Phospholipase Dα1 mediates the high-Mg^{2+} stress response partially through regulation of K^+ homeostasis

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June 2, 2020

Abstract

Intracellular levels of Mg^{2+} are tightly regulated, as Mg^{2+} deficiency or excess affects normal plant growth and development. In Arabidopsis, we determined that phospholipase Dα1 (PLDα1) is involved in the stress response to high-magnesium conditions. The T-DNA insertion mutant pldα1 is hypersensitive to increased concentrations of magnesium, exhibiting reduced primary root length and fresh weight. PLDα1 activity increases rapidly after high-Mg^{2+} treatment, and this increase was found to be dose-dependent. Two lines harboring mutations in the HKD motif, which is essential for PLDα1 activity, displayed the same high-Mg^{2+} hypersensitivity of pldα1 plants. Moreover, we show that high concentrations of Mg^{2+} disrupt K^+ homeostasis, and that transcription of K^+ homeostasis-related genes CIPK9 and HAK5 is impaired in pldα1. Additionally, we found that the akt1, hak5 double mutant is hypersensitive to high-Mg^{2+}. We conclude that in Arabidopsis, the enzyme activity of PLDα1 is vital in the response to high-Mg^{2+} conditions, and that PLDα1 mediates this response partially through regulation of K^+ homeostasis.

Keywords

Phospholipase D; magnesium; potassium; homeostasis; *Arabidopsis thaliana* ; HAK5; CIPK9

Acknowledgments

The Czech Science Foundation (grant No. 17-00522S) and the Ministry of Education, Youth and Sports of the Czech Republic (European Regional Development Fund-Project 'Centre for Experimental Plant Biology' no. CZ.02.1.01/0.0/0.0/16_019/0000738) funded this study. The authors wish to thank Kateřina Vltavská for technical assistance, as well as Francisco Rubio from the Departamento de Nutrición Vegetal, Centro de Edafología y Biología Aplicada del Segura-CSIC, Murcia, Spain for providing *akt1, hak5* seeds. The authors declare that they have no conflicts of interest.

Introduction

Magnesium (Mg) is an essential macronutrient. As a cofactor for many enzymes, Mg is required for fundamental cellular processes including energy metabolism, photosynthesis, and the synthesis of nucleic acids and

proteins (Guo, Nazim, Liang & Yang, 2016). Mg is also involved in stress resistance (Huber & Jones, 2013, Mengutay, Ceylan, Kutman & Cakmak, 2013) and well-balanced Mg fertilization enhances crop yield and quality (Wang *et al.* , 2019). The intracellular level of Mg^{2+} is tightly regulated, and its deficiency or excess affects plant growth and development. Although there is a relatively good understanding of the physiological mechanisms responding to magnesium deficiency, not much is known about cellular response to high levels of Mg^{2+}. For example, serpentine soils expose plants to high amounts of Mg and low levels of calcium. Similarly, in semi-arid regions, water stress can lead to the accumulation of Mg^{2+}. For non-adapted plants, high Mg^{2+} conditions inhibit growth. In Arabidopsis, high-Mg^{2+} treatment results in a reduction of primary root length, fresh weight, and epicotyl length (Niu *et al.* , 2018). Plants grown in high-Mg^{2+} soil may avoid Mg^{2+} toxicity by limiting internal Mg^{2+} accumulation and/or Mg^{2+}excretion from leaves. Sequestration of excess Mg^{2+} in the vacuole under high-Mg^{2+} conditions seems to play a pivotal role in Mg^{2+} tolerance. Vacuoles in leaf mesophyll cells can hold up to 80 mM of Mg^{2+} (Hermans, Conn, Chen, Xiao & Verbruggen, 2013).

As with other essential nutrients, magnesium (in its ionic form) is absorbed by plants from the soil. Recently, the signaling mechanism behind the response to high-Mg^{2+} was described. Network of calcineurin B-like calcium sensor proteins (CBL) CBL2/3, CBL-interacting protein kinases (CIPK) CIPK3/9/23/26, and sucrose nonfermenting-1-related protein kinase2 (SnRK2) SRK2D/E/I participate in the regulation of unknown downstream target(s) to confer Mg^{2+} tolerance. Knockout mutants *cbl2/3,cipk3/9/23/26* , and *srk2dD/E/I* showed hypersensitivity to high levels of Mg^{2+}. *srk2d/e/i* showed reduced shoot growth, and *cbl2/3* and *cipk3/9/23/26* showed reduced shoot and root growth under high Mg^{2+} conditions compared to wild type (wt). Moreover, *cbl2/3* and*cipk3/9/23/26* showed significantly less vacuolar Mg^{2+} influx than wt plants, which resulted in a decrease in the cellular concentration of Mg^{2+}. Additionally, SRK2D protein kinase, which is involved in abscisic acid (ABA)-mediated drought response, physically interacts with CIPK3, 9, 23, and 26 (Chen, Peng, Li & Liao, 2018b, Mogami *et al.* , 2015, Tang*et al.* , 2015).

In addition to this signaling network, there is another group of proteins that are involved in high-Mg^{2+} response. Increased ABA content and expression of ABA biosynthesis genes have been reported under high-Mg^{2+} conditions (Guo *et al.* , 2014, Visscher *et al.* , 2010). Moreover, the ABA-insensitive mutant *abi1-1* is less sensitive to high-Mg^{2+}treatment than wt (Guo *et al.* , 2014). These results suggest that ABA signaling is involved in the response to high-Mg^{2+}conditions. Additionally, several other proteins were identified by the increased sensitivity to high-Mg^{2+} of the corresponding knockout mutants. Vacuolar-type H^+-pyrophosphatase (AVP1) (Yang *et al.* , 2018), magnesium transporter 6 (MGT6) (Yan *et al.* , 2018) and mid1-complementing activity (MCA) (MCA1/2 (Yamanaka *et al.* , 2010) are required for high Mg^{2+} tolerance because their knockout mutants are hypersensitive to high-Mg^{2+}. In contrast, knock out mutants of cation exchanger 1 (CAX1) (Bradshaw, 2005, Cheng, Pittman, Barkla, Shigaki & Hirschi, 2003) and nucleoredoxin 1 (NRX1) (Niu *et al.* , 2018) were more resistant to high Mg^{2+}. Interestingly, the last four proteins, MCA1/2, CAX1, and NRX1, are involved in the regulation of cytosolic Ca^{2+} concentration, but the exact molecular mechanisms of their involvement in high Mg^{2+} response are not yet understood. However, CAX1 serves as a calcium-proton antiporter localized in the tonoplast and helps maintain cytoplasmic Ca^{2+} levels (Cheng *et al.* , 2003). The authors speculated that the *cax1* might have higher calcium content, which may have a positive effect under high-Mg^{2+} conditions. Additionally, supplementation of high-Mg^{2+} growth media with calcium alleviates the growth defects typically observed under excess Mg^{2+} (Tang *et al.* , 2015, Yamanaka*et al.* , 2010). Similar to magnesium-calcium, an antagonistic relationship has also been described for magnesium – potassium (Senbayram, Gransee, Wahle & Thiel, 2015). Potassium (K^+) is an essential macronutrient, and its homeostasis is involved in response to abiotic stress caused by salt (Maathuis & Amtmann, 1999, Sun, Kong, Li, Liu & Ding, 2015) or high iron (Zhang *et al.* , 2018). K^+ uptake in Arabidopsis roots is largely controlled by two channels, HAK5 and Arabidopsis K^+ transporter 1 (AKT1) (Santa-Maria, Oliferuk & Moriconi, 2018). HAK5 is activated when the external potassium concentration is below 20 μM (Pyo, Gierth, Schroeder & Cho, 2010). At K^+ concentrations higher than 0.5 mM, AKT1 is crucial (Nieves-Cordones, Martinez, Benito & Rubio, 2016). CIPKs/CBLs are important regulators of K^+ uptake. In yeast (*Saccharomyces cerevisiae*), HAK5 has been shown to be activated by CIPK23-CBL1/8/9/10 complexes. HAK5 is activated after phosphorylation by CIPK23 (Ragel *et al.* , 2015). CBL1/9-CIPK23 also interacts with and activates AKT1 via

phosphorylation (Li, Kim, Cheong, Pandey & Luan, 2006). Additionally, translocation of the Shaker-type K^+ *Arabidopsis thaliana* channel AKT2 from the endoplasmic reticulum to the plasma membrane as well as its activity is modulated by the CBL4-CIPK6 complex (Held *et al.* , 2011).

Plant phospholipase D (PLD) cleaves common phospholipids, such as phosphatidylcholine, to release phosphatidic acid (PA) and free head groups. PA can act as a signaling molecule (Pokotylo, Kravets, Martinec & Ruelland, 2018). In Arabidopsis, there are 12 members of the PLD family, which are sorted by domain structure and biochemical properties. PLDα1, the most abundant PLD in Arabidopsis, reportedly plays a role in stress response, including plant-microbe interactions, wounding, freezing, dehydration, and salinity (Hong *et al.* , 2016, Ruelland*et al.* , 2015, Wang, Guo, Wang & Li, 2014). The protein levels of PLDα1 remain unchanged, but its activity and the amount of PA increase transiently after treatment with NaCl (Zhang *et al.* , 2012). Additionally, transcript levels of *ΠΛΔδ* increase with NaCl treatment (Katagiri, Takahashi & Shinozaki, 2001). Compared to wt, plants with genetically impaired *ΠΛΔα1* have decreased seedling root growth in high-salt medium. A similar phenotype was observed in*πλδδ* plants. However, observation of the double mutant*πλδα1, πλδδ* suggested that individual PLDs act in distinct pathways in the salt stress-response (Bargmann *et al.* , 2009). Moreover, RNAi suppression of both *ΠΛΔγ1* and *ΠΛΔγ2* confers aluminum resistance (Zhao *et al.* , 2011) while genetic manipulation of *ΠΛΔϵ* expression revealed its role in nitrogen signaling (Hong *et al.* , 2009). To summarise, PLDs are involved in a range of abiotic stress responses, including ion toxicity and nutrient sensing, though the exact molecular mechanisms are mostly unknown. However, PA, the product of PLD activity, seems to play a pivotal role.

Ιν τηις στυδψ, ωε σηοω τηατ Αραβιδοπσις πλδα1 ις ηψπερσενσιτιε το ηιγη λεελς οφ μαγνεσιυμ, ανδ τηατ ενζψματισαλλψ-αστιε ΠΛΔα1 ις νεσεσσαρψ φορ Αραβιδοπσις το ρεσπονδ το ηιγη-Μγ $^{2+}$

σονδιτιονς. Ωε αλσο ρεεαλ τηατ ΠΛΔα1 μεδιατες τηις ρεσπονσε παρτιαλλψ τηρουγη ρεγυλατιον οφ K^+ *ηομεοστασις.*

Materials and Methods

Plant materials

All plants were *Arabidopsis thaliana* , of the ecotype Col-0. Knockout lines *πλδα1-1* (SALK_067533), *πλδα1-2*(SALK_053785), *πλδα1-3* (GABI_332D11), *πλδα1-4*(GABI_738H09), and *hak5* (SALK_130604) were obtained from the NASC. The *hak5, akt1* double mutant (Ragel *et al.* , 2015) was provided by Francisco Rubio, at the Departamento de Nutrición Vegetal, Centro de Edafología y Biología Aplicada del Segura-CSIC, Murcia, Spain.

Preparation of transgenic lines

To make complementation lines, *AτΠΛΔα1* (from 3,300 bp upstream of the start codon to the end of the 3' UTR) was amplified from wt genomic DNA with Phusion polymerase (New England Biolabs) and cloned into the pENTR3C vector (Invitrogen). To create mutants mutated in both HKD motifs, megaprimers MP334-F and MP663-R were generated first from wt cDNA using AtPLDa1-F/AtPLDa1-K334R and AtPLDa1-K663R/AtPLDa1-R primers, respectively. Next, primers MP334-F and MP663-R were used to produce AtPLDa1-K334R K663R. The part of DNA containing mutations was cut with HindIII and used to replace corresponding part in wt sequence cloned in pENTR3C. Both entry clones were recombined into the Gateway binary vector pGWB601 (Nakamura *et al.*, 2010) using LR Clonase II (Invitrogen). Final constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was used to transform *plda1-1*plants by floral dip (Clough & Bent, 1998). Transformants were selected by spraying with BASTA. The presence of PLDα1 or mutated PLDα1 in transgenic lines was confirmed by western blotting using anti-PLDα1 antibodies (Fig. 2a).

Growth phenotype analysis

Seeds were sterilized with 30% bleach for 10 min, followed by washing 5 times with sterile water. Plants were grown for 5 d on half-strength MS media, after which they were transferred to agar plates with indicated nutrients for 7 d. They were grown in a growth chamber at 22°C during the day, 21°C at night, under long day (16 h of light) conditions at 100 µmol m^{-2} s^{-1}. Plates were scanned, after which primary root length (primary root growth after the 7 d treatment) was measured from the resulting images in ImageJ (version 1.50b). Plants of the same genotype from one plate (3-6 plants) were pooled and weighed to determine seedlings fresh weight. All experiments were performed with three biological replicates. For the growth response of adult plants to high Mg^{2+} concentration in growth medium, wt and *πλδα1-1* were grown hydroponically for 3 weeks in modified half-strength Hoagland´s media (Hoagland & Arnon, 1950) followed by 10 d in media with or without 10 mM $MgSO_4$. Nutrient concentrations were as follows: $NH_4H_2PO_4$, KNO_3, $Ca(NO_3)_2.4H_2O$, $MgSO_4.7H_2O$, 24.5 µM ferric citrate, 0.45 µM KI, 4.85 µM H_3BO_3, 5.92 µM $MnSO_4.4H_2O$, 0.7 µM $ZnSO_4.7H_2O$, 0.1 µM $Na_2MoO_4.2H_2O$, 0.01 µM $CuSO_4.5H_2O$, 0.01 µM $CoCl_2.6H_2O$, 10.02 µM Na_2EDTA, 10 µM $FeSO_4.7H_2O$, 55.51 µM myo-inositol, 0.81 µM nicotinic acid, 0.49 µM pyridoxin, and 2.97 µM thiamin. Aeration of the hydroponic media was performed every 3 h for 15 min using an aquarium air pump. Nutrient solution was replaced once weekly. Plants were grown in a growth chamber at 22°C during the day, 21 °C at night, under short-day conditions (10 h light per day).

Gene expression analysis

Plants were grown hydroponically, as described above. Four-week-old plants were treated with half-strength modified Hoagland´s media with or without 10 mM $MgSO_4$ for 24 h, after which root and leaf tissue was collected and immediately frozen in liquid nitrogen. RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), DNA was removed using the Turbo DNA-free Kit (Applied Biosystems), and cDNA synthesis was done with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Reverse transcription was done with anchored-oligo(DT)18 primers and 1 µg RNA. Quantitative PCR was done with a LightCycler 480 system (Roche) using the LightCycler 480 SYBR Green I Master mix (Roche). The primers used are listed in Table S1. SAND family protein (At2g28390) was used to normalize target gene transcription values.

Measurement of nutrient content

Seedlings were grown for 7 d on half-strength MS media, after which they were transferred to agar plates with indicated nutrients for 10 d. Plates were kept in a growth chamber at 22°C during the day, 21°C at night, under long day (16 h of light) conditions at 100 µmol m^{-2} s^{-1}. Mg^{2+}-, K^+-, and Ca- content in samples (pooled plants, ˜100 mg dry weight) was determined with inductively coupled plasma optical emission spectroscopy (Spectroblue, Spectro, Germany) analysis in the laboratory of Ekolab Žamberk, Czech Republic).

Western blot analysis

Plants were grown on agar plates for ten days. Protein-extracts from whole seedlings were prepared as described by Novák *et al.*(2018). Proteins were separated on 10% SDS-PAGE and blotted onto nitrocellulose membranes by wet transfer. Membranes were blocked in 5% low fat milk in TBS-T for 1 h, and probed with 1:2,000 anti-PLDα1/2 (AS12 2364, Agrisera, Sweden) in 3% low fat milk in TBS-T for 1 h as well as 1:5,000 goat anti-rabbit (Bethyl) in 5% low fat milk in TBS-T for 1 h. Precision plus protein WesternC standard (Bio-Rad) was used to estimate molecular weights, and this lane was separated from the membrane after blotting and incubated separately in Blocking reagent (Qiagen) in TBS-T for 1 h, followed by 1:10,000 Precision Protein StrepTactin-HRP Conjugate (Biorad) for 1 h. For loading control, the membrane was stained with Novex reversible membrane protein stain (Invitrogen) according to manufacturer´s instructions.

Preparation of the protein soluble fraction

Four-week-old hydroponically grown plants were either kept under control conditions or treated with $MgSO_4$ (10 and 40 mM) for 10, 30, and 180 min. Roots from 6 plants were used to prepare one sample. The soluble fraction was prepared according to Janda *et al.*(2019). Briefly, roots were homogenized using homogenization buffer (50 mM HEPES-NaOH, pH 7.5, 0.4 M sucrose, 0.1 M KCl, 0.1 M $MgCl_2$) with a protease inhibitor cocktail (P 9599, Sigma) and Pierce Phosphatase Inhibitor Mini Tablets (A23957, Thermo Fisher Scientific).

The homogenate was filtered and centrifuged at 6,000 × g for 15 min at 4°C, and the supernatant was centrifuged at 200,000 × g for 1 h at 4°C. The resulting supernatant (the soluble fraction) was collected.

Δετερμινατιον οφ ΠΛΔα αστιτψ ιν προτειν σολυβλε φρασταιονς

Protein concentration of the soluble fraction was determined using a Coomassie Plus Protein Assay (Thermo Scientific). To measure PLDα activity, 15 μg protein were used per assay. The substrate solution consisted of 8 μM fluorescent PC (BODIPY-PC, D3792, Life Technologies), 50 μM 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (Avanti Polar Lipids), 0.015% sodium deoxycholate (MP Biomedicals), and 50 mM MES buffer (pH 6.5). The substrate solution was shaken gently at 23°C for 30 min, followed by sonication for 10 min. The reaction solution contained 20 mM $CaCl_2$, 0.4% *n* -butanol (Lachema), and 25 μL of substrate solution. The reaction was initiated by adding protein solution, and incubated at 28°C with 300 rpm for 30 min. Lipids were extracted and run through HP-TLC silica gel-60 plates as in Krčková*et al.* (2018). Plates were developed in a mobile phase of acetone/ethanol (1/1, by vol.) and laser-scanned using a Fuji FLA-7000 fluorescence scanner. Phosphatidylbutanol spots were quantified using the Fuji FLA-7000 quantification software Multigauge (version (Fujifilm, Japan). BODIPY-phosphatidylbutanol was identified after comparison to the standard, prepared using commercially available PLD (Sigma) (Pejchar*et al.* , 2010).

Results

πλδα1 ις ηψπερσενσιτιε το ηιγη λεελς οφ $Mγ^{2+}$

We grew *πλδα1-1* (Bargmann *et al.* , 2009) seedlings in varying concentrations of diverse nutrients including Mg^{2+}. *πλδα1-1* was hypersensitive to magnesium, with reduced primary root length, fresh weight (Fig. 1a,b,c), and number and length of lateral roots (Fig. S1.). A significant decrease in*πλδα1-1* primary root length was observed after application of 1 mM $MgCl_2$; at this conditions, *πλδα1-1* primary roots were found to be 6% shorter than wt (Fig. 1a,b). Concentrations of 5 mM $MgCl_2$ and higher had a severe effect on the growth of wt plants; however, in all studied concentrations,*πλδα1-1* was more sensitive. The greatest difference in primary root length between wt and *πλδα1-1* was observed in plants treated with 15 mM $MgCl_2$, where *πλδα1-1* roots were 40% shorter (Fig. 1b). The greatest difference in fresh weight between wt and *πλδα1-1* was observed with 10 mM $MgCl_2$, where*πλδα1-1* was half the weight of wt (Fig. 1c).

To determine whether the $MgCl_2$ hypersensitivity observed in seedlings persists in mature plants, wt and *πλδα1-1* were grown hydroponically. $MgSO_4$ (at 10 mM) was added to the hydroponic solution and the plants were maintained for 10 days. Reduced growth in *πλδα1-1* compared to wt plants was markedly visible (Fig. 1d). However, because magnesium sulfate was used instead of magnesium chloride, it was necessary to rule out the possible effects of other ions. Plants were treated with 10 mM $MgCl_2$, $MgSO_4$, or $Mg(NO_3)_2$, and growth was assessed. Although there were visible variations in the effect of individual anions, the significant difference between wt and *πλδα1-1* was clearly detectable in all cases (Fig. S2). Therefore, it is possible to rule out that the anion is responsible for the observed *πλδα1-1* phenotype.

To ensure that the observed Mg^{2+} hypersensitivity was due to an insertion in *ΠΛΔα1* and no other genes, we used three additional T-DNA insertion lines for *ΠΛΔα1* , including*πλδα1-2* (described by Bargmann et al. (2009)), *πλδα1-3*(SALK), and *πλδα1-4* (GABI-KAT). We also made complementation lines by transforming *πλδα1-1* plants with *ΠΛΔα1* driven by its native promoter. The levels of PLDα1 (PLDα1-Com) protein in seedling extracts was verified using anti-PLDα1/2 antibody. PLDα1 was not detected in any of the *πλδα1* lines (Fig. 2a). Levels of PLDα1 in the complementation lines were lower than in wt; therefore, the two PLDα1-Com lines with the highest PLDα1 protein levels were used for subsequent analyses. *πλδα1-2, πλδα1-3* , *πλδα1-4,* and complementation lines were phenotyped for Mg^{2+}sensitivity. Primary root length was 23, 25, and 26% lower in*πλδα1-2* , *πλδα1-3,* and *πλδα1-4* , respectively. Fresh weight was 52, 53, and 54% lower in *πλδα1-2* , *πλδα1-3,* and*πλδα1-4* , respectively, compared to wt when treated with high Mg^{2+} (Fig. 2 c, d, e). Overall, all PLDα1 mutants were similarly Mg^{2+}-sentitive to *πλδα1-1* . In contrast, primary root length and

fresh weight in lines *πλδα1-1* -Com1 and *πλδα1-1* -Com2 were similar to wt when treated with high Mg^{2+} (Fig. S3).

Arabidopsis *πλδα1* plants are more sensitive to high-Mg^{2+} conditions than wt; thus, PLDα1 appears to be involved in response to high-Mg conditions. These results uncovered a novel physiological role of PLDα1 in the context of Mg^{2+}-homeostasis.

ΠΛΔα1 αςτιτψ ινςρεασες αφτερ τρεατμεντ ωιτη Μγ²⁺

Next, we investigated whether high levels of Mg^{2+}could trigger changes in PLDα1 activity. Arabidopsis has 12 genes encoding PLDs, which differ biochemically and require different *in vitro* conditions for activation (Hong *et al.* , 2016). PLDs cleave ordinary phospholipids such as phosphatidylcholine, releasing PA and free head group, e.g. choline. PA is also the product of diacylglycerol kinase activity, as well as the substrate for PA phosphatase, among other enzymes (Ruelland *et al.* , 2015). Hence, PA levels do not necessarily correlate with PLD activity. A unique feature of PLDs is their so-called transphosphatidylation activity, where, in the presence of primary alcohols such as *n* -butanol, PLD transfers the phosphatidyl group from its substrate to *n* -butanol, releasing phosphatidylbutanol (PBut). PBut-formation therefore directly corresponds to PLD activity (deVrije & Munnik, 1997).

PLDα1 is known to be both membrane-associated and cytosolic (Fan, Zheng, Cui & Wang, 1999). Predominant cytosolic localization was reported by Novák *et al.* (2018) in Arabidopsis expressing PLDα1-YFP, therefore we determined PLDα activity in the soluble fraction. Plants were treated with 10 or 40 mM $MgSO_4$, after which root samples were taken at 10, 30, and 180 min. The soluble fraction was prepared, and the activity of PLDα was determined using fluorescently labeled phosphatidylcholine as a substrate under conditions optimal for PLDα (Hong, Zheng & Wang, 2008). Lipids, including PBut, were extracted and separated using high-performance thin-layer chromatography (HP-TLC), and the amount of fluorescently labeled PBut was quantified (Fig. 3). PLDα activity was also measured in samples prepared from *πλδα1-1*plants, where PLDα activity was found to be negligible (Fig. 3a). Hence, we concluded that the quantity of released PBut corresponds to PLDα1 activity.

PLDα1 activity in the soluble fraction increased after $MgSO_4$ treatment (Fig. 3b). The increase was concentration-dependent, as higher concentrations of $MgSO_4$ consistently led to an increase in PLDα1 activity (Fig. 3b,c). Interestingly, the increase in PLDa1 activity was transient, reaching 2.5-fold after 30-minutes of treatment with 10 mM $MgSO_4$ (Fig. 3d).

An increase in PLDα1 activity could be due to higher rates of transcription of *ΠΛΔα1* , activation of PLDα1, or a combination of the two. Thus, we measured transcriptional levels of *ΠΛΔα1* in control and high-Mg^{2+} treated (10 mM $MgSO_4$, for 24h) plants using quantitative RT-PCR. We found no increase in *ΠΛΔα1* transcript levels following Mg^{2+} treatment (Fig. 3e).

These results demonstrate that PLDα1 is activated by Mg^{2+} shortly after treatment, though not at the transcriptional level.

ΠΛΔα1 αςτιτψ ςονντριβυτες το ηιγη- Μγ²⁺τολερανςε

To confirm that the activity of PLDα1 is essential for high-magnesium tolerance in wt plants, we introduced an inactive mutant for*ΠΛΔα1* into *πλδα1-1* plants (*p* PLDα1::PLDα1-Mut/*πλδα1*). Members of the PLD superfamily retain the highly conserved HKD motif, which is encoded twice in higher-plant PLDs (Wang *et al.* , 2014). Point mutations in HKD motifs result in the complete loss of PLD activity in *Brassica oleracea* (Lerchner, Mansfeld, Kuppe & Ulbrich-Hofmann, 2006), as well as in humans and mice (Sung *et al.* , 1997).

Transgenic *πλδα1-1* plants expressing*πΠΛΔα1* ::*ΠΛΔα1* $_{K334R;K663R}$ (lines Mut1 and Mut2) at levels consistent with wt (Fig. 2a) showed similar sensitivity to $MgCl_2$ as *πλδα1-1* , for both primary root length (Fig. 4a) and fresh weight (Fig. 4b).

These results demonstrate that Arabidopsis PLDα1 activity is critical in mediation of the response to high-magnesium conditions.

πλδα1 ασσυμυλατες λεσς $Mγ^{2+}$ ανδ K^+ υνδερ ηιγη-$Mγ^{2+}$σονδιτιονς

To elucidate the possible mechanism responsible for the higher susceptibility of *πλδα1* , we measured Mg^{2+}-content in wt and mutant plants under control and high-Mg^{2+} conditions. After high-Mg^{2+} (10 mM) treatment, seedling Mg^{2+} levels were elevated by about five times in wt and *πλδα1-1.* Nevertheless, *πλδα1* showed significantly lower Mg^{2+}-content than wt (Fig. 5a).

There is known to be an antagonistic relationship between the uptake of Mg^{2+} and Ca^{2+} (Guo, Babourina, Christopher, Borsic & Rengel, 2010, Yamanaka *et al.* , 2010). Moreover, increased levels of Mg^{2+} application in rice results in lower uptake of calcium and potassium (Fageria, 2001), and transcription of the potassium transporter *HAK5* increases following treatment with Mg^{2+} in Arabidopsis (Tang & Luan, 2017, Visscher *et al.* , 2010). Therefore, we measured Ca^{2+} and K^+ content in the wt and*πλδα1-1* plants to investigate these relationships.

In agreement with Mg-Ca antagonism, seedling Ca^{2+}content was lower when 10 mM $MgCl_2$ was added to the agar medium. However, we did not observe any difference between wt and*πλδα1-1* (Fig. 5b). Interestingly, K^+ levels in wt and *πλδα1-1* were lower in Mg^{2+}-treated plants, with *πλδα1-1* plants retaining even less K^+ than wt (Fig. 5c).

These results demonstrate that PLDα1 is involved in the regulation of Mg^{2+}- and K^+- content in Arabidopsis seedlings grown in high-Mg^{2+} conditions.

Αδδιτιον οφ $ä^{2+}$ ανδ K^+ αλλειατες $Mγ^{2+}$-ηψπερσενσιτιτψ ιν πλδα1 πλαντς

Aware that there is an antagonistic relationship between some of the essential nutrients, we investigated whether excess Ca^{2+} or K^+ could affect *πλδα1*hypersensitivity to Mg^{2+}. Application of both Ca^{2+} and K^+ ameliorate growth in*πλδα1-1* in high-Mg^{2+} (Fig. 6a). Addition of Ca^{2+} completely restored the growth of *πλδα1-1*to wt levels, in both root length and fresh weight. Roots from both*πλδα1-1* and wt were smaller (Fig. 6b), while fresh weight for both was similar, compared to control conditions (Fig. 6c). Application of K^+ lowered the root-length difference between wt and *πλδα1* . With Mg^{2+}, root length of*πλδα1-1* was 82.5% that of wt. However, with K^+, root length of *πλδα1-1* increased to 94.9% that of wt. Fresh weight under high-Mg^{2+} in*πλδα1-1* was brought up to wt levels with K^+(Fig. 6c), though root length and fresh weight in both *πλδα1-1*and wt were lower compared to the control conditions (Fig. 6).

Addition of Ca^{2+} increased plant growth in both wt and*πλδα1-1* plants (Fig. 6). However, no difference in Ca^{2+} content between wt and *πλδα1-1* was detected (Fig 5b). These results, along with what is known about the antagonistic relationship between Ca and Mg, suggest that Ca^{2+} deficiency is not the underlying factor behind growth defects in *πλδα1* grown under high-Mg^{2+}, but that high Mg^{2+} or low K^+ content is responsible. Under high-Mg^{2+} conditions,*πλδα1-1* ρεταινεδ λεσς $Mγ^{2+}$ τηαν ωτ, τηουγη τηε λοωερ $Mγ^{2+}$-σοντεντ ωας μορε τοξις το *πλδα1-1*than the higher-Mg^{2+} content in wt. This phenomenon may be explained by impairment of Mg^{2+} sequestration in *πλδα1-1* , which would result in higher cytosolic concentrations of Mg^{2+}. Additionally, lower K^+content may contribute to impaired growth in *πλδα1-1* , or a combination of the two mechanisms.

K^+-ρελατεδ γενες ‘ΙΠΚ9 ανδ HAK5 αρε νοτ τρανσςριπτιοναλλψ υπρεγυλατεδ ιν πλδα1

Ten members of the MGT family were identified in the Arabidopsis genome, (Li, Tutone, Drummond, Gardner & Luan, 2001). Therefore, we investigated transcriptional response of MGT family genes, of which Arabidopsis has 10 (Li *et al.* , 2001), to high-magnesium stress. Transcript levels were determined using

quantitative RT-PCR in roots and leaves of wt and *πλδα1-1* plants, separately (Fig. 7a). Transcript levels of *MGT1* in roots and*MGT7* in leaves was slightly elevated in Mg^{2+}-treated plants, though there was no difference between wt and *πλδα1-1* (Fig. 7a). Transcript levels for two genes are not shown, as *MGT5* was under the detection limit and*MGT8* was found to be a pseudogene (Zhang *et al.* , 2019).

In Arabidopsis, CAX1 is known to be involved in high-Mg^{2+} resistance (Bradshaw, 2005). Moreover, transcription of *CAX1* is downregulated under high-Mg^{2+} conditions (Visscher *et al.* , 2010). Hence, we determined transcriptional levels of *CAX1* in control and Mg^{2+}-treated wt and *πλδα1-1* plants.*CAX1* transcription was decreased in the roots and leaves of Mg^{2+}-treated plants; however, as in case of MGT genes, there was no difference between wt and *πλδα1-1* (Fig. 7b).

Next, we looked at transcription of *CIPK9* and *HAK5* , both of which are known to be involved in potassium homeostasis under low-potassium conditions (Coskun, Britto & Kronzucker, 2014), and are reportedly upregulated in high-magnesium conditions (Tang *et al.* , 2015, Visscher *et al.* , 2010). Furthermore, Arabidopsis CIPK9 has also been shown to participate in high-Mg^{2+} response (Tang *et al.* , 2015). In agreement with Visscher et al. (2010), we observed high upregulation of *CIPK9* and *HAK5* in wt roots after Mg^{2+} treatment. However, the increase in*CIPK9* and *HAK5* transcript levels was almost completely abolished in *πλδα1-1* roots (Fig. 7b). In Mg^{2+}-treated *πλδα1-1* leaves, *CIPK9*transcript levels were slightly increased (˜doubled), and *HAK5* was not detected; however, there was no difference between wt and *πλδα1-1* (Fig. 7b).

These results indicate that PLDα1 is essential in a signaling mechanism which leads to an increased expression of *HAK5* and *CIPK9*in roots upon high-Mg^{2+} treatment.

The hak5, akt1 double mutant is hypersensitive to high-magnesium

Based on our previous observations, we speculated that proper regulation of potassium homeostasis is essential in high Mg^{2+}-conditions. We examined whether *hak5*plants are hypersensitive to high-magnesium, and found no difference between *hak5* and wt (Fig. 8). Next, we tested the sensitivity of a *hak5* , *akt1* double mutant to high-$Mg^{2+}$and found that it was significantly more sensitive than wt. Root length in *hak5* , *akt1* plants was 9.5% (Fig. 8b), and fresh weight 14.5%, less than in wt (Fig. 8c). Under control conditions,*hak5, akt1* growth did not differ from wt (Fig. 8).

However, there was still a significant difference between *πλδα1*and *hak5, akt1* sensitivity to high-Mg^{2+}.*πλδα1-1* roots were 24%, and fresh weight 47%, less than wt; thus, *hak5, akt1* is less sensitive to high-Mg^{2+} compared to *plda1-1*(Fig. 8b,c).

These results revealed that plants impaired in K^+uptake are also compromised in their tolerance to high levels of Mg^{2+}. Therefore, appropriate regulation of potassium homeostasis is key to that of magnesium.

We conclude that in Arabidopsis, K^+-homeostasis is involved in response to high-Mg^{2+}, and that this mechanism is at least partially mediated by PLDα1.

Discussion

Magnesium is an essential and abundant macronutrient, though its importance has been overlooked. Magnesium is the eighth-most widespread element on Earth, and is well soluble and highly mobile in soil (Guo, 2017). Typically, Arabidopsis is cultivated *in vitro* on full-strength (containing 1.5 mM Mg^{2+}) or half-strength (with 0.75 mM Mg^2+) MS media. We used half-strength MS in our study, and root growth differences between wt and *πλδα1* were observed with 1 mM more Mg^{2+}added to the media (Fig. 1).

High extracellular concentrations of Mg^{2+} lead to higher intracellular concentrations (Guo *et al.* , 2014, Mogami*et al.* , 2015, Tang *et al.* , 2015, Yan *et al.* , 2018). The concentration of Mg^{2+} in the plant cell differs significantly between cellular compartments. Less than 1 mM Mg^{2+} is found in mitochondria (0.2-0.5

mM), the cytosol (0.2-0.4 mM), and apoplast (0.2-0.5 mM). The vacuole (5-80 mM) and chloroplasts (1-5 mM) contain the highest concentrations (Hermans*et al.* , 2013). Sequestration of excess Mg^{2+} to the vacuole, or possibly to the endoplasmic reticulum, is presumably a key mechanism for Mg^{2+} tolerance. The plant-specific Mg^{2+}/H^{+} exchanger (MHX), MGT2, and MGT3 reportedly play a role in the sequestration of Mg^{2+} in vacuoles (Conn *et al.* , 2011, Shaul*et al.* , 1999), though knockout lines for these genes exhibit wt-like response to high-Mg^{2+} conditions. Hence, the participation of these proteins in Mg^{2+} tolerance is not clear. Detoxification of high-Mg^{2+} via Mg^{2+}-vacuolar sequestration is regulated by calcium sensors CBL2/3 and their downstream component CIPK3/9/23/26 protein kinases (Tang *et al.* , 2015). The *cbl2, cbl3* double mutant is hypersensitive to high-Mg^{2+}. Interestingly, when grown under under high-Mg^{2+} conditions, *cbl2, cbl3* was found to retain lower concentrations of Mg^{2+}compared to wt, though the lower Mg^{2+} concentration appeared to be more toxic to *cbl2, cbl3* , as it grew less than wt (Tang *et al.* , 2015). This observation may be explained by reduced vacuolar-sequestration. We also found lower levels of Mg^{2+} in *πλδα1* plants (Fig. 5). Thus, reduced vacuolar Mg^{2+}-sequestration should be considered. Interplay between PLDα1 and CIPK9 likely contributes to the Mg^{2+} hypersensitivity observed in *πλδα1* , as supported by the observed decrease in transcript levels of *CIPK9*in *πλδα1* roots (Fig. 7).

mgt6 plants were also found to be hypersensitive to high-Mg^{2+}, and contained less Mg^{2+}in the shoot, compared to wt. Under high-Mg^{2+}conditions, MGT6 likely mediates the transport of Mg^{2+}into shoot tissues (Yan *et al.* , 2018). Additionally, the MGT7 mutant *mrs2-7* is more sensitive to high-Mg^{2+}than wt (Oda *et al.* , 2016). As MGT6 and MGT7 reportedly localize to the endoplasmic reticulum, it has been suggested that they also act as a bi-directional transporters, thus maintaining cytosolic concentrations of Mg^{2+} using the ER as a storage location (Oda *et al.* , 2016).

Nutrient antagonism and ion homeostasis in plant

More than the Mg^{2+} concentration alone, the ratios of Mg^{2+} to Ca^{2+} and Mg^{2+} to K^{+} appear to contribute to the *plda1* phenotype (Fig. 6). Interference in the uptake of Mg^{2+}, Ca^{2+}, and $K^{+}$by plants (sometimes called "nutrient antagonism") has been widely reported (Diem & Godbold, 1993, Fageria, 2001, Pathak & Kalra, 1971). However, the molecular mechanism of nutrient antagonism is not yet fully understood.

High levels of external Ca^{2+} result in reduced uptake of Mg^{2+}, and vice versa (Fageria, 2001, Mogami*et al.* , 2015, Tang *et al.* , 2015, Yan *et al.* , 2018). In agreement with these reports, we found that Arabidopsis seedlings accumulate less Ca^{2+} upon treatment with high-Mg^{2+}. We also observed that addition of Ca^{2+} alleviates the reduction in growth typically seen under high-Mg^{2+} conditions (Fig. 6). Moreover, altered sensitivity to high-Mg^{2+} of plants with genetically-impaired Ca^{2+} homeostasis proteins MCA1/2, CAX1, and NRX1 has been demonstrated (Bradshaw, 2005, Niu *et al.* , 2018, Yamanaka *et al.* , 2010). However, Ca^{2+}content in *πλδα1* does not appear to differ from wt under high-Mg^{2+} (Fig. 5); thus, *πλδα1*Mg^{2+} hypersensitivity is most likely not caused by altered Ca^{2+} homeostasis.

Likewise, high levels of external K^{+} result in reduced uptake of Mg^{2+} (Ding, Luo & Xu, 2006, Fageria, 2001), and an effect of high-Mg^{2+} on K^{+}uptake has also been reported in Arabidopsis (Mogami *et al.* , 2015), though a more in-depth study of this phenomenon is needed. Authors observed lower K^{+}-content in the aerial parts of plants growth under high external concentrations of Mg^{2+}. It is possible that K^{+} and Mg^{2+} compete for the use of Mg^{2+}transporters, as it has been reported that the monocot K^{+} transporters *Os* HKT2;4 and *Ta* HKT2;1 can transport Mg^{2+} (Horie *et al.* , 2011). Shabala and Hariadi (2005) suggest that at least two mechanisms are involved in Mg^{2+}-uptake through the plasma membrane, one of which allows for uptake of K^{+} and Ca^{2+}. Later, Guo et al. (2010) observed in Arabidopsis that suppression of the cyclic nucleotide-gated channel (CNGC10) led to decreased influx of K^{+}, Ca^{2+}, and Mg^{2+}, implicating involvement of CNGC10 in Ca^{2+} and Mg^{2+} transport, and by extension, K^{+} transport.

We found that wt seedlings treated with high-Mg^{2+} had lower concentrations of K^{+} (Fig. 5), and that K^{+} was even lower in *πλδα1* . Additionally, we impaired transcription of *HAK5* and *CIPK9* (genes involved in K^{+} homeostasis) in *πλδα1* treated with high-Mg^{2+} (Fig. 7). In low-K^{+}conditions, CIPK9 regulates K^{+} homeostasis (Liu, Ren, Chen, Wang & Wu, 2013, Pandey *et al.* , 2007), while HAK5 is largely responsible

for its uptake (Rubio, Aleman, Nieves-Cordones & Martinez, 2010). We hypothesize that high external Mg^{2+} concentrations lead to a decrease in intracellular K^+ concentrations; thus, activating a not yet fully understood compensation mechanism regulated by PLDα1, HAK5, and potentially CIPK9. The significance of the K^+ compensation mechanism is seen in Arabidopsis mutants for two proteins involved in K^+ uptake, HAK5 and AKT1, which display increased sensitivity to high-Mg^{2+} (Fig. 8). Importance of AKT1 and HAK5 for K^+ uptake in high-Mg^{2+} conditions was also shown by Caballero et al. (2012), where a significant decrease in K^+ uptake in mature *akt1, hak5* Arabidopsis plants was found. However, altered K^+-accumulation in the *πλδα1* vacuole cannot be excluded as well.

ΠΛΔα1 ανδ ΠΑ αρε ινολεδ ιν στρεσς ρεσπονσες

We found that PLDα1 activity (prepared from Arabidopsis roots) was rapidly and transiently increased in response to high-Mg^{2+} (Fig. 3). Phospholipase Dα1 belongs to the C2 subfamily of plant PLDs, and is activated by millimolar concentrations of Ca^{2+}. PLDα1 prefers phosphatidylcholine to phosphatidylethanolamine as a substrate (Kolesnikov *et al.* , 2012, Wang *et al.* , 2014). Protein phosphorylation may also regulate PLDα1 activity, as it is predicted to have phosphorylation sites (Takáč *et al.* , 2016) and phosphorylated PLDα1 has been detected in response to drought stress (Umezawa *et al.* , 2013). Phospholipase Dα1 localizes predominantly to the cytosol; however, when stressed (such as through wounding or dehydration), it translocates to membranes (Chen *et al.* , 2018a, Wang *et al.* , 2000).

PLDα1 releases PA, which serves as an important secondary messenger and as a precursor in lipid biosynthesis. Elevated levels of PA have been described in response to many abiotic stresses, including salinity, drought, cold, injury, and heat, as well as biotic stresses (Hou, Ufer & Bartels, 2016, Testerink & Munnik, 2005, Vergnolle *et al.* , 2005, Wang *et al.* , 2014, Zhao, 2015). The molecular mechanism of PA as a signaling molecule appears fairly diverse, as a wide range of PA-binding proteins have been identified, including lipid transporters, protein kinases, and enzymes such as NADPH oxidase respiratory burst oxidase homologs D and F (RbohD/F) (Hong *et al.* , 2016, Pokotylo*et al.* , 2018, Yao & Xue, 2018).

Possible mechanisms of PLDa1 activity in magnesium and potassium homeostasis

We found that high-Mg^{2+} hypersensitivity of Arabidopsis *πλδα1* and plants producing inactive PLDα1 protein was the same (Fig. 4), demonstrating that PLDα1 activity is key in regulating the response to increased Mg^{2+}concentrations. Although it cannot be ruled out that choline also plays a role, we assume that PA functions as a key molecule. Several molecular mechanisms for PA regulation have been hypothesized. ABA is known to be involved in response to high-Mg^{2+} conditions in Arabidopsis (Guo *et al.* , 2014), and PA is known to participate in ABA signaling in various ways. The PA produced through PLDα1 activity interacts with protein phosphatase 2C (PP2C), heterotrimetric GTP-binding protein, and RbohD/F (Mishra, Zhang, Deng, Zhao & Wang, 2006, Zhang, Qin, Zhao & Wang, 2004, Zhang *et al.* , 2009), all of which mediate ABA signaling. PA produced by PLDα1 also interacts directly with regulator of G-protein signaling 1 (RGS1), modulates the level of active Gα, and consequently, ABA signaling (Choudhury & Pandey, 2017, Zhao & Wang, 2004). Phytosphingosine-1-phosphate (phyto-S1P) has been identified as a lipid messenger, generated by sphingosine kinases (SPHKs), that mediates ABA response. PA binds to SPHK1 and SPHK2, stimulating their activity; thus, regulating ABA response (Guo, Mishra, Taylor & Wang, 2011). Arabidopsis PA has also been shown to interact directly with class 1 protein kinases SnRK2.4, and SnRK2.10 (McLoughlin *et al.* , 2012). Proteins from the same family (but in class 3), SnRK2d (SnRK2.2), SnRK2e (SnRK2.6), and SnRK2i (SnRK2.3) are known to be part of the high-Mg^{2+}response. However, further research is needed to confirm the participation the proteins discussed here in the Arabidopsis PA-high Mg^{2+} response.

There are also several ways in which PA affects K^+homeostasis. PA has been shown to bind to potassium channel β subunit 1 (KAB1) (McLoughlin *et al.* , 2013), which, in Arabidopsis, physically associates with the inward-rectifying potassium channel 1 (KAT1) (Tang, Vasconcelos & Berkowitz, 1996). KAT1 appears

to be crucial for turgor-pressure changes in guard cells (Pilot *et al.* , 2001). Whether KAT1 is functional in the roots has yet to be investigated. Recently, PA-mediated inhibition of Shaker K^+ channel AKT2 in Arabidopsis and rice was reported (Shen *et al.* , 2020).

The mode of action of PA in the regulation of the rat voltage-gated potassium channel Kv1 has been studied in detail, with experiments revealing two effects of PA on Kv1 gating. The first method is generic, where the negative charge in PA shifts the membrane voltage. The second method is more specific to phosphatidic acid, where the negatively-charged end of the molecule interacts with the part portion of the channel that senses voltage changes in order to keep the pore closed. Whether a similar mechanism is used in the regulation of plant K^+ channels remains to be investigated (Hite, Butterwick & MacKinnon, 2014).

In conclusion, we found that Arabidopsis PLDα1 is involved in response to high-Mg^{2+} conditions. We also demonstrate that PLDα1 activity is an essential part of this response. Moreover, high external concentrations of Mg^{2+} were found to disrupt K^+ homeostasis, and PLDα1 is involved in the response to this disruption (Fig. 9).

Author contributions

DK and JM designed the study and wrote the manuscript. DK, ZK, PP, KK, TP, and MD performed the experiments. All authors reviewed and edited the manuscript.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Φιγ. Σ1 *πλδα1-1* ις ηψπερσενσιτιε το ηιγη λεελς οφ εξτερναλ $Mγ^{2+}$· τηε εφφεςτ ον λατεραλ ροοτς

Φιγ. Σ2. Ηιγη αμουντς οφ $Mγ^{2+}$ συππλιεδ βψ διφφερεντ σαλτς προδυςεδ σιμιλαρ πηενοτψπες ιν *πλδα1-1* .

Fig. S3 Insertion of PLDα1 into *πλδα1-1* line restores growth to wt-like levels under high-Mg^{2+}conditions

Table S1 : Primers used in the study.

Figure legends

Fig. 1 *πλδα1-1* is hypersensitive to high external levels of Mg^{2+}. Plants were grown on half-strength MS for 5 days, after which they were transferred to plates supplemented with 0 (Control), 1, 5, 10, 15, and 20 mM $MgCl_2$ for 7 days. (a) Growth of *πλδα1-1* and wt seedlings on agar plates with excess Mg^{2+}. (b) Root length of *πλδα1-1* and wt seedlings 7 d after transfer. Values represent mean ± SD, n=24 plants. (c) Fresh weight of *πλδα1-1* and wt 7 d after transfer. Values represent means ± SD, n=4 (pools of 6 plants). (c) Wt and *πλδα1-1*plants were grown hydroponically for 3 weeks in half-strength Hoagland´s media, followed by 10 d with or without 10 mM $MgSO_4$. The experiment was repeated three times with similar results. Asterisks indicate significant differences compared to wt (Student's t-test, *$p<0.05$, **$p<0.01$).

Fig. 2 Detection of PLDα1 by western blot in the various lines used. Knockout lines *plda1-2* , *plda1-3,* and *plda1-4* had the same phenotype as *plda1-1* under high-Mg^{2+}. (a) Western blot detection of PLDα1 in protein extracts from 10-day-old seedlings. Each lane was run with 9.5 µg of protein. (b) Loading control stained with Novex. (c) Plants grown on half-strength MS for 5 d, followed by transfer to agar plates with or without 10 mM $MgCl_2$ for 7 d. (d) Root length in *πλδα1-2, πλδα1-3, πλδα1-4,* and wt plants. Values represent mean ± SD, n=24. (e) Fresh weight of *πλδα1-2, πλδα1-3, πλδα1-4,* and wt. Values represent means ± SD, n=4 (pools of 6 plants). Asterisks indicate significant differences compared to wt (Student´s t-test, *p<0.05, **p<0.01).

Fig. 3 High-Mg^{2+} treatment triggers a transient increase in PLDα1 activity in a dose-dependent manner, but and does not induce transcription of *ΠΛΔα1* . 4-week-old hydroponically grown plants were treated with $MgSO_4$ and sampled at 10, 30, and 180 min. (a) Thin layer plate showing phosphatidyl butanol (PBut) levels in plants treated with $MgSO_4$. -*n*- But indicates the control sample, where +*n* -But was omitted. (b) Thin layer plate showing accumulation of fluorescently-labeled PBut after $MgSO_4$ treatment over time. (c) Quantification of PBut accumulation in response to $MgSO_4$ over time. (d) Relative increase in PLDα1 activity with $MgSO_4$ treatment over time. Values represent mean ± SD, n=3. (e) Transcription analysis of *ΠΛΔα1* in roots and leaves of wt plants after treatment with 10 mM $MgSO_4$ for 24 h. Transcript levels were measured in roots (R) and leaves (L) by quantitative RT-PCR. Transcription was normalized to the reference gene SAND, and transcription of non-treated plants was set to one. Values represent the mean ± SD, n=3; C, control; R, roots; L, leaves;*n*- But, *n* -butanol; PBut, phosphatidyl butanol.

Fig. 4 Growth of Arabidopsis seedlings expressing inactive PLDα1 is the same that of *πλδα1* on agar plates with excess Mg^{2+}. Plants were grown on half-strength MS for 5 d and transferred to agar plates with or without 10 mM $MgCl_2$ for 7 d. (a) Root length of plants after Mg^{2+} treatment. Values represent mean ± SD, n=24. (b) Fresh weight of plants after Mg^{2+} treatment. Values represent mean ± SD, n=8 (pools of 3 plants). Asterisks indicate significant differences compared to wt (Student´s t-test, ** p<0.01).

Fig. 5 Under high-Mg^{2+} conditions, concentrations of Mg^{2+} and K^+ are lower in *πλδα1-1* compared to wt. Seven-day-old seedlings were transferred on $\frac{1}{2}$ MS agar plates with or without 10 mM $MgCl_2$ and grown for 10 days. Bars represent mean ± SD, n=5 (Student´s t-test, ** p<0.01). Asterisks indicate significant differences compared to wt.

Fig. 6 Addition of Ca^{2+} and K^+ alleviates *πλδα1-1* Mg^{2+}-hypersensitivity. Plants were grown on half-strength MS for 5 d and transferred to agar plates supplemented with 10 mM $MgCl_2$, 10 mM $MgCl_2$ + 10 mM $CaCl_2$, or 10 mM $MgCl_2$ + 50 mM KCl for 7 d. (a) Growth of plants on agar plates with additional Mg^{2+} and Ca^{2+}, or K^+. (b) Root length of plants after transfer to supplemented plates. Values represent mean ± SD, n=24. (c) Fresh weight of plants after transfer to supplemented plates. Values represent means ± SD, n=4 (pools of 6 plants). Asterisks indicate significant differences compared to wt (Student´s t-test, * p<0.05, ** p<0.01).

Fig. 7 Transcription of *CIPK9* and *HAK5* is reduced in the roots of *πλδα1* under high-Mg^{2+}conditions. Transcription levels of (a) *MGT* , (b) *CAX1* ,*CIPK9* , and *HAK5* genes in roots (R) and leaves (L) after high-Mg^{2+} treatment. 4-week-old hydroponically grown plants were treated with 10 mM $MgSO_4$ for 24 h. Transcript levels were measured in roots by quantitative RT-PCR. Transcription was normalized to the reference gene SAND, and the transcription of non-treated plants was set to one. Values represent means ± SD, n=3, (Student´s t-test, *p<0.05). Asterisks indicate significant differences compared to wt. nd indicated not detected.

Fig. 8 The double knockout line *hak5, akt1* is hypersensitive to high-Mg^{2+} conditions. Growth of*hak5* and *hak5, akt1* on agar plates with added Mg^{2+}. Plants were grown on half-strength MS for 5 d and transferred to plates with or without 10 mM $MgCl_2$for 7 d. (a) Growth of plants. (b) Root length in plants after treatment. Values represent mean ± SD, n=24. (c) Fresh weight of plants after treatment. Values represent mean ± SD, n=8 (pools of 3 plants). Asterisks indicate significant differences compared to wt (Student´s t-test, * p<0.05, ** p<0.01).

Fig. 9 Proposed model for PA-mediated response to high-Mg^{2+} in Arabidopsis. High concentrations of extracellular Mg^{2+} results in excess intracellular Mg^{2+} and reduced K^+-uptake, leading to a lower intracellular concentrations of K^+. Meanwhile, PLDα1 is activated and produces PA and polar head group such as choline (Cho). Activation of PLDα1 leads to transcription of the K^+ channel *HAK5* and protein kinase*CIPK9* , possibly activating K^+-uptake. CIPK9 is reported to be involved in Mg^{2+} sequestration via the CBL2/3-CIPK3/9/23/26 network and an unknown tonoplast-localized Mg^{2+} transporter. Additionally, CIPK9 is involved in the regulation of K^+ homeostasis. PLDα1 activity may also interact with machinery regulating K^+ vacuole homeostasis. AKT1 – Arabidopsis K^+ transporter 1, CBL - calcineurin B-like calcium sensor protein, CIPK-CBL-interacting protein kinase, Cho – choline, HAK5 – high-affinity K^+ transporter 5, PA – phosphatidic acid, PLDα1 – phospholipase Dα1, arrows in black solid lines – this study, arrows in black dotted lines – possible interaction based on this study, arrows in gray broken lines – reported study, arrows in gray dotted lines – possible interaction.

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