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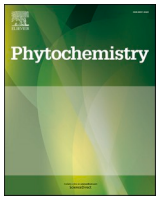

Stress- and phospholipid signalling responses in Arabidopsis PLC4-KO and -overexpression lines under salt- and osmotic stress

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ABSTRACT

Several drought and salt tolerant phenotypes have been reported when overexpressing (OE) phospholipase C (*PLC*) genes across plant species. In contrast, a negative role for Arabidopsis *PLC4* in salinity stress was recently proposed, showing that roots of *PLC4-OE* seedlings were more sensitive to NaCl while *plc4* knock-out (KO) mutants were more tolerant. To investigate this apparent contradiction, and to analyse the phospholipid signalling responses associated with salinity stress, we performed root growth- and phospholipid analyses on *plc4-KO* and *PLC4-OE* seedlings subjected to salinity (NaCl) or osmotic (sorbitol) stress and compared these with wild type (WT). Only very minor differences between *PLC4* mutants and WT were observed, which even disappeared after normalization of the data, while in soil, *PLC4-OE* plants were clearly more drought tolerant than WT plants, as was found earlier when overexpressing Arabidopsis *PLC2, -3, -5, -7* or *-9*. We conclude that *PLC4* plays no opposite role in salt-or osmotic stress and rather behaves like the other Arabidopsis PLCs.

1. Introduction

Plants are sessile organisms and as such have to continuously respond and adapt to their local environment. Understanding how these acclimation reactions take place is of prime importance to secure the world food supply. One of the biggest struggles in agriculture is the quality and availability of water (Boretti and Rosa, 2019). Drought and soil salinization are two main problems that are often related, as local salt concentrations rise when soils dry out. Hence, pathways involved in drought- and salinity responses often overlap, including the signalling molecules and hormones activating them (Gamalero and Glick, 2022; Golldack et al., 2014; Hao et al., 2022; Marusig and Tombesi, 2020; Verma et al., 2022).

Salt and osmotic stress typically trigger the formation of two lipid second messengers, i.e. phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA) (Han and Yang, 2021; Hou et al., 2016; Munnik and Vermeer, 2010; Testerink and Munnik, 2011; Yao and Xue, 2018; Verslues et al., 2023), which are part of the phospholipase C (PLC) pathway. Normally, the concentrations of these lipids are relatively low, in particular of PIP_2, but *in vivo* $^{32}P_i$-radiolabelling experiments revealed that these lipids are rapidly produced in response to salt (NaCl) or osmotic (sorbitol, PEG, mannitol) stress, reacting within seconds to minutes and reaching a maximum at ~30–60 min (DeWald et al., 2001; Konig et al., 2007; Meijer et al., 2001, 2017; Munnik et al., 2000; Takahashi et al., 2001; Zonia and Munnik, 2004; van Leeuwen et al., 2007). The formation of both lipids has been associated with important cellular processes, including vesicular trafficking, cytoskeletal reorganization, and transport of molecules across membranes, which are regulated through recruitment and/or activation of specific protein targets, like protein kinases, phosphatases, small G-proteins and membrane transporters (Doumane et al., 2021; Hou et al., 2016; Ischebeck et al., 2013; Lebecq et al., 2022; Naramoto et al., 2009; Noack and Jaillais, 2017; Pleskot et al., 2013; Pokotylo et al., 2014, 2018; Synek et al., 2021; Testerink and Munnik, 2011; Ufer et al., 2017; Wang et al., 2019; Wu et al., 2017; Yao and Xue, 2018; Yperman et al., 2021; Zhao et al., 2010).

For salt and osmotic stress, a direct interaction between PA and NADPH oxidases, Respiratory burst oxidase homolog D and F (RbohD and RbohF), was uncovered, linking PA with reactive oxygen species) ROS) production (Chapman et al., 2019; Kadota et al., 2015; Zhang et al., 2009). Similarly, PA has been connected to Salt Overly Sensitive 1 (SOS1), an Na^+/H^+ antiporter that pumps Na^+ out of the cell (Qiu et al., 2004). PA binds and activates MAPK kinase 7 and 9(MKK7 and MKK9), which leads to the phosphorylation and activation of Mitogen-activated

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protein kinase (MAPK6) (Shen et al., 2019). Alternatively, PA can directly stimulate the phosphorylation, and activation, of SOS1 through MPK6 (Yu et al., 2010).

For PIP_2, several protein domains that specifically bind its lipid headgroup have been identified, including proteins involved in endo- and exocytosis by interacting with clathrin and EXO70 (de Jong and Munnik, 2021). PIP_2 formation has been shown to be important for membrane identity and creates cell polarity in the tip of root hairs, pollen tubes and cell plate formation during cell division, having consequences for PIN localization, and the organization of the actin cytoskeleton, in particular at the plasma membrane, though PIP_2 has also been found in the nucleus (de Jong and Munnik, 2021; Dieck et al., 2012; Guo et al., 2020; Ischebeck et al., 2010, 2013; Kato et al., 2019; Mei et al., 2012; Noack and Jaillais, 2020; Song et al., 2021; Wu et al., 2017; Xing et al., 2021; Zhao et al., 2010).

The formation of PIP_2 is triggered by activation of PIP 5-kinase (PIP5K), which phosphorylates phosphatidylinositol 4-phosphate (PIP) into PIP_2. For salinity stress, this has recently been shown to involve PIP5K7, -8 and -9 (Kuroda et al., 2021). PLC can hydrolyse both PIP and PIP_2 as substrates, generating diacylglycerol (DAG) and the water-soluble headgroup, i.e. inositol 1,4 phosphate (IP_2) or inositol 1,4, 5 phosphate (IP_3), respectively (Munnik, 2014). DAG is rapidly phosphorylated into PA by DAG kinase (DGK) (Arisz and Munnik, 2013), while IP_2 and IP_3 can be phosphorylated into higher inositolpolyphosphates (IPPs), like IP_5 and IP_6, and even into pyrophosphorylated forms, IP_7 and IP_8 (Laha et al., 2016; Munnik, 2014). Abscisic acid (ABA) has been shown to trigger IP_6 formation in minutes and to release Ca^{2+} from an intracellular store in guard cells, resulting in the closure of stomata (Flores and Smart, 2000; Lemtiri-Chlieh et al., 2000, 2003). A potential role for PLC herein has been confirmed by gene silencing and knock-out (KO) analyses (Hunt et al., 2003; Mills et al., 2004; van Wijk et al., 2018; Zhang et al., 2018a, 2018b).

Besides the PLC/DGK route, PA can also be generated through activation of phospholipase D (PLD), which hydrolyses structural phospholipids, like phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), into PA and the respective alcohol group (choline, ethanolamine or glycerol). Arabidopsis contains 12 PLDs, of which PLDα1 and PLDδ have been implicated in salt- and osmotic stress, as well as in drought (Katagiri et al., 2001; Munnik and Testerink, 2009; Uraji et al., 2012).

Overexpression (OE) of *PLC* has been shown to improve drought tolerance in various plant species. These include *ZmPLC1* in maize (Wang et al., 2008), *BnPLC2* in canola (Georges et al., 2009), *NtPLCδ1* in tobacco (Tripathy et al., 2012), *OsPLDα1* and *OsPLC4* in rice (Abreu et al., 2018; Deng et al., 2019), *GmPLC7* in soybean (Chen et al., 2021a), and *AtPLC2, AtPLC3, AtPLC5* and *AtPLC7* in Arabidopsis (van Wijk et al., 2018; Zhang et al., 2018a; Zhang et al., 2018b, van Hooren et al., 2023). In rice, overexpression of *PLC* also improved the plant's tolerance to salt- and osmotic stress (Deng et al., 2019; Li et al., 2017). Similarly, OE of wheat *PLC1* (TaPLC1) in Arabidopsis increased its salt- and osmotic stress tolerance (Wang et al., 2020).

Xia et al. (2017) recently reported that dexamethasone (DEX)-inducible OE of At*PLC4* in Arabidopsis seedlings resulted in reduced primary root growth under salt stress, while *plc4*-knock-out (KO) mutants showed an improved growth performance under salinity, implying a negative role for PLC4 in salt stress. Considering the above, we found this rather counterintuitive, especially since of all nine Arabidopsis PLCs, *PLC4* has the highest root expression and the highest fold-increase in response to salt stress (Tasma et al., 2008). Hence, we repeated the primary root growth of *plc4*-KO and *PLC4*-OE lines and also added studies on the phospholipid signalling responses when subjected to salt- or osmotic stress and tested the effect of *PLC4*-OE on the drought tolerance of Arabidopsis plants in soil.

2. Results

2.1. PLC4 expression in KO- and OE mutants

In addition to the *plc4-3* (SALK_201150) line used by Xia et al. (2017) we used the *plc4-2* (CS876876/SAIL_791_G05) and two lines with 35S promoter*PLC4-GFP* i.e. *PLC4-OE2*, *PLC4-OE4* constructs that were previously generated (Riveras et al., 2015). The last three of these constructs were not earlier described and thus, to measure the expression level of *PLC4* in the various mutant backgrounds, RT-qPCR was used (Fig. 1). *PLC4-OE2* and *PLC4-OE4* displayed 2.7- and 4.2-times higher expression levels than WT, respectively, while the expression in the *plc4-3* line, was severely reduced and close to zero. Surprisingly, however, *plc4-2*, was found to exhibit *PLC4* levels like WT. Upon further inspection, the T-DNA insertion of p*lc4-2* was found to be positioned at an intron, which may explain why there is no reduced expression. For the rest of the experiments, we disregarded this mutant.

2.2. Primary root growth under salt- and osmotic-stress

Next, we compared the primary root length of *plc4*-KO and *PLC4*-OE lines with WT for their growth under control, salt or osmotic stress conditions. Four-day old plants were transferred to agar plates with or without 100 mM NaCl and grown for another eight days (Fig. 2). Plants exposed to salinity stress had significantly smaller roots than control plants (64–72% of control).

In contrast to what was found by Xia et al. (2017), where *plc4* seedlings grew longer than WT and their DEX inducible *PLC4*-OE grew shorter, we found no significant differences between WT, *plc4-3* and

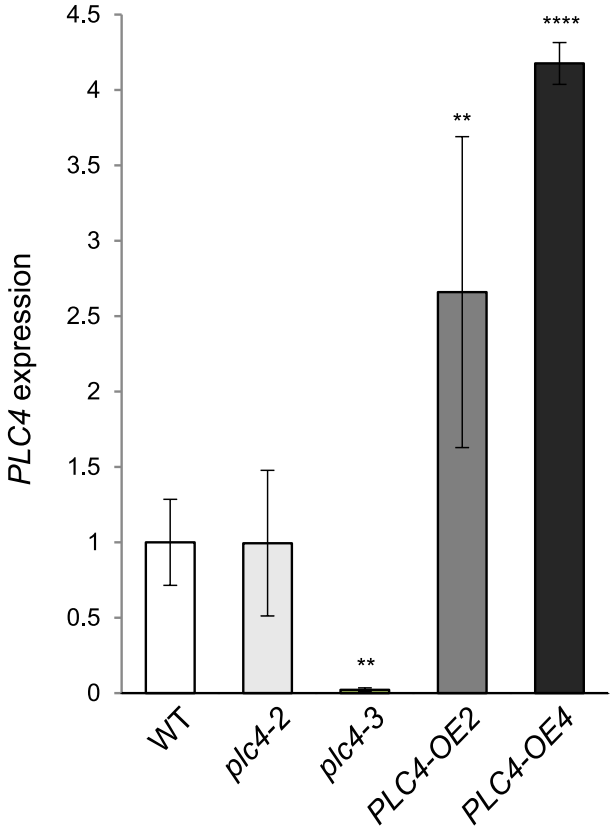

Fig. 1. *PLC4* expression in wild-type and *PLC4* mutants. Expression levels measured by RT-qPCR and normalized to *SAND*. Values are means ± SE of 2 independent experiments with 3 biological replicates each. ANOVA test: **, $P < 0.01$; ****, $P < 0.0001$.

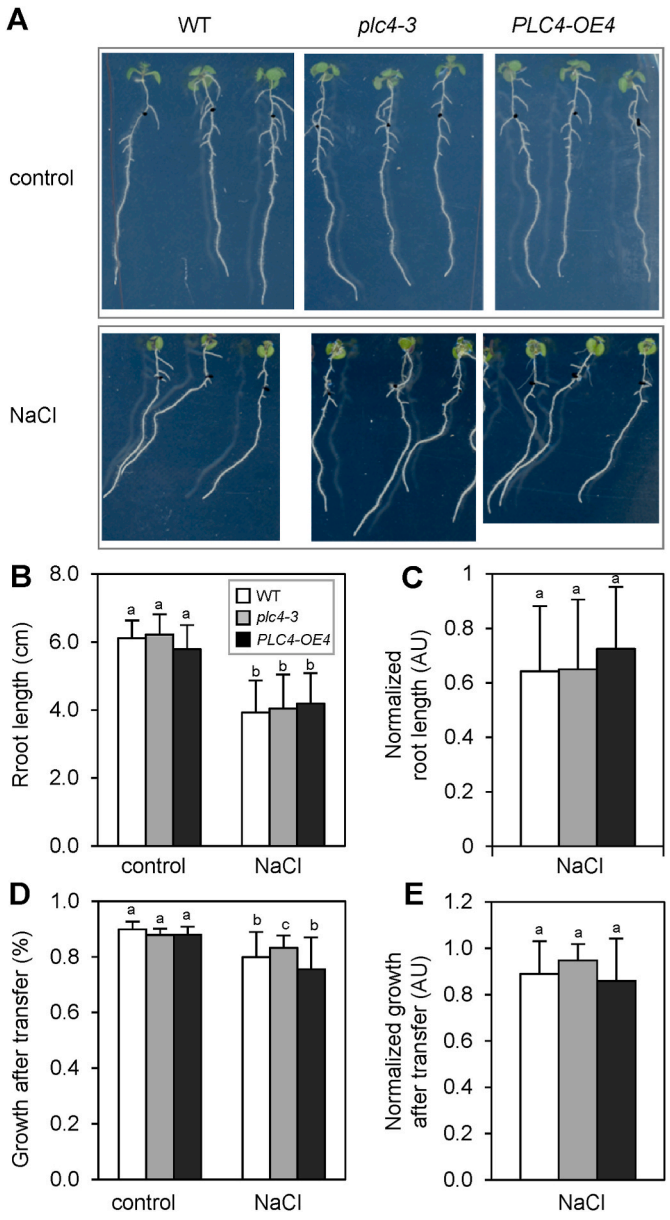

Fig. 2. Effect of salt stress on primary root growth of Arabidopsis WT and *PLC4* mutants. Seedlings were grown for 4 days on ½MS-agar plates and then transferred to ½MS plates supplemented with 100 mM NaCl or control plates without salt. A) Seedlings, 8 days after transfer (DAT). **B**) Primary root length 8 DAT (12 days old). C) Primary root length normalized to their size at control conditions. **D**) Primary root growth after transfer as ratio of total main root length. E) Relative root length normalized to the control conditions in. N = 75–99 from 2 independent experiments with error bars representing SE. Statistics were done using two-way ANOVA test and post hoc TUKEY test. Significance group(s) are indicated with letters ($P < 0.05$).

PLC4-OE4. Small, non-significant changes were observed at control conditions by us, but even then, when normalized, no significant changes were observed (Fig. 2B). Repeated experiments with *PLC4-OE2* instead of *PLC4-OE4* gave similar results, also showing no significant difference in response. As an alternative to total primary root growth, we measured how much primary root growth occurred after the transfer to the salt plates, and visualized this as a percentage of total primary root length (Fig. 2D). As such, a small but significant increase of 4% for the *plc4-3* mutant compared to WT at the salt condition was found. At control conditions, there were small changes too but these were not significant. Normalizing for these small changes, removed all significant changes for the *plc4-3* under salt conditions (Fig. 2E).

Performing the experiments with 200 mM sorbitol, which is osmotically similar to 100 mM NaCl but without the ionic stress, we found *PLC4-OE4* roots were slightly (4.4%), but significantly, smaller at sorbitol conditions (Fig. 3A). However, the significance was lost when normalizing for their size at control conditions (Fig. 3B). As such, no significant changes were found when measuring primary root-growth responses after transferring (Fig. 3C and D).

2.3. Phospholipid responses under salt- and osmotic stress

Previously, salt (NaCl) and osmotic (sorbitol, PEG, mannitol) stress have been described to rapidly trigger changes in the phospholipid profile. More precisely, PIP_2 and PA levels were found to increase while PIP levels decreased (DeWald et al., 2001; Konig et al., 2007; Meijer et al., 2001, 2017; Mishkind et al., 2009; Munnik et al., 2000; Takahashi et al., 2001; van Leeuwen et al., 2007; Zhang et al., 2018a, 2018b).

To investigate the lipid responses in the *plc4-KO* and *PLC4-OE* backgrounds, five-day old WT and mutant seedlings were prelabelled O/N with $^{32}P_i$ and the next day treated for 30 min with either NaCl or sorbitol. Lipids were extracted, run on a TLC and the levels of PIP_2, PIP, and PA measured by phosphoimaging as percentage of total ^{32}P-labelled lipids (Fig. 4, Supplemental table S1A). In response to salt stress, PIP_2 levels went up significantly (Fig. 4A). Less significant but still clear was the increase in PA and the small decrease in PIP (Fig. 4B and C). Interestingly, however, no significant differences between WT, *plc4* or *PLC4-OE* genotypes were observed, neither at control nor at salt stress conditions (Fig. 4A–C).

Earlier, enhanced PIP_2 responses with sorbitol were found for

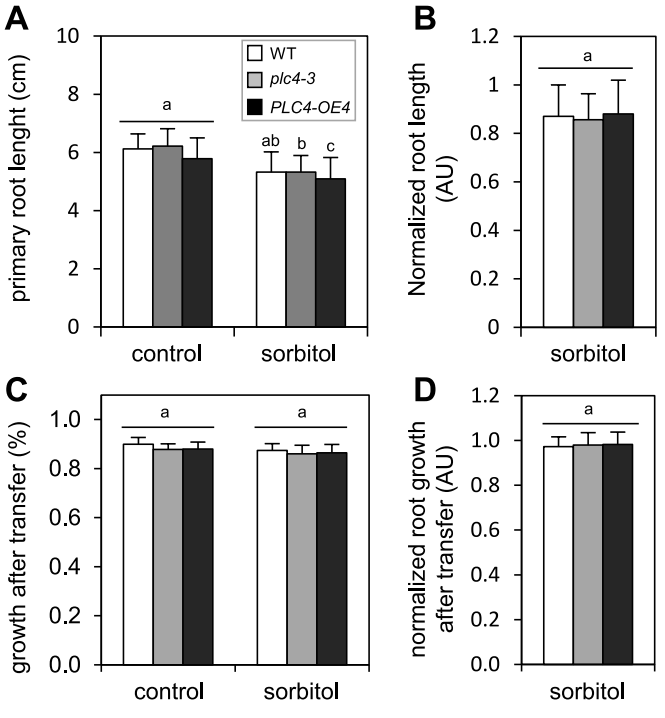

Fig. 3. Effect of hyperosmotic stress on primary root growth of WT and *PLC4* mutants. Seedlings were grown for 4 days on ½MS plates and then transferred to ½MS ± 200 mM sorbitol, after which primary root length was measured 8 DAT. A) Primary root length normalized to their size at control conditions. B) Primary root length normalized to size of genotypes at control conditions. **C**) Primary root growth after transfer as ratio of total main root length. D) Relative root length normalized to the control conditions in N=75-99 from 2 independent experiments with error bars representing SE. Statistics were done using two-way ANOVA test and post hoc TUKEY test. Significance group(s) are indicated with letters ($P<0.05$).

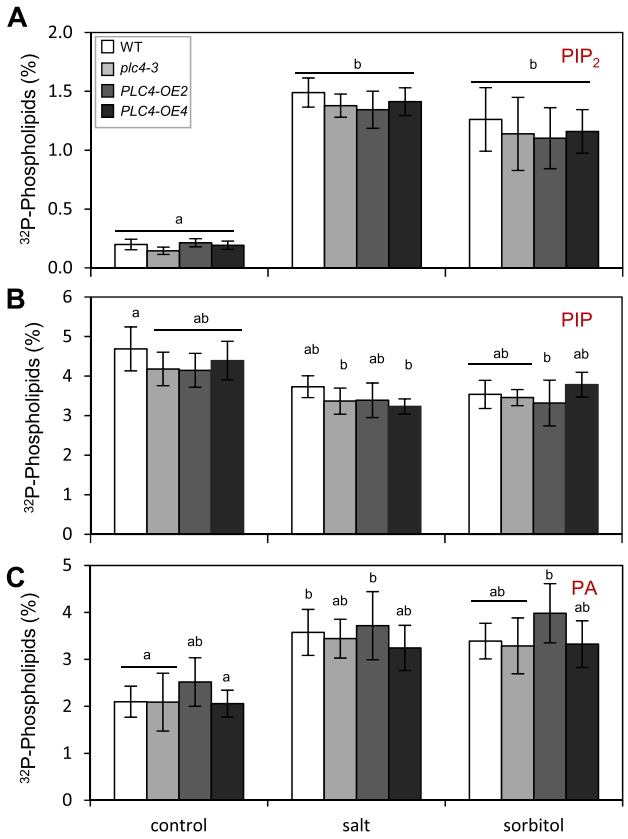

Fig. 4. PPI- and PA levels in WT and *PLC4*-KO and -OE mutants in response to salt- or osmotic stress. Three, Five-day-old seedlings, were grouped, labelled overnight with $^{32}PO_4^{3-}$ and the next day treated with ½ MS medium ±300 mM NaCl or 600 mM for 30 min. Lipids were then extracted, separated by TLC, and quantified by phosphoimaging.
^{32}P-levels of PIP_2 (**A**), PIP (**B**) and PA (**C**) in WT, *plc4-3, PLC4*-OE lines #2 and #4 under control conditions salt- or sorbitol-stress N = 6–13, from 3 to 5 different independent experiments. Error bars are SE. Samples were tested for significance using a two-way ANOVA test and post hoc TUKEY test. Significance group(s) are indicated with letters ($P < 0.05$).

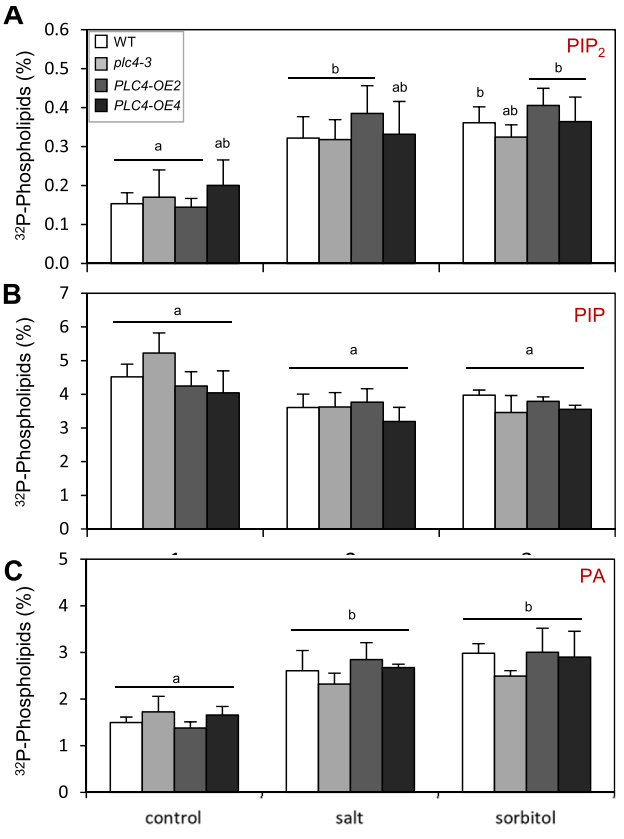

Fig. 5. Rapid PPI- and PA responses in WT and PLC4-KO and -OE mutants in response to salt- or osmotic stress. Three, Five-day-old seedlings, were grouped, labelled overnight with $^{32}PO_4^{3-}$ and the next day treated with ½ MS medium ±300 mM NaCl or 600 mM sorbitol for 5 min. Lipids were then extracted, separated by TLC, and quantified by phosphoimaging.
Data shown are the ^{32}P-levels of PIP_2 (**A**), PIP (**B**) and PA (**C**) in WT, *plc4-3,* and *PLC4* OE lines #2 and #4 under control conditions salt- or sorbitol-stress, with N = from 5 to 10, from 2 to 4 different independent experiments. Error bars are SE. Samples were tested for significance using a two-way ANOVA test and post hoc TUKEY test. Significance group(s) are indicated with letters ($P < 0.05$).

Arabidopsis lines overexpressing *PLC3* and *PLC5,* but not with *PLC7*, (van Wijk et al., 2018; Zhang et al., 2018a, 2018b). Here, sorbitol treatment was found to stimulate PIP_2 and PA formation, and reduced the PIP levels, but again, no significant differences between WT and the various *PLC4* mutants were observed, as was with salt stress (Fig. 4A–C).

In order to make sure earlier effects were not missed, lipid responses were also measured after 5 min of salt or sorbitol stress (Fig. 5, Supplemental table S1B). While the PA responses became stronger, because they are faster than the PIP_2 response, again no significant differences between WT and *PLC4* genotypes were obtained.

In summary, while PLC4 had been implicated in salinity stress earlier (Xia et al., 2017), we were unable to find significant differences in either primary root growth, or in the lipid signalling responses related to PLC, neither in salt nor osmotic stress conditions.

2.4. *Overexpression of PLC4 increases drought stress survival in soil*

Earlier, our lab showed that ectopic overexpression of *PLC2*, *PLC3*, *PLC5*, *PLC7* or *PLC9* in Arabidopsis increased the survival rates when plants were exposed to drought stress (van Wijk et al., 2018; Zhang et al., 2018a, 2018b; van Hooren et al., 2023). Testing this drought survival for *PLC4-OE* lines #2 and #4 grown on soil, a ~2.5 times higher survival rate than for WT plants was obtained (Fig. 6). These results again confirm that *PLC* overexpression in general leads to an improved dehydration tolerance for plants, and that *PLC4* is no exception.

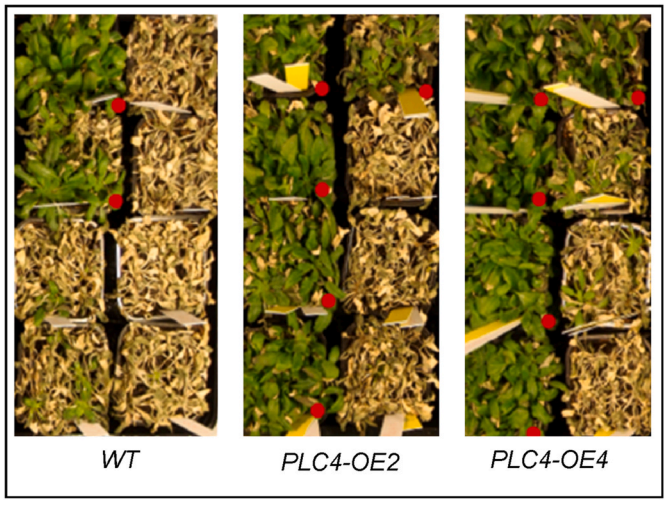

Fig. 6. Overexpression of *PLC4* improves drought tolerance in Arabidopsis. Three weeks old plants were withheld from water for three weeks, after which they were rewatered again. Five days after rewatering they were ordered by survivors per pot and photographed. Red dots indicate surviving plants.

3. Discussion

Overexpression of *PLC* has been shown to improve drought tolerance in various plant species, including Arabidopsis *PLC2, -3, -5, -7, and -9*, of which some also showed improved tolerance to salt and osmotic stress (Deng et al., 2019; Georges et al., 2009; Tripathy et al., 2012; van Wijk et al., 2018; Wang et al., 2008, 2020; Zhang et al., 2018a, 2018b; van Hooren et al., 2023). Recently, Xia et al. (2017) reported an opposite effect on salt tolerance for Arabidopsis *PLC4*: overexpressors being more sensitive to NaCl, while KO mutants were more tolerant. Since this observation was rather striking and unexpected, we decided to test these lines ourselves. Also, because they would make an excellent exception to the rule. However, we found no significant changes in *PLC4*-OE or *plc4*-KO lines compared to WT were found with respect to primary root growth, nor in the lipid responses related to PLC signalling, i.e. PA, PIP and PIP_2.

Differences between our results might be explained by the mode of overexpression. We used constitutive overexpression with the 35S promoter, while Xia et al. (2017) used inducible overexpression with dexamethasone. Our constitutively overexpressed-*PLC4* lines might have adapted to new equilibria (e.g. phosphoinositides, PA and other downstream processes. The inducible lines used by Xia et al. (2017) on the other hand would not have had the time to adapt to the overexpression before being exposed to the salt stress. The combination of sudden *PLC4* OE with salt stress might result in them finding decreased growth for these plants.

Another difference is that our *PLC4-OE* lines contained a C-terminal *GFP* fusion, whereas the line from Xia et al. did not. Theoretically, GFP might interfere with PLC4's function and therefore undo the OE effects. However, we could not find any GFP fluorescence for these lines under the confocal microscope, while we did find increased *PLC4* transcript levels by RT-qPCR (Fig. 1). Moreover, increased survival rates upon drought were found for both independent *PLC4*-OE lines, indicating that the PLC4 is functional.

Xia et al. (2017) proposed that overexpression of *PLC4* would lead to enhanced PIP_2 hydrolysis, which would increase intracellular IP_3- and IP_6 concentrations and release Ca^{2+} under salt stress. Furthermore, they proposed that this increase in Ca^{2+} would lead to a decrease in salt tolerance. Besides the fact that we found no change in PIP or PIP_2 hydrolysis, we would instead argue that an increased Ca^{2+} response would result in an increased stress response and that this increased stress response would enable plants to survive for longer periods under severe stress conditions, as seen in our, but also in other people's, salt- and osmotic stress experiments (Kollist et al., 2019; Kudla et al., 2018; Liu et al., 2020). Earlier, differences in the basal level of PIP_2 or its responses have been found for Arabidopsis *PLC-OE* lines. For example, *PLC3-OE* showed a stronger PIP_2 responses upon sorbitol treatment (Zhang et al., 2018a), while *PLC5-OE* lines contained much lower PIP_2 levels at control conditions, leading to relatively increased PIP_2 responses to sorbitol as compared to WT (Zhang et al., 2018b). For *PLC7-OE* lines, no differences were found (van Wijk et al., 2018), even though *PLC3* and *PLC7* belong to the same clade within the *PLC* gene family (Tasma et al., 2008). *PLC4* and *PLC5* also belong to the same clade, but no difference in the basal or response levels of PIP_2 in the *PLC4-OE* lines were observed. *PLC5-OE* showed a strong root hair phenotype (less and shorter), which turned out to be linked to the disappearance of PIP_2 from the tip of the growing root hair (Zhang et al., 2018b). Such phenotype was not observed for *PLC4-OE* lines, nor for *PLC2, PLC3-*, *PLC7-* and *PLC9*-OE lines (van Wijk et al., 2018; Zhang et al., 2018a, 2018b; van Hooren et al., 2023).

The reason for the observed drought tolerance is still unclear. ABA has been implicated to play a role, as expression of various *PLC* genes has been found to be induced by ABA (Zhang et al., 2018a). Furthermore, all *PLC*-OEs have been found to be more compact than WT plants during drought stress, potentially reducing their water evaporation rate (van Hooren, 2023). Recent transcriptomics and metabolomics have also implicated control of the circadian rhythm and phosphate signalling as potential avenues though which *PLC*-OE might increase drought tolerance, these results have been extensively discussed (van Hooren, 2023).

Besides OE of *PLC2, -3, -4, -5, -7, and -9* in Arabidopsis, the increase in drought tolerance has been found in various other plant systems, including maize (Wang et al., 2008), canola (Georges et al., 2009), tobacco (Tripathy et al., 2012), rice (Deng et al., 2019), wheat (Wang et al., 2020), and soybean (Chen et al., 2021a), strongly suggesting that this is a general effect of *PLC* overexpression. That plant PLCs can play an important role in stress signalling is also evident from other lines of research. Arabidopsis contains nine different *PLC* genes, which show specific and differential inductions by various biotic- and abiotic stresses (Tasma et al., 2008), providing enough redundancy to fine-tune responses and to also compensate effects of KO mutants. Aside from *plc4*-KO mutants, KO mutants of *plc2, plc3, plc7* and *plc9* and KD mutants of *plc5* have been investigated. For *plc9* mutants, reduced thermotolerance has been reported, while PLC9-OE lines revealed increased tolerance (Zheng et al., 2012). For *plc3* and *plc5* mutants, small effects on primary and lateral root formation were found, without getting additive effects in *plc3 plc5*-double mutants (Zhang et al., 2018a, 2018b). In contrast, *plc5 plc7*-double mutants gave several additional phenotypes, including changes in stomatal movement, seed mucilage and leaf serration (van Wijk et al., 2018; Zhang et al., 2018a, 2018b), while the *plc3 plc7* combination was found to be homozygous lethal (van Wijk et al., 2018). Similarly, *plc2* KO is homozygous lethal (Di Fino et al., 2017). A few 'mutant escapes' revealed a defect in female gametogenesis, making it sterile (Di Fino et al., 2017). *PLC2* is also required for plant defence (D'Ambrosio et al., 2017) and is the only *PLC* gene of Arabidopsis that is constitutively expressed (Tasma et al., 2008; van Hooren et al., 2023). There appears to be ecotype-specific differences as well, since a *plc2* KO in the Arabidopsis *Ws* background is viable (Kanehara et al., 2015); all other described *plc* mutants are within the *Col-0* background. In general, issues of lethality, or lack of strong phenotypes might be overcome by generating inducible-KO lines, to find instant disruptive phenotypes in the processes in which the particular PLC is directly involved with rather than working with a mutant who had a chance to compensate its disabilities. Since the effect of PLC could be very local, substrate and product formation may be better linked by expressing and imaging the various lipid biosensors (van Leeuwen et al., 2007; Vermeer et al., 2006; Vermeer et al., 2009; Simon et al., 2014; Vermeer et al., 2017; Li et al., 2023). Unfortunately, there are no biosensors available for IPPs yet (de Jong and Munnik, 2021).

Many of the investigated *PLCs*, when constitutively overexpressed, have been associated with drought and salt, but also heat stress (Abreu et al., 2018; Chen et al., 2021b; Deng et al., 2019; Gao et al., 2014; Georges et al., 2009; Li et al., 2017; Ren et al., 2017; Tripathy et al., 2012; van Wijk et al., 2018; Wang et al., 2008, 2020; Zhang et al., 2018a, 2018b; Zheng et al., 2012), abiotic stresses that often occur together in natural conditions. As the mutants show relatively few phenotypes, we think that the balance in phospholipid metabolism, as well as other pathways, might be shifted in these mutants so growth is not hampered.

4. Conclusion

While Xia et al. (2017) reported a negative role for PLC4 in salt stress tolerance, with roots of *plc4*-KO seedlings being more tolerant to salinity stress while those overexpressing *PLC4* being more sensitive, we were unable to confirm such data. We found no significant differences between WT and *PLC4*-KO- or -OE mutants, nor in their response to osmotic stress using sorbitol. Similarly, no significant differences between WT and *PLC4* mutants in PLC-related lipid responses (PIP, PIP_2 and PA) were observed. However, PLC4-OE plants did show increased drought survival, as has been found for other Arabidopsis PLCs (i.e. *PLC2, -3, -5, -7* and *-9*) and for PLCs in other plant species. We conclude that PLC4 is not specifically, nor negatively, involved in salt- or osmotic stress tolerance and that OE of any PLC seems to promote drought tolerance.

How the latter is generated is still unclear.

5. Experimental

5.1. Arabidopsis lines

Arabidopsis thaliana (Col-0) was used as wild type (WT), and this ecotype is also the genetic background for all mutant lines used in this study. KO lines *plc4-2* (CS876876/SAIL_791_G05) was obtained from Nakamura's lab (Kanehara et al., 2015) while *plc4-3* (SALK_201150) was obtained from the SALK collection (singal.salk.edu) and was previously described by the Ren lab (Xia et al., 2017). Two lines with 35S promoter*PLC4-GFP* i.e. *PLC4-OE2*, *PLC4-OE4* constructs were generously provided by Rodrigo Gutiérrez (Universidad Catolica de Chile) (Riveras et al., 2015).

5.2. Plant growth conditions

Plants were grown either on agar plates or in soil. On agar plates, plants were grown essentially as described earlier (Zhang et al., 2018b). In short, seeds were surface sterilized, stratified for two days in the dark at 4 °C, and then transferred to ½MS medium, supplemented with 1% Daishin agar and 1% sucrose. Plates were cultivated in growth chambers with 16-h light/8-h dark, 100–125 μmol photons m_s^{-2}, at 21 °C. To test the effect of salt- or osmotic stress, seedlings were first grown on normal medium for four days and then transferred to plates with or without 100 mM NaCl or 200 mM sorbitol. Eight days after transfer, seedlings were scanned with an Epson Perfection V700 digital scanner and the primary root length determined using FIJI software with the SmartRoot plugin (Lobet et al., 2011; Schindelin et al., 2012). For each data point 75–99 replicates were measured over two separate experiments. Changes were verified using two-way analysis of variance (ANOVA) followed by post hoc Tukey tests.

For drought tolerance experiments, nine plants per pot (4.5 × 4.5 × 7.5 cm) were grown in soil (Zaaigrond nr. 1, SIR 27010-15, JongKind BV, The Netherlands) for three weeks and watered every other day for constant water availability in the tray. After these three weeks, excess of water was removed and plants were withheld of water for another three weeks. Five days after rewatering, survivors would become green again and scored.

5.3. PLC4 expression analysis

Expression levels of *PLC4* in OE- and KO lines were measured using RT-qPCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad,CA). 0.5 μg of RNA isolated from six-day old seedlings was converted into cDNA using oligo-dT18 primers, dNTPs, and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. An AB 7300 Real-Time PCR system (applied Biosystems) was used for the RT-qPCR. Relative expression levels were determined by comparing the threshold cycle values of *PLC4* to the housekeeping gene, *SAND* (*AT2G28390*). Primers that were used are: *PLC4_qPCR_fwd:* 'CGGAGCTCAAATGATTGC', *PLC4*_qPCR_rev: 'GTCCATTAGGACTTGCATCC'; *SAND_qPCR_fwd:* 'AACTCTATGCAGCATTTGATCCACT' and SAND_qPCR_rev: 'TGATTGCATATCTTTATCGCCATC'. Three technical replicates and three biological replicates were used. Changes were verified using a student T-test.

5.4. $^{32}P_i$-labelling and lipid responses

Experiments were essentially performed as described earlier (Munnik and Zarza, 2013). Per sample, 3 five-day old seedlings were labelled overnight (O/N) with 0.3–0.6 MBq ^{32}P-orthophosphate ($^{32}P_i$). The next day, seedlings were treated for 5 or 30 min in labelling buffer with or without 300 mM NaCl or 600 mM sorbitol for salt- or osmotic stress, respectively. These concentrations have similar osmolalities and are commonly used in such short-term experiments (Munnik and Vermeer, 2010). Lipids were then extracted, separated by thin layer chromatography (TLC) using an alkaline TLC solvent system that separates PIP, PIP_2 and PA from the rest of the phospholipids (Munnik et al., 1994), quantified by phosphoimaging (Typhoon FLA 7000; GE healthcare), and expressed as a percentage of total ^{32}P-labelled phospholipids. Changes were verified using two-way analysis of variance (ANOVA) followed by post hoc Tukey tests.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2023.113862.

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