

Phospholipase D Is a Negative Regulator of Proline Biosynthesis in *Arabidopsis thaliana**

Received for publication, August 1, 2003, and in revised form, January 22, 2004
Published, JBC Papers in Press, January 23, 2004, DOI 10.1074/jbc.M308456200

Laurent Thiery‡, Anne-Sophie Leprince‡, Delphine Lefebvre‡, Mohamed Ali Ghars§¶, Elise Debarbieux‡, and Arnould Savouré‡¶

From the ‡Unité de "Physiologie Cellulaire et Moléculaire des Plantes," UMR 7632 CNRS, Université Pierre & Marie Curie, Case 156, 4 place Jussieu, 75252 Paris cedex 05, France and §Laboratoire "Adaptation des Plantes aux Stress Abiotiques," Institut National de la Recherche Scientifique, Hamman lif 2050, Tunisia

Accumulation of proline has been observed in a large number of plant species in response to drought and salt stresses, suggesting a key role of this amino acid in plant stress adaptation. Upstream components of the proline biosynthesis signal transduction pathways are still poorly defined. We provide experimental evidence that phospholipase D (PLD) is involved in the regulation of proline metabolism in *Arabidopsis thaliana*. The application of primary butyl alcohols, which divert part of PLD-derived phosphatidic acid by transphosphatidylation, stimulated proline biosynthesis even without hyperosmotic constraints. Moreover, application of primary butyl alcohols enhanced the proline responsiveness of seedlings to mild hyperosmotic stress. These data indicate that some PLDs are negative regulators of proline biosynthesis and that plants present a higher proline responsiveness to hyperosmotic stress when this regulator is abolished. We clearly demonstrate that PLD signaling for proline biosynthesis is similar to *RD29A* gene expression and different from the abscisic acid-dependent *RAB18* gene expression. Our data reveal that PLDs play positive and negative roles in hyperosmotic stress signal transduction in plants, contributing to a precise regulation of ion homeostasis and plant salt tolerance.

Accumulating evidence suggests a major role for phospholipids to serve as precursors for the generation of secondary messengers in animal and plant cell transduction pathways. Recent reports have indicated that phospholipase D (PLD)¹ is also involved in water stress signaling through the hydrolysis of phospholipids to generate phosphatidic acid (PA) and free head groups. Water deficit triggers PLD activity and promotes stomatal closing and more recently it was shown that PLD activation mediates ABA signal transduction cascades (1–4). Twelve PLD genes were identified in the *Arabidopsis* genome and tentatively grouped into five classes based on sequence similarity and biochemical properties (5). Biochemical requirements of these different plant PLDs, particularly for Ca²⁺ concentration, pH, and substrate lipids, clearly showed distinct

features, suggesting different metabolic and physiological functions in plants.

Drought and high salinity are common environmental stresses that affect plant growth and crop productivity (6). They both produce osmotic stress by decreasing the chemical activity of water and leading to a loss of cell turgor. The presence of salt has an additional effect causing ion toxicity because high intracellular concentration of sodium is deleterious to cellular metabolism (7). Plants are sessile organisms, and their survival in a changing environment requires rapid responses to adverse conditions. Water deficit begins with stress perception, which initiates signal transduction pathway(s) to trigger a complex set of adaptive responses.

Both drought and salt stresses stimulate the accumulation of compatible solutes that include ions such as K⁺ or organic compounds such as sucrose, betaines, and proline in the cytosol as a protective mechanism. Accumulation of proline is a widespread plant response to environmental stresses, and proline is thought to play a role in the adaptive response (8). The significance of proline accumulation in osmotic adjustment and tolerance is still a matter of debate, and recently, the proline metabolism intermediate pyrroline-5-carboxylate (P5C) was suggested to be highly toxic to the cell by either directly or indirectly triggering apoptosis (9). Proline has also been proposed to serve as a scavenger of radicals, a transient storage of nitrogen, and a source of redox equivalent (10).

Higher plants synthesize proline via two different pathways. The first pathway is from glutamate that is converted to P5C by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) catalyzing the rate-limiting step in proline biosynthesis upon osmotic stress conditions (11, 12). P5C is then reduced to proline by Δ^1 -pyrroline-5-carboxylate reductase. The second pathway is from ornithine that is transformed to P5C through δ -transamination by ornithine- δ -aminotransferase (OAT), and P5C is subsequently reduced to proline. Upon relief from hyperosmotic stress, proline is rapidly degraded by the sequential action of the mitochondrial enzymes proline dehydrogenase (ProDH) and P5C dehydrogenase. Genes encoding enzymes catalyzing proline biosynthesis and degradation have been cloned from various plant species. Strong positive correlation between *P5CS* transcript and proline levels have been abundantly reported (10) with a reciprocal transcriptional regulation between *P5CS* and *ProDH* genes upon and after hyperosmotic stress (13–15).

Among factors involved in the regulation of proline metabolism, ABA and calcium were previously shown to play an important role (10). ABA-dependent and independent signaling cascades have been identified between the initial water stress signal and the expression of drought-responsive genes like genes involved in proline biosynthesis, *RAB18* and *RD29A*

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by the Tunisian Ministry of Education, Scientific Research and Technology.

¶ To whom correspondence should be addressed. Fax: 33-1-44-27-26-72; E-mail: savoure@ccr.jussieu.fr.

¹ The abbreviations used are: PLD, phospholipase D; ABA, abscisic acid; PA, phosphatidic acid; PBut, phosphatidylbutanol; P5C, Δ^1 -pyrroline-5-carboxylate; P5CS, Δ^1 -pyrroline-5-carboxylate synthetase; ProDH, proline dehydrogenase; OAT, ornithine- δ -aminotransferase.

(16–18). The two pathways may interact and converge because genes like *RD29A* possess an ABRE-responsive complex mediating ABA regulation and a dehydration-responsive element that can be activated by osmotic stress but not by ABA (19).

Calcium also plays a role as a signaling molecule in a number of responses to drought and salinity. Water and salt stresses have been shown to induce transient free Ca^{2+} cytosolic waves derived from either influx from the apoplastic space or release from internal stores. Using *Arabidopsis* plants expressing cytosolic aequorin, calcium was shown to be necessary but not sufficient for drought and salt induction of *P5CS*, indicating that an additional signaling factor is involved (20).

Despite the importance of the proline accumulation in the adaptive response of plants to osmotic stress and in contrast to metabolic events involved in proline accumulation, the signaling cascades regulating proline metabolism are still poorly characterized. In this report, we describe how PLD negatively regulates the proline biosynthesis pathway in *Arabidopsis*. We also show that calcium is involved in proline biosynthesis during osmotic stress and provide molecular evidence that this signaling cascade is different from the ABA-dependent pathway.

EXPERIMENTAL PROCEDURES

Plant Material—*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia seeds were surface-sterilized and grown on $0.5\times$ Murashige-Skoog (MS) agar medium (21) in 14-cm-diameter Petri dishes as previously described (22). After an overnight period at 4 °C to raise dormancy, the seedlings were placed under continuous illumination with 45 μmol of photons $\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 22 °C for 12 days.

Hyperosmotic Stress and Inhibitory Treatments—Twelve-day-old seedlings were removed from $0.5\times$ MS agar plates and put onto a liquid $0.5\times$ MS medium supplemented with different butanol isomers, verapamil, or EGTA or with water as control for 1 h. Then an equal volume of water, NaCl (200 mM), or mannitol (400 mM) was added to the medium.

When plants were treated with 1-butanol (0.5% v/v) and calcium (10 or 50 mM) or with both 1-butanol (0.5% v/v) and potassium (10 or 50 mM), no pretreatment was made. After different incubation times, the seedlings were collected and immediately frozen in liquid nitrogen and stored at -80 °C prior to analysis.

In Vivo Phospholipase D Activity—Phospholipids were metabolically labeled by incubating 12-day-old seedlings for 24 h in [^{33}P]orthophosphate (53 MBq $\cdot\text{liter}^{-1}$). Primary butyl alcohols were then added to the medium supplemented with NaCl or mannitol to measure PLD activity by transphosphatidylation through phosphatidylbutanol (PBut) formation. The incubations were stopped by immersing seedlings in boiling water for 5 min. The lipids were then extracted and separated as described by Refs. 4 and 23, respectively. Radiolabeled PA and PBut were quantified using a PhosphorImager (Amersham Biosciences). PLD activity is presented as the formation of [^{33}P]PBut counts, which is expressed as the percentage compared with nonstressed control plants. The means were compared using analyses of variance followed by protected *t* tests with the LSMEANS instruction of the SAS GLM procedure.

Northern Blot Analysis—Total RNAs were isolated from seedlings harvested in liquid nitrogen by the guanidinium thiocyanate-CsCl purification method (24). RNA samples were denatured and separated by electrophoresis in a 1.2% agarose-formaldehyde gel. After transfer to nylon membrane, the RNAs were fixed by UV cross-linking. The membranes were hybridized at 65 °C with either specific 3'-untranslated regions of *AtP5CS1*, *AtP5CS2*, or *RD29A* or with full length of *At- δ OAT*, *ProDH*, or *RAB18* according to Ref. 25. The fragments were labeled with [^{32}P]dCTP using Ready-To-Go™ DNA labeling beads (Amersham Biosciences). Before hybridization, the membranes were stained with methylene blue as a control for RNA loading. The hybridization signals were quantified using a PhosphorImager (Amersham Biosciences).

Gel Electrophoresis, Electroblothing, and Immunological Detection—The proteins were extracted as described (26), separated by SDS-PAGE (27), and transferred electrophoretically to a nitrocellulose filter in a solution of 48 mM Tris, 39 mM glycine, 0.04% (w/v) SDS, and 20% (v/v) methanol at 50 mA for 1 h. For immunodetection, the nitrocellulose filter was incubated in TBS (TBS-T) with 5% nonfat dry milk and 0.05% (v/v) Tween 20 for 1 h at room temperature and then in TBS-T with

0.1% (v/v) rabbit antiserum for 16 h at room temperature. The antisera were obtained by immunization of rabbits with the following proteins: AtP5CS1 (amino acids 5–717), AtProDH (amino acids 1–522), and At δ OAT (amino acids 52–474). The blots were washed with TBS-T. Detection was performed with an ECL assay using horseradish peroxidase-conjugated antibodies (Amersham Biosciences). Equal protein loading and integrity of protein samples were verified by Ponceau S red staining of the blot membrane.

Proline Determination—Free proline content was measured using L-proline as standard according to Ref. 28.

RESULTS

Primary Butanol Triggers Proline Accumulation—Recently, PLDs were shown to be involved in drought signaling (29, 30), reinforcing the need to investigate their putative role in the regulation of proline metabolism. PLD readily transfers the phosphatidyl moiety of a phospholipid to a primary alcohol rather than water producing PBut at the expense of PA, and because PBut is an inactive lipid formed, butanol treatment will inhibit PA signaling from PLD. Using this tool, we investigated the implication of PLDs in water stress-induced proline accumulation in *A. thaliana*.

In response to 24-h treatments with either 200 mM NaCl or 400 mM mannitol, proline was accumulated to 2.5-fold compared with control in *A. thaliana* seedlings (Fig. 1A). Interestingly, the proline level was 2-fold increased in 1-butanol-treated plants without osmoticum (Fig. 1A). *sec*-Butanol, an activator of G protein activity (23), and *ter*-butanol, an inactive analogue of 1- and 2-butanol (23), did not have any effect on proline accumulation (Fig. 1A). To analyze the biological function of PLD in *Arabidopsis*, PLD activity was measured by transphosphatidylation reactions in the presence of primary butyl alcohols. The seedlings were incubated with [^{33}P] for 24 h to label structural phospholipids and subsequently treated with NaCl or mannitol. Lipids were then extracted and separated by TLC. Because PBut was formed after the addition of 1-butanol, the amount of PBut reflects a cumulative PLD activity under each treatment. The PA level was higher in seedlings upon hyperosmotic stresses compared with control plants, suggesting a higher PLD activity in stress conditions (Fig. 2). When 1-butanol was added, PBut was formed at the expense of PA upon normal growth as well as in hyperosmotic stress conditions. A lower PLD activity was present in seedlings grown in control medium than with NaCl and mannitol because PBut levels were increased by 2.5- and 1.5-fold, respectively, compared with control. In contrast, the increase in PBut formation was not accompanied by a significant decrease in PA formation, suggesting a low impact of the activated PLDs to this PA pool. The fact that 1-butanol stimulated proline accumulation could result from an activation of PLD by 1-butanol or through a diminution of PA signaling from PLDs. Therefore, the effect of different concentrations of 1-butanol was analyzed on proline and PBut levels (Fig. 3). Accumulation of proline increased in a dose-dependent manner in the presence of low 1-butanol concentrations with a maximum at 0.5%. Interestingly 1-butanol concentration above 0.75% diminished proline to lower levels than control. This result contrasted with PBut levels that strongly increased with 1-butanol concentrations above 0.5%, suggesting a stimulation of PLDs by primary alcohol as already reported by 23. We concluded, therefore, that stimulation of proline by 1-butanol was not due to an increase in PLD activity but rather to inhibition of a negative regulation by specific PLDs.

Molecular Analysis of Hyperosmotic Stress-responsive Genes—Next we investigated the effect of 1-butanol on three markers of proline biosynthesis, namely *AtP5CS1*, *AtP5CS2*, and *OAT*, and one marker of proline catabolism, *ProDH*. *AtP5CS1*, *OAT*, and *ProDH* transcript levels were increased by

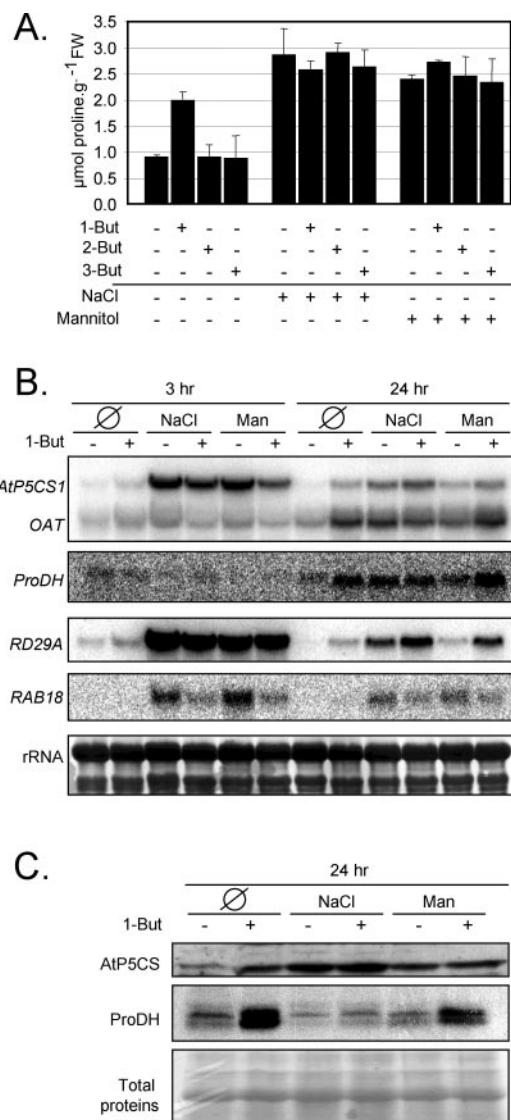


FIG. 1. Primary butanol triggers proline biosynthesis and differentially regulates the expression of two drought marker genes. *A*, 12-day-old seedlings grown on 0.5× Murashige and Skoog medium were transferred to 0.5× Murashige and Skoog liquid medium for treatment. The plants were preincubated with 0.5% (*v/v*) 1-, 2-, or 3-butanol for 1 h and then treated with 200 mM NaCl or 400 mM mannitol for 3 or 24 h. Control plants were treated with the same amount of water under the same conditions. The results are shown as the means ± S.E. of six independent experiments. *B*, Northern blot analysis of total RNA (10 µg) extracted from seedlings treated as described in *A*. The blots were hybridized with DNA fragments specific for *AtP5CS1*, *δOAT*, *ProDH*, *RD29A*, and *RAB18*. Methylene blue staining of rRNAs is shown as a loading control. *C*, P5CS and ProDH immunoblots of protein extracts from seedlings treated as described in *A*. The proteins (20 µg/lane) were separated on a 8% SDS-PAGE gel. The detection of P5CS and ProDH were by autoradiography using an ECL kit. In the bottom panel, Total proteins refers to protein staining with Ponceau Red as a control for loading and transfer of proteins onto the membrane. *FW*, fresh weight; *1-But*, 1-butanol.

hyperosmotic treatments with different kinetics (Fig. 1*B*). *AtP5CS1* transcript level was higher 3 h after treatment with either 200 mM NaCl or 400 mM mannitol than it was after 24 h. In contrast, *OAT* transcript level peaked at 24 h, whatever the treatment. Interestingly, we also observed a significant increased of *ProDH* RNA level after a 24-h NaCl treatment and to a lesser extent after a 24-h mannitol treatment. Under the same experimental conditions, the *AtP5CS2* transcript level did not show any variation, whatever the treatment (data not shown).

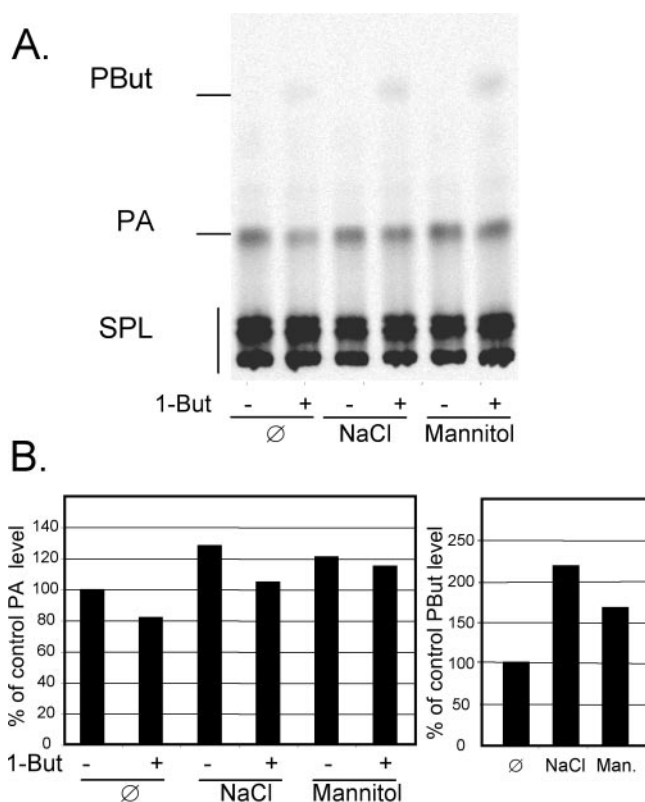


FIG. 2. In vivo PLD activity in Arabidopsis seedlings in response to various treatments. The seedlings were labeled with ³³P for 24 h and then treated with or without (control) 200 mM NaCl or 400 mM mannitol in the presence or absence of 0.5% 1-butanol. The lipids were extracted, separated by TLC, and quantified by phosphorimaging as described under "Experimental Procedures." *A*, representative autoradiography from a TLC plate after separation of phospholipids from seedlings treated with or without NaCl or mannitol. The position of PBut, PA, and structural phospholipids are indicated. *B*, quantification of PA and PBut levels by PhosphorImager. The values are the means of four independent treatments. The results for the PA and PBut are expressed as percentages with respect to nonstressed control seedlings. Based on variance analyses, NaCl and mannitol stresses had a significant effect on PA and PBut levels compared with control ($p < 0.05$), although 1-butanol did not affect PA level compared with the corresponding control, whatever the treatment ($p < 0.05$). *1-But*, 1-butanol; *SPL*, structural phospholipids.

Application of 1-butanol without osmoticum increased *AtP5CS1* transcript levels by 1.5-fold after 3 h and over 3-fold after 24 h (Fig. 1*B*). In NaCl- or mannitol-treated plants, 1-butanol increased *AtP5CS1* and *OAT* transcript levels at 24 h. A slight decrease in *P5CS1* transcript level was always observed at 3 h in the presence of 1-butanol, indicating that other PLDs may be partially involved in short time responses. In contrast to NaCl, 1-butanol alone or together with mannitol triggered *ProDH* transcript accumulation at 24 h.

We also investigated protein levels of P5CS, OAT, and ProDH using specific antibodies. P5CS accumulated to the same extent at 24 h of treatment with NaCl or mannitol. 1-Butanol alone triggered P5CS accumulation, although it had no clear effect on P5CS level in NaCl- or mannitol-treated plants. No significant accumulation of ProDH proteins was observed after a 24-h NaCl or mannitol treatment. Two proteins detected by the ProDH antibody were probably reflecting mature and immature forms of the enzyme that localized in mitochondria (14). However, accumulation of ProDH was observed at 24 h with 1-butanol alone or with mannitol. OAT proteins remained unchanged to a low level, whatever the treatment (data not shown). In contrast to ProDH and OAT, a positive correlation was observed between P5CS transcript and protein levels.

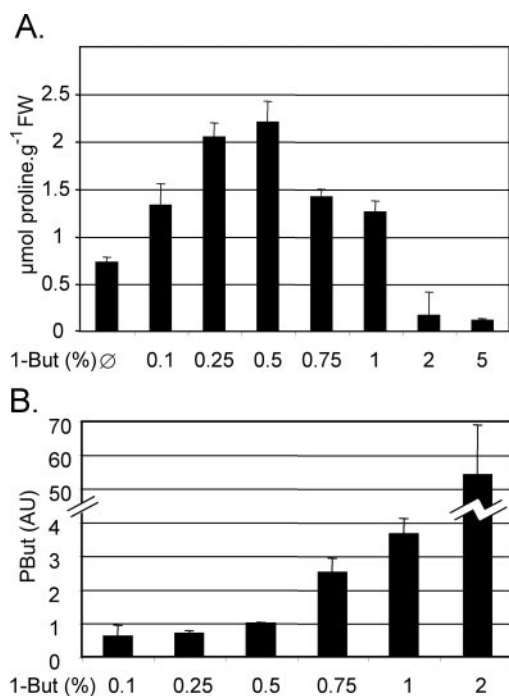


FIG. 3. Proline and PBut formation are stimulated by 1-butanol in a different dose-dependent manner. The seedlings were prepared as described in the legend to Fig. 2. The proline (A) and PBut (B) levels were measured in seedlings treated with different concentrations of 1-butanol for 24 h. The results are shown as the means \pm S.E. of three independent experiments. FW, fresh weight; 1-But, 1-butanol; AU, arbitrary units.

sec- and *ter*-butanol did not show any effect on protein and transcript accumulation, as compared with control (data not shown), suggesting the implication of a PLD in negative regulation of proline biosynthesis in normal conditions. However, the inhibition of PA biosynthesis from PLD is apparently not sufficient to activate proline biosynthesis to the level observed during water stress.

The effect of PLD on proline metabolism prompted us to investigate its role on the ABA-dependent *RAB18* and ABA-independent *RD29A*-regulated genes. The transcript level of *RAB18* was increased by NaCl and mannitol treatments at a higher level after 3 h and diminished when 1-butanol was added (Fig. 1B). Interestingly, PLD also negatively regulated *RD29A*, because its transcript level closely matched that of *P5CS1*, whatever the treatment.

Proline Biosynthesis Is Mediated by Calcium—Several reports provided evidence pointing to calcium as an important secondary messenger leading to proline accumulation (for review see Ref. 10). We tested here the effect of verapamil, a phenylalkylamine-type calcium channel inhibitor that predominantly blocks L-type calcium channels (31), and EGTA, an impermeant calcium chelator, on proline accumulation upon osmotic stress. A strong inhibition of proline accumulation was observed upon treatment of seedlings with 1 mM verapamil in the presence of 200 mM NaCl and to a lesser extent in the presence of 400 mM mannitol (Fig. 4).

In NaCl-treated plants, EGTA completely inhibited proline accumulation, whereas the proline level was only reduced by 50% after mannitol treatment. These results show that calcium is a key component for the biosynthesis of proline in plants after a NaCl treatment and to a lesser extent after a mannitol treatment.

Primary Butanol Enhanced Proline Responsiveness of Seedlings to Low Hyperosmotic Stress—Because 1-butanol stimulated proline accumulation, we tested whether calcium was another

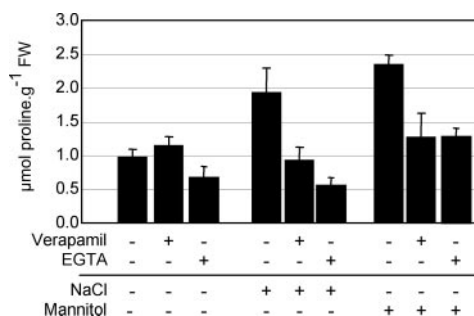


FIG. 4. Proline accumulation is dependent on extracellular calcium stores. The seedlings were prepared as described in the legend to Fig. 1. The plants were preincubated with 1 mM verapamil, 10 mM EGTA for 1 h and then treated with 200 mM NaCl or 400 mM mannitol for 24 h. The control plants were treated under the same conditions with the same amount of water. The results are shown as the means \pm S.E. of six independent experiments.

factor involved in proline biosynthesis (20). Calcium alone did not have any effect on proline level at 50 mM (Fig. 5A). Interestingly, the addition of both CaCl_2 50 mM with 1-butanol increased the proline content to $2.5 \mu\text{mol}\cdot\text{g}^{-1}$ fresh weight, which corresponds to the levels observed in NaCl- or mannitol-stressed plants.

Like CaCl_2 , 50 mM KCl alone did not have any impact on proline levels (Fig. 5A). Interestingly, the application of both 10 mM KCl and 1-butanol increased proline levels 2-fold, as was observed with 1-butanol alone. Treatment of seedlings with both 50 mM KCl and 1-butanol caused the same induction of proline biosynthesis as did NaCl or mannitol treatments ($2.5 \mu\text{mol}\cdot\text{g}^{-1}$ fresh weight). These results indicate that the observed increase of proline level is probably due to an osmotic effect and that calcium alone is not a limiting factor for the induction of proline synthesis upon the inhibition of PLD by 1-butanol.

50 mM CaCl_2 or KCl did not have any measurable effect on P5CS level (Fig. 5B). However, the addition of 1-butanol together with CaCl_2 dramatically increased the P5CS level, this effect being specific and dependent on the dose of calcium, because the application of KCl, even at 50 mM, with 1-butanol did not have any effect on P5CS. However, this increase of P5CS was not correlated with the corresponding accumulation of proline. Interestingly, the ProDH levels decreased when concentrations of CaCl_2 or KCl were raised to 50 mM, even with butanol-1.

The application of calcium stimulated the increase of *AtP5CS1* transcript levels (Fig. 5C) as was already shown in Ref. 20. *AtP5CS1* and *ProDH* accumulation is correlated with their corresponding protein levels in plants treated with both 1-butanol and calcium.

When seedlings were treated with both 1-butanol and 50 mM CaCl_2 , a strong increase of *OAT* and *RD29A* transcript levels were detected at 24 h (Fig. 5C), whereas these treatments did not have any effect on *RAB18* transcript levels. However, *OAT* levels were not affected by any treatment with butanol isomers (data not shown). Taken together, these data illustrate fine regulation of proline metabolism by independent negative and positive regulators involving calcium and PLD.

DISCUSSION

Recently, several reports suggested the implication of phospholipids in the mediation of plant responses to environmental stresses, especially drought and salinity. Several PLD genes have been isolated from various plant species, and their molecular characterization has shown their role in early signaling events. In this paper, we have established that PLDs during osmotic stress play a role as negative regulators of proline biosynthesis in *A. thaliana*. To our knowledge, this is the first

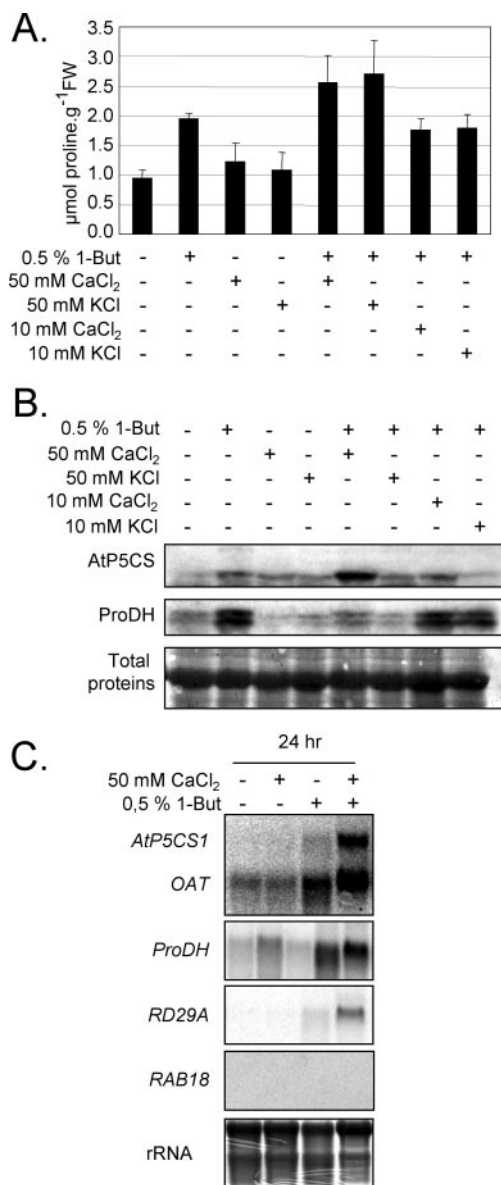


FIG. 5. Inhibition of phosphatidic acid biosynthesis from phospholipase D enhances proline synthesis in response to mild hyperosmotic stress. A, seedlings were prepared as described in the legend to Fig. 1. The plants were incubated with 1-butanol 0.5% (v/v) and 10 and 50 mM of CaCl₂ or KCl for 24 h. The results are shown as the means \pm S.E. of six independent experiments. B, P5CS and ProDH immunoblots from protein extracts of seedlings treated as described for A. The immunoblots were prepared as described for Fig. 1C. C, Northern blot analysis of total RNA (10 μ g) extracted from seedlings treated as described for A. The blots were hybridized as described for Fig. 1B.

case of the implication of PLDs as negative regulators of gene expression in plants.

In response to hyperosmotic stress triggered by NaCl, mannitol, or sucrose, an increase of PA level and its conversion to diacylglycerol pyrophosphate has been reported in plants (29). This PA may come from phospholipase C and/or PLD activities. PLC generates diacylglycerol and indirectly PA because of diacylglycerol kinase activity. However, a recent work demonstrated that PA mainly derives from AtPLD δ activity during dehydration because AtPLD δ antisense transgenic plants are affected in this PA accumulation in response to water stress (30). PLD α was also shown to play a crucial role in regulating transpiration water loss. *Arabidopsis* depleted in PLD α are impaired in stomatal closure and show reduced response to ABA (32). Hyperosmotic stress and dehydration stimulated

PLD activity in tomato, alfalfa, and resurrection plant *Craterostigma plantagineum* (29, 33). Higher PLD activity was also detected in the drought-sensitive cultivar of cow pea than in the resistant one (34). Recently, it was also established that PLDs are key signaling components of the regulation of the expression of the ABA-dependent *RAB18* gene (2, 4).

Therefore, we investigated the role of PLD in the regulation of proline metabolism in *A. thaliana*. *In vitro* studies showed the ability of plant PLDs to transfer the phosphatidyl moiety of structural phospholipids to short chain primary alcohols rather than water, producing PBut instead of PA. No other plant enzyme possesses this biochemical property. This enzymatic activity may be used to monitor PLD activity by measuring the accumulation of PBut but also to inhibit PA biosynthesis from PLD. This experimental approach has been successfully used in plants for the investigation of PLD in biotic and abiotic signaling (35).

Using 1-butanol, a stimulation of proline biosynthesis was observed in seedlings grown without osmoticum, although this alcohol did not have any effect on proline accumulation with 200 mM NaCl or 400 mM mannitol treatment. However, the proline level was lower than that observed with NaCl or mannitol (Fig. 1A). This may be explained by a stimulation of proline oxidation by ProDH, because an increase in its protein level was detected and extracellular applied proline was shown to stimulate transcript and protein levels of ProDH in the absence of hyperosmotic stress (13). Interestingly, treatments with mild hyperosmotic medium like 50 mM KCl or CaCl₂ (Fig. 5A) or even 100 mM mannitol (data not shown) were not able to trigger proline biosynthesis, although when 1-butanol was added, proline increased to levels observed with 200 mM NaCl or 400 mM mannitol.

The formation of PBut caused by the addition of 1-butanol provides a relative measure of PLD activity according to treatment conditions in contrast to PA measurement because PA may originate from PLC activity by diacylglycerol kinase or be an intermediate in the biosynthetic pathway of phospholipids. Seedlings responded to NaCl or mannitol treatment by increasing PLD activity as indicated by the significant increase of PBut formation by 2.5- and 1.5-fold, respectively. However, the PA levels were not significantly modified, suggesting that the activated PLDs contributed very little to the PA pool and/or that PA formation resulted from different pathways. These results corroborate previous observations showing that PLD becomes activated by hyperosmotic stress treatments (29, 30). Interestingly, a low PLD activity reflected by PBut levels in seedlings in control growth medium was detected. One may argue that higher proline levels observed with 1-butanol in optimal growth condition could be a consequence of a stimulation of PLD activity by alcohols. However, when different 1-butanol concentrations were used, the PBut levels did not significantly change until 0.5% in contrast to treatments with higher 1-butanol concentrations, which on the one hand strongly increased PBut levels and on the other hand diminished those of proline. These results indicate that a specific PLD activity negatively regulated proline metabolism under optimal growth conditions. In addition to PA synthesis, PLD activity hydrolyzes membrane lipids to produce polar head groups. These changes may also potentially alter cellular responses. However, head groups are present in the millimolar concentration range in plant cells, which may be incompatible with signaling. Transgenic tobacco plants that overexpress phosphoethanolamine *N*-methyltransferase leading to higher endogenous choline free levels by 5-fold appeared to have normal development (36). Actually, with the exception of alanine, only proline levels changed in the pool of amino acids in the presence of 1-butanol

in *A. thaliana* upon osmotic stress (data not shown). Because the addition of 1-butanol enhanced the sensitivity of proline accumulation in response to mild hyperosmotic stress triggered by either ionic or non ionic osmoticum, these PLDs may play a key role in the negative regulation of proline biosynthesis when the growth conditions are optimal and in the fine regulation of proline metabolism during osmotic stress.

These PLDs may be activated through G proteins as reported in *C. plantagineum* (33) because the increase of PLD activity by water deficit involved heterotrimeric G proteins (23). The G protein signaling cascades can be artificially activated by alcohols, such as secondary butanol. *sec*-Butanol did not have any effect in proline accumulation (Fig. 1) nor in proline biosynthesis gene expression, whatever the treatment (data not shown). Therefore, these data suggest that G proteins may not be implicated in the regulation of the PLDs involved in proline biosynthesis.

We also established that calcium is a key component in the water stress-induced signaling cascade preceding proline biosynthesis and that it acts downstream of PLD. Earlier findings clearly provide a valuable basis for further detailed characterization of the role of calcium in proline accumulation upon water stress (20). Moreover, some PLDs have been shown to be regulated by calcium. The increase of cytosolic free calcium is one of the earliest responses to drought and salinity, suggesting its important role in the mediation of water stress signals. Calcium oscillations may originate from intracellular stores through inositol 1,4,5-trisphosphate (1, 4, 5) via inositol 1,4,5-trisphosphate-dependent calcium channels or from extracellular stores through plasma membrane calcium channels. We showed here that inhibition of calcium signaling from the extracellular stores using a calcium chelator (EGTA) or a calcium channel inhibitor (verapamil) has a direct inhibitory effect on proline levels in NaCl- and mannitol-treated plants. The calcium involved in proline biosynthesis appears to be mainly derived from extracellular stores, because EGTA and verapamil strongly decreased proline accumulation upon hyperosmotic stress. The addition of extracellular calcium did not induce the accumulation of *AtP5CS1* transcripts (Ref. 20 and our results). We have shown that calcium added with 1-butanol triggered the accumulation of P5CS, although KCl with 1-butanol did not have any effect. These results suggest that this effect is calcium-specific. Moreover, 1-butanol-treated plants upon mild hyperosmotic stress accumulate proline to levels observed with higher osmotic stress conditions. These results strengthen the conclusion of Knight *et al.* (20) that calcium is not sufficient for *AtP5CS* expression. Actually our data suggest that PLD may be the other signaling component involved with calcium in proline biosynthesis, acting as a negative sensor, because plants present a higher sensitivity to hyperosmotic stress when this sensor is abolished (Fig. 5A).

Molecular and genetic analyses of dehydration-responsive genes have suggested that ABA plays a major role in plant response to water stress (37). Expression data suggest the existence of ABA-dependent and ABA-independent water stress signaling pathways that interact and converge (38). During these experiments, we also addressed the question of the implication of ABA in proline level regulation. Previous studies have shown that proline biosynthesis may not be regulated by endogenous ABA in hyperosmotic stress conditions (16, 39), although contradictory results have also been published (40). It has been shown that PLD is required for ABA-dependent activation of *RAB18* in barley aleurone protoplast and in *A. thaliana* cell suspension, respectively (2, 4). Moreover, the addition of PA evoked ABA responses, supporting its role as a signal (1). Therefore, we investigated the pattern of expression of two

marker genes, *RAB18* and *RD29A* activated by ABA-dependent and -independent pathways, respectively. *RAB18* activation is inhibited by 1-butanol after 3 and 24 h of stresses as already shown (2). This regulation contrasts with the one observed with *AtP5CS1* and *RD29A*, whose transcripts clearly accumulated after 24 h of stress in 1-butanol-treated plants. From this work, it is clear that the *RAB18* signaling pathway is different from that of *AtP5CS* and *RD29A*. Interestingly, *AtP5CS* and *RD29A* have very close patterns of expression. *AtP5CS1* and *RAB18* transcript levels suggest that the hyperosmotic stress regulation of these two genes may be independent from each other and that activation of *AtP5CS1* is ABA-independent. Our data clearly demonstrated that PLDs control ABA-dependent and ABA-independent signaling pathways in response to hyperosmotic stress.

Plants produce a wide range of PA from PLDs because they may be formed from structural lipids at different locations in the cell, therefore having various fatty acid composition. The PLD family appears to be more complex in plants than in animals. For example, only two mammalian PLD isoforms have been identified in human cells in contrast to the 12 *Arabidopsis* PLDs having different biochemical requirements (5, 41). Plant PLDs seem to play specific roles, and the inhibition of one PLD might not be compensated by another. A future challenge will be to identify the PLD(s) involved in this negative regulation as opposed to water stress phospholipid signaling, thus providing a very precise regulation of proline metabolism, and to find the signal that can inhibit PLD activity upon hyperosmotic stress.

Acknowledgments—We thank Emmanuel Grenier (Institut Supérieur Agricole de Beauvais) for statistical analysis and, Dr. Michael G. Palmgren, Dr. Margaret Ahmad, and Prof. François Guerinneau for critical reading of the manuscript. We are grateful to our colleagues in the laboratory for helpful discussion on lipid signaling.

REFERENCES

- Jacob, T., Ritchie, S., Assmann, S. M., and Gilroy, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12192–12197
- Ritchie, S., and Gilroy, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2697–2702
- Gampala, S. S., Hagenbeek, D., and Rock, C. D. (2001) *J. Biol. Chem.* **276**, 9855–9860
- Hallouin, M., Ghelis, T., Brault, M., Bardat, F., Cornel, D., Miginiac, E., Rona, J. P., Sotta, B., and Jeannette, E. (2002) *Plant Physiol.* **130**, 265–272
- Quin, C., and Wang X. (2002) *Plant Physiol.* **128**, 1057–1068
- Boyer, J. S. (1982) *Science* **218**, 443–448
- Serrano, R., and Gaxiola, R. (1994) *Crit. Rev. Plant Sci.* **13**, 121–138
- Delauney, J. A., and Verma, D. P. D. (1993) *Plant J.* **4**, 215–223
- Deuschle, K., Funck, D., Hellmann, H., Daschner, K., Binder, S., and Frommer, W. B. (2001) *Plant J.* **27**, 345–356
- Hare, P. D., Cress, W. A., and Van Staden, J. (1999) *J. Exp. Bot.* **50**, 413–434
- Szoke, A., Miao, G. H., Hong, Z., and Verma, D. P. (1992) *Plant Physiol.* **99**, 1642–1649
- Kavi Kishor, P. B., Hong, Z., Miao, G. H., Hu, C. A. A., and Verma, D. P. S. (1995) *Plant Physiol.* **108**, 1387–1394
- Verbruggen, N., Hua, X. J., May, M., and Van Montagu, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8787–8791
- Kiyosue, T., Yoshida, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1996) *Plant Cell* **8**, 1323–1335
- Peng, Z., Lu, Q., and Verma, D. P. (1996) *Mol. Gen. Genet.* **253**, 334–341
- Savouré, A., Hua, X. J., Bertauche, N., Van Montagu, M., and Verbruggen, N. (1997) *Mol. Gen. Genet.* **254**, 104–109
- Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993) *Mol. Gen. Genet.* **236**, 331–340
- Lang, V., and Palva, E. T. (1992) *Plant Mol. Biol.* **20**, 951–962
- Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994) *Plant Cell* **6**, 251–264
- Knight, H., Trethewey, A. J., and Knight, M. R. (1997) *Plant J.* **12**, 1067–1078
- Murashige, T., and Skoog, F. (1962) *Physiol. Plant.* **15**, 473–497
- Verbruggen, N., Villarreal, R., and Van Montagu, M. (1993) *Plant Physiol.* **103**, 771–781
- Munnik, T., Arisz, S. A., de Vrije, T., and Musgrave, A. (1995) *Plant Cell* **7**, 2197–2210
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 4.2.1–4.2.6, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Church, G., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1991–1995
- Martinez-Garcia, J. F., Monte, E., and Quail, P. H. (1999) *Plant J.* **20**, 251–257
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Bates, L. S., Waldren, R. P., and Teare, I. D. (1973) *Plant Soil* **39**, 205–207
- Munnik, T., Meijer, H. J., Ter Riet, B., Hirt, H., Frank, W., Bartels, D., and

- Musgrave, A. (2000) *Plant J.* **22**, 147–154
30. Katagiri, T., Takahashi, S., and Shinozaki, K. (2001) *Plant J.* **26**, 595–605
31. White, P. J. (1996) *J. Exp. Bot.* **47**, 713–716
32. Sang, Y., Zheng, S., Li, W., Huang, B., and Wang, X. (2001) *Plant J.* **28**, 135–144
33. Frank, W., Munnik, T., Kerkmann, K., Salamini, F., and Bartels, D. (2000) *Plant Cell* **12**, 111–124
34. El Maarouf, H., Zuily-Fodil, Y., Gareil, M., d'Arcy-Lameta, A., and Pham-Thi, A. T. (1999) *Plant Mol. Biol.* **39**, 1257–1265
35. Wang, X. (2002) *Curr. Opin. Plant Biol.* **5**, 408–414
36. McNeil, S. D., Nuccio, M., Ziemak, M. J., and Hanson A. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10001–10005
37. Xiong, L., Schumaker, K. S., and Zhu, J. K. (2002) *Plant Cell* **14**, S165–S183
38. Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J. K. (1997) *Plant Cell* **9**, 1935–1949
39. Yoshida, Y., Nanjo, T., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) *Biochem. Biophys. Res. Commun.* **261**, 766–772
40. Strizhov, N., Abraham, E., Okresz, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C., and Szabados, L. (1997) *Plant J.* **12**, 557–569
41. Exton, J. H. (2002) *FEBS Lett.* **531**, 58–61