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Phospholipases C and D Modulate Proline Accumulation in *Thellungiella halophila/salsuginea* Differently According to the Severity of Salt or Hyperosmotic Stress

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Proline accumulation is one of the most common responses of plants to environmental constraints. *Thellungiella halophila/salsuginea*, a model halophyte, accumulates high levels of proline in response to abiotic stress and in the absence of stress. Recently, lipid signaling pathways have been shown to be involved in the regulation of proline metabolism in *Arabidopsis thaliana*. Here we investigated the relationship between lipid signaling enzymes and the level of proline in *T. salsuginea*. Inhibition of phospholipase C (PLC) enzymes by the specific inhibitor U73122 demonstrated that proline accumulation is negatively controlled by PLCs in the absence of stress and under moderate salt stress (200 mM NaCl). The use of 1-butanol to divert some of the phospholipase D (PLD)-derived phosphatidic acid by transphosphatidylation revealed that PLDs exert a positive control on proline accumulation under severe stress (400 mM NaCl or 400 mM mannitol) but have no effect on its accumulation in non-stress conditions. This experimental evidence shows that positive and negative lipid regulatory components are involved in the fine regulation of proline metabolism. These signaling pathways in *T. salsuginea* are regulated in the opposite sense to those previously described in *A. thaliana*, revealing that common signaling components affect the physiology of closely related glycophyte and salt-tolerant plants differently.

Keywords: Lipid signaling • Phospholipase C • Phospholipase D • Proline • Salt and mannitol stresses • *Thellungiella salsuginea*.

Abbreviations: DAG, diacylglycerol; DMSO, dimethylsulfoxide; MS, Murashige and Skoog; NPC, non-specific phospholipase C; PA, phosphatidic acid; PBut, phosphatidylbutanol; P5C, pyrroline-5-carboxylate; P5CS, pyrroline-5-carboxylate synthetase; PLC, phospholipase C; PLD, phospholipase D; ProDH, proline dehydrogenase; TLC, thin-layer chromatography.

Introduction

Sessile plants need to adapt to withstand environmental changes and have evolved mechanisms to sense the nature and severity of stresses and to respond accordingly. Drought and salinity both contribute to water stress and are two of the environmental constraints that most dramatically affect plant growth and distribution as well as crop productivity. Both factors cause a decrease in soil water potential that disrupts uptake of water by the plant. Changes in salinity also influence plant physiology by inducing cellular ion imbalances resulting in ion toxicity and poorer nutrition. Compensatory adaptations to water stress may involve activation of ion channels, reorganization of membrane trafficking, gene expression and biosynthesis of osmoprotectants (Hasegawa et al. 2000).

The amino acid proline is an osmolyte that accumulates in a wide range of plant species in response to water stress (Delauney and Verma 1993, Szabados and Savouré 2010). However, in some species such as *Arabidopsis thaliana*, the level of free proline that accumulates is too low to achieve osmotic adjustment (Liu and Zhu 1997, Ghars et al. 2008). Therefore, the exact role of proline accumulation in stress tolerance is still puzzling (Szabados and Savouré 2010). Several studies have suggested that proline may play other non-exclusive roles possibly to limit or repair damage due to stress. Proline can serve as a hydroxyl radical scavenger, interact with enzymes preserving protein structure and activity, maintain pH and redox balance in response to environmental change and act as a reserve of carbon, nitrogen and energy for when stress is relieved (Hare et al. 1999). Proline has also been implicated as a stress-related signaling molecule in yeasts as growth is slower in mutants that overaccumulate proline (Maggio et al. 2002).

In response to water stress, plants produce proline from glutamate in two steps. First glutamate is phosphorylated and

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reduced by the bifunctional pyrroline-5-carboxylate (P5C) synthetase (P5CS) enzyme producing P5C. P5C is then reduced to proline by P5C reductase. Upon recovery from stress, proline is rapidly degraded through the sequential action of the two mitochondrial enzymes, proline dehydrogenase (ProDH) and P5C dehydrogenase (for review, see Szabados and Savouré 2010, Servet et al. 2012). Understanding the mechanisms by which the plant transduces stress signals to trigger proline accumulation, i.e. by increasing proline synthesis and limiting its degradation, would be a step towards elucidating the role of this amino acid in the acquisition of stress tolerance.

While late responses to water stress are relatively well documented, there is more and more focus on identifying components involved early in stress signaling pathways. Various signals including phytohormones, such as ABA, and intracellular messengers, such as calcium, phosphoinositides, kinases or phosphatases, have been shown to mediate and coordinate stress responses. Several phospholipid-based signaling pathways are rapidly activated in plants in response to water stress, bringing into play enzymes such as phospholipases, lipid kinases and phosphatases under spatial and temporal control. Phospholipase D (PLD) and phosphoinositide-specific phospholipase C (PLC) pathways contribute directly or indirectly to the production of phosphatidic acid (PA), now considered as an important lipid second messenger in plants, in response to water stress (Mueller-Roeber and Pical 2002, Meijer and Munnik 2003, Wang 2004, Bargmann and Munnik 2006, Testerink and Munnik 2005, Testerink and Munnik 2011). Little is known about signaling cascades involved in the regulation of proline metabolism (Szabados and Savouré 2010, Servet et al. 2012). Some key signaling components have been identified in the glycophyte Arabidopsis. ABA-independent signaling cascades are known to regulate *P5CS* gene expression (Savouré et al. 1997, Sharma and Verslues 2010). Thiery et al. (2004) demonstrated that PLDs are negative regulators of proline biosynthesis in Arabidopsis. More recently Parre et al. (2007) showed that calcium signaling via PLCs is essential for proline accumulation during ionic but not non-ionic hyperosmotic stress in *A. thaliana*.

Over recent years, the salt cress *Thellungiella salsuginea*, previously named *T. halophila*, ecotype Shandong, a close relative of *A. thaliana*, has attracted growing interest as a model for research in plant salt stress tolerance (Bressan et al. 2001, Inan et al. 2004, Amtmann et al. 2005, Amtmann 2009). Unlike *A. thaliana*, *T. salsuginea* grows and reproduces in extreme conditions of salt, drought and cold stress; because of its high tolerance of environmental constraints, this species is considered an extremophile (Inan et al. 2004, Amtmann 2009). *Thellungiella salsuginea* displays constitutively elevated transcript levels of paralogs of *A. thaliana* stress genes and metabolites, suggesting that *T. salsuginea* somehow anticipates stress (Taji et al. 2004, Gong et al. 2005, Bohnert et al. 2006).

Here we investigated the lipid signaling pathways involved in the regulation of proline biosynthesis in *T. salsuginea* by measuring changes in proline and lipid levels in response to stress induced by either salt or mannitol. We demonstrate that PLCs act as negative regulatory components in non-stress and mild stress conditions, while PLDs play a key role as positive regulatory components of proline biosynthesis in response to more severe stress. The way proline metabolism is regulated in response to water stress in *T. salsuginea* is the direct opposite of what has previously been described for its close relative *A. thaliana*.

Results

Salt and mannitol stresses trigger proline accumulation in *Thellungiella*

The accumulation of proline is a common stress response in plants. To investigate this response in stress-tolerant plants, we measured proline levels in 15-day-old *T. salsuginea* seedlings in response to 24 h of water stress, caused by either mannitol or salt, or no stress. As shown in **Fig. 1**, *T. salsuginea* contains up to 3 μmol proline g^{-1} FW in non-stress conditions. Treatments from 100 to 300 mM NaCl triggered proline accumulation in a dose-dependent manner. No further increase in proline content was observed at higher salt concentrations. The response to mannitol treatment is different; there is a slight accumulation of proline at 200 mM mannitol. At higher mannitol concentrations, proline levels reach a plateau at around 15 μmol proline g^{-1} FW, similar to the maximum measured in response to high salt concentrations. To induce maximum proline accumulation for experiments in the rest of the study, concentrations of 200 mM NaCl (moderate salt conditions), 400 mM NaCl (high salt conditions) and 400 mM mannitol (high mannitol conditions) were chosen.

PLCs control proline accumulation in *T. salsuginea*

Water stress activates PLCs, which hydrolyze phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] to generate inositol

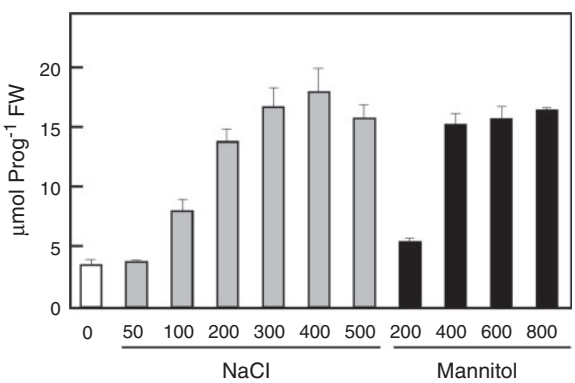

Fig. 1 NaCl and mannitol stresses induce proline accumulation in *Thellungiella salsuginea*. Fifteen-day-old seedlings grown on 0.5× MS medium were transferred to 0.5× MS liquid medium and treated with different concentrations of NaCl or mannitol for 24 h. The results shown are the means ± SD of three independent experiments.

1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG) (Hirayama et al. 1995, Drobak and Watkins 2000, DeWald et al. 2001, Takahashi et al. 2001). As a second messenger, $InsP_3$, or most probably its more highly phosphorylated derivatives, can diffuse into the cytosol activating receptors coupled to calcium channels, leading to the local release of calcium from intracellular stores (Munnik and Testerink 2009). DAG can be rapidly phosphorylated to second messenger PA by DAG kinases (DAGKs).

Here we investigated the involvement of PLCs in the regulation of proline accumulation in *T. salsuginea* seedlings. For this, the aminosteroid U73122, a specific PLC inhibitor, was applied to *T. salsuginea* seedlings in stress or non-stress condition. More proline accumulated in non-stressed plants after treatment with a range of U73122 concentrations (**Fig. 2**). For example, treatment with 100 μM U73122 resulted in a 4.8-fold increase in proline content compared with the inhibitor-free control. When seedlings treated with 400 mM NaCl for 24 h were incubated with increasing concentrations of U73122, a dose-dependent decrease in proline levels was observed, although there was only a 20% decrease at 100 μM U73122. The inactive analog U73343 did not have any effect on proline levels (**Fig. 2**). The concentration of 100 μM U73122 was used for the rest of the study.

Thellungiella salsuginea seedlings were treated with U73122 when salt or mannitol stress was applied. Proline accumulation was higher in U73122-treated seedlings grown under non-stress and moderate salt conditions than in the corresponding inhibitor-free control (**Fig. 3**). In contrast, at high salt or mannitol concentrations, 20–26% less proline accumulated in seedlings treated with U73122.

The inhibitory effect of U73122 on proline accumulation in response to high concentrations of either salt or mannitol suggests that calcium signaling mediated by PLCs may be involved. This hypothesis was investigated by treating seedlings with exogenous calcium in order to reverse the effect of the PLC inhibitor. As shown in **Fig. 3**, calcium treatment largely compensated for the U73122-induced decrease in proline accumulation due to inhibition of PLCs. Addition of 10 mM calcium increases proline levels compared with the corresponding Murashige and Skoog (MS) control medium for all conditions tested, even slightly increasing the proline content in non-stress conditions.

PLDs regulate proline accumulation upon severe NaCl or mannitol stress

PLDs, which hydrolyze glycerophospholipids to generate PA and free head groups, are involved in water stress signaling (for reviews, see Meijer and Munnik 2003, Bargmann and Munnik 2006, Hong et al. 2010). Genetic and pharmacological evidence show that PLDs are activated in response to drought and salinity stresses (Munnik et al. 2000, Katagiri et al. 2001).

Here the involvement of PLDs in water stress signaling was investigated in *T. salsuginea*. In the presence of small amounts of a primary alcohol, PLDs catalyze a transphosphatidylation reaction that produces novel phosphatidylalcohols instead of PA. For example, in the presence of 1-butanol, PLDs produce phosphatidylbutanol (PBut), an inactive compound in the plant cell. Using this unique enzyme property, PA signaling can be uncoupled from PLD activity and the cumulative PLD activity assessed by quantifying the formation of PBut. The optimal time for labeling phospholipids in *T. salsuginea* seedlings was first determined in a time-course of carrier-free

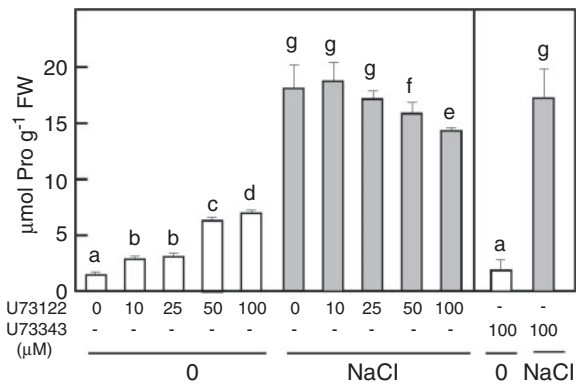

Fig. 2 Dose-dependent effects of the PLC antagonist U73122 on proline levels. Fifteen-day-old seedlings grown on 0.5× MS medium were transferred to 0.5× MS liquid medium for treatment. After 1 h pre-incubation with the indicated concentration of the aminosteroid PLC inhibitor U73122, plants were treated with or without 400 mM NaCl plus the inhibitor for 24 h as described in the Materials and Methods. Control plants were treated with the same amount of solvent DMSO or with the inactive analog U73343 at 100 μM under the same conditions. Means with the same letter do not differ significantly at $P < 0.05$ ($n = 4 \pm$ SD).

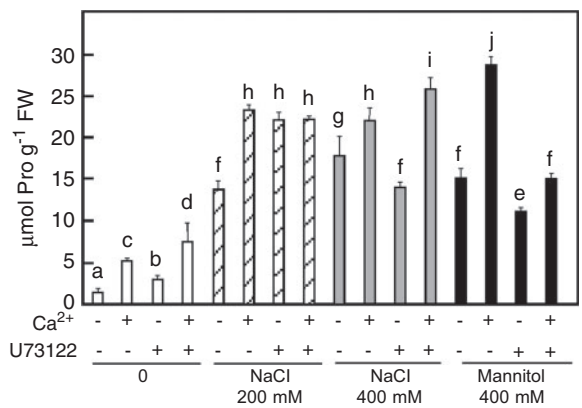

Fig. 3 Inhibition of proline accumulation by U73122 upon severe salt or mannitol stress can be reversed by extracellular calcium. Seedlings were prepared as described in the legend to **Fig. 1**. After 1 h pre-incubation with either 100 μM U73122, 10 mM $CaCl_2$, or a combination of U73122 and $CaCl_2$, seedlings were treated with or without 200 mM NaCl, 400 NaCl or 400 mM mannitol together with drug/calcium combinations for 24 h. Means with the same letter do not differ significantly at $P < 0.05$ ($n = 3 \pm$ SD).

[^{33}P]orthophosphate incorporation. As observed in **Supplementary Fig. S1**, structural lipids began to be metabolically labeled after 2 h. Steady-state conditions of incorporation were reached at 24 h, and this labeling time point was used for the experiments that follow.

To measure PLD activity uncoupled from PA signaling, *T. salsuginea* seedlings were treated with a range of 1-butanol concentrations. PBut accumulation increases with concentrations of 1-butanol, increasing up to 0.75%, indicating a diversion of PLD activity (**Fig. 4**). No correlation between 1-butanol concentration and PBut accumulation was observed with concentrations of 1-butanol >0.75%. These high 1-butanol concentrations lead to toxicity manifest as leaf necrosis (for 2 and 5% 1-butanol) (data not shown) and a reduction in proline levels (**Fig. 4**). For this reason, as in Arabidopsis (Thiery et al. 2004), we used 0.5% 1-butanol to quantify PLD activity related to PA signaling. Proline levels were not affected by 1-butanol up to a concentration of 0.5% in non-stress conditions (**Fig. 4**). Controls *sec*-butanol and *tert*-butanol, inactive isomers of 1- and 2-butanol (Munnik et al. 1995), and ethanol did not have any effect on proline levels. Therefore, the proline content of *T. salsuginea* grown in non-stress conditions does not depend on PLD activity.

Proline metabolism is regulated by lipid signaling upon salt or mannitol stress

We further investigated the role of PLDs and PLCs in proline metabolism in seedlings in response to either salt or mannitol stress when treated with either 1-butanol or U73122 alone or in combination (**Fig. 5**). Application of 1-butanol did not have any effect on proline levels in non-stress conditions, but a 42% decrease in proline was observed in response to 200 mM NaCl stress. Following 1-butanol application, proline levels decreased by 50 and 65% in response to NaCl (400 mM) and mannitol stresses, respectively. Treatments of *Thellungiella* seedlings with U73122 triggered higher proline accumulation in non-stress condition and in response to mild salt stress (**Fig. 5**), as observed in **Fig. 3**. At high salt (400 mM) or mannitol (400 mM) concentrations, less proline accumulated in seedlings treated with U73122. The application of U73122 and 1-butanol simultaneously decreased proline levels in all the stress conditions tested, but increased proline levels in non-stress conditions.

We investigated the effect of PLC and PLD inhibitors on key proline metabolism genes in *T. salsuginea*, namely *P5CS* for proline biosynthesis and *ProDH* for proline catabolism (**Fig. 5**).

P5CS transcript levels were much higher in plants responding to either salt or mannitol stress; in response to salt, the more severe the salt treatment the higher were the *P5CS* transcript levels. Interestingly, the *ProDH* transcript level remained very low, similar to the level observed in non-stressed plants, whatever stress was applied.

Application of 1-butanol increased *P5CS* transcript levels in normal growth conditions and after 24 h of mild salt stress. However 1-butanol slighly affected *P5CS* transcript levels in high salt and hyperosmotic responses. In contrast to this, U73122 lowered *P5CS* transcript levels in all conditions, with the exception of 400 mM NaCl. Application of both 1-butanol and U73122 fully restored *P5CS* transcript levels in non-stress

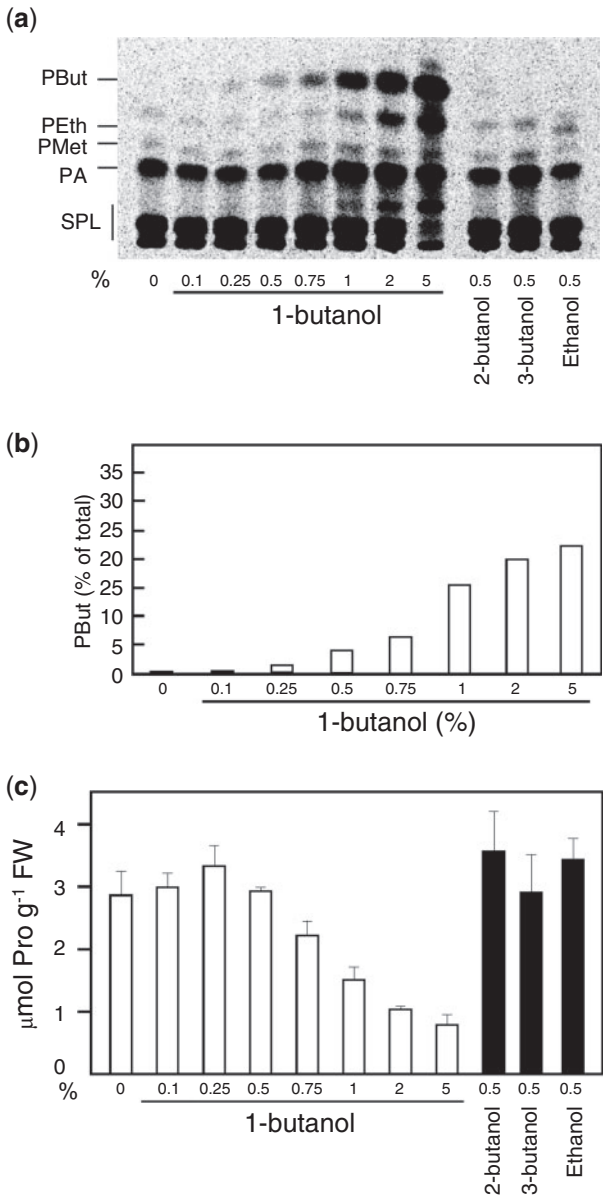

Fig. 4 Mild concentrations of 1-butanol generate PBut but have no effect on proline accumulation. Fifteen-day-old seedlings were treated for 24 h with the same volume of various alcohols or water (control). (a and b) Seedlings were labeled with [^{33}P]orthophosphate for 24 h and then treated with different concentrations of 1-butanol or 0.5% *sec*- or *tert*-butanol for controls. Lipids were extracted, separated by the ethyl acetate TLC system (a) and quantified by phosphorimaging (b) as described in the Materials and methods. The results are given as the percentage of total radioactivity found in phospholipids. (c) Proline levels. The results shown are the means ± SD of four independent experiments. PBut, phosphatidylbutanol; PA, phosphatidic acid; PEth, phosphatidylethanol; PMet, phosphatidylmethanol; SPL, structural phospholipid.

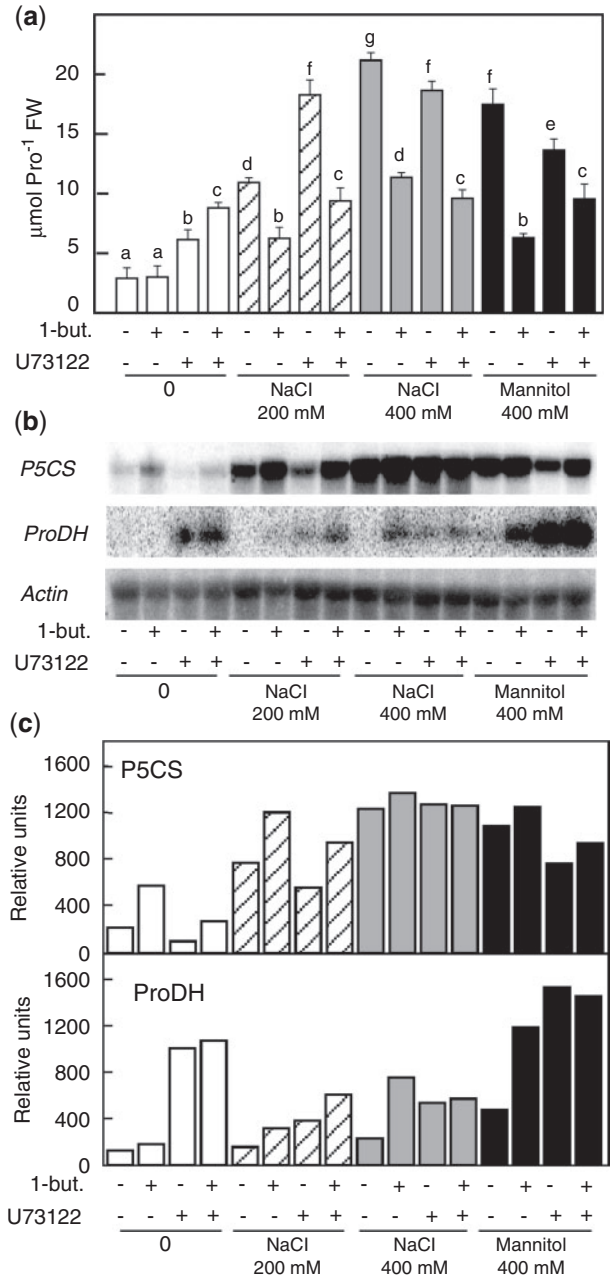

Fig. 5 A primary alcohol and the U73122 PLC antagonist differentially modulate proline content. Fifteen-day-old seedlings were treated with either water, 200 or 400 mM NaCl, or 400 mM mannitol in the presence or absence of 0.5% 1-butanol and/or 100 µM U73122 for 24 h as described in the Materials and methods. (a) Proline levels correspond to means ± SD of four independent experiments. The same letter (a–g) above bars indicates values that do not differ significantly at $P < 0.05$. (b) Representative Northern blots of total RNA (10 µg) extracted from seedlings. The blots were hybridized with cDNA fragments specific for *P5CS*, *ProDH* and actin, and detected with a PhosphorImager. (c) Quantification of the *P5CS* and *ProDH* transcript abundance. Values are the signal intensities of the bands as in (b) relative to the actin transcript levels.

condition and in the presence of 200 mM NaCl, but only partially in the presence of 400 mM mannitol. However in response to 400 mM NaCl, application of both inhibitors did not have any effect on *P5CS* transcript levels.

ProDH steady-state transcript levels were almost undetectable in both stressed and non-stressed plants. However, there was a sharp increase in the abundance of *ProDH* transcripts in response to 1-butanol and U73122 in plants subjected to mannitol stress, and to a lesser extent in those subject to salt stress. In the same way, U73122 alone or in combination with 1-butanol also increased *ProDH* transcript levels in non-stressed seedlings, but to a lesser extent than in stressed seedlings.

There is a good correlation between *P5CS* transcript levels and proline accumulation in the different growth conditions without any inhibitor treatment (**Fig. 5**). However, the effects of 1-butanol or U73122 applied individually or in combination do not fit this correlation, suggesting that the corresponding lipid signaling pathways may have an impact on some post-transcriptional mechanisms.

Lipid signaling in *T. salsuginea* upon either salt or mannitol stress

To investigate PLD signaling in *T. salsuginea* further, structural phospholipids were labeled by incubating seedlings with carrier-free [^{33}P]orthophosphate for 24 h. Seedlings were then treated with inhibitors in medium containing NaCl or mannitol. PA levels were higher in plants subjected to either high salt (400 mM) or mannitol stress (**Fig. 6**, **Supplementary Fig. S2**), but not in those subjected to mild salt stress. When 1-butanol was added, PBut was also produced (**Fig. 6**). The highest level of PBut was observed in seedlings treated with 400 mM mannitol, indicative of higher PLD activity. An increase in PA was also detected in plants treated with the PLC antagonist U73122 in each stress condition tested. The combined effects of 1-butanol and U73122 enhanced PBut and PA content in all stress conditions tested.

Discussion

Accumulation of proline in plants exposed to water stress has been very well documented (for reviews, see Aspinall and Paleg 1981, Delauney and Verma 1993). Proline accumulation results from an increase in proline biosynthesis and repression of proline catabolism (Savouré et al. 1995, Kiyosue et al. 1996, Peng et al. 1996, Verbruggen et al. 1996, Armengaud et al. 2004). The effects of genetic modification on the expression level of *P5CS* and *ProDH* genes indicate that proline accumulation could play a role in stress adaptation (Kishor et al. 1995, Nanjo et al. 1999, Szekely et al. 2008) although opposite conclusions have also been reached using antisense *AtProDH* Arabidopsis plants and proline-overproducing carrot cell lines (Maggio et al. 1997, Mani et al. 2002). Several studies have shown that *T. salsuginea* has a high capacity to accumulate proline upon water stress (Inan et al. 2004, Gong et al. 2005, Kant et al. 2006,

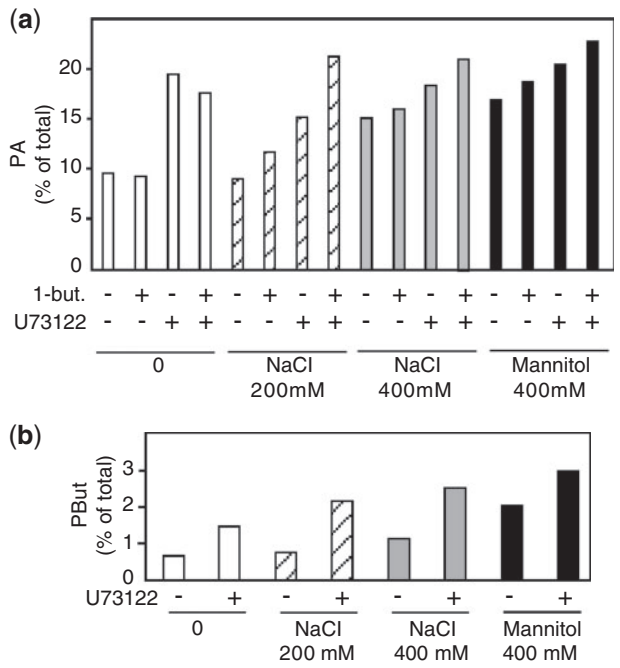

Fig. 6 Lipid signaling in *Thellungiella salsuginea* seedlings in response to salt or mannitol stress. Seedlings were labeled with ^{33}P for 24 h and then treated with water, 200 mM or 400 mM NaCl, or 400 mM mannitol in the presence or absence of 0.5% 1-butanol and/or 100 μM U73122. Lipids were extracted, separated by the methods of Lepage (1967) for PA quantification or of Lepage (1967) for PBut measurement, and quantified by phosphorimaging as described in the Materials and methods. Quantification of PA (a) and PBut (b) levels by PhosphorImager. After normalization, the results are expressed as the percentage of the total radioactivity found within the phospholipids. The data shown correspond to one representative experiment out of three performed.

Ghars et al. 2008). Here we measured up to 8-fold more proline in *T. salsuginea* when not stressed than in its close relative *A. thaliana*, suggesting that stress tolerance mechanisms are constitutively active in this halophyte even in the absence of stress (Taji et al. 2004). In *T. salsuginea*, we found that the proline content increased with the intensity of salt or hyperosmotic stress. However, a maximum of around 15 μmol proline g^{-1} FW was reached with either 300 mM NaCl or 400 mM mannitol. This value is about double that measured in Arabidopsis grown in the same conditions in response to 200 mM NaCl or 400 mM mannitol (Thiery et al. 2004, Parre et al. 2007).

Proline accumulates because the balance between its biosynthesis and its catabolism is altered in a complex way (Szekely et al. 2008, Verbruggen and Hermans 2008, Szabados and Savouré 2010). Compartmentalization of this metabolism between the cytosol, chloroplasts and mitochondria adds another level of complexity, as the flux of precursors, end-products and redox status would exert additional control. Amid this complexity, proline content positively correlates with *P5CS* steady-state transcript levels in *T. salsuginea*. Also, whatever the conditions, i.e. non-stress or stress, very low levels of *ProDH* transcripts were observed, consistent with the fact that *T. salsuginea* has a low capacity for proline catabolism (Kant et al. 2006). However, the lack of correlation we found between proline accumulation and *P5CS* and *ProDH* transcript levels when lipid signaling pathways were pharmacologically disrupted suggests that additional regulatory mechanisms of proline metabolism are in place at least in *A. thaliana* and *T. salsuginea*. Although these mechanisms are still poorly investigated, a post-transcriptional mechanism of *P5CDH* expression by small interfering RNAs (siRNAs) upon salt stress in Arabidopsis was described by Borsani et al. (2005). Mattioli et al. (2008) also suggested that *P5CS1* transcript levels may be regulated by a similar post-transcriptional mechanism because this gene is potentially overlapping with a 3′-untranslated region of an antisense gene. While other indicators could thus be used, proline accumulation, as the sum total of all these individual mechanisms, remains a meaningful physiological parameter with which to investigate stress signaling pathways.

We found that the aminosteroid PLC inhibitor U73122 slightly inhibited proline accumulation in high salt- and mannitol-stressed seedlings. Addition of calcium reversed the inhibitory effect of U73122, indicating that PLCs positively regulate proline accumulation, at least in part, through intracellular calcium signaling in response to severe osmotic stresses. In contrast, U73122 stimulated proline accumulation in non-stress and mild salt conditions, and addition of calcium further enhanced this proline response. These results suggest that in these conditions PLCs negatively regulate proline accumulation via the DAG and PA signaling pathway without the involvement of calcium.

Uncoupling PA signaling from PLD by primary alcohols is an experimental approach that has been successfully used to investigate PLDs in biotic and abiotic signaling in plants (Wang 2002, Meijer and Munnik 2003). In *T. salsuginea*, proline biosynthesis was inhibited in 1-butanol-treated seedlings grown in the presence of NaCl (200 and 400 mM) or mannitol (400 mM), but it had no effect on proline accumulation in the absence of osmoticum. These data suggest that PLDs are positive regulators of proline metabolism under stress conditions in *T. salsuginea*. The increase in PBut content in response to severe NaCl and mannitol stresses indicates that PLD activity is higher the more severe the stress is. An intriguing observation is the high PA and PBut contents measured when a combination of 1-butanol and U73122 was applied to seedlings. Phosphoinositides, particularly PIP_2, are key regulators and activators of certain PLD isoforms (Qin and Wang 2002). One should also underline that 1-butanol does not inhibit PLD but only diverts its activity. Therefore, these data, especially the increase in PBut content, may suggest an activation of PLD activity by the accumulation of PIP_2 due to the inhibition of PLC. However we cannot exclude the possibility that when PLD activity is diverted and PLC activity inhibited, there is a compensation of this lipid signaling pathway by another non-specific PLC (NPC) family. These enzymes, which generate

DAG through glycerophospholipid hydrolysis, were recently identified in Arabidopsis (Nakamura et al. 2005). Arabidopsis NPC4 was demonstrated to play an important role in plant response to ABA and drought (Peters et al. 2010) as well as high salinity (Peters et al. 2010, Kocourkova et al. 2011). Interestingly Peters et al. (2010) reported that NPC4 contributes to the production of DAG used for PA formation and the NPC4/DAG pathway promotes plant growth in response to water deficit in Arabidopsis. Further experiments are required to establish the role of this NPC family in response to salt stress in *Thellungiella*.

Here we demonstrated that PLC and PLD signaling pathways interact either to induce or to reduce proline accumulation under non-stress or stress conditions in *T. salsuginea*. These combined results allow us to propose a model of how proline metabolism is tightly regulated in *T. salsuginea* cells by PLD and PLC components (**Fig. 7**). (i) In the absence of stress, PLCs negatively regulate proline accumulation whereas PLDs do not play a role. (ii) Under moderate salt stress (e.g. 200 mM NaCl), PLCs mediate negative regulation of proline biosynthesis and PLDs positively regulate proline accumulation. (iii) Under severe salt- and mannitol-induced stress, PLDs are major positive regulators of the proline response, while PLCs may have a minor role as positive regulators via calcium signaling. The *Thellungiella* genome is expected to be completely sequenced over the next couple of years. Such knowledge will facilitate the analysis of NPC, PLC and PLD gene family organization in *Thellungiella* and give us a better understanding of the contribution each enzyme isoform makes in the regulation of proline metabolism.

Much of what is known of stress tolerance and stress signaling has been learnt through studying *A. thaliana*. While it is an unsurpassed model for plant genetics, *A. thaliana* is a true glycophyte and so the outcome of using this species as a model to study water and salinity stress is limited. As we show here, PLC and PLD signaling pathways involved in the regulation of proline metabolism appear to act in opposite ways in *A. thaliana* and *T. salsuginea* despite their phylogenetic similarity. We speculate that *Thellungiella* adaptation to environmental stress conditions may be driven by global changes in gene regulation at transcriptional and post-transcriptional levels more than by the acquisition of novel functions. Through changes in regulatory networks, a more robust response to environmental stress can be achieved and may partially explain why *T. salsuginea* is more able to withstand severe environmental constraints than *A. thaliana*. Further characterization and comparison of the signaling pathways in these two species should extend our knowledge of plant stress adaptation in general.

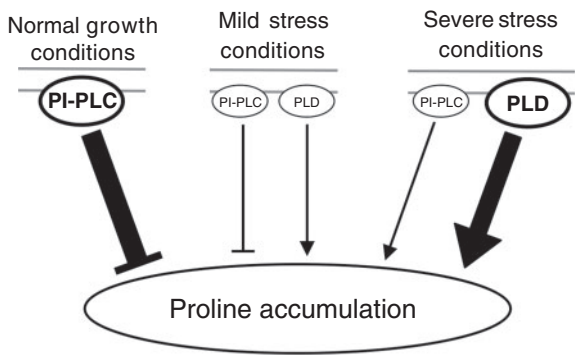

Fig. 7 Working model of how lipid signaling pathways regulate proline accumulation in different growth conditions in *Thellungiella salsuginea*. PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D.

Materials and Methods

Plant material

Seeds of *T. halophila/salsuginea* ecotype Shandong kindly provided by R. Bressan from Purdue University of West Lafayette (Bressan et al. 2001) were surface-sterilized and sown onto grids placed on 0.5× MS agar medium (Murashige and Skoog 1962) in 14 cm diameter Petri dishes. After 15 d at 4°C to break dormancy, seedlings were allowed to grow for 15 d at 22°C under continuous light with a luminosity of 90 µmol photons $m^{-2} s^{-1}$.

Pharmacological effectors and stress treatments

All inhibitors were purchased from Sigma Aldrich. Fifteen-day-old seedlings (at the 5–6 leaf stage) were transferred for 1 h pre-treatments onto 0.5× MS liquid medium supplemented with inhibitors or with the same amount of solvent [dimethylsulfoxide (DMSO)] as a control. Pre-treated seedlings were then transferred to Petri dishes containing 0.5× MS medium with various concentrations of NaCl or mannitol supplemented with either the inhibitor used for pre-treatment or solvent only. After different incubation times, seedlings were collected and frozen immediately in liquid nitrogen, and stored at −80°C until further analysis. At least three independent experiments each with three replicates were conducted for each treatment.

Lipid extraction and separation

Phospholipids were metabolically labeled by incubating 15-day-old seedlings for 24 h in growth medium supplemented with [^{33}P]orthophosphate (53 MBq l^{-1}). Inhibitors were then added to the medium with or without NaCl or mannitol.

Treatments were stopped by immersing seedlings in boiling water for 5 min to denature phospholipases (Douce 1964). Lipids were extracted according to the method of Folch et al. (1957) modified by Bligh and Dyer (1959). Seedlings were homogenized in chloroform–methanol (2 : 1, v/v) with a Waring blender. The homogenate was centrifuged at 3,000 × *g* for 15 min. The lower phase containing lipids was removed by aspiration and the chloroform solvent evaporated with

nitrogen gas. The residue was redissolved in 200 µl of toluene–ethanol (4 : 1, v/v) for storage. The lipids were then separated using the acidic solvent chloroform–acetone–methanol–acetic acid–water (50 : 20 10 : 10 : 5, by vol.) (Lepage 1967). The presence of PBut was visualized by separating it from other phospholipids by a modified ethyl acetate thin-layer chromatography (TLC) system (De Vrije and Munnik 1997). Unlabeled phospholipid standards run alongside samples were visualized by exposing the plate to iodine vapor. The radiolabeled structural phospholipids PA and PBut were quantified using a Storm PhosphorImager (GE Healthcare). After normalization and subtraction of the background signal, PBut and PA levels were calculated as percentages of the total radioactive counts per lane.

Northern blot analysis

Total RNA was extracted, separated on formaldehyde–agarose gels and blotted onto nylon membranes as described by Thiery et al. (2004). Membranes were stained with methylene blue to control for RNA loading and transfer. Membranes were hybridized at 65°C with either full-length *AtP5CS1* or *AtProDH1* cDNAs (Thiery et al. 2004) labeled with [^{32}P]dCTP using Ready-To-Go DNA labeling beads (Amersham Biosciences). As controls for each sample, actin transcripts were analyzed from the same amount of total RNA. Quantification was performed using a Storm PhosphorImager (GE Healthcare), and results were normalized with respect to the actin transcripts.

Proline assay

The amount of free proline in fresh plant material was measured as described by Bates et al. (1973) using L-proline as the standard.

Statistical analysis

Analysis of variance (ANOVA) using the AV1W MSUSTAT program with orthogonal contrasts and mean comparison procedures was performed to detect differences between treatments. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD) ($P < 0.05$).

Supplementary data

Supplementary data are available at PCP online.

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