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

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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

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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 srk2d/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 absicic acid (ABA)-mediated drought response, physically interacts with CIPK3, 9, 23, and 26 (Chen, Peng, Li & Liao, 2018b, Mogani 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 caz1 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, Yanamakae 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, Oliveruk & 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.

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, ΑτΠΛΔα1 (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 Mg²⁺ concentration in growth medium, wt and πλδλ1-I 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 MgSO₄. Nutrient concentrations were as follows: NH₄H₂PO₄, KNO₃, Ca(NO₃)₂·4H₂O, MgSO₄·7H₂O, 24.5 μM ferric citrate, 0.45 μM KI, 4.85 μM H₃BO₃, 5.92 μM MnSO₄·4H₂O, 0.7 μM ZnSO₄·7H₂O, 0.1 μM Na₂MoO₄·2H₂O, 0.01 μM CuSO₄·5H₂O, 0.01 μM CoCl₂·6H₂O, 10.02 μM Na₂EDTA, 10 μM FeSO₄·7H₂O, 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 MgSO₄ 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. Mg²⁺, 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-PLDz1/2 (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 MgSO₄ (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 MgCl₂) 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.

**Results**

**πλδα1 ic ηψηρεσενσιτε το ηιγη λεελς οφ Mγ2+**

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 \(\Pi D\alpha\, 1\) and no other genes, we used three additional T-DNA insertion lines for \(\Pi D\alpha\, 1\), including \(\pi\lambda\delta\alpha\, 1-2\) (described by Bargmann et al. (2009)), \(\pi\lambda\delta\alpha\, 1-3\) (SALK), and \(\pi\lambda\delta\alpha\, 1-4\) (GABI-KAT). We also made complementation lines by transforming \(\pi\lambda\delta\alpha\, 1-1\) plants with \(\Pi D\alpha\, 1\) driven by its native promoter. The levels of PLD\(\alpha\) (PLD\(\alpha\)-Com) protein in seedling extracts was verified using anti-PLD\(\alpha\)/2 antibody. PLD\(\alpha\) was not detected in any of the \(\pi\lambda\delta\alpha\, 1\) lines (Fig. 2a). Levels of PLD\(\alpha\) in the complementation lines were lower than in wt; therefore, the two PLD\(\alpha\)-Com lines with the highest PLD\(\alpha\) protein levels were used for subsequent analyses. \(\pi\lambda\delta\alpha\, 1-2\), \(\pi\lambda\delta\alpha\, 1-3\), and \(\pi\lambda\delta\alpha\, 1-4\) were phenotyped for Mg\(^{2+}\) sensitivity. Primary root length was 23, 25, and 26% lower in \(\pi\lambda\delta\alpha\, 1-2\), \(\pi\lambda\delta\alpha\, 1-3\), and \(\pi\lambda\delta\alpha\, 1-4\), respectively. Fresh weight was 52, 53, and 54% lower in \(\pi\lambda\delta\alpha\, 1-2\), \(\pi\lambda\delta\alpha\, 1-3\), and \(\pi\lambda\delta\alpha\, 1-4\), respectively, compared to wt when treated with high Mg\(^{2+}\) (Fig. 2c, d, e). Overall, all PLD\(\alpha\) mutants were similarly Mg\(^{2+}\)-sensitive to \(\pi\lambda\delta\alpha\, 1-1\). In contrast, primary root length and...
fresh weight in lines $\pi \lambda \delta a1$-Com1 and $\pi \lambda \delta a1$-Com2 were similar to wt when treated with high Mg$^{2+}$ (Fig. S3).

Arabidopsis $\pi \lambda \delta a1$ plants are more sensitive to high-Mg$^{2+}$ conditions than wt; thus, PLD$\alpha 1$ appears to be involved in response to high-Mg conditions. These results uncovered a novel physiological role of PLD$\alpha 1$ in the context of Mg$^{2+}$-homeostasis.

$\Pi \Lambda \Delta a1$ αστιτψ νυσρεαςες αφτερ τρεατμεν πουτι Μγ$^{2+}$

Next, we investigated whether high levels of Mg$^{2+}$ could trigger changes in PLD$\alpha 1$ 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).

PLD$\alpha 1$ 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 PLD$\alpha 1$-YFP, therefore we determined PLD$\alpha$ 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 PLD$\alpha 1$ was determined using fluorescently labeled phosphatidylcholine as a substrate under conditions optimal for PLD$\alpha 1$ (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). PLD$\alpha$ activity was also measured in samples prepared from $\pi \lambda \delta a1$-1 plants, where PLD$\alpha$ activity was found to be negligible (Fig. 3a). Hence, we concluded that the quantity of released PBut corresponds to PLD$\alpha 1$ activity.

An increase in PLD$\alpha 1$ 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 PLD$\alpha 1$ activity (Fig. 3b,c). Interestingly, the increase in PLD$\alpha 1$ activity was transient, reaching 2.5-fold after 30-minutes of treatment with 10 mM MgSO$_4$ (Fig. 3d).

An increase in PLD$\alpha 1$ activity could be due to higher rates of transcription of $\Pi \Lambda \Delta a1$, activation of PLD$\alpha 1$, or a combination of the two. Thus, we measured transcriptional levels of $\Pi \Lambda \Delta a1$ in control and high-Mg$^{2+}$ treated (10 mM MgSO$_4$, for 24h) plants using quantitative RT-PCR. We found no increase in $\Pi \Lambda \Delta a1$ transcript levels following Mg$^{2+}$ treatment (Fig. 3e).

These results demonstrate that PLD$\alpha 1$ is activated by Mg$^{2+}$ shortly after treatment, though not at the transcriptional level.

$\Pi \Lambda \Delta a1$ αστιτψ σοντριβυτες το ηιγη- Μγ$^{2+}$-τολερανςε

To confirm that activity of PLD$\alpha 1$ is essential for high-magnesium tolerance in wt plants, we introduced an inactive mutant for $\Pi \Lambda \Delta a1$ into $\pi \lambda \delta a1$-1 plants ($p$ PLD$\alpha 1$::PLD$\alpha 1$-Mut/$\pi \lambda \delta a1$). 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 $\pi \lambda \delta a1$-1 plants expressing $\pi \Pi \Lambda \Delta a1$ ::$\Pi \Lambda \Delta a1$ $K334R,K663R$ (lines Mut1 and Mut2) at levels consistent with wt (Fig. 2a) showed similar sensitivity to MgCl$_2$ as $\pi \lambda \delta a1$-1, for both primary root length (Fig. 4a) and fresh weight (Fig. 4b).
These results demonstrate that Arabidopsis PLDz1 activity is critical in mediation of the response to high-magnesium conditions.

**πλδα1 αργυμυλατες λες Μγ2+ ανδ Κ+ υνδερ ηιγη-Μγ2+ ςοντιονς**

To elucidate the possible mechanism responsible for the higher susceptibility of πλδα1, we measured Mg2+-content in wt and mutant plants under control and high-Mg2+ conditions. After high-Mg2+ (10 mM) treatment, seedling Mg2+ levels were elevated by about five times in wt and πλδα1-1. Nevertheless, πλδα1 showed significantly lower Mg2+-content than wt (Fig. 5a).

There is known to be an antagonistic relationship between the uptake of Mg2+ and Ca2+ (Guo, Babourina, Christopher, Borsic & Rengel, 2010, Yamanaka et al., 2010). Moreover, increased levels of Mg2+ application in rice results in lower uptake of calcium and potassium (Fageria, 2001), and transcription of the potassium transporter HAK5 increases following treatment with Mg2+ in Arabidopsis (Tang & Luan, 2017, Visscher et al., 2010). Therefore, we measured Ca2+ and K+ content in the wt and πλδα1-1 plants to investigate these relationships.

In agreement with Mg-Ca antagonism, seedling Ca2+ content was lower when 10 mM MgCl2 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 Mg2+-treated plants, with πλδα1-1 plants retaining even less K+ than wt (Fig. 5c).

These results demonstrate that PLDz1 is involved in the regulation of Mg2+ and K+ content in Arabidopsis seedlings grown in high-Mg2+ conditions.

**Αδδιτιον οφ α2+ ανδ Κ+ αλλειατες Μγ2+ -ηψπερσενσιτψ ιν πλδα1 πλαντς**

Aware that there is an antagonistic relationship between some of the essential nutrients, we investigated whether excess Ca2+ or K+ could affect πλδα1 hypersensitivity to Mg2+. Application of both Ca2+ and K+ ameliorate growth in πλδα1-1 in high-Mg2+ (Fig. 6a). Addition of Ca2+ completely restored the growth of πλδα1-1to 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 Mg2+, 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-Mg2+ 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 Ca2+ increased plant growth in both wt and πλδα1-1 plants (Fig. 6). However, no difference in Ca2+ 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 Ca2+ deficiency is not the underlying factor behind growth defects in πλδα1 grown under high-Mg2+, but that high Mg2+ or low K+ content is responsible. Under high-Mg2+ conditions, πλδα1-1 ρεταινεδ λεσς Μγ2+ τηουγη τηε λοωερ Μγ2+ -ςοντεν ωας μορε τοζις το πλδα1-1θαν τον τυε ρεταινεδ Μγ2+ κοντεν τον πλδα1-1, which would result in higher cytosolic concentrations of Mg2+. Additionally, lower K+ content may contribute to impaired growth in πλδα1-1, or a combination of the two mechanisms.

**Κ+-ρελατεδ γενες "ΙΠΚ9 ανδ ΗΑΚ5 αρε νοτ τρανσριπτιοναλψ 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 πδα1-1 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 πδα1-1 (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 πδα1-1 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 πδα1-1 (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 πδα1-1 roots (Fig. 7b). In Mg^{2+}-treated πδα1-1 leaves, CIPK9 transcript levels were slightly increased (~doubled), and HAK5 was not detected; however, there was no difference between wt and πδα1-1 (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 πδα1 and hak5, akt1 sensitivity to high-Mg^{2+}. πδα1 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 πδα1 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 (Hermanset 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 plantspecific Mg$^{2+}$/H$^+$ exchanger (MHX), MGT2, and MGT3 reportedly play a role in the sequestration of Mg$^{2+}$ in vacuoles (Conn et al., 2011, Shaulet 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 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 πδα1 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 πδα1, as supported by the observed decrease in transcript levels of CIPK9 in πδα1 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 πδα1 does not appear to differ from wt under high-Mg$^{2+}$ (Fig. 5); thus, πδα1Mg$^{2+}$ 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 Tn 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 πδα1. Additionally, we impaired transcription of HAK5 and CIPK9 (genes involved in K$^+$ homeostasis) in πδα1 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\textsuperscript{2+} concentrations lead to a decrease in intracellular K\textsuperscript{+} concentrations; thus, activating a not yet fully understood compensation mechanism regulated by PLD\textalpha{}1, HAK5, and potentially CIPK9. The significance of the K\textsuperscript{+} compensation mechanism is seen in Arabidopsis mutants for two proteins involved in K\textsuperscript{+} uptake, HAK5 and AKT1, which display increased sensitivity to high-Mg\textsuperscript{2+} (Fig. 8). Importance of AKT1 and HAK5 for K\textsuperscript{+} uptake in high-Mg\textsuperscript{2+} conditions was also shown by Caballero et al. (2012), where a significant decrease in K\textsuperscript{+} uptake in mature akt1, hak5 Arabidopsis plants was found. However, altered K\textsuperscript{+}-accumulation in the πλδα1 vacuole cannot be excluded as well.

ΠΛΔα1 ανδ ΠΑ αρε νολεδ υν στρεσς ρεσπονσες

We found that PLD\textalpha{}1 activity (prepared from Arabidopsis roots) was rapidly and transiently increased in response to high-Mg\textsuperscript{2+} (Fig. 3). Phospholipase D\textalpha{}1 belongs to the C2 subfamily of plant PLDs, and is activated by millimolar concentrations of Ca\textsuperscript{2+}. PLD\textalpha{}1 prefers phosphatidylcholine to phosphatidylethanolamine as a substrate (Kolesnikov et al., 2012, Wang et al., 2014). Protein phosphorylation may also regulate PLD\textalpha{}1 activity, as it is predicted to have phosphorylation sites (Takáč et al., 2016) and phosphorylated PLD\textalpha{}1 has been detected in response to drought stress (Umezawa et al., 2013). Phospholipase D\textalpha{}1 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).

PLD\textalpha{}1 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 PLD\textalpha{}1 activity in magnesium and potassium homeostasis

We found that high-Mg\textsuperscript{2+} hypersensitivity of Arabidopsis πλδα1 and plants producing inactive PLD\textalpha{}1 protein was the same (Fig. 4), demonstrating that PLD\textalpha{}1 activity is key in regulating the response to increased Mg\textsuperscript{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\textsuperscript{2+} conditions in Arabidopsis (Guo et al., 2014), and PA is known to participate in ABA signaling in various ways. The PA produced through PLD\textalpha{}1 activity interacts with protein phosphatase 2C (PP2C), heterotrimeric 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 PLD\textalpha{}1 also interacts directly with regulator of G-protein signaling 1 (RGS1), modulates the level of active Gz, 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\textsuperscript{2+} response. However, further research is needed to confirm the participation the proteins discussed here in the Arabidopsis PA-high Mg\textsuperscript{2+} response.

There are also several ways in which PA affects K\textsuperscript{+} 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-Mg2+ conditions. We also demonstrate that PLDα1 activity is an essential part of this response. Moreover, high external concentrations of Mg2+ 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.

Figure legends

**Fig. 1** πλδα1-1 is hypersensitive to high external levels of Mg2+. 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 MgCl2 for 7 days. (a) Growth of πλδα1-1 and wt seedlings on agar plates with excess Mg2+. (b) Root length of πλδα1-1 and wt seedlings 7 d after transfer. Values represent mean ± SD, n=24 plants. (c) Root length of πλδα1-1 and wt seedlings 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 MgSO4. 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-Mg²⁺. (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 MgCl₂ for 7 d. (d) Root length in πλδα1-2, πλδα1-3, πλδα1-4, and wt plants. Values represent mean ± SD, n=24. (b) Fresh weight of plants after Mg²⁺ treatment. Values represent means ± SD, n=24. (c) Fresh weight of plants after transfer to supplemented plates. Values represent means ± 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. 3 High-Mg²⁺ treatment triggers a transient increase in PLDα1 activity in a dose-dependent manner, but does not induce transcription of IIAΔα1. 4-week-old hydroponically grown plants were treated with MgSO₄ and sampled at 10, 30, and 180 min. (a) Thin layer plate showing phosphatidyl butanol (PBut) levels in plants treated with MgSO₄. -n- But indicates the control sample, where +n -But was omitted. (b) Thin layer plate showing accumulation of fluorescently-labeled PBut after MgSO₄ treatment over time. (c) Quantification of PBut accumulation in response to MgSO₄ over time. (d) Relative increase in PLDα1 activity with MgSO₄ treatment over time. Values represent mean ± SD, n=3. (e) Transcription analysis of IIAΔα1 in roots and leaves of wt plants after treatment with 10 mM MgSO₄ 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 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 on agar plates with excess Mg²⁺. Plants were grown on half-strength MS for 5 d and transferred to agar plates with or without 10 mM MgCl₂ for 7 d. (a) Root length of plants after Mg²⁺ treatment. Values represent mean ± SD, n=24. (b) Fresh weight of plants after Mg²⁺ 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-Mg²⁺ conditions, concentrations of Mg²⁺ and K⁺ are lower in πλδα1-1 compared to wt. Seven-day-old seedlings were transferred on ½ MS agar plates with or without 10 mM MgCl₂ 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 Ca²⁺ and K⁺ alleviates πλδα1-1Mg²⁺-hypersensitivity. Plants were grown on half-strength MS for 5 d and transferred to agar plates supplemented with 10 mM MgCl₂, 10 mM MgCl₂ + 10 mM CaCl₂, or 10 mM MgCl₂ + 50 mM KCl for 7 d. (a) Growth of plants on agar plates with additional Mg²⁺ and Ca²⁺, 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-Mg²⁺ conditions. Transcription levels of (a) MGT, (b) CAX1, CIPK9, and HAK5 genes in roots (R) and leaves (L) after high-Mg²⁺ treatment. 4-week-old hydroponically grown plants were treated with 10 mM MgSO₄ 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-Mg²⁺ conditions. Growth of hak5 and hak5, akt1 on agar plates with added Mg²⁺. Plants were grown on half-strength MS for 5 d and transferred to plates with or without 10 mM MgCl₂ 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, PLD\(\alpha\)1 is activated and produces PA and polar head group such as choline (Cho). Activation of PLD\(\alpha\)1 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. PLD\(\alpha\)1 activity may also interact with machinery regulating K\(^{+}\) vacuole homeostasis. AKT1 – Arabidopsis K\(^{+}\) transporter 1, CBL - calcieurin B-like calcium sensor protein, CIPK-CBL-interacting protein kinase, Cho – choline, HAK5 – high-affinity K\(^{+}\) transporter 5, PA – phosphatidic acid, PLD\(\alpha\)1 – phospholipase D\(\alpha\)1, 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.

References


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