by approximately 50 to 80 mV, over a period of 60 to 90 min. In the presence of 0.01 mM FC (after a 90-min pretreatment), introduction of nitrate still led to a transient depolarization, followed by a net hyperpolarization (Fig. 9A). However, under these conditions, the removal of nitrate did not bring about a net depolarization; rather the membrane potential remained hyperpolarized.

Treatment with 0.1 mM KCl caused a rapid depolarization of the membrane potential (about 30–50 mV), within 5 min. In this state, however, the normal electrical response to nitrate was observed in nitrate-grown roots (Fig. 9B). The membrane potential depolarized upon the addition of NH_4Cl in a similar manner to the response to KCl. As in the presence of K^+, the electrical response to nitrate in the presence of NH_4^+ was qualitatively the same as the response in the absence of the depolarizing cation (*cf.* Figs. 1B and 9C).

DISCUSSION

We have observed that the electrical response of nitrate-grown maize roots to nitrate was comprised of two sequential parts: a rapid and transient depolarization of the cell membrane potential, followed by a net hyperpolarization (Fig. 1; Table I). This electrical response displays nitrate-inducibility which correlates with the well-documented nitrate-inducibility of net nitrate uptake by maize roots (13, 14, 17, 19). Upon first exposure to 0.1 mM nitrate, roots of seedlings grown in the absence of nitrate displayed a smaller response than that of nitrate-grown roots (Table II; Figs. 4 and 5). Within 1.5 to 3 h of initial exposure to nitrate, however, the magnitude of the electrical response increased in noninduced roots (Fig. 4).

Our observations of a two-part electrical response to nitrate do not completely agree with the report that nitrate caused only a net hyperpolarization of the membrane potential in maize roots (24). It is possible that differences in experimental materials (excised roots *versus* roots of intact seedlings) and/or techniques may be responsible for the discrepancy between

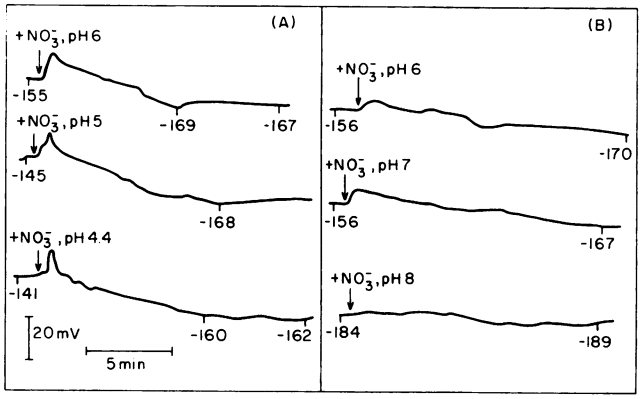

Figure 6. Effect of external pH on the electrical response to nitrate in nitrate-grown roots. The traces in panel A depict membrane potential measurements made in one plant, while those in panel B were measured in a second seedling. Solutions were buffered with 5 mM Mes-Tris for pH 4.4 to pH 6, and with 5 mM Hepes-Tris for pH 7 and 8. After the initial electrical response to nitrate was determined at pH 6, further responses to nitrate were determined in solutions of either ascending or descending pH value. After monitoring the electrical response to nitrate at a given pH [*e.g.* 0.15 mM $CaCl_2$, 0.05 mM $Ca(NO_3)_2$, and 5 mM Mes-Tris (pH 6)], the bathing solution was changed to nitrate-free solutions (0.2 mM $CaCl_2$) buffered at the new pH value as indicated.

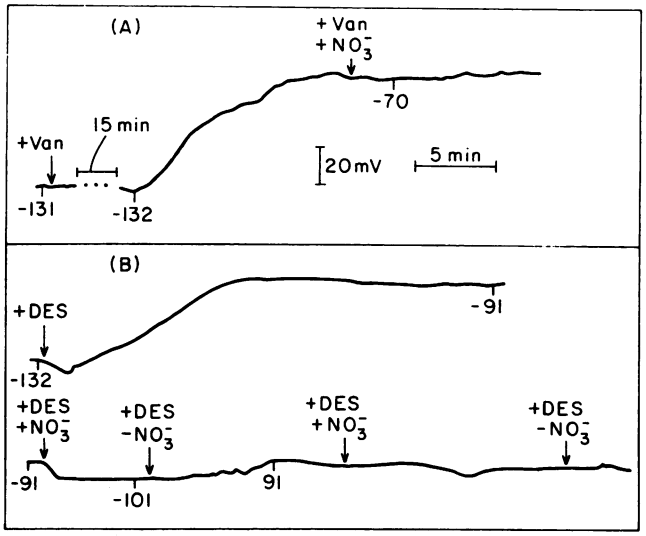

Figure 7. Influence of inhibitors of proton-translocating ATPases (vanadate, DES) on the response of the membrane potential to nitrate. Roots of nitrate-grown seedlings were impaled in 0.2 mM $CaCl_2$. After a 30-min exposure to either (A) 0.2 mM $CaCl_2$ and 0.2 mM sodium orthovanadate (Na_3VO_4) or (B) 0.2 mM $CaCl_2$ and 0.05 mM DES, the response to nitrate was monitored by replacing the pretreatment solution with a solution containing 0.15 mM $CaCl_2$, 0.05 mM $Ca(NO_3)_2$ and the respective inhibitor. For the experiments with vanadate in A, the root was exposed to a solution containing 0.6 mM Na^+ (as 0.3 mM Na_2SO_4 + 0.2 mM $CaCl_2$) prior to the solution change to 0.2 mM Na_3VO_4 + 0.2 mM $CaCl_2$.

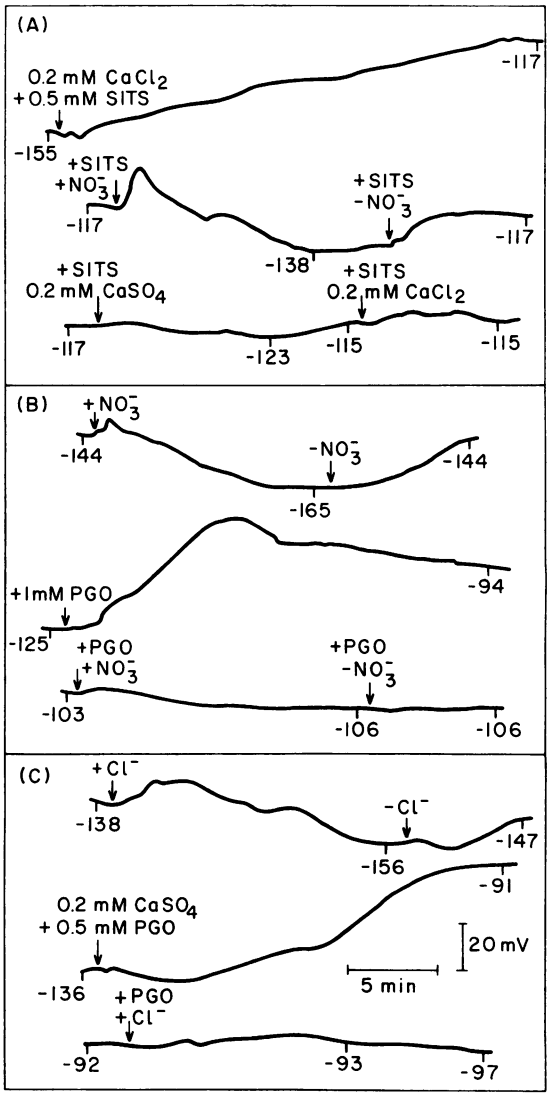

Figure 8. Influence of SITS and PGO on the response of the membrane potential to nitrate and chloride. Roots of nitrate-grown seedlings were impaled in 0.2 mM $CaCl_2$ (panels A and B) or 0.2 mM $CaSO_4$ (panel C). Panel A represents a continuous monitoring of the membrane potential, and depicts the response to: (a) top trace, exposure to 0.5 mM SITS and 0.2 mM $CaCl_2$; (b) middle trace, sequential addition and withdrawal of 0.1 mM nitrate in the presence of SITS [0.15 mM $CaCl_2$, 0.05 mM $Ca(NO_3)_2$ and 0.5 mM SITS]; and (c) bottom trace, withdrawal (0.2 mM $CaSO_4$) and reintroduction of 0.2 mM $CaCl_2$ in the presence of 0.5 mM SITS. Panel B depicts the response of the membrane potential to: top trace, addition [0.15 mM $CaCl_2$, 0.05 mM $Ca(NO_3)_2$] and withdrawal (0.2 mM $CaCl_2$) of nitrate before PGO treatment; middle trace, a 30 min treatment with 1 mM PGO and 0.2 mM $CaCl_2$; and bottom trace, addition and withdrawal of nitrate after initial PGO-treatment, but still in the presence of 1 mM PGO. Panel C shows the response of the membrane potential to addition (0.2 mM $CaCl_2$) and withdrawal (0.2 mM $CaSO_4$) of chloride, both before and after treatment with 0.5 mM PGO (top and bottom traces, respectively). The middle trace of panel C records the electrical response of this root to 0.5 mM PGO in the presence of 0.2 mM $CaSO_4$.

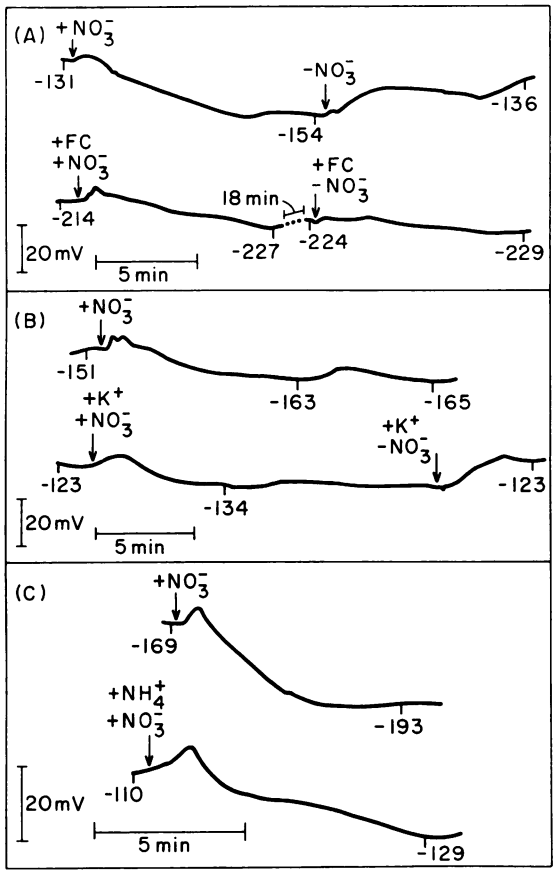

Figure 9. Influence of FC, K^+, and NH_4^+ on the electrical response of maize roots to nitrate. Seedlings were grown in 0.15 mM $CaCl_2$ and 0.05 mM $Ca(NO_3)_2$ (panels A and B), or in 0.15 mM $CaSO_4$ and 0.05 mM $Ca(NO_3)_2$ (panel C). Impalements were made in 0.2 mM $CaCl_2$ (panels A and B) or 0.2 mM $CaSO_4$ (panel C). The top trace in panel A shows changes in the membrane potential due to the addition [0.2 mM $CaCl_2$ and 0.05 mM $Ca(NO_3)_2$] and withdrawal (0.2 mM $CaCl_2$) of nitrate in the absence of FC. Bottom trace of panel A shows changes in the membrane potential due to the addition and withdrawal of nitrate in the presence of 0.01 mM FC after a 90-min pretreatment in 0.2 mM $CaCl_2$ plus 0.01 mM FC. Panel B shows the response of the membrane potential to the addition of 0.1 mM nitrate in the absence of potassium (top trace) and in the presence of 0.1 mM KCl after a 15-min pretreatment in 0.2 mM $CaCl_2$ plus 0.1 mM KCl (bottom trace). Panel C shows the response of the membrane potential to the addition of 0.1 mM nitrate [0.15 mM $CaSO_4$ and 0.05 mM $Ca(NO_3)_2$] in the absence of ammonium, (top trace), and in the presence of 0.1 mM $(NH_4)_2SO_4$ after a 15-min pretreatment in 0.2 mM $CaSO_4$ and 0.1 mM $(NH_4)_2SO_4$ (bottom trace).

the two studies. We were prompted to search for nitrate-inducible, transient, nitrate-dependent depolarizations of the membrane potential by the convincing reports of their existence in *Lemna gibba* and other organisms (9, 20, 25, 26). In spite of their small magnitude, nitrate-dependent depolarizations occurred in our studies with sufficient frequency and regularity to convince us of their existence in maize roots as well (Tables I and II).

Maize roots displayed a similar two-part electrical response to chloride that was distinct from the response to nitrate, as indicated by the results in Figures 5 and 8. We found that the presence of chloride did not interfere with the nitrate response in nitrate-grown roots, and nitrate pretreatment, while affecting the electrical response to nitrate, did not affect the response to chloride (Fig. 5). Furthermore, treatment of roots with SITS completely inhibited the electrical response to chloride, but did not alter the response to nitrate (Fig. 8). Previous studies with maize root protoplasts and root segments (16) have shown that chloride transport is inhibited by SITS and, in an accompanying paper, we report that SITS-treatment had little effect on net nitrate uptake by roots identical to those used in the present study (19). Taken together, these results support the idea that maize roots have distinct transport mechanisms for each of these anions. Additional support is provided by the report that both DIDS and FITC inhibited chloride uptake by maize roots, but neither inhibited nitrate uptake (8).

Treatment of roots with phenylglyoxal, a diketone which binds the guanidinium group of arginine residues, completely abolished the electrical response to either nitrate or chloride (Fig. 8). In an accompanying paper (19), we report that such treatment of identical seedling roots also inhibited net nitrate uptake. Dhugga *et al.* (8) previously reported that phenylglyoxal inhibited both nitrate and chloride uptake by maize roots, and proposed that proteins in both transport systems possess essential arginine residues which are accessible to phenylglyoxal. It should be noted that Kasamo (15), and Gildensoph and Briskin (10), reported that fairly high concentrations of phenylglyoxal (10–20 mM) also inhibited the activity of the plasma membrane H^+-ATPase in membrane vesicles isolated from mung bean roots and red beet storage tissue. The time course and magnitude of depolarization of the membrane potential by PGO observed in our study was similar to the electrical effects elicited by the H^+-ATPase inhibitors, vanadate and DES (*cf.* Figs. 7 and 8B, C). Therefore, the possibility exists from our experiments, that PGO is acting both on anion transport proteins and the H^+ pump. Upon initial exposure, PGO could bind to arginine residues residing in plasmalemma anion transport proteins (for both NO_3^- and Cl^- transport) that are exposed to the external solution. With longer exposure, sufficient PGO could accumulate in the cytoplasm to bind to arginine residues in the plasmalemma H^+ pump that are exposed to the cytoplasm. Therefore, it is impossible to determine from the experiments presented here, whether PGO is inhibiting NO_3^- uptake via a direct interaction with the nitrate transporter, indirectly, via an effect on the H^+ pump, or by acting at both sites.

To explain the observation of a nitrate-inducible, two-part electrical response to nitrate in cells of *Lemna* fronds, Ullrich and Novacky (26) proposed that nitrate-dependent transient depolarizations of the membrane potential were directly caused by the operation of a NO_3^-/H^+ symport mechanism, and that subsequent hyperpolarizations of the membrane potential were due to plasma membrane proton pumps which were secondarily stimulated by the operation of the symport. Our results with maize roots generally support this model. It should be noted that it is not possible to experimentally differentiate between a NO_3^-/H^+ symport mechanism and a NO_3^-/OH^- antiporter (and it may not be possible to discrim-

inate between these two systems mechanistically due to the ability of water to dissociate to yield H^+ and OH^-). We have chosen to describe the system in the maize root plasmalemma as a proton cotransport system in keeping with recent convention concerning proton-coupled transport systems.

Alkaline external pH values significantly inhibited the electrical response to nitrate in *Lemna* fronds (26) and in maize roots as well (Fig. 6). These results are theoretically consistent with the operation of a plasma membrane NO_3^-/H^+ symport. Correlative evidence linking pH effects on the electrical response to nitrate with those on nitrate uptake itself is provided in the accompanying paper (19). We observed that net nitrate uptake by maize seedling roots, as measured with nitrate-specific microelectrodes, was progressively inhibited by increases in pH from pH 4.5 to pH 8. At pH 8 and above, inhibition of nitrate uptake was nearly complete. These observations are in agreement with previous observations of acidic pH optima for nitrate uptake in maize plants of different genetical, developmental and cultural origins (18, 27).

When *Lemna* fronds were incubated in darkness (*versus* in the light), Ullrich and Novacky observed that the magnitude of nitrate-dependent depolarizations of the membrane potential increased by two- to threefold (26). These authors proposed that, in the light, the depolarizing activity of a NO_3^-/H^+ symport was masked by the hyperpolarizing activity of a stimulated plasma membrane proton pump. In this view, the pump was taken to be stimulated already in the light, either by increased energy (*i.e.* ATP) availability from photosynthesis or by direct light activation. In order to test if depletion of energy supplies in maize seedlings might unmask nitrate-dependent depolarizations of the membrane potential and increase their magnitude, we have removed the shoot and endosperm for 24 h prior to monitoring the electrical response to nitrate. This treatment did not significantly alter the electrical response to nitrate; no significant increases in the magnitude of nitrate-dependent transient depolarizations were observed (LV Kochian, PR McClure, unpublished data).

Nitrate-dependent depolarizations of the membrane potential in *Lemna* fronds displayed substrate saturation kinetic parameters similar to those of nitrate uptake (26), thus providing futher correlative evidence linking the electrical response to nitrate with uptake. We could not observe substrate concentration dependence of the transient depolarization in maize roots with nitrate concentrations between 0.01 and 10 mM (Figs. 2 and 3). In contrast, the nitrate-dependent hyperpolarization of the membrane potential displayed a saturable dependence upon external nitrate concentrations with an apparent K_m of 0.05 mM (Figs. 2 and 3). Such a value for the K_m is similar to estimates of apparent K_m values for net nitrate uptake in maize roots (19, 22). From this similarity and the apparent concentration-independence of the nitrate-dependent depolarization of the membrane potential, it may be argued that, in addition to a depolarizing NO_3^-/H^+ symport system, a separate hyperpolarizing nitrate transport system could exist in the plasma membrane of maize root cells. We do not agree with this argument because: (a) nitrate-dependent hyperpolarizations, in our studies, were preceded consistently by transient depolarizations; (b) the hyperpolarizing process may have masked any concentration dependence of the depolarizing process; and (c) it would be expected that a hyperpolarization directly attributable to nitrate would proceed more rapidly than the relatively slow responses observed in this study.

Thibaud and Grignon (24) reported that DES, an inhibitor of H^+-ATPases, did not alter the hyperpolarizing influence of nitrate in maize roots that they observed, and they therefore argued that nitrate transport was directly responsible for the hyperpolarization of the membrane potential. This argument and its experimental support are in opposition to the Ullrich and Novacky model (25, 26) which proposes that nitrate-dependent hyperpolarizations are due to a stimulation of the plasma membrane proton pump. Furthermore, the DES results presented by Thibaud and Grignon are in contradiction to the results presented here for both DES and vanadate. One possible explanation for the differences between our results and those of Thibaud and Grignon is that the limited time of exposure to DES in the experiments of Thibaud and Grignon (<15 min) was insufficient to completely inhibit the proton pump and render it insensitive to stimulatory effectors. We have demonstrated that longer exposures (>30 min) of maize roots to DES and vanadate, essentially abolished both parts of the electrical response to nitrate (Fig. 7). However, this explanation can be questioned when one considers the results of Balke and Hodges (1), which demonstrated that both K^+ and Cl^- uptake into oat roots are significantly inhibited by DES within 2 min. If these inhibitions are due to an effect of DES on the H^+ pump, then the results of Thibaud and Grignon are difficult to explain in relation to the results presented here.

Despite these contradictions, our results with H^+-ATPase inhibitors suggest that the coupling between the electrical response to nitrate and the operation of the plasma membrane H^+-ATPase is more direct than that suggested by the previous study of Thibaud and Grignon. Furthermore, our results do not support the idea that nitrate transport is directly responsible for the nitrate-dependent hyperpolarization of the membrane potential. However, they can not be used logically to discard this concept, because these longer pretreatments with vanadate or DES essentially abolish nitrate uptake, as demonstrated by data presented in an accompanying paper (19).

If multiple mechanisms of nitrate transport exist in roots of higher plants and if they are responsible for different components of the electrical response to nitrate, it may be possible to differentially modify their activities by chemical, environmental, or genetic means. Subsequently, alteration of each of the components of the electrical response to nitrate might be observed. To date, however, we have been unable to identify potential chemical and environmental effectors of nitrate transport which specifically alter different components of the electrical response to nitrate, other than external nitrate concentration (Figs. 2 and 3). All of the other potential effectors which we have studied, (*i.e.* nitrate pretreatment [Table I *versus* Table II], pH [Fig. 6], vanadate and DES [Fig. 7], SITS and PGO [Fig. 8], FC, K^+, and NH_4^+ [Fig. 9]) either did not alter the electrical response to nitrate or modified both parts of the response.

In conclusion, we propose that, for maize roots, the simplest model of nitrate transport which would explain (a) the two-

part electrical response to nitrate, (b) the nitrate inducibility of the response, (c) the obligatory coupling of both parts of the response with the activity of the plasmalemma H^+-ATPase, and (d) our inability to modify differentially each part of the response, is one in which transport across the plasma membrane is carried out by a nitrate-inducible NO_3^-/H^+ symport. In this model, the transient depolarization of the membrane potential is caused by a H^+:NO_3^- stoichiometry greater than one, and the subsequent net hyperpolarization is due to stimulated proton pumping by the plasma membrane H^+-ATPase. Based on the available electrophysiological data for higher plants, it is not necessary to invoke a more complicated model involving an additional electrogenic nitrate transport mechanism which would hyperpolarize the membrane potential. Further tests of this model will benefit from an approach integrating biophysical techniques with a molecular identification and characterization of the protein(s) involved in nitrate transport across the plasma membrane of higher plant cells.

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