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The externally derived portion of the hyperosmotic shock-activated cytosolic calcium pulse mediates adaptation to ionic stress in suspension-cultured tobacco cells

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Signal transduction

Summary

The influx of Ca^{2+} into the cytosol has long been suggested to serve as a signaling intermediate in the acquisition of tolerance to hyperosmotic and/or salinity stresses. Here we use aequorin-transformed suspension-cultured tobacco cells to directly assess the role of cytosolic calcium (Ca^{2+}_{cyt}) signaling in salinity tolerance acquisition. Aequorin luminescence recordings and ^{45}Ca influx measurements using inhibitors of Ca^{2+} influx (Gd^{3+} and the Ca^{2+}-selective chelator EGTA), and modulators of organellar Ca^{2+} release (phospholipase C inhibitors U73122 or neomycin) demonstrate that hyperosmolarity, whether imposed by NaCl or by a non-ionic molecule sorbitol, induces a rapid (returning to baseline levels of Ca^{2+} within 10 min) and complex Ca^{2+}_{cyt} pulse in tobacco cells, deriving both from Gd^{3+}-sensitive externally derived Ca^{2+} influx and from U73122- and neomycin-sensitive Ca^{2+} release from an organelle. To determine whether each of the two components of this brief Ca^{2+} signal regulate adaptation to hyperosmotic shock, the Ca^{2+} pulse was modified by the addition of Gd^{3+}, U73122, neomycin, or excess Ca^{2+}, and then cells were treated with salt or sorbitol. After 10 min the cell culture medias were diluted with additional hyperosmotic media to reduce the toxic affects of the modulators, and the growth of cells was measured after 1 week. Gd^{3+} treatment reduced growth in salt relative

Abbreviations: Ca^{2+}_{cyt}, cytosolic calcium; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*, *N*, *N′N′*-tetraacetic acid; IP_3, inositol-1, 4, 5-trisphosphate; MES, 2-(*N*-morpholino)-ethanesulfonic acid; $PI(4, 5)P_2$, phosphatidylinositol-(4, 5)-bisphosphate; PLC, phospholipase C

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to control cells but not in sorbitol, and exposure to excess Ca^{2+} increased growth in salt but not in sorbitol. In contrast, exposure to inhibitors of IP_3 formation had no effect on growth in salt or sorbitol. Therefore, although hyperosmotic treatment stimulates both Ca^{2+} influx and Ca^{2+} release from an internal Ca^{2+} depot, only Ca^{2+} influx has a measurable impact on ionic stress tolerance acquisition in tobacco cell suspensions. In contrast, osmoadaptation in these cells appears to occur independent of Ca^{2+} signaling.
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Introduction

Plant cells in high saline environments must respond immediately to more than one stress: hyperosmolarity (osmotic stress) and the toxicity imposed by an excess of sodium ions (ionic stress) (Pasternak, 1987; Hasegawa et al., 2000; Kawasaki et al., 2001; Tester and Davenport, 2003). Thus, cells adapt to saline conditions by not only increasing their production of intracellular osmolytes (e.g. proline), thereby regulating their osmotic pressure and preventing plasmolysis, but also by increasing their capacity to extrude Na^+ from the cytoplasm to thereby maintain ionic homeostasis (Pasternak, 1987; Hasegawa et al., 2000; Tester and Davenport, 2003).

Nearly all environmental stresses stimulate cytosolic Ca^{2+} transients in the plant cell cytosol that theoretically lead to stress resistance by way of Ca^{2+}-dependent signal transduction (Knight et al., 1991, 1997; Trewavas and Malho, 1997; Rudd and Franklin-Tong, 2001; Sanders et al., 2002; White and Broadley, 2003). Molecular and genetic analyses in *Arabidopsis* have identified several calcium-binding signaling proteins including SOS3 (Salt Overly Sensitive) (Ishitani et al., 2000), calcineurin B-like (CBL) proteins (Cheong et al., 2003), and one or more calcium-dependent protein kinases (CDPKs) (Sheen, 1996; Harmon et al., 2000) that are activated in response to salt treatments (Zhu, 2003). SOS3 transmits the Ca^{2+} signal to activate other downstream protein kinases such as SOS2, which mediate ionic stress resistance by regulating transcription factors for stress tolerance genes, and directly activating enzymatic activities such as plasma membrane (SOS1) and vacuolar (NHX) Na^+/H^+ antiporters, resulting in increased tolerance to salt (Hasegawa et al., 2000; Zhu, 2003; Chinnusamy et al., 2004; Boudsocq and Lauriere, 2005; Kaur and Gupta, 2005). In contrast, osmotic stress tolerance (e.g. osmotic adjustment via osmolyte synthesis) appears to arise by way of Ca^{2+}-independent signaling through Ca^{2+}-independent protein kinases (Boudsocq and Lauriere, 2005; Kaur and Gupta, 2005).

While much is known about the events occurring downstream of Ca^{2+} during salt-activated signaling, relatively little is known about the events occurring upstream of Ca^{2+} entry. Cell-wall and plasma membrane-embedded turgor receptor-protein kinases may initiate the osmotic signaling cascade (Urao et al., 1999; Tamura et al., 2003; Chinnusamy et al., 2004; Kacperska, 2004), and stretch-activated Ca^{2+} channels might be turgor-sensors, initiating Ca^{2+} influx directly (Kacperska, 2004). In addition, changes have been observed in membrane phosphoinositide metabolism in hyperosmotically challenged plant cells that are coincident with Ca^{2+} pulses, leading to the hypothesis that inositol-1,4,5-trisphosphate (IP_3)-regulated Ca^{2+} channels in an internal organelle(s) might be responsible for the observed cytosolic calcium (Ca^{2+}_{cyt}) increases (Drobak and Watkins, 2000; Takahashi et al., 2001; DeWald et al., 2001; Chinnusamy et al., 2004; Kaur and Gupta, 2005). Thus, it can be reasonably hypothesized that both trans-plasma membrane Ca^{2+} entry and internal organelle Ca^{2+} release are stimulated by hyperosmotic shock. It remains unclear, however, whether both influx and organelle release of Ca^{2+} are involved in signaling to ionic and/or osmotic tolerance.

Hyperosmotic (manitol)- and NaCl-activated cytosolic Ca^{2+} signaling has previously been demonstrated to activate three genes (*P5CS*, *LTI78*, and *RAB18*) that were presumed to be involved in osmotic stress tolerance (Knight H. et al., 1997), but the direct relevance of those genes to osmotic adjustment was not assessed. In contract, we have previously utilized genetic mutants, inhibitors, supplemental Ca^{2+}, and hyperosmotic stress to demonstrate that externally derived Ca^{2+} is responsible for ion homeostasis but not osmotic stress tolerance in yeast (Matsumoto et al, 2002).

The purpose of this study is thus two-fold: first, to clearly delineate the origins of the Ca^{2+} entering the cytosol during hyperosmotic treatment in *Nicotiana tabacum* suspension cultures, and in the process to identify signaling modulators of the hyperosmotically activated Ca^{2+} pulse. Secondly, to

use the same signaling modifiers to determine whether the Ca^{2+} entering from a particular cellular location specifies ionic or osmotic stress tolerance acquisition in tobacco cells. We find that while the hyperosmotically induced Ca^{2+} transient in tobacco suspension cells is derived from both Gd^{3+}-sensitive externally derived Ca^{2+} influx and from U73122- and neomycin-sensitive Ca^{2+} release from an organelle, neither Ca^{2+} influx nor organelle-derived Ca^{2+} contributes to osmotic stress tolerance. Rather, similar to yeast, only the influx of Ca^{2+} leads to ionic tolerance. To the best of our knowledge, this is the first study to correlate a salt tolerance phenotype with modulation of the Ca^{2+} transient in a plant system.

Materials and methods

Plant material and cell culture

Suspension cultures of aequorin-transformed tobacco (*N. tabacum* L. cv. Wisconsin-38) were established and maintained as previously reported (Cessna and Low, 2001) by continuous shaking in Murashige and Skoog (MS) basal growth medium (Sigma-Aldrich, St. Louis, MO), with the addition of 3% w/v sucrose, 10 nM kinetin, and 13.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The osmolarity of the medium was measured at 189 ± 12 mOsM with a vapor pressure osmometer. The total Ca^{2+} in the medium was 2.99 mM, and the estimated free $[Ca^{2+}]$ was 2.7 mM.

For cell growth assays, cell cultures were treated isotonically with the indicated signaling modulator (each obtained from Sigma-Aldrich, St. Louis, MO, USA) followed by hypertonic treatment with either NaCl or sorbitol solutions made in MS, such that the increase in osmolarity was 100 mOsM above normal. After 10 min, these cultures were diluted 1:20 into fresh hypertonic (300 mOsM) culture medium. Cultures were then grown for one week, after which time they were vacuum filtered and dried in an oven (40 °C) overnight, and weighed on an analytical balance. Because of the long duration of exposure to modulators, each of which may be toxic to cell growth, tests for the affects of each modulator were done in combination with a matched control, in which cells were treated first with osmolyte, then diluted 1:20 into fresh hypertonic media containing the modulator at 1/20 the concentration to which the test cells were first exposed. Thus, after the first 10 min of the experiment, test and control cultures contained identical concentrations of modulators and osmolytes, the only difference being that test cells were exposed to modulators in high concentrations prior to osmotic stress, while control cultures were exposed to modulators in low concentrations only after osmolyte addition. When the dilution of the signaling modulator-containing media was performed in the luminometer, no change in $[Ca^{2+}]_{cyt}$ was observed (data not shown), demonstrating that release of the pharmacological blockade was not sufficient 10 min after application to still activate Ca^{2+} influx, and any effect of the modulator on growth was limited to its effect on the first 10 min of signaling, i.e. during the Ca^{2+} pulses shown in Fig. 1A.

To further rule out any toxicity effects of any of the modulators, after 1 d of treatment 1 mL aliquots of cells from each treatment group were withdrawn in the sterile hood and assessed with visible microscopy. No visible difference in the number of plasmolyzed cells or the amount of accumulated cellular debris could be seen in any of the treatment groups (data not shown), indicating that our protocol for modulator treatment was mild enough to only report their effects on the Ca^{2+} pulses, and did not cause significant cell death or plasmolysis.

Luminometry and Ca^{2+} quantization

Luminometry was performed as previously described, with some minor changes (Cessna and Low, 2001). Briefly, cultures were incubated in 3 μM coelenterazine

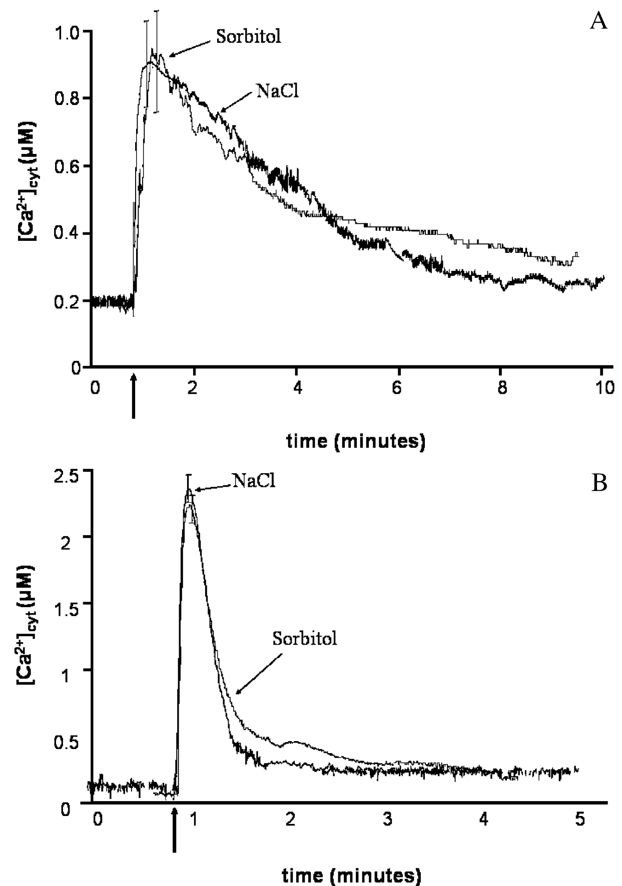

Figure 1. Hyperosmotic shock activates Ca^{2+}_{cyt} pulses in *N. tabacum* cells. Aequorin-transformed coelenterazine-reconstituted *N. tabacum* cells were treated with either (A) 50 mM NaCl or 100 mM sorbitol, or (B) with 250 mM NaCl or 500 mM sorbitol. Data shown are averages of four independent experiments. Error bars are standard deviations of the $[Ca^{2+}]_{cyt}$ at the peaks.

(Nanolight Technologies, Pinetop, AZ) for 6–14 h immediately after subculture into fresh medium. 0.25 mL of cell culture was then transferred to a luminometer cuvette and placed in a Turner Designs TD 20/20 luminometer chamber. Luminescence was then integrated five times per second. Salts, inhibitors, and all other treatments were added as indicated from stock solutions made to match the osmolarity of the cell culture medium. No measurable change in medium pH or osmolarity occurred after modulator, chelator, or $CaCl_2$ additions to cell cultures (data not shown). To determine the amount of un-discharged aequorin remaining at the end of each experiment, cell membranes were solublized by injecting 0.25 mL of a solution containing 5% Nonidet P40 and 50 mM $CaCl_2$ into the luminometer chamber, and residual luminescence was recorded. Subsequent additions of 0.25 mL aqueous detergent or ethanol solutions containing 200 mM $CaCl_2$ were also made to ensure complete discharge of all remaining functional aequorin. Luminescence traces were then transformed into micromolar $[Ca^{2+}]_{cyt}$ using the equation described by Allen et al. (1977).

To assure that no luminescence arose from the cells in a manner independent of Ca binding to reconstituted aequorin (Plieth, 2005), non-transformed cells were first treated with the same concentration of coelenterazine as the aequorin-transformed cells for the same duration, and then treated with salt or sorbitol. No increases in luminescence could be detected in either case (data not shown).

^{45}Ca uptake measurements

Three milliliter of suspension-cultured tobacco cells transferred at twice their usual density were used for determination of ^{45}Ca influx, as previously described (Cessna et al., 1998). Cells were first treated with gadolinium chloride, or left untreated (control). After 1 min, 0.1 μCi of aqueous $^{45}CaCl_2$ was added to the cell culture medium immediately prior to hypersaline or iso-osmotic treatment. Cells were incubated with gentle shaking for 7 min, suction filtered through Whatman filter paper at 20 mm of Hg, and immediately rinsed with 3 mL of normo-osmotic buffer containing 10 mM $CaCl_2$, 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), and 160 mM sucrose (pH 5.6) to displace any externally bound ^{45}Ca. Cells were resuspended in 3 mL of buffer and shaken for 20 min followed by suction filtering and a final rinse with 3 mL of buffer. Cells were scraped into pre-weighed scintillation vials containing 3 mL of ICN Ecolite scintillation liquid. Radioactivity was assessed by counting the radioactivity in the vials using a Packard 1600CA liquid scintillation analyzer (Meriden, CT) and weighing the vials. Data are presented as fold increase in counts/mg in NaCl-treated cells relative to that measured in isotonically treated controls.

Results

Hyperosmolarity stimulates Ca^{2+}_{cyt} pulses in suspension-cultured tobacco cells

Cytosolic Ca^{2+} content was measured in suspension-cultured aequorin-transformed tobacco cells responding to the addition of NaCl or sorbitol to the cell culture media (Fig. 1). In both cases, cytosolic Ca^{2+} levels increased rapidly, and then returned to near baseline levels by 10 min (see also, Knight et al., 1997). Because treatments with NaCl and sorbitol at the same osmolarities induced Ca^{2+}_{cyt} pulses that were indistinguishable in amplitude and duration, and because NaCl- and sorbitol-induced Ca^{2+} transients displayed similar sensitivities to numerous modulators (Table 1), it appears that the Ca^{2+} signals are osmotically activated rather than specific to NaCl in tobacco (see also Hasegawa et al., 2000).

Osmotically stimulated cytosolic Ca^{2+} pulses involve both the influx of extracellular Ca^{2+} and phospholipase C (PLC) activation

To characterize the cellular origins of the osmotically activated Ca^{2+} pulses, and to identify

Table 1. Correlation between the effects of various modulators on the NaCl- and the sorbitol-stimulated Ca^{2+}_{cyt} pulses

Modulator	Conc (mM)	Putative activity	% inhibition of Ca^{2+}_{cyt} pulse[a]	
			NaCl-induced pulse	Sorbitol-induced pulse
EGTA	3	Ca^{2+} chelator	80.2 (10.3)	83.5 (11)
$GdCl_3$	1	Ca^{2+} channel agonist	76.7 (7.2)	81.4 (6.9)
U73122	0.1	PLC agonist	26.6 (2.9)	28.1 (4.8)
Neomycin	0.1	PLC agonist	30.1 (5.5)	ND

ND = no data.
[a]NaCl- or sorbitol-activated Ca^{2+}_{cyt} transients were monitored as in Fig. 1. Modulators (or control solvents, water or DMSO) were added to the cell culture medium 2 min prior to treatment with 125 mM NaCl or 250 mM sorbitol in the luminometer cuvette. The Ca^{2+}_{cyt} pulses generated in the presence of each modulator were compared to those generated in control cells. The difference in amplitude of the resulting Ca^{2+}_{cyt} pulses above background was used to calculate percent inhibition. Each experiment was performed at least three times; standard deviations are reported in brackets.

signaling modulators to test the role of externally versus internally derived Ca^{2+} in signaling to NaCl tolerance, we first examined the affect of modulating medium $[Ca^{2+}]$, and then applying NaCl and measuring the resulting changes in cytosolic Ca^{2+}. When external $[Ca^{2+}]$ was reduced to ~100 μM by the addition of 3 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N′N′*-tetraacetic acid (EGTA) to cell culture medium, the amount of Ca^{2+} entering the cytosol after salt stress was reduced dramatically (Fig. 2A). In contrast, tripling the $[Ca^{2+}]$ in the culture medium by the addition of $CaCl_2$ immediately prior to osmotic stress led to a greater amount of Ca^{2+} entering the cytoplasm (Fig. 2A); presumably, a steeper $[Ca^{2+}]$ gradient across the plasma membrane facilitates greater Ca^{2+} uptake when channels open. Furthermore, both NaCl-activated and sorbitol-activated Ca^{2+}_{cyt} pulses were found to be highly sensitive to the calcium channel blocker gadolium chloride (Gd^{3+}) (Fig. 2B and Table 1).

To independently confirm that Gd^{3+}-sensitive externally derived influx of Ca^{2+} is initiated after hyperosmotic stress, ^{45}Ca influx experiments were also performed. As shown in Fig. 2C, exposure to NaCl initiates a more than a 2.5-fold increase in ^{45}Ca label relative to iso-osmotic conditions, and Gd^{3+} inhibits this increase by nearly 70%. Similar results were found when the stress was imposed with sorbitol instead of NaCl (data not shown).

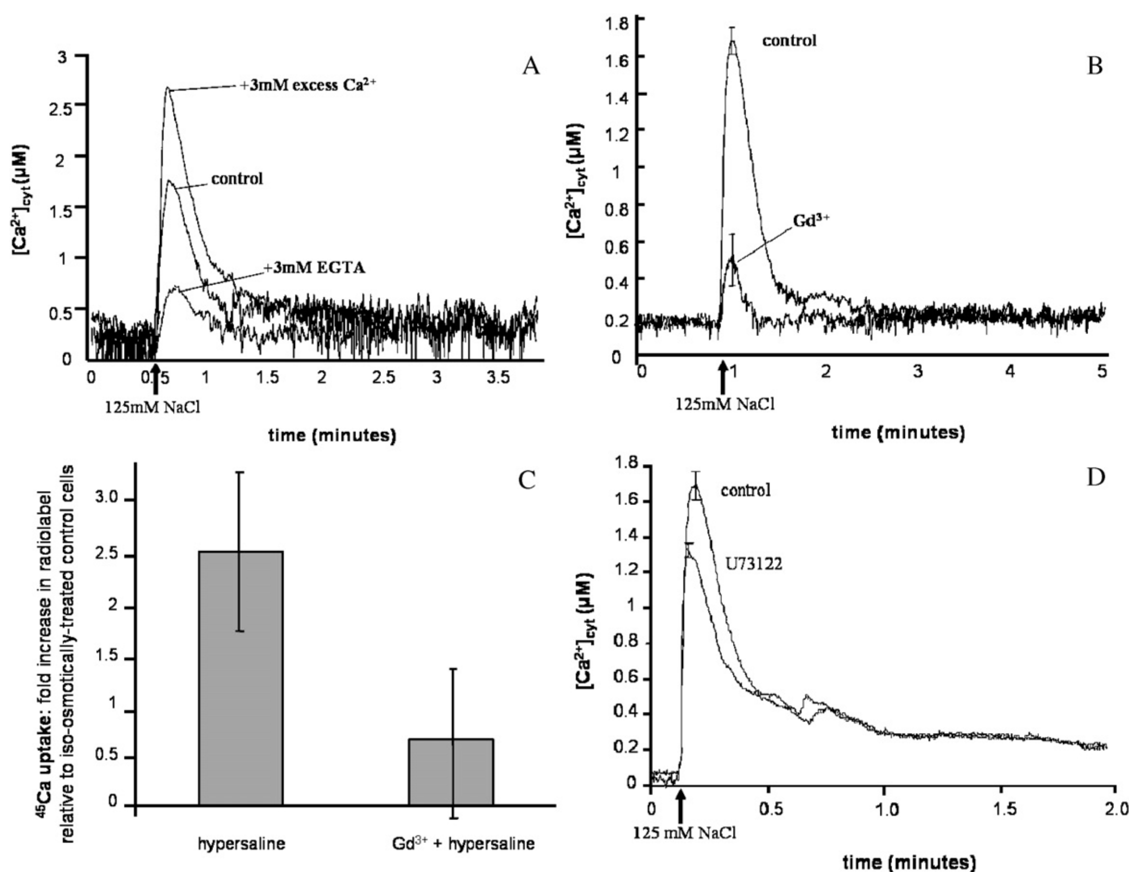

Figure 2. The Ca^{2+}_{cyt} is generated from both externally derived influx and PLC-dependent release from an organelle. (A) Cells were treated with 125 mM NaCl immediately after either enhancing $[Ca^{2+}]_{ext}$ by addition of 3 mM $CaCl_2$, or by reducing $[Ca^{2+}]_{ext}$ by addition of 3 mM of the Ca^{2+} chelator EGTA, and $[Ca^{2+}]_{cyt}$ was recorded (as in Fig. 1A). The trace labeled 'control' was exposed to 125 mM NaCl without prior alteration in the medium $[Ca^{2+}]$. Data shown are representative of three independent experiments in which similar results were obtained. (B) Cells were treated with 1 mM $GdCl_3$, or with solvent alone (control) 5 min prior to recording $[Ca^{2+}]_{cyt}$ in the luminometer. NaCl was then added at the indicated time. Data shown are the average responses of four independent experiments; error bars are standard deviations. (C) Cells were exposed to ^{45}Ca-containing medium followed immediately by either iso-osmotic treatment (180 mM sucrose; control), treatment with 125 mM NaCl (hypersaline), or 1 mM Gd^{3+} followed by 125 mM NaCl (Gd^{3+}+hypersaline). Data are presented as the mean (±SD) fold increase in radiolabel over and above that measured in control cells ($n = 3$). (D) Cells were treated with 125 mM NaCl at the times indicted by the arrows after five minutes of treatment with 0.1% DMSO (control) or 100 μM U73122. The data shown are the average responses recorded in four different experiments; error bars are standard deviations.

Taken together, these data indicate that hyperosmolarity induces an influx of Ca^{2+} from across the plasma membrane by way of Gd^{3+}-sensitive channels.

To determine whether phospholipase C (PLC) activation and the release of Ca^{2+} from an organelle also occurs during hyperosmotic stress, signaling modulators known to inhibit Ca-signaling upstream of IP_3 formation (U73122 and neomycin; Takahashi et al., 2001) were tested for their ability to block the salt or sorbitol-activated Ca^{2+}_{cyt} pulses. U73122 significantly reduced the amount of Ca^{2+} entering the cytosol after salt or sorbitol treatments (Fig. 2D and Table 1), implying that phosphoinositide turnover and IP_3-gated Ca^{2+} channels are activated by hyperosmolarity (see also DeWald et al., 2001; Takahashi et al., 2001). Prior treatment with neomycin, a chemically distinct PLC inhibitor, also inhibited inhibition the Ca^{2+} pulse to a similar level (Table 1). Taken together, we conclude that PLC activation is required for full expression of the hyperosmotically induced Ca^{2+} pulse, most likely by activating IP_3-mediated organellar release.

Externally derived Ca^{2+} influx, but not PLC-mediated Ca^{2+} release, is a signaling requirement for maintaining growth during ionic stress

In order to clarify the role of Ca^{2+} in the alleviation of NaCl and/or sorbitol stress, cell culture growth experiments were performed. Sorbitol-treated cell cultures (with no Ca^{2+} modulation) grew to 43±5% greater mass in the same time span as NaCl-treated cells at the same osmolarity (Fig. 3A), indicating that the combination of osmotic and ionic stresses imposed by NaCl is in fact more problematic to cellular function and growth than osmotic stress alone (see also Hasegawa et al., 2000).

In order to assess the roles of externally and internally derived Ca^{2+} in NaCl and sorbitol tolerances, further experimentation was then performed in which tobacco cells were stressed with either salt or sorbitol before or after the addition of

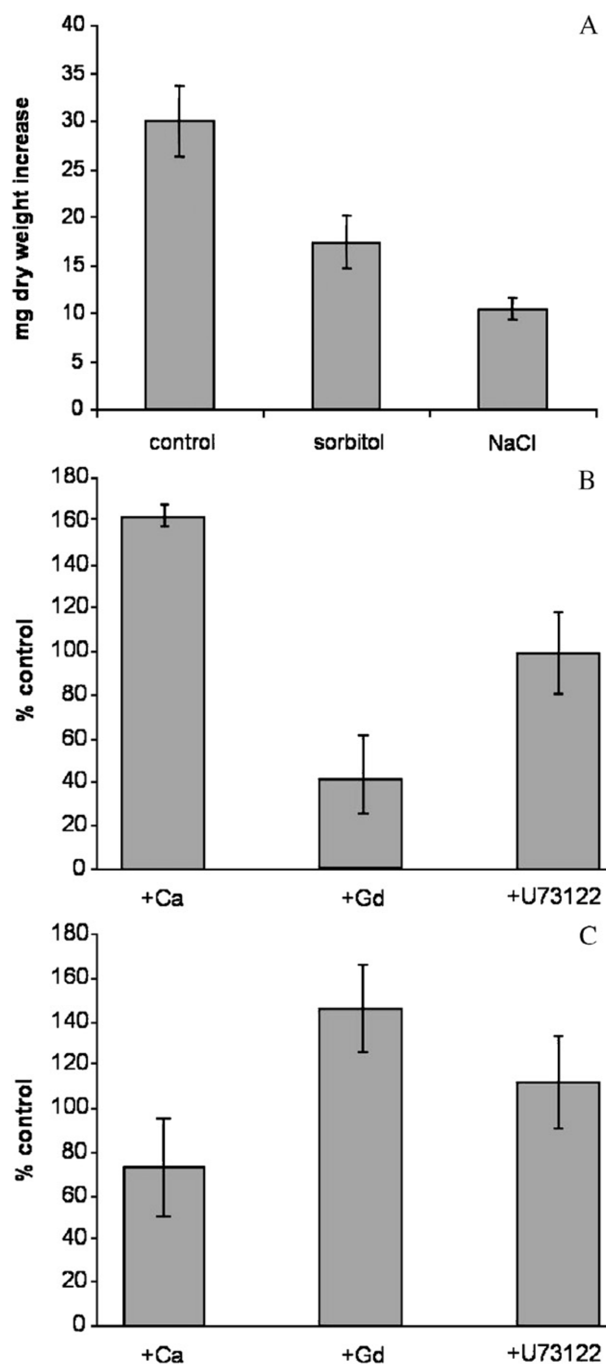

Figure 3. Growth in NaCl, but not sorbitol, depends on Ca^{2+} influx. (A) 25 mL of cell culture was either untreated (control) or treated with 100 mM sorbitol or 50 mM NaCl. After 7 d, cells were filtered, dried in an oven overnight at 40 °C, and weighed. Data are average increases in mass over untreated cells on day 0±standard deviations ($n = 4$). (B) 25 mL suspension cultures (at day 13 of their 14-d culture cycle) were treated with either 5 mM excess $CaCl_2$, 0.2 mM $GdCl_3$, 100 μM U73122 or no treatment (controls) followed immediately by the addition of 50 mM NaCl (total [NaCl] increase). After 10 min, the suspensions were diluted 1:20 with fresh cell culture medium enhanced with 50 mM NaCl. Untreated control cultures were diluted 1:20 into fresh NaCl-enhanced medium, which was supplemented with additions of $CaCl_2$, $GdCl_3$, or U73122 to match the conditions of the treated flasks (see materials and methods). After 7 d, each flask was filtered, dried in an oven, and weighed. Data are presented as average % difference in growth (g dry-weight/week) of treated/untreated matched control±SD ($n = 3$). (C) As in (B), except osmotic stress was applied as 100 mM sorbitol.

Gd^{3+}, U73122, neomycin sulfate, or excess Ca^{2+}, and then growth was measured. To reduce the affects of modulator treatments on normal cell division and growth, cells were diluted 1:20 into fresh hyperosmotic media after 10 min of treatment to assure that the concentrations of the modulators were low enough for growth to continue normally. Growth of these cells was compared to growth of cells in matched control conditions. In this manner, only the first 10 min of the hyperosmotic response (i.e. the duration of the Ca^{2+} pulses) was assessed for its role in osmotic and ionic stress tolerance (see "Materials and methods" section). As can be seen in Fig. 3B, prevention of Ca^{2+} influx with Gd^{3+} during the initial phase of salt treatment had an inhibitory effect on cell growth in salt, but increasing the amount of Ca^{2+} entering from outside the cell during the first few minutes of salt exposure by enhancing the culture media's Ca^{2+} content lead to enhanced growth in salt. Thus, it appears that externally derived Ca^{2+} influx is a signaling intermediate in the development of NaCl tolerance. In contrast, U73122 pretreatment at concentrations shown to be inhibitory to the Ca^{2+} pulse (Fig. 2D) had no appreciable effect on the cells' ability to grow in hypersaline media (Fig. 3B), indicating that the U73122-sensitive portion of the salt-activated Ca^{2+} signal is not a signaling requirement for gaining NaCl tolerance. Nearly identical results were found when cells were first treated with neomycin (data not shown), further confirming that the organelle-derived release of Ca^{2+} to the cytosol is less critical to NaCl tolerance than Gd^{3+}-sensitive influx.

Because NaCl treatment leads to both osmotic and ionic disequilibria, and because the Ca^{2+}-dependent osmotic signaling pathway in yeast mediates only responses to the ionic stress (Matsumoto et al., 2003), cell growth in sorbitol-enhanced media (which imposes only an osmotic disequilibrium) was also measured in the presence and absence of Ca^{2+} modulators. In contrast to salt-stressed cells, the growth of sorbitol-stressed cells was not enhanced by the addition of excess Ca^{2+} to the culture medium, nor was it reduced by blockade of Ca^{2+} influx with Gd^{3+} (Fig. 3C). In fact, there was a slight reversal of both trends. However, similar to cells responding to NaCl, U73122 did not alter the growth of cells in sorbitol (Fig. 3C), nor did neomycin sulfate treatment have any measurable affect on growth in sorbitol (data not shown). Together, these data indicate that Ca^{2+} influx is a signaling intermediate in the acquisition of ionic stress tolerance, but is not involved in osmotic adjustment

Conclusions

A signaling role for Ca^{2+} ions in the regulation of salinity tolerance in plants has long been evident: the addition of Ca^{2+} to soils or culture media has been shown to increase the ability of plants or plant cultures to grow in salt (Hasegawa et al., 2000), Ca^{2+}-regulated signaling enzymes have been identified that are required for survival in salt (Sheen, 1996; Chinnusamy et al., 2004; Boudsocq and Lauriere, 2005), and salt-induced cytosolic Ca^{2+} pulses have been measured in maize root cells (Lynch et al., 1989), and in *Arabidopsis* seedlings (Knight et al., 1997) and root tips (DeWald et al., 2001). Knight et al. (1997) also found that both externally derived influx and organelle-derived (vacuolar), PLC-mediated Ca^{2+} release are activated by hyperosmotic stress. Our findings in this study are three-fold: (1) we confirm the above-mentioned findings, and demonstrate their relevance in a different plant system, suspension-cultured tobacco cells; (2) more remarkably, we find that ionic stress tolerance, but not osmotic stress tolerance, is mediated by Ca^{2+} signaling; and (3) we report that while PLC-mediated Ca^{2+} signaling is activated by NaCl stress in tobacco, its role in signaling to NaCl tolerance is insignificant relative to the role of externally derived Ca^{2+} influx.

There are at least three possible explanations for this last finding. First, it may be that inhibiting PLC with U73122 or neomycin simply does not reduce the NaCl-induced Ca^{2+}_{cyt} pulse below a threshold level required for signaling to NaCl tolerance. However, this argument seems to make PLC activation superfluous in the signaling cascade. A second explanation could be that PLC serves an alternate signaling role outside of its effects on Ca^{2+} channels. In support of this second explanation, DeWald et al. (2001) demonstrated that the elevation of IP_3 occurring in *Arabidopsis* after osmotic treatment lasts for an extended period (>1 h), much longer than the Ca^{2+}_{cyt} pulse, and phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$) levels are elevated to a far greater extent than those of IP_3 (DeWald et al., 2001). Thus, they proposed that PLC activation in response to hyperosmolarity might be related to the formation of IP_5 or IP_6 and related signaling in addition to the activation of a Ca^{2+} signal (Odom et al., 2000; DeWald et al., 2001). Because our salinity tolerance cell growth assay was performed by the application of PLC inhibitors for only 10 min, followed by relief of the blockade by dilution, it is likely that we inhibited only the IP_3-mediated Ca^{2+} transient, and not any other aspect of

phosphoinositide signaling that might occur on a slower time-scale.

Our findings are very similar to what we previously found in yeast (Matsumoto et al., 2002), and closely align with molecular genetic reports of signaling requirements that lie downstream of Ca^{2+} influx in ionic versus osmotic regulation (Hasegawa et al., 2000; Zhu, 2003). In both yeast and plants, there is evidence that two osmosensors are activated during hyperosmotic stress, one osmosensor triggers trans-plasma-membrane Ca^{2+} influx leading to ionic stress tolerance, most likely by way of calcineurin- or related Ca-binding protein-dependent activation of membrane-embedded ion transporters (i.e. SOS pathway in *Arabidopsis*) (Hasegawa et al., 2000; Matsumoto et al., 2002; Zhu, 2003; Cheng et al., 2004; Chinnusamy et al., 2004), and another osmosensor triggers parallel, but Ca^{2+}-independent, protein kinase-mediated signaling pathways leading to osmotic stress tolerance (Hasegawa et al., 2000; Kiegerl et al., 2000; Zhang and Klessig, 2001; Hohmann, 2002). While it might seem odd that plants and yeast measure osmolarity and then respond by regulating ionic stress tolerance. It should be noted that plant roots in nature seldom if ever are exposed to osmotic stress in the absence of ionic stress; reduction in water potential is inevitably coincident with increases in ion concentrations (Hasegawa et al., 2000; Pasternak, 1987). Thus, evolutionarily, plants would have no ability and no need to distinguish osmotic and ionic stresses, and could thus activate tolerance to one in response to the other (see also Boudsocq and Lauriere, 2005).

Finally, we recognize that this study is confirmatory of a significant published report, in which *Arabidopsis* seedlings were found to respond to hyperosmolarity with a Gd^{3+}- and U73122-sensitive Ca^{2+} pulse (Knight et al., 1997). However, our findings are at odds with one important conclusion made in that paper, in which the hyperosmotically triggered Ca^{2+}_{cyt} pulse was demonstrated to impact the transcription of genes that were thought to be involved in osmotic stress tolerance (*P5CS*, *LTI78*, and *RAB18*; Knight et al., 1997). Based on those data, and opposite to our findings, they concluded that Ca^{2+} influx signals to osmoregulation, but not to ionic stress tolerance. It remains to be seen whether the genes they monitored have any impact on osmoregulation, whether similar, or perhaps even more significant results, would have been found if they had measured the activation of genes involved in ionic stress tolerance (e.g. *SOS1*, *NHX*, or *HKT*), or whether tobacco and *Arabidopsis* fundamentally differ at the cellular level. In any case, we are confident that with further experimentation and analysis their findings could be reconciled to ours and to the large body of recent literature supporting our conclusions that has been published since the publication of their seminal study (e.g. Hasegawa et al., 2000; Ishitani et al., 2000; Matsumoto et al., 2002; Cheong et al., 2003; Cheng et al., 2004; Zhu, 2003; Chinnusamy et al., 2004; Boudsocq and Lauriere, 2005).

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