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## Phospholipid Signaling Responses in Salt-Stressed Rice Leaves

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Salinity is one of the major environmental factors limiting growth and productivity of rice plants. In this study, the effect of salt stress on phospholipid signaling responses in rice leaves was investigated. Leaf cuts were radiolabeled with <sup>32</sup>P-orthophosphate and the lipids extracted and analyzed by thin-layer chromatography, autoradiography and phosphoimaging. Phospholipids were identified by co-migration of known standards. Results showed that <sup>32</sup>P<sub>i</sub> was rapidly incorporated into the minor lipids, phosphatidylinositol bisphosphate (PIP<sub>2</sub>) and phosphatidic acid (PA) and, interestingly, also into the structural lipids phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), which normally label relatively slowly, like phosphatidylcholine (PC) and phosphatidylinositol (PI). Only very small amounts of PIP<sub>2</sub> were found. However, in response to salt stress (NaCl), PIP<sub>2</sub> levels rapidly (<30 min) increased up to 4-fold, in a time- and dose-dependent manner. PA and its phosphorylated product, diacylglycerolpyrophosphate (DGPP), also increased upon NaCl stress, while cardiolipin (CL) levels decreased. All other phospholipid levels remained unchanged. PA signaling can be generated via the combined action of phospholipase C (PLC) and diacylglycerol kinase (DGK) or directly via phospholipase D (PLD). The latter can be measured *in vivo*, using a transphosphatidylation assay. Interestingly, these measurements revealed that salt stress inhibited PLD activity, indicating that the salt stress-induced PA response was not due to PLD activity. Comparison of the <sup>32</sup>P-lipid responses in salt-tolerant and salt-sensitive cultivars revealed no significant differences. Together these results show that salt stress rapidly activates several lipid responses in rice leaves but that these responses do not explain the difference in salt tolerance between sensitive and tolerant cultivars.

**Keywords:** Lipid signaling • Phosphatidic acid • Phosphoinositides • Phospholipase • Rice • Salt stress.

**Abbreviations:** CL, cardiolipin; DAG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerolpyrophosphate; IP<sub>2</sub>, inositol bisphosphate; IP<sub>3</sub>, inositol triphosphate; IP<sub>6</sub>, inositol hexaphosphate; O/N, overnight; PA, phosphatidic acid; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-phosphate; PIP<sub>2</sub>, phosphatidylinositol bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; TLC, thin-layer chromatography.

### Introduction

Salt stress is one of the most important abiotic stresses that adversely affect natural productivity and causes significant crop loss worldwide. Almost every aspect of the plant's physiology and biochemistry is affected. Salinity leads to dehydration and osmotic stress, resulting in stomatal closure, reduced supply of carbon dioxide and a high production of reactive oxygen species, causing irreversible cellular damage and photoinhibition. Photoinhibition coupled with salinity stress causes serious damage to many cellular and physiological processes including photosynthesis, nutrient uptake, water absorption, root growth and cellular metabolism, which all obviously lead to yield reduction (Dhariwal et al. 1998, Hasegawa et al. 2000, Zheng and Shannon 2000, Zhu 2001). Salinity also causes imbalance of the cellular ions, resulting in ion toxicity (Khan et al. 2002, Khan and Panda 2002, Panda and Khan 2003, Demiral and Turkan 2005, Mandhania et al. 2006). Specific effects of salt

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stress on plant metabolism, especially on leaf senescence, have been related to the accumulation of toxic Na<sup>+</sup> and Cl<sup>-</sup> ions and to K<sup>+</sup> and Ca<sup>2+</sup> depletion (Al-Karaki 2000).

The most important cereal crop in the world is rice, yielding one-third of the total carbohydrate source. Three billion people consider rice as their staple food, accounting for 50–80% of their daily calorie intake. Rice is a salt-sensitive monocot (Maas and Hoffman 1997, Francois and Mass 1994). In particular, seedlings and the reproductive stages are very sensitive to salinity, while germination is relatively tolerant (Lutts et al. 1996, Zheng and Shannon 2000, Zheng et al. 2001). Rice cultivars vary in their ability to tolerate salt stress, with both salt-tolerant and salt-sensitive lines being available (Zeng et al. 2004, Zeng 2005).

Understanding the mechanisms by which plants perceive salt stress signals, and convey them to the cellular machinery to activate adaptive responses, is important for the development of classical breeding programs as well as for biotechnological methods to improve the salt tolerance of crops. Salt tolerance depends on a complex signaling network, involving multiple signal transduction pathways, allowing plants to respond rapidly and properly to salt stress (Zhu 2001). These include reactive oxygen species, nitric oxide, calcium, protein kinases, including calcium-dependent protein kinase (CDPK), sucrose non-fermenting-related kinases (SnRKs) and mitogen-activated protein kinases (MAPKs), and various lipid second messengers (Munnik and Meijer 2001, Zhu 2001, Xiong et al. 2002).

One of these lipid signals is phosphatidic acid (PA). Normally, PA only constitutes a minor proportion of the cellular lipid pool but, in responses to stress, PA levels can increase significantly (Munnik 2001, Testerink and Munnik 2005, Wang 2005). PA can be formed by phospholipase D (PLD), which hydrolyzes structural phospholipids to generate PA and a free head group, but can also be produced indirectly, via the sequential action of phospholipase C (PLC) and diacylglycerol (DAG) kinase (DGK). PLC hydrolyzes polyphosphoinositides, such as phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P<sub>2</sub>] into DAG and inositol bisphosphate (IP<sub>2</sub>) or inositol triphosphate (IP<sub>3</sub>). While DAG remains in the membrane and is rapidly phosphorylated to PA by DGK, IP<sub>3</sub> was always considered to release Ca<sup>2+</sup> from intracellular stores. However, it has now become clear that higher plants do not contain IP<sub>3</sub> receptors such as those conserved in mammalian cells or green algae (Zonia and Munnik 2006, Wheeler and Brownlee 2008). Instead IP<sub>3</sub> or IP<sub>2</sub> may be converted to inositol hexaphosphate (IP<sub>6</sub>) which is a much stronger elicitor than IP<sub>3</sub> in mobilizing [Ca<sup>2+</sup>]<sub>cyt</sub> from intracellular stores of stomatal guard cells (Lemtiri-Chlieh et al. 2000, Lemtiri-Chlieh et al. 2003). Hyperosmotic stress has been shown to affect IP<sub>3</sub> and phosphatidylinositol bisphosphate (PIP<sub>2</sub>) levels in Arabidopsis and tobacco cells (Pical et al. 1999, Drøbak

and Watkins 2000, DeWald et al. 2001, Takahashi et al. 2001, Zonia and Munnik 2004, Williams et al. 2005, Vermeer et al. 2006, van Leeuwen et al. 2007), and for the putative phosphorylation of IP<sub>3</sub> to IP<sub>6</sub>, two dual-specificity inositolpolyphosphate kinases have been identified (Stevenson-Paulik et al. 2002). It should be noted that PIP<sub>2</sub> is not only a precursor for PLC signaling but is also emerging as a second messenger itself (see Van Leeuwen et al. 2007, and references therein).

Hyperosmotic stress has been shown to increase PA levels in the green alga *Chlamydomonas* and in cell suspension cultures of tomato, tobacco and alfalfa (Munnik et al. 2000, Munnik and Meijer 2001, Arisz et al. 2003, Zonia and Munnik 2004). Both the PLD and PLC–DGK pathways have been shown to be responsible for this (Munnik et al. 2000, Katagiri et al. 2001, Sang et al. 2001, Munnik and Meijer 2001, Testerink and Munnik 2005, Bargmann et al. 2009b). The mechanism by which PA works as a signaling molecule is still not clear. Proposed functions include increasing membrane charge and curvature (e.g. Roth 2008) to facilitate vesicular trafficking and to recruit target proteins to the membrane and influence their activity (Testerink and Munnik 2005, Wang 2005).

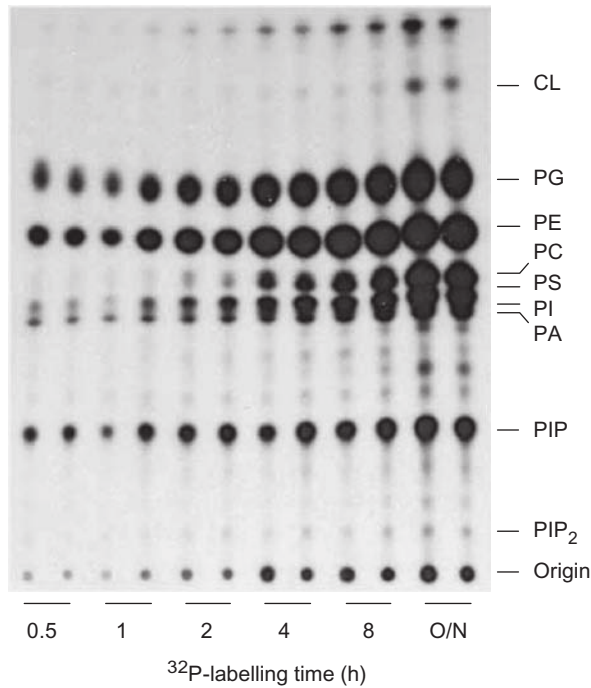
Diacylglycerolpyrophosphate (DGPP) is the phosphorylated product of PA which is produced by a novel enzyme, PA kinase (reviewed by van Schooten et al. 2006). Interestingly, DGPP is only found in plants and yeast; it is lacking in higher animals. DGPP is a minor lipid, present in very low amounts, but in response to hyperosmotic stress its levels have been found to increase in *Chlamydomonas*, the resurrection plant *Craterostigma plantagineum*, and in cell suspension cultures of tomato, alfalfa and Arabidopsis (Munnik et al. 1996, Pical et al. 1999, Munnik et al. 2000, Meijer et al. 2001).

While the data above imply that salt stress can trigger various lipid signaling responses in plants, for rice this is still unknown. Here, the effect of salinity stress on potential lipid signals in rice is investigated.

## Results

### <sup>32</sup>P incorporation into phospholipids of rice leaf

To investigate phospholipid signaling responses in rice leaves, we first studied the radiolabeling kinetics of <sup>32</sup>P<sub>i</sub> in leaf cuts when floating on 5 mM MES buffer (pH 5.7). A typical result is illustrated in Fig. 1, showing a time-course of <sup>32</sup>P<sub>i</sub> incorporation into rice phospholipids. During the first hour, the structural lipids phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) were remarkably well labeled, followed by phosphatidylinositol (PI) and phosphatidylserine (PS) and, relatively slowly, by phosphatidylcholine (PC). Concerning the minor signaling lipids, phosphatidylinositol monophosphate (PIP) and PA were labeled relatively quickly, whereas hardly any <sup>32</sup>P-PIP<sub>2</sub> was observed. Overnight (O/N)



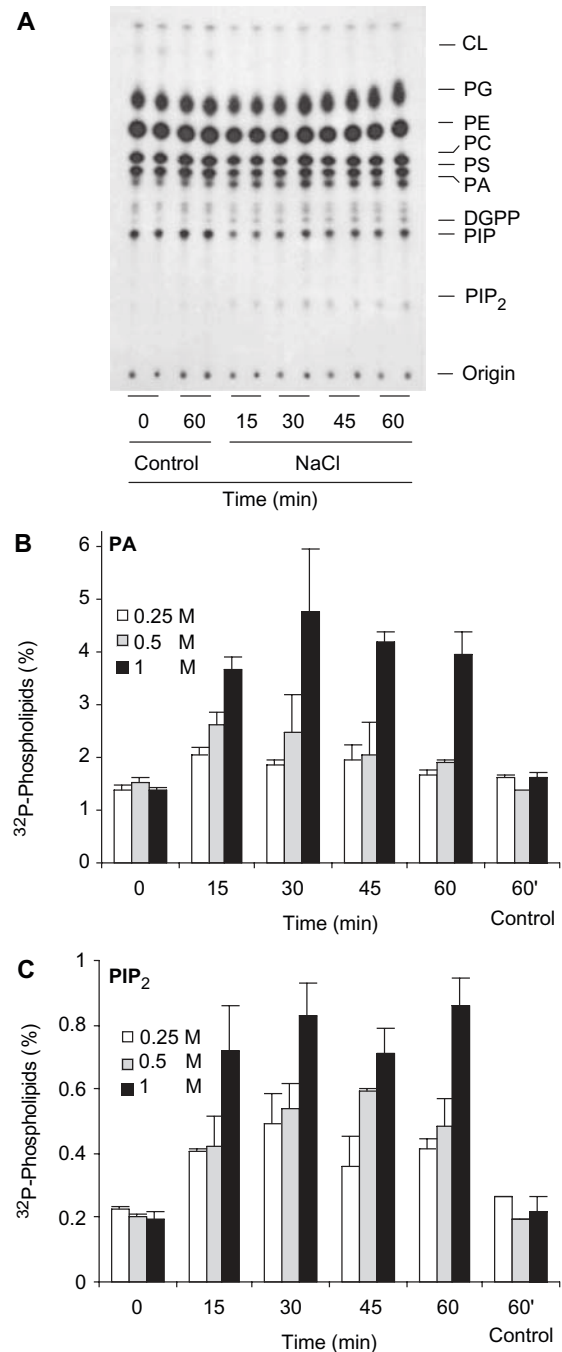
**Fig. 1**  $^{32}\text{P}$  incorporation into phospholipids of rice leaf. Leaf cuts of rice (*Oryza sativa*) were labeled with  $^{32}\text{P}_i$  for the times indicated, after which their lipids were extracted and subjected to alkaline TLC to separate the different species. Radioactivity was detected by autoradiography. The results of a typical experiment are shown. CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PIP, phosphatidylinositol monophosphate;  $\text{PIP}_2$ , phosphatidylinositol bisphosphate.

labeling gave appropriate amounts of signal and this became our routine labeling time for the remainder of the experiments.

### NaCl stress rapidly stimulates the accumulation of PA and $\text{PIP}_2$

In order to study the effect of salinity, O/N labeled leaf cuts were treated with different concentrations of NaCl, which was added in the same buffer for the times indicated. Routinely, leaf cuts were incubated with 0–1,000 mM NaCl for 0–60 min. Incubations were stopped by adding perchloric acid (5% final concentration) and lipids were extracted, separated by thin-layer chromatography (TLC) and quantified by phosphoimaging.

The results of a typical experiment are presented in **Fig. 2**. Two main responses were observed: an increase in PA and  $\text{PIP}_2$ . Responses were time- and dose dependent, with PA increases of up to 3.4-fold (**Fig. 2B**) and  $\text{PIP}_2$  increases of up to 4-fold (**Fig. 2C**), depending on the time and dose. In addition, two minor responses were picked up. DGPP levels



**Fig. 2** Salt stress stimulates PA and  $\text{PIP}_2$  formation in a time- and dose-dependent manner. Rice leaf cuts were labeled O/N with  $^{32}\text{P}_i$  and then subjected to different concentrations of NaCl (0, 250, 500 and 1,000 mM) for different time periods (0, 15, 30, 45 and 60 min). The 60 min control sample was incubated for 60 min in buffer without salt. Lipids were extracted and subjected to alkaline TLC to separate the different phospholipid species. Radioactivity was visualized by autoradiography and quantified by phosphoimaging. (A) Autoradiograph of alkaline TLC showing lipids from leaf cuts of rice plants treated with 1 M NaCl for the times indicated. (B, C) Radioactivity levels of PA (B) and  $\text{PIP}_2$  (C) after NaCl treatment at the concentrations and for the times indicated.



increased whereas cardiolipin (CL), a minor structural phospholipid which is predominantly present in mitochondria, was found to decrease in response to salt stress (Fig. 2A).

To investigate how fast the lipid responses were, leaf cuts were subjected to salt stress for only 5 min. As shown in Fig. 3, PA and PIP<sub>2</sub> levels increased in a dose-dependent manner, except that 250 mM produced no response (Fig. 3A–C). The DGPP response was also visible (Fig. 3A) but the CL response was not apparent yet, indicating that this was a relatively slower response.

### In vivo PLD Activity

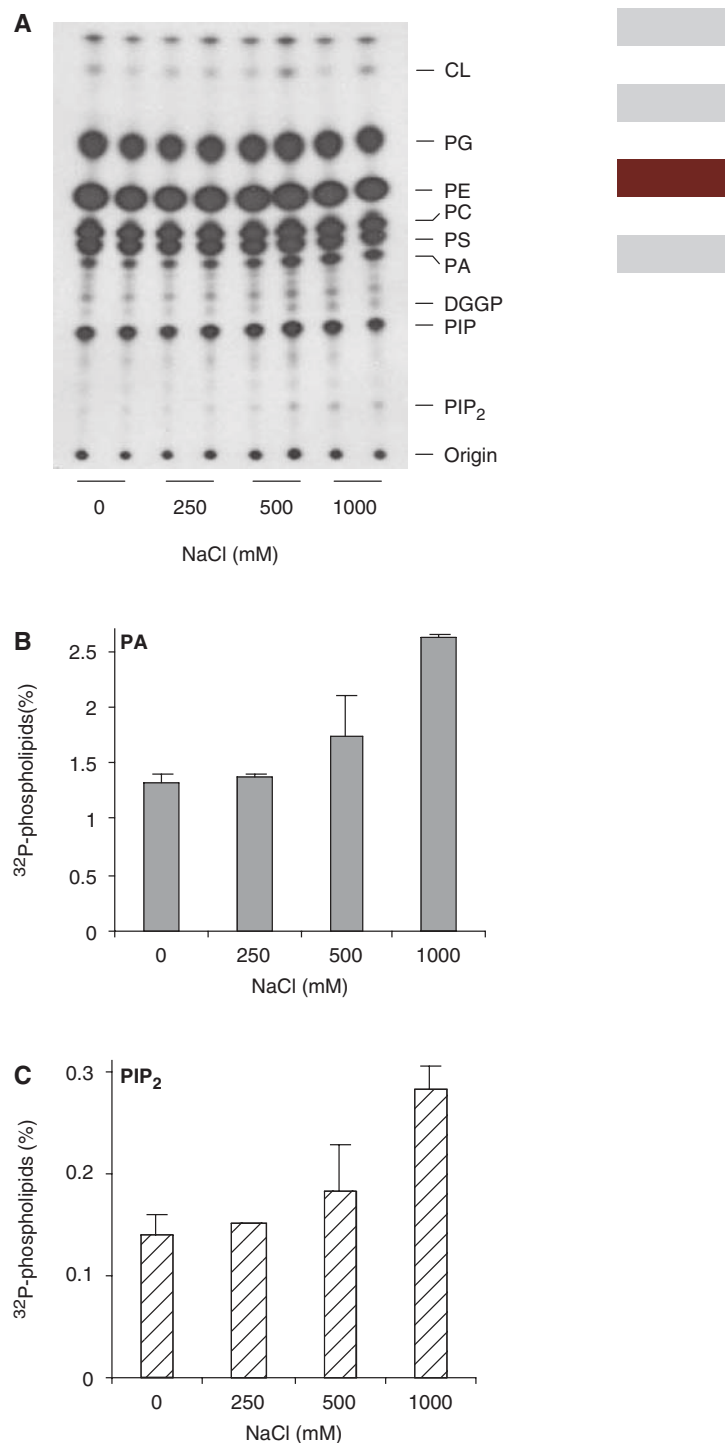
Since PLD can generate PA directly, and salt stress has repeatedly been reported to activate PLD activity (Testerink and Munnik 2005, Wang 2005, Bargmann and Munnik 2006), PLD's contribution to the salt-induced PA response in rice leaves was investigated. To investigate this, the in vivo transphosphatidylating activity of PLD was used (Munnik et al. 1995). For this, leaf cuts were labeled O/N and then subjected to different concentrations of salt in the presence of 0.5% (v/v) *n*-butanol. After 30 min, reactions were stopped, and lipids were extracted and separated by ethyl acetate TLC to monitor the PLD-catalyzed phosphatidylbutanol (PBut) formation by phosphoimaging (Munnik et al. 2000).

Interestingly, and in contrast to what had been found before in *Chlamydomonas* (Munnik et al. 2000), Arabidopsis seedlings (Katagiri et al. 2001), tomato cell suspension cultures (Munnik et al. 2000) and Arabidopsis leaf discs (Bargmann et al. 2009b), salt did not activate PLD in rice leaves (Fig. 4). In contrast, salt was even found to inhibit PLD activity, as seen by the dose-dependent decrease in PBut formation (Fig. 4A) while PA levels clearly increased (Fig. 4B).

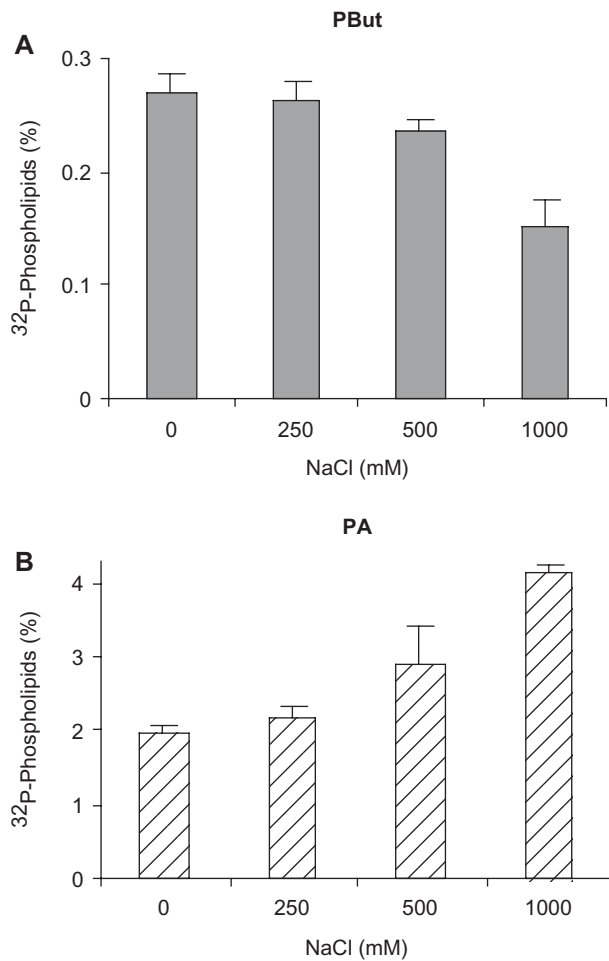
To study the activity of PLD under longer salt stress conditions, pre-labeled leaf cuts were incubated with 500 mM NaCl for 4, 8 or 24 h. *n*-Butanol [0.5% (v/v)] was only added for the last 30 min of the incubation. As shown in Fig. 5, increased PBut levels were found after 8 h (1.8-fold increase), and, after 24 h, PLD activity was still 1.3-fold higher than in the control situation without salt. These results indicate that the fast increase of PA within the first half hour is not a consequence of PLD activation, but that PLD is activated at later time points (hours vs. minutes). In tobacco pollen tubes, PLD was also found to be inhibited by salt stress in the minute range (Zonia and Munnik 2004).

### Salt sensitivity test of tolerant and sensitive rice cultivars

In order to compare the salt stress response in a salt-tolerant (Sakha 104) and salt-sensitive cultivar (Sakha 102), a hydroponics system with different concentrations of NaCl was used. First, we tested the response of both cultivars under a wide range of NaCl concentrations, i.e. 125, 250, 500 and

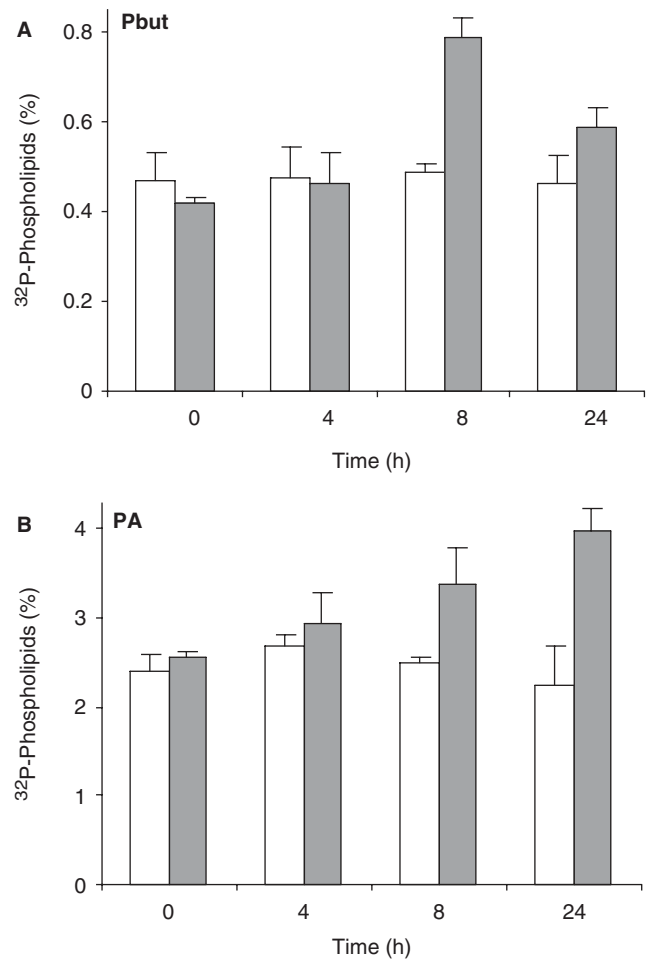


**Fig. 3** NaCl triggers PA and PIP<sub>2</sub> responses within 5 min. Leaf cuts of rice (*Oryza sativa*) were pre-labeled with <sup>32</sup>P, O/N and then subjected to different concentrations of NaCl (i.e. 0, 250, 500 and 1,000 mM) for 5 min. Lipids were then extracted and separated by alkaline TLC. Radioactivity was visualized by autoradiography and quantified by phosphoimaging. (A) Autoradiograph of alkaline TLC. (B, C) Radioactivity levels of PA (B) and PIP<sub>2</sub> (C).



**Fig. 4** Salinity stress inhibits PLD activity in rice leaves. Rice leaf cuts were pre-labeled O/N with  $^{32}\text{P}_i$  and then subjected to different concentration of NaCl (i.e. 0, 250, 500 and 1,000 mM) for 30 min, in the presence 0.5% (v/v) *n*-butanol as transphosphatidylase substrate for PLD (see Materials and Methods). Lipids were extracted and separated by ethyl acetate TLC. Radioactivity was quantified by phosphoimaging. Radioactivity levels of PBut (A) and PA (B) are shown.

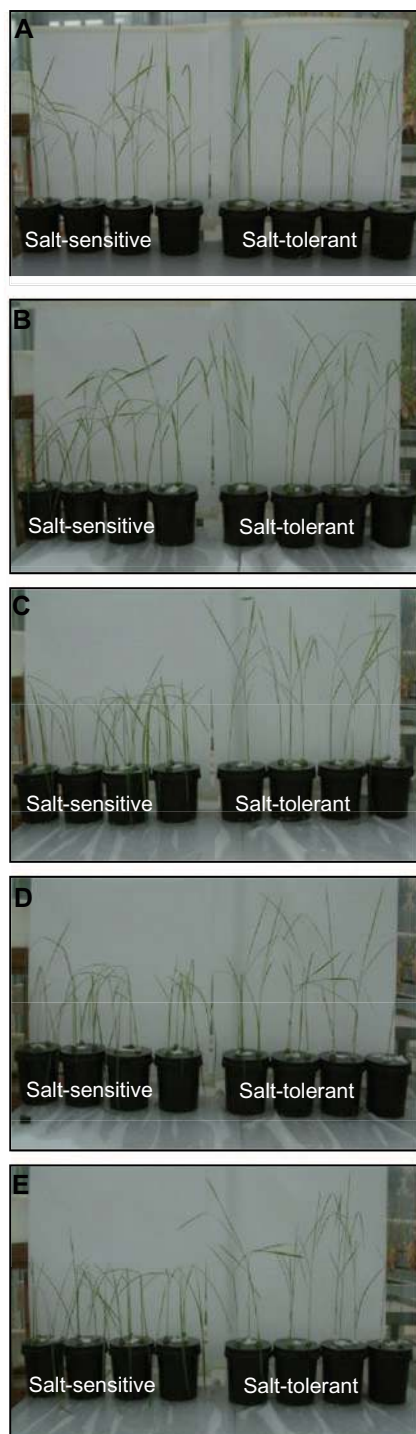
1,000 mM. Interestingly, the plants were found to respond quickly to salinity. Within 30 min, the salt-sensitive cultivar had already started to wilt, while the salt-tolerant cultivar still appeared normal. After 1 week, most plants had died, except at 125 mM NaCl, but no difference in salinity tolerance between the two cultivars was observed. When plants were subjected to a narrower range of salt stress, i.e. 125, 150, 175, 200 and 225 mM NaCl, we found that the growth of both cultivars was inhibited, but that the salt-sensitive cultivar was affected more strongly. At 175 mM NaCl, the salt-tolerant cultivar was still alive while the salt-sensitive cultivar was already dead. Above this concentration, both cultivars died (Figs. 6, 7).



**Fig. 5** Salt stress activates PLD activity after prolonged incubation. Leaf cuts of rice were pre-labeled O/N with  $^{32}\text{P}_i$  and then subjected to 500 mM NaCl for 0, 4, 8 or 24 h. In this case, 0.5% (v/v) *n*-butanol was added 30 min prior to the end of the treatment. Lipids were then extracted, separated by ethyl acetate TLC and quantified by phosphoimaging. Radioactivity levels of PBut (A) and PA (B) are shown. Control (open bars); NaCl (filled bars).

### Salt stress stimulates a PA and PIP<sub>2</sub> response in salt-tolerant and salt-sensitive cultivars

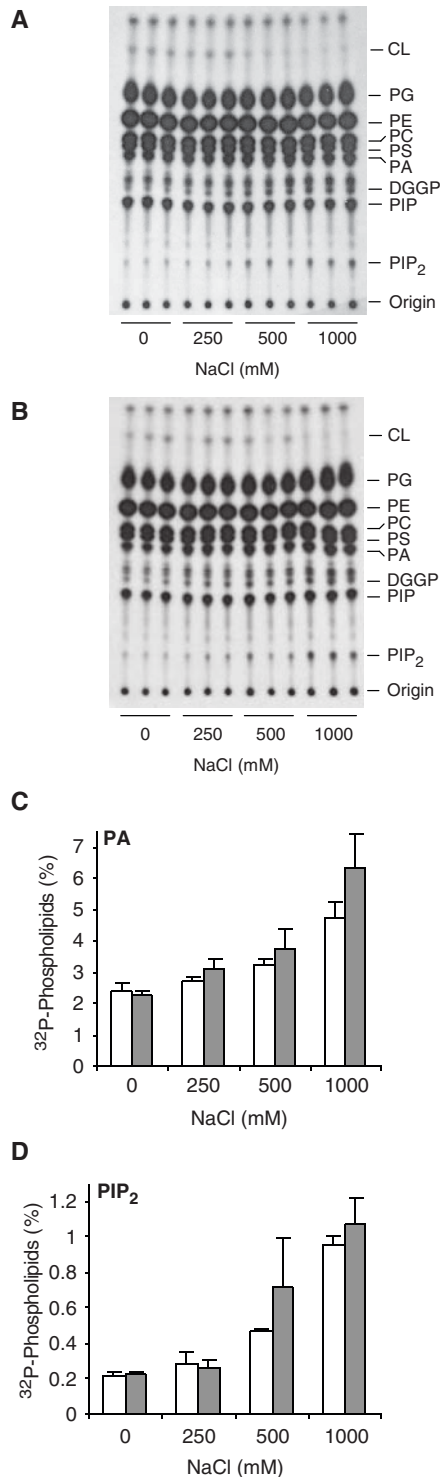
To investigate whether the salt-tolerant and salt-sensitive cultivars exhibited a different lipid composition and/or lipid signaling response, O/N labeled leaf cuts from both cultivars were subjected to different concentrations of NaCl for 30 min. As shown in Fig. 8A and B, the labeling pattern was very similar and also the responses of PA, PIP<sub>2</sub>, DGPP and CL appeared to be the same. Quantification of the PA and PIP<sub>2</sub> responses revealed a slightly higher response for the salt-tolerant cultivar; however, this was not statistically significant (Fig. 8C, D).



**Fig. 6** Short-term salt stress response in salt-tolerant and salt-sensitive rice cultivars. Two-week-old rice plants of both cultivars, grown on soil, were transferred to a hydroponics system containing 1/2 HGI nutrient solution. After 1 week, they were transferred to hydroponics containing different concentrations of NaCl (mM). Plants were monitored after 30 min. (A) Control, (B) 125 mM, (C) 250 mM, (D) 500 mM, (E) 1,000 mM.



**Fig. 7** Long-term salt stress response in salt-tolerant and salt-sensitive rice cultivars. Two-week-old rice plants of both cultivars, grown on soil, were transferred to a hydroponics system containing 1/2 HGI nutrient solution. After 1 week, plants were transferred to hydroponics containing different concentrations of NaCl (mM). Plants were monitored after 1 week. (A) Control, (B) 125 mM, (C) 150 mM, (D) 175 mM, (E) 200 mM, (F) 225 mM.



**Fig. 8** Salt stress stimulates PA and PIP<sub>2</sub> responses in both salt-tolerant and salt-sensitive cultivars. Leaf cuts of salt-tolerant and salt-sensitive rice cultivars (filled and open bars, respectively) were pre-labeled O/N with <sup>32</sup>P, and then subjected for 30 min to different concentrations of NaCl (0, 250, 500 and 1,000 mM). Radioactivity was detected by autoradiography and quantified by phosphoimaging. Autoradiographs

## Discussion

This study investigated early lipid signaling responses in salt-stressed rice leaves. We found that PA and PIP<sub>2</sub> levels increased within 5 min, and that the responses were time and dose dependent. These results are in agreement with those found in dicot plant species such as Arabidopsis and tobacco, although most of those data were obtained from cell suspensions (Pical et al. 1999, Munnik, 2001, van Leeuwen et al. 2007, Bargmann et al. 2009b), and Arabidopsis leaf cuts (Bargmann et al. 2009a, b). PA has been implicated in intracellular signaling (Testerink and Munnik 2005, Wang 2005). PA formed in response to salt stress has been suggested to function as a signaling molecule guiding the plant's acclimation responses to salt stress. PA can bind and affect the activity of various signaling proteins, including protein kinases and phosphatases (Testerink and Munnik 2005, Wang 2005). The protein kinase SnRK2 was found in a proteomic screen for PA targets (Testerink et al. 2004) and has been shown to be activated in response to salt stress (Munnik et al. 1999, Hrabak et al. 2003, Boudsocq et al. 2004). PA has also been suggested to regulate the activity of vacuolar H<sup>+</sup>-ATPases upon high salt treatment, which may help maintain the proton gradient that drives Na<sup>+</sup>/H<sup>+</sup> antiporter activity (Zhang et al. 2006).

The accumulation of PIP<sub>2</sub> in response to salt stress seems to be typical for osmotic stress; both salinity and hyperosmotic stress, but no other stresses, have been shown to increase PIP<sub>2</sub> in plant cells, including Arabidopsis cell cultures (Pical et al. 1999) and seedlings (DeWald et al. 2001, van Leeuwen et al. 2007), tobacco pollen (Zonia and Munnik 2004) and cell cultures (van Leeuwen et al. 2007), and various green algae (Einspahr et al. 1988, Meijer et al. 1999, Heilmann et al. 2001). Three PIP<sub>2</sub> isomers have been reported for plant cells: PI(3,5)P<sub>2</sub>, PI(4,5)P<sub>2</sub> and PI(3,4)P<sub>2</sub> (Brearley and Hanke 1993, Meijer et al. 1999, Pical et al. 1999, DeWald et al. 2001). The 3,5- and 4,5-phosphorylated isomers have been identified in a number of plant tissues and species (Meijer et al. 1999, Meijer and Munnik 2003), whereas the 3,4-phosphorylated isomer has only been identified in the aquatic plant *Spirodela polyrrhiza* (Brearley and Hanke 1993) and may actually be PI(3,5)P<sub>2</sub> (Meijer et al., 1999). While PIP<sub>2</sub> is only a very minor phospholipid, accounting for 30–100 times less than its precursor PI4P (which accounts for approximately 0.5% of total phospholipids), 99% is typically the PI(4,5)P<sub>2</sub> isoform (Meijer and Munnik, 2003). Meanwhile, PI(4,5)P<sub>2</sub> is no longer considered to function exclusively as a precursor of IP<sub>3</sub> signaling, but rather it is

of alkaline TLCs showing lipids of a salt-tolerant (A) and salt-sensitive cultivar (B). Radioactivity levels of PA (C) and PIP<sub>2</sub> (D) of a salt-tolerant (filled bars) or salt-sensitive (open bars) cultivar.



emerging as a signalling molecule in its own right. As such, PLC would still play a signaling role as it would switch off PI(4,5)P<sub>2</sub> signaling and generate the DAG for PA production via DGK (Munnik and Testerink 2009). In mammalian systems, PI(4,5)P<sub>2</sub> has been shown to function in a number of cellular processes, including the reorganization of the cytoskeleton, vesicular trafficking and regulating ion channels (see van Leeuwen et al. 2007). Each of those processes seems very relevant to salt stress signaling in plants and deserves further investigation. The rapid accumulation of PI(4,5)P<sub>2</sub> is most probably due to activation of a PI4P 5-kinase, which has been shown to be induced upon salt stress (Heilmann et al. 2001, Im et al. 2007).

The Arabidopsis genome encodes 11 putative PI4P 5-kinase genes (Mueller-Roeber and Pical 2002, Kusano et al. 2008) of which at least one is up-regulated in response to hyperosmotic stress (Takahashi et al. 2001). Future T-DNA knockout mutants may help us to identify the PI4P 5-kinases involved in the salt stress response and to study the consequences downstream. Using a green fluorescent protein (GFP)-based PI(4,5)P<sub>2</sub> biosensor in tobacco BY-2 cells and Arabidopsis seedlings revealed that the increase of PI(4,5)P<sub>2</sub> in response to salt is most likely to occur at the plasma membrane (van Leeuwen et al. 2007).

DGPP also increased in response to salt stress. Earlier, this was found for various dicots and the green alga *Chlamydomonas* (Munnik et al. 1996, Pical et al. 1999, Munnik et al. 2000, Meijer et al. 2001). Whether DGPP simply functions to attenuate PA signaling or is also playing a role as a signaling molecule itself still remains to be shown (van Schooten et al. 2006). Interestingly, DGPP has recently been reported to function as a phospholipid second messenger in ABA signaling. DGPP was shown to mimic ABA effect on anion currents and to stimulate RAB18 gene expression (Zalejski et al. 2005, Zalejski et al. 2006).

What has not been observed earlier is the decrease of CL in response to salt stress. CL is an anionic phospholipid with a unique tetra-acyl structure, found in the inner and outer membrane of mitochondria in eukaryotes (Hovius et al. 1990, Ardail et al. 1990, Simbeni et al. 1991, Hovius et al. 1993). While it is a minor structural phospholipid, accounting for approximately 3% of total phospholipids, CL can constitute 20–30% of the mitochondrial lipids. CL plays a role in respiration and oxidative phosphorylation (Daum 1985, Hoch 1992, Robinson 1993) and has been implicated in targeting cytosolic-synthesized pre-proteins to the mitochondria (Leenhouts et al. 1993, Robinson 1993). It is unclear what the decrease in CL in response to salt stress means. Is it degraded to PA? A specific mitochondrial PLD which hydrolyzes CL to PA and PG has been reported, and is suggested to promote mitochondrial fusion (Schlame et al. 2000). For plants this is not known. The fact that salt stress inhibited PLD activity would not be in agreement with this,

although we do not know whether such a presumed mitochondrial PLD would belong to the class of PLDs able to transphosphatidylate (Bargmann and Munnik 2006). Alternatively, the apparent reduction in CL might be due to a decrease in mitochondria vs. extramitochondrial membranes.

Several previous studies regarding plants have reported on PLD activity being stimulated by salt stress. These include *Chlamydomonas* (Munnik et al. 2000), Arabidopsis seedlings (Katagiri et al. 2001), leaf discs of *C. plantagineum* (Munnik et al. 2000), suspension-cultured tomato and tobacco cells (Munnik et al. 2000, Bargmann et al. 2009b) and Arabidopsis leaf discs (Bargmann et al. 2009b). Nevertheless, in tobacco pollen tubes, PLD was also found to be inhibited by salt stress (Zonia and Munnik 2004). In rice leaves, we found that PA accumulated in a dose-dependent manner while PBut levels decreased. Therefore, the increase in PA is likely to be generated via the DGK pathway (Munnik et al. 2000, Munnik 2001, Testerink and Munnik 2005) although the involvement of a non-transphosphatidylating PLD (e.g. against CL) cannot be excluded. When rice leaves were subjected to longer exposures to salt stress, an increase in PLD activity was indeed found. Possibly, this reflects a change in PLD gene expression and/or an adaptive response to salt stress.

When we compared the phospholipid labeling pattern and the salt-induced lipid responses in a salt-tolerant and salt-sensitive cultivar, no major differences were found. In general, salt-tolerant plant cultivars accumulate more osmoprotectants and less Na<sup>+</sup> than salt-sensitive cultivars (Rhodes and Samaras 1994, Bohnert and Jensen 1996, Nuccio et al. 1999, Hasegawa et al. 2000, Roychoudhury et al. 2008). The increase in compatible solutes raises the osmotic pressure, helping to maintain turgor and the uptake of water (Rhodes and Samaras 1994, Hasegawa et al. 2000). The main problem with salt stress is the lowering of the water potential and the direct toxicity of the Na<sup>+</sup> and Cl<sup>-</sup> absorbed. When ions accumulate over time, a difference in salt tolerance appears (Munns 1993). Sensitive cultivars accumulate ions more quickly than tolerant cultivars, and the ion accumulation leads to leaf death and, progressively, to death of the whole plant (Munns 2002). While differences within the first 30 min could be observed between both cultivars, the differences in their phospholipid signaling responses were not statistically significant. This indicates that the difference in salt tolerance lies downstream of lipid signaling, but it is also possible that our method is not sensitive enough to pick up the differences. For example, different PA and/or PIP<sub>2</sub> pools may be involved, at different cellular and subcellular locations. Our method only measures the total PA and PIP<sub>2</sub> responses. The same holds for the PBut accumulation. Rice is predicted to contain 17 PLDs (Li et al. 2007). With our methodology, it is still possible that a PLD with a much lower activity is involved in salt tolerance. We have also tried to label rice seedlings,

but these gave a huge variation in  $^{32}\text{P}_i$  uptake and hence  $^{32}\text{P}$ -lipid responses (data not shown) and, therefore, could not be used. Future knockout mutants and gene silencing strategies will be helpful in investigating this.

All lipid responses that we described were dose dependent and we realize that some high salt concentrations are not physiologically relevant. However, there is a big difference between 'sensing' a certain salt concentration (minutes–hours) and 'living' at a certain salt concentration (days), and we emphasize that the observed lipid responses do also occur at physiological concentrations. Besides, numerous laboratories have used the same concentrations to look at protein kinases, phosphatases, calcium responses, gene expression, etc., so in this way lipid responses are informative and comparable (for example, see Munnik and Meijer 1999, Munnik et al. 2000, Katagiri et al. 2001, Hrabak et al. 2003, Boudsocq et al. 2004, and references therein).

## Materials and Methods

### Plant material

Seeds of salt-sensitive (Sakha 102) and salt-tolerant (*Oryza sativa* cv. *japonica*) rice lines (Sakha 104) were provided by the Faculty of Agriculture, Cairo, Egypt (Bradbury et al. 2005). Plants were grown on soil in a growth chamber using a 14 h light/10 h dark regime at 28°C. Leaf cuts (0.5 cm) from 4-week-old plants were routinely taken.

### $^{32}\text{P}_i$ labeling and salt treatment

Leaf pieces of ~0.5 cm, containing the midrib, were cut from the middle of the leaf using a pair of scissors. Leaf cuts were metabolically labeled by floating them O/N on 200 µl of 5 mM MES buffer pH 5.7 (KOH) and 1 mM KCl, supplemented with 5 µCi of carrier-free  $\text{PO}_4^{3-}$  in a 2 ml Eppendorf microcentrifuge tube. Samples were subjected to salt stress by adding 200 µl of NaCl in MES buffer for the times and at the concentrations indicated. Three leaf cuts for every replicate were taken.

### Lipid extraction and analysis

Treatments were stopped by adding 400 µl of 10% perchloric acid. After 30 min shaking on a Eppendorf shaker, the material was transferred to a new tube containing 400 µl of  $\text{CHCl}_3/\text{MeOH}/\text{HCl}$  [50:100:1 (by vol.)], where lipids were extracted while shaking for 30 min. A two-phase system was induced by adding 400 µl of  $\text{CHCl}_3$  and 200 µl of 0.9% (w/v) NaCl. The rest of the extraction was performed as described previously (van der Luit et al. 2000). Lipids were routinely separated on silica TLC plates (Merck), using an alkaline solvent system: chloroform/methanol/20% ammonium/water [90:70:4:16 (by vol.); Munnik et al. 1998]. For identification, lipids were subjected to a second dimension using an acid TLC solvent as in Munnik et al. (1994). CL was identified by

co-migration with a lipid standard. Phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA).

### In vivo PLD activity

O/N labeled leaf cuts were subjected to different concentration of salt for 30 min in the presence of 0.5% (v/v) *n*-butanol as transphosphatidylolation substrate (Munnik et al. 1995). To study PLD activity after long salt exposures (i.e. 4, 8 and 24 h), 0.5% (v/v) *n*-butanol was only present during the last 30 min of the salt treatment. Lipids were extracted as described above and separated on TLC plates using the organic phase of an ethyl acetate system containing: ethyl acetate/isooctane/formic acid/water [12:2:3:10 (by vol.); Munnik et al. 1998]. Phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics).

### Salt sensitivity of rice cultivars

Salt-tolerant (Sakha 104) and salt-sensitive (Sakha 102) cultivars were grown on soil under the same conditions as described above. After 2 weeks, plants were transferred to a hydroponics system containing 1/2 Hoagland nutrient solution. After 1 week, plants were hydroponically transferred to the same nutrient solution containing different concentrations of NaCl (0, 125, 150, 175, 200, 225, 250, 500 and 1,000 mM). The effect of salt was monitored after 30 min and after 1 week.

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