Running head: Protein kinases in plant growth under high Mg$^{2+}$

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Two Distinct Families of Protein Kinases Are Required for Plant Growth under High External Mg$^{2+}$ Concentrations in Arabidopsis

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Summary:
ABA-activated SnRK2 protein kinases and their interacting partners, members of CBL-interacting protein kinases are required for plant growth under high external Mg$^{2+}$ concentrations in Arabidopsis.
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Abstract

Protein phosphorylation events play key roles in maintaining cellular ion homeostasis in higher plants, and the regulatory roles of these events in Na\(^+\) and K\(^+\) transport have been studied extensively. However, the regulatory mechanisms governing Mg\(^{2+}\) transport and homeostasis in higher plants remain poorly understood, despite the vital roles of Mg\(^{2+}\) in cellular function. SRK2D/SnRK2.2, a member of subclass III sucrose non-fermenting-1 related protein kinase 2s (SnRK2s), functions as a key positive regulator of abscisic acid (ABA)-mediated signaling in response to water-deficit stresses in Arabidopsis (*Arabidopsis thaliana*). Here, we used immunoprecipitation coupled with liquid chromatography-tandem mass spectrometry analyses to identify Calcineurin B-like (CBL)-interacting protein kinase 26 (CIPK26) as a novel protein that physically interacts with SRK2D. In addition to CIPK26, three additional CIPKs (CIPK3, CIPK9, and CIPK23) can physically interact with SRK2D in planta. The *srk2d/e/i* triple mutant lacking all three members of subclass III SnRK2s and the *cipk26/3/9/23* quadruple mutant lacking CIPK26, CIPK3, CIPK9, and CIPK23 showed reduced shoot growth under high external Mg\(^{2+}\) concentrations. Similarly, several ABA biosynthesis-deficient mutants, including *aba2-1*, were susceptible to high external Mg\(^{2+}\) concentrations. Taken together, our findings provided genetic evidence that SRK2D/E/I and CIPK26/3/9/23 are required for plant growth under high external Mg\(^{2+}\) concentrations in Arabidopsis. Furthermore, we showed that ABA, a key molecule in water-deficit stress signaling, also serves as a signaling molecule in plant growth under high external Mg\(^{2+}\) concentrations. These results suggested that SRK2D/E/I and CIPK26/3/9/23-mediated phosphorylation signaling pathways maintain cellular Mg\(^{2+}\) homeostasis.
Introduction

As sessile organisms, plants have evolved multiple adaptive mechanisms to control growth and development under continuously changing environmental conditions. Plant hormones coordinate cellular and physiological responses to adjust growth and development. Abscisic acid (ABA), a key phytohormone involved in multiple biological processes, including plant development and responses to water-deficit stresses caused by drought and high salinity, functions in a dose-dependent manner. At basal levels, endogenous ABA regulates the density of stomata during epidermal development (Tanaka et al., 2013). However, high levels of ABA, which are synthesized in response to water-deficit stresses, induce the expression of stress-responsive genes and stomatal closure (Iuchi et al., 2001; Urano et al., 2009).

The sucrose non-fermenting-1 related protein kinase 2s (SnRK2s) form a unique family of plant-specific protein kinases involved in cellular signaling in response to water-deficit stresses. In particular, subclass III SnRK2s play a pivotal role in coping with drought stress via regulating ABA-controlled biochemical and physiological responses in Arabidopsis (Fujii and Zhu, 2009; Fujita et al., 2009). The srk2d/e/i triple mutant, in which all three subclass III SnRK2s (SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1, and SRK2I/SnRK2.3) are mutated, displays near-perfect ABA-insensitivity and exhibits greatly reduced tolerance to drought stress (Fujii and Zhu, 2009; Fujita et al., 2009). Under water-deficit conditions, subclass III SnRK2s are activated in an ABA-dependent manner (Boudsocq et al., 2004; Yoshida et al., 2006). In recent years, substantial progress has been made in understanding how subclass III SnRK2s are activated in response to water-deficit stresses: Cellular dehydration caused by water deficit stresses leads to increased levels of endogenous ABA, and then ABA-bound pyrabactin resistance1/PYR1-like/regulatory components of ABA receptor (PYR/PYL/RCAR) proteins potentiate the formation of group-A protein phosphatase 2C (PP2C)-PYR/PYL/RCAR-ABA ternary complexes. These complexes inhibit PP2C activity, thereby enabling activation of subclass III SnRK2s (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). The activated subclass III SnRK2s then positively regulate downstream transcription factors such
as ABA-responsive elements (ABREs)-binding proteins/ABRE-binding factors (AREB/ABFs) by phosphorylating them, which results in the activation of ABA-responsive gene expression or other ABA-related responses (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2009; Fujita et al., 2009; Yoshida et al., 2010).

Besides their key roles in regulating gene expression, at least one of the subclass III SnRK2s, namely SRK2E/OST1, plays a key role in stomatal closure in response to water-deficit stresses (Mustilli et al., 2002). SRK2E/OST1 activates the slow anion channel-associated 1 (SLAC1) via its phosphorylation (Geiger et al., 2009; Lee et al., 2009). The activated SLAC1 mediates anion release, leading to depolarization of the guard cell plasma membrane. In turn, the depolarization-activated guard cell outward rectifying K⁺ channel (GORK) mediates the release of K⁺ (Hosy et al., 2003). These changes lead to a decrease in turgor pressure in guard cells, resulting in stomatal closure. Despite the key roles of SRK2E/OST1 in regulating ion flux in guard cells, little is known about potential roles of subclass III SnRK2s (including SRK2D and SRK2I) in regulating ion flux or susceptibility to inorganic ions in tissues other than guard cells.

In this research, to explore a novel signaling pathway mediated by subclass III SnRK2s, we sought to identify proteins that physically interact with subclass III SnRK2s in planta. We focused mainly on SRK2D/SnRK2.2, because of its important role in ABA signaling during the vegetative growth stage (Fujii et al., 2007; Fujita et al., 2009). Using a combined immunoprecipitation (IP) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach, we identified Calcineurin B-like (CBL)-interacting protein kinase 26 (CIPK26) as a novel protein that physically interacts with SRK2D. We showed that in addition to CIPK26, three other closely related protein kinases (CIPK3, CIPK9, and CIPK23) could also physically interact with SRK2D in planta. We generated multiple loss-of-function mutants including a cipk26 cipk3 cipk9 cipk23 (cipk26/3/9/23) quadruple mutant, and found that this quadruple mutant is hypersusceptible to high external Mg²⁺ concentrations. Our results demonstrated that the srk2d/e/i triple mutant and some ABA-deficient mutants also show reduced shoot growth under high external Mg²⁺
concentrations. Based on our results, we discuss the novel roles of subclass III SnRK2s and CIPK26/3/9/23 in plant growth under high external Mg$^{2+}$ concentrations, and their potential roles in maintaining Mg$^{2+}$ homeostasis in Arabidopsis.

RESULTS

Identification of SRK2D-interacting proteins by co-immunoprecipitation coupled with LC-MS/MS analyses

To gain a further understanding of the subclass III SnRK2s-mediated signaling pathway, we used IP coupled with LC-MS/MS to identify novel subclass III SnRK2-interacting proteins in Arabidopsis. Recently, studies integrating genetics with phosphoproteomics have identified undescribed downstream targets of protein kinases of interest, including targets of subclass III SnRK2s (Umezawa et al., 2013; Wang et al., 2013). However, the methods used in those studies cannot be used to identify associated proteins that are not phosphorylated by the protein kinases of interest, for example, proteins that function as upstream regulators of the protein kinases. Conversely, a recently developed method combining IP with LC-MS/MS has been used to identify proteins that interact with a protein of interest, including adaptor proteins and upstream regulators (Nishimura et al., 2010; Pauwels et al., 2010). Thus, the method combining IP with LC-MS/MS is complementary to the combined genetics and phosphoproteomics approach, and can be used to further elucidate signaling pathways mediated by protein kinases. We focused mainly on SRK2D/SnRK2.2 because of its broad expression patterns in vegetative tissues and its important role in ABA responses (Fujii et al., 2007; Fujita et al., 2009), and we sought to identify SRK2D-interacting proteins in planta. We have generated transgenic Arabidopsis plants constitutively expressing the synthetic green fluorescent protein (sGFP)-tagged SRK2D protein (SRK2D-sGFP) or sGFP alone under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the wild-type background (Fujita et al., 2009). Fluorescence microscopy analyses showed that these two transgenic lines expressed SRK2D-sGFP and sGFP proteins, respectively, as previously reported (data not shown) (Fujita et al., 2009). The growth
of these transgenic plants was similar to that of wild-type plants on germination medium (GM) agar plates (Supplemental Fig. S1, A and B). We confirmed that the expressed SRK2D-sGFP proteins were activated in response to ABA treatment or to hyperosmotic stress induced by mannitol treatment by in-gel kinase assay (Supplemental Fig. S1C). Constitutive expression of SRK2D-sGFP alleviated the impaired drought tolerance observed in the srk2d/e/i mutant (Supplemental Fig. S1D). These results indicate that the expressed SRK2D-sGFP proteins are functional in planta.

Next, we used the co-immunoprecipitation (co-IP) method to isolate SRK2D-sGFP protein complexes in planta by using an anti-GFP antibody. Detergent-solubilized fractions from the sGFP- or SRK2D-sGFP-expressing lines grown on GM plates for 3 weeks were subjected to co-IP. The immunoprecipitates were separated by SDS-PAGE, followed by either the immunoblot analysis with the anti-GFP antibody or silver staining. A single band on the immunoblot confirmed the presence of intact sGFP or SRK2D-sGFP in each immunoprecipitate (Fig. 1A). Visualization by silver staining demonstrated that the SRK2D-sGFP samples contained numerous bands that were absent from the sGFP samples (Fig. 1B), suggesting that SRK2D-interacting proteins may be included in these bands. Subsequently, the regions of the gels corresponding to these bands in each lane (including lanes containing sGFP and SRK2D-sGFP samples) were excised and subjected to in-gel trypsin digestion (Supplemental Fig. S1, E–G). The products of in-gel trypsin digestion were analyzed with an LTQ-Orbitrap LC-MS/MS instrument. MS and MS/MS spectra were assigned to specific peptide sequences by the MASCOT search engine.

Our three independent LC-MS/MS analyses (of two independent untreated samples and an independent mannitol-treated sample) allowed identification of numerous candidate proteins as interactors of SRK2D-sGFP. We further screened the proteins to narrow down the candidate SRK2D-interacting proteins using the following criteria: (i) the protein should contain more than two unique peptides (with confidence > 95%), (ii) the peptides should be specifically detected in the
SRK2D-sGFP samples, but not in the sGFP samples (at least two of the three independent analyses), (iii) the protein should be predicted to localize in the cytoplasm, nucleus, or plasma membrane by the SUBA3 program (Tanz et al., 2013), based on the fact that SRK2D-sGFP localizes both in the cytoplasm and nucleus (Fujita et al., 2009) (see also Supplemental Fig. S2B). In total, 25 proteins met these criteria (Table S1). Importantly, ABA INSENSITIVE 1 (ABI1), which is known to be a negative regulator of subclass III SnRK2s (Fujii et al., 2009; Umezawa et al., 2009), met all of these criteria. This indicated that the SRK2D-ABI1 protein complex remained at least partially intact during the purification step in the co-IP, and implied that this method would be useful to identify known or previously undescribed interactors of SRK2D. Next, we tested whether SRK2D was able to physically interact with the candidates using a yeast two-hybrid assay. As far as we tested, in addition to ABI1, CBL-interacting protein kinase 26 (CIPK26) could physically interact with SRK2D in yeast, but TUDOR-SN protein 2 (TSN2) and ATP-citrate lyase B-1 (ACLB-1) could not (Fig. 1C). These results suggest that the plant-specific protein kinase, CIPK26, is a potent and novel interactor of SRK2D.

**Physical interactions between CIPK26 and SnRK2s**

We evaluated the tissue-specific expression profiles of *CIPK26* by generating transgenic Arabidopsis plants carrying the *CIPK26* promoter fused to the β-glucuronidase gene (*GUS*). GUS activity was widely observed in both aerial parts and roots of the *CIPK26* /*GUS* plant (Supplemental Fig. S2A), suggesting that the *CIPK26* gene is broadly expressed in vegetative tissues. The subcellular localization of CIPK26 was then analyzed in *Nicotiana benthamiana* leaves transiently co-expressing both sGFP-tagged CIPK26 (sGFP-CIPK26) and mCherry-tagged SRK2D (SRK2D-mCherry). Confocal microscopic analyses revealed that sGFP-CIPK26 accumulated mainly in the cytoplasm, whereas SRK2D-mCherry accumulated in both the cytoplasm and the nucleus (Supplemental Fig. S2B). Also, GFP fluorescence was observed mainly in the cytoplasm in the transgenic Arabidopsis plants expressing *sGFP-CIPK26* under the control of its own promoter.
(Supplemental Fig. S2C). These observations suggest that CIPK26 is mainly localized in the cytoplasm, whereas SRK2D is localized in both the cytoplasm and the nucleus.

To further investigate the physical interaction between SRK2D and CIPK26, we conducted a co-IP assay using untreated, ABA-treated, or mannitol-treated plants expressing both 4×myc-tagged CIPK26 (4×myc-CIPK26) and sGFP, or SRK2D-sGFP under the control of the CaMV 35S promoter. We observed that the 4×myc-CIPK26 proteins were co-immunoprecipitated with the SRK2D-sGFP proteins, but not with sGFP proteins, in extracts from untreated plants (Fig. 2A). The 4×myc-CIPK26 proteins were also co-immunoprecipitated with SRK2D-sGFP proteins with an apparent enhancement upon ABA or mannitol treatment (Fig. 2A). This result suggests that CIPK26 can physically interact with SRK2D both in the presence and absence of stress conditions. To narrow down the regions of SRK2D responsible for the physical interaction with CIPK26, we generated SRK2D derivatives and tested the physical interaction between each of the SRK2D derivatives and CIPK26 using a yeast two-hybrid assay. The yeast two-hybrid assay showed that the regulatory domain of SRK2D (SRK2D RD) is necessary and sufficient for the physical interaction with CIPK26 (Fig. 2B). In more detail, both domain I and domain II in the regulatory domain are sufficient for the physical interaction with CIPK26 (Fig. 2B). The Arabidopsis SnRK2 family consists of 10 members, which are classified into subclass I, II, or III (Boudsocq et al., 2004). Considering that subclass III SnRK2s (SRK2D, SRK2E/OST1, and SRK2I) have distinct but overlapping functions in regulating ABA-mediated physiological responses (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009), we wondered whether CIPK26 could physically interact with SnRK2s other than SRK2D. The yeast two-hybrid assay showed that in addition to SRK2D, CIPK26 could physically interact with members of subclass II SnRK2s (SRK2C/SnRK2.8 and SRK2F/SnRK2.7) and members of subclass III SnRK2s (SRK2E and SRK2I), but not with members of subclass I SnRK2s (SRK2A/SnRK2.4, SRK2B/SnRK2.10, SRK2G/SnRK2.1, SRK2H/SnRK2.5, and SRK2J/SnRK2.9) in yeast (Fig. 2C). The
bimolecular fluorescence complementation (BiFC) analyses showed that in addition to SRK2D, SRK2E and SRK2C could physically interact with CIPK26, but not with CIPK24/SOS2, an outer group of CIPK, in *N. benthamiana* leaves (Fig. 2D; see also Supplemental Fig. S3B). BiFC visualization showed that the physical interactions occurred mainly in the cytoplasm. These results indicate that CIPK26 can physically interact with subclass II and subclass III SnRK2s, mainly in the cytoplasm.

**CIPK26/3/9/23 can physically interact with SRK2D**

CIPK26 belongs to the CBL-interacting protein kinase family (Weinl and Kudla, 2009). Four Arabidopsis CIPKs, CIPK26, CIPK3, CIPK9, and CIPK23 (CIPK26/3/9/23), were grouped in the same clade in a phylogenetic tree of CIPKs from land plants (Supplemental Fig. S3, A and B), implying that CIPK26/3/9/23 have some overlapping functions *in planta*. To investigate whether in addition to CIPK26, CIPK3, CIPK9, and CIPK23 can physically interact with SRK2D, we performed yeast two-hybrid assays and BiFC assays. The yeast two-hybrid assays showed that SRK2D RD, which is necessary and sufficient for the interaction with CIPK26 (Fig. 2B), could interact with CIPK3 and CIPK9, but not with CIPK23 and CIPK24 (Fig. 2E), suggesting that CIPK3 and CIPK9 can physically interact with SRK2D in yeast. Also, BiFC assays in *N. benthamiana* leaves demonstrated that in addition to CIPK26, CIPK3, CIPK9, and CIPK23 could physically interact with SRK2D, whereas there was no evidence of an interaction between SRK2D and CIPK24 in the BiFC assay (Fig. 2F). Taken together, these data suggest that in addition to CIPK26, CIPK3, CIPK9, and CIPK23 are potent and novel interactors of SRK2D *in planta*.

**CIPK26 can phosphorylate SRK2D *in vitro***

To explore the functional relevance of the physical interaction between SRK2D and CIPK26 at the molecular level, we next investigated whether recombinant SRK2D proteins can phosphorylate CIPK26 proteins or conversely, whether recombinant CIPK26 proteins can phosphorylate SRK2D proteins *in vitro*. The recombinant maltose-binding protein (MBP)-tagged SRK2D (SRK2D-MBP)
proteins and glutathione S-transferase (GST)-tagged CIPK26 proteins (CIPK26-GST) showed autophosphorylation activity and protein kinase activity toward myelin basic protein (Supplemental Fig. S4A; lane 1, 3, 6 and 8). This result indicates that both SRK2D-MBP and CIPK26-GST are functional protein kinases. To clarify whether SRK2D and CIPK26 phosphorylate each other, we generated kinase-inactive forms of SRK2D and CIPK26, because their autophosphorylation activities make it difficult to assess the possibility of trans-phosphorylation events between these protein kinases. Lys-42 of CIPK26 and Lys-52 of SRK2D correspond to a highly conserved residue that is required for activity in most protein kinases (Hanks et al., 1988). The Lys-42 to Asn mutation of CIPK26 and the Lys-52 to Asn mutation of SRK2D abolished their autophosphorylation activities and their kinase activities toward myelin basic protein (Supplemental Fig. S4A; lane 2, 4, 7, and 9). This result confirmed that the mutated GST-tagged CIPK26 proteins (CIPK26<sup>K42N</sup>-GST) and the mutated MBP-tagged SRK2D proteins (SRK2D<sup>K52N</sup>-MBP) were dysfunctional protein kinases. We conducted in vitro phosphorylation reactions using CIPK26<sup>K42N</sup>-GST or SRK2D<sup>K52N</sup>-MBP as substrates. The SRK2D-MBP proteins could not phosphorylate the CIPK26<sup>K42N</sup>-GST proteins (Fig. 3A, lane 3), whereas CIPK26-GST proteins phosphorylated SRK2D<sup>K52N</sup>-MBP proteins (Fig. 3A, lane 6). These results suggest that CIPK26 cannot be a phosphorylation substrate for SRK2D, but rather that SRK2D is a potential substrate for CIPK26 in vitro.

To narrow down the region of SRK2D subjected to phosphorylation by CIPK26, we tested whether the kinase domain (SRK2D KD) and regulatory domain (SRK2D RD) of SRK2D could be phosphorylated by CIPK26 in vitro. CIPK26-GST could efficiently phosphorylate the SRK2D RD, whereas only a very weak phosphorylation signal from the SRK2D KD was detected when co-incubated with CIPK26-GST (Fig. 3B). This result indicated that the SRK2D RD could be phosphorylated by CIPK26. Based on a previous report that CIPK26 can activate the activity of the NADPH oxidase, RBOHF, only when together with the calcineurin B-like calcium sensors CBL1 or CBL9 (Drerup et al., 2013), it is possible that these
CBLs affect the phosphorylation of SRK2D by CIPK26. We tested the effects of co-incubation of CBL1-GST or CBL9-GST with CIPK26-GST on the phosphorylation level of SRK2D^{K52N}-MBP. The addition of CBL1-GST or CBL9-GST did not enhance, but rather slightly reduced, the phosphorylation level of SRK2D^{K52N}-MBP by CIPK26-GST (Fig. 3C). These results suggest that, at least under *in vitro* conditions, CIPK26 is capable of recognizing SRK2D as a substrate, and phosphorylating it, independent of CBL1/CBL9. Competitive binding of CBL1/CBL9-GST to CIPK26-GST with SRK2D^{K52N}-MBP might explain the slightly reduced phosphorylation of SRK2D^{K52N}-MBP.

We then tested whether co-incubation of CIPK26 and CBL1/CBL9 with SRK2D could enhance SRK2D activity *in vitro*. No obvious synergistic effect on the phosphorylation level of myelin basic protein was observed when CIPK26-GST and CBL1/CBL9 were co-incubated with SRK2D-MBP *in vitro* (Supplemental Fig. S4B; lane 6, 7, and 8). To dissect the phosphorylation of myelin basic protein by SRK2D-MBP from that by CIPK26-GST, we further tested the SRK2D activity using an in-gel kinase assay (Fig. 3D). After co-incubation of SRK2D-MBP with CIPK26-GST and CBL1/CBL9-GST in the presence of ATP, but not [γ-^{32}P]ATP, the kinase activity of SRK2D-MBP toward myelin basic protein was tested by an in-gel kinase assay in the presence of [γ-^{32}P]ATP. Despite pre-incubation of SRK2D-MBP with CIPK26-GST and CBL1/CBL9-GST, the phosphorylation level of myelin basic protein by SRK2D-MBP remained unchanged (Fig 3D). We could not detect the kinase activity of CIPK26-GST in our experimental conditions, probably because of misfolding of CIPK26-GST proteins during the renaturation step after SDS-PAGE (Fig 3D). Taken together, these results suggest that CIPK26 can phosphorylate SRK2D *in vitro*; however, despite the presence of CBL1/CBL9, CIPK26 is unlikely to substantially enhance the kinase activity of SRK2D *in vitro*. While we did not observe that CIPK26 activated SRK2D *in vitro*, it is still possible that CIPK26 is involved in modulating the activity of SRK2D *in vivo*.

*cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants show severely impaired
growth phenotypes

To gain insight into the functional relationship between CIPK26/3/9/23 and subclass III SnRK2s in planta, we first examined the effect of disruptions in these CIPKs on the growth of Arabidopsis plants. We obtained T-DNA insertion lines of CIPK26, CIPK3, CIPK9, and CIPK23 in the Columbia accession from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. We isolated homozygous mutants for each line (Supplemental Fig. S5A), and confirmed that the expression of the relevant gene was completely interrupted by the T-DNA insertion in each homozygous mutant by reverse transcription (RT)-PCR (Supplemental Fig. S5B). Considering that CIPK26/3/9/23 formed a monophyletic group (Supplemental Fig. S3B) and could physically interact with SRK2D in planta (Fig. 2F), we considered that CIPK26, CIPK3, CIPK9 and CIPK23 could be functionally redundant to some extent. Hence, we generated and analyzed multiple mutants of cipk26, cipk3, cipk9, and cipk23 (Fig. 4, A–F; Supplemental Fig. S5, B–H). We confirmed that the expression of CIPK26/3/9 was disrupted in the corresponding multiple mutants (Supplemental Fig. S5B). However, in contrast to the complete disruption of CIPK23 expression in the cipk23 single mutant, we detected weak, but significant, expression of CIPK23 in the cipk23/9 double mutant, the cipk26/3/23, cipk26/9/23, and cipk3/9/23 triple mutants, and the cipk26/3/9/23 quadruple mutant (Supplemental Fig. S5B), despite the homozygous T-DNA insertions in CIPK23. A similar phenomenon has been reported in other multiple loss-of-function mutants (Tokunaga et al., 2012). Quantitative RT-PCR analyses showed that the expression level of CIPK23 in each mutant was reduced to 5% to 21% of that in wild type (Supplemental Fig. S5C), indicating a significant reduction in CIPK23 expression in these mutants. Compared with wild type, all single and double mutants showed similar growth phenotypes with respect to the growth of rosette leaves on GM agar plates (Supplemental Fig. S5, D and E), and the growth of rosette leaves and inflorescence height when grown in soil in pots (Fig. 4, A–E; Supplemental Fig. S5, F and G). By contrast, when grown in soil, the cipk26/3/9 triple mutant and the cipk26/3/9/23 quadruple mutant displayed severely impaired
growth phenotypes, represented by small rosettes and necrotic symptoms on the leaf tips at the vegetative growth stage (Fig. 4, A–C), reduced inflorescence height and necrotic symptoms on the shoot apex at the reproductive growth stage (Fig. 4, D–F), and reduced seed yield (Supplemental Fig. S5H), whereas they grew normally on GM agar plates (Supplemental Fig. S5, D and E). The cipk26/9/23 triple mutant showed a moderately impaired growth phenotype when grown in soil, whereas the other triple mutants (cipk3/9/23 and cipk26/3/23) grew normally both on GM agar plates and in soil (Fig. 4, A–E; Supplemental Fig. S5, D and E).

Subclass III SnRK2s play a pivotal role in ABA signaling (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009), and it has been reported that the srk2d/e/i triple mutant showed a near-perfect ABA-insensitive phenotype during the germination and vegetative growth stages (Fujita et al., 2009; Nakashima et al., 2009). Thus, it is possible that CIPK26/3/9/23 participate in the ABA signaling pathway. Accordingly, we tested the ABA-sensitivity of seedlings of the cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants. Unlike that of the srk2d/e/i mutant, the ABA-sensitivity of the cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants was similar to that of the wild type (Supplemental Fig. S6, A and B). This result suggested that CIPK26/3/9/23 are unlikely to play a key role in ABA signaling during the vegetative growth stage. Consistent with this observation, the activation patterns of subclass III SnRK2s in response to ABA or mannitol treatment in the cipk26/3/9/23 quadruple mutants were comparable with those in the wild-type plants (Supplemental Fig. S6C).

cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants are hypersusceptible to high external Mg$^{2+}$ concentrations

To gain further insight into the physiological functions of CIPK26, CIPK3, CIPK9, and CIPK23 in planta, we next focused our attention on the impaired growth phenotypes of the cipk26/3/9 triple mutant and the cipk26/3/9/23 quadruple mutant (Fig. 4, A–F). Hitherto, similar phenotypes (necrotic symptoms on the leaf tips and shoot apex) have been reported for a cax1/cax3 double mutant, in which CAX1 and...
CAX3, which encode tonoplast-localized Ca\(^{2+}/H^+\) antiporters, are disrupted (Cheng et al., 2005). The cax1/cax3 double mutant is impaired in vacuolar H\(^+\)/Ca\(^{2+}\) antiport and H\(^+\)-ATPase activity, and is hypersensitive to high external Ca\(^{2+}\) concentrations, but tolerant to high external Mg\(^{2+}\) concentrations (Cheng et al., 2005). Considering the apparently similar phenotypes of the cipk26/3/9 triple mutant, the cipk26/3/9/23 quadruple mutant, and the cax1/cax3 double mutant, it is possible that the impaired growth phenotypes in these cipk mutants resulted from the external Ca\(^{2+}\)–Mg\(^{2+}\) conditions.

Accordingly, we used a hydroponic culture system to evaluate the growth of seedlings of wild type, the cipk26/3/9 triple mutant, and the cipk26/3/9/23 quadruple mutant in media containing various concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) (Fig. 4, G and H). Under normal growth conditions (media supplemented with 2 mM CaCl\(_2\) or 2 mM MgCl\(_2\); i.e., 2 mM Ca\(^{2+}\) or Mg\(^{2+}\)), the cipk26/3/9 triple mutant and the cipk26/3/9/23 quadruple mutant showed growth retardation similar to that observed when they were grown in soil. Contrary to our expectations, the growth retardation of the cipk26/3/9 triple mutant and cipk26/3/9/23 quadruple mutant became more severe at a higher external Mg\(^{2+}\) concentration (4 mM MgCl\(_2\); Fig. 4, G and H, right) and at lower external Ca\(^{2+}\) concentrations (0.1 mM or 0.2 mM CaCl\(_2\); Fig. 4, G and H, left), whereas the growth retardation was rescued under low external Mg\(^{2+}\) concentrations (0.1 mM or 0.2 mM MgCl\(_2\); Fig. 4, G and H, right). These results indicate that the cipk26/3/9 triple mutant and cipk26/3/9/23 quadruple mutant are hypersusceptible to external Ca\(^{2+}\) and Mg\(^{2+}\) concentrations. Furthermore, considering the growth retardation of these cipk mutants was not rescued under a high external Ca\(^{2+}\) concentration (4 mM CaCl\(_2\); Fig. 4, G and H, left), these results suggest that the growth retardation in the cipk26/3/9 triple mutant and the cipk26/3/9/23 quadruple mutant is not simply because of Ca\(^{2+}\) deficiency, but rather, because of Mg\(^{2+}\) toxicity. Consistent with these observations, the impaired growth phenotype (reduced inflorescence height) observed in the cipk26/3/9 triple mutant and the cipk26/3/9/23 quadruple mutant grown in soil was partially rescued by decreasing the concentration of MgCl\(_2\) in the liquid medium from 2 mM to 0.1 mM (Supplemental Fig. S7, A and
To investigate whether ion homeostasis was affected in the *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants grown in the hydroponic culture system, we measured contents of Mg, Ca, K and Na in aerial parts of these mutants by inductively coupled plasma-mass spectrometry (ICP-MS). ICP-MS analyses revealed that the growth retardation in the *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants was accompanied by a significant reduction in either Ca or Mg content, and an increase in Na content, compared with those of wild type (Supplemental Fig. S8, A and B). The K content in these mutants was comparable to that of wild type, with a few exceptions (Supplemental Fig. S8, A and B). Under a low external Mg\(^{2+}\) concentration (0.1 mM MgCl\(_2\)), in which the growth retardation of the *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants was rescued, the contents of Ca, Mg, and Na were comparable to those of wild type (Supplemental Fig. S8, A and B). These results suggest that the growth retardation of the *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants can be at least partly attributed to a disruption in Mg\(^{2+}\) and Ca\(^{2+}\) homeostasis. The impaired growth phenotypes (small rosettes and reduced inflorescence height) of the *cipk26/3/9/23* quadruple mutant grown in soil were rescued by expression of *CIPK26* under the control of its own promoter (Supplemental Fig. S9, A-E). In addition, the reduced shoot growth of the *cipk26/3/9/23* quadruple mutant under relatively higher external Mg\(^{2+}\) concentrations (2 mM or 4 mM MgCl\(_2\)) in hydroponic culture was also rescued by expressing *CIPK26* under the control of its own promoter (Supplemental Fig. S9, F and G).

To assess whether overexpression of *CIPK26* affects susceptibility of shoot growth to high external Mg\(^{2+}\) concentrations or low external Ca\(^{2+}\) concentrations, we generated transgenic Arabidopsis plants overexpressing *CIPK26* under the control of the CaMV 35S promoter. We evaluated the growth of the transgenic plants under high external Mg\(^{2+}\) concentrations or low external Ca\(^{2+}\) concentrations. The expression of *CIPK26* in two independent overexpressors was confirmed to be higher than that in wild-type or vector-control plants by quantitative RT-PCR (Supplemental Fig. S10A). Then we tested the plants’ susceptibility to high external...
Mg\(^{2+}\) concentrations on agar plates. The *CIPK26*-overexpressing plants were significantly more tolerant than vector-control plants to a high external Mg\(^{2+}\) concentration (25 mM MgCl\(_2\)) on agar plates (Supplemental Fig. S10, B and C). These *CIPK26*-overexpressing plants also grew better under a low external Ca\(^{2+}\) concentration (0.1 mM CaCl\(_2\)) than did vector-control plants in the hydroponic culture system (Supplemental Fig. S10, D and E). Taken together, these results support the view that *CIPK26* plays an important role in plant growth under both high external Mg\(^{2+}\) and low external Ca\(^{2+}\) conditions in a dose-dependent manner.

Hypersusceptibility of *srk2d/e/i* triple and *srk2d/e/i/cipk26/3/9/23* septuple mutants to a high external Mg\(^{2+}\) concentration

Considering that CIPK26 was identified as a novel interactor of subclass III SnRK2s (Fig. 2, A–D), we considered whether subclass III SnRK2s are also required for plant growth under high external Mg\(^{2+}\) concentrations. First, we investigated whether SRK2D can still physically interact with CIPK26 under a high external Mg\(^{2+}\) concentration by co-IP. We observed that the 4×myc-CIPK26 proteins were co-immunoprecipitated with the SRK2D-sGFP proteins in extracts from plants treated with or without 20 mM MgCl\(_2\) (Supplemental Fig. S11). This result indicates that SRK2D forms a protein complex with CIPK26 under high external Mg\(^{2+}\) concentrations.

To test the susceptibility of the *srk2d/e/i* mutant to high external Mg\(^{2+}\) concentrations, we used an assay system on agar plates, because it was difficult to grow the *srk2d/e/i* mutant hydroponically in view of its extremely drought-sensitive phenotype (Fujii and Zhu, 2009; Fujita et al., 2009). Consistent with the patterns of plant growth in the hydroponic culture system (Fig. 4G), the *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants showed increased susceptibility (defined as increased susceptibility to inhibition of shoot growth) to high external Mg\(^{2+}\) concentrations on agar plates. Using this experimental system, we found that, as well as the *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants, the *srk2d/e/i* mutant also showed increased susceptibility to 20 mM MgCl\(_2\) (Fig. 5, A and B). This
observation indicated that, besides CIPK26/3/9/23, subclass III SnRK2s play an important role in plant growth under high external Mg\(^{2+}\) concentrations. In addition, ICP-MS analyses demonstrated that the Mg and K contents in the aerial parts of the \textit{srk2d/e/i} mutant grown with 20 mM MgCl\(_2\) were significantly lower than those of the wild type, as was the case in the \textit{cipk26/3/9} triple and \textit{cipk26/3/9/23} quadruple mutants (Fig. 5C, orange bars). In contrast, the Na content in the aerial parts of the \textit{srk2d/e/i} mutant grown with 20 mM MgCl\(_2\) was similar to that of the wild type (Fig. 5C, orange bars).

To analyze the functional redundancy among CIPK26/3/9/23 and among subclass III SnRK2s in modulating Mg\(^{2+}\)-susceptibility (“Mg\(^{2+}\)-susceptibility” is defined as susceptibility to shoot growth inhibition in response to increased external Mg\(^{2+}\) concentrations), we tested the susceptibility of the various mutants to a high external Mg\(^{2+}\) concentration. We analyzed the \textit{cipk26}, \textit{cipk3}, \textit{cipk9} and \textit{cipk23} single mutants and multiple \textit{cipk} mutants, and \textit{srk2d}, \textit{srk2e} and \textit{srk2i} single mutants and multiple \textit{snrk2} mutants. All of the tested single and double \textit{cipk} mutants, except for the \textit{cipk26/3} double mutant, showed a similar susceptibility to a high external Mg\(^{2+}\) concentration as that of the wild type (Supplemental Fig. S12A). In contrast, the \textit{cipk26/3} double mutant, and the \textit{cipk26/3/9}, \textit{cipk26/3/23} and \textit{cipk26/9/23} triple mutants showed greater Mg\(^{2+}\)-susceptibility than that of wild type, whereas the \textit{cipk3/9/23} triple mutant did not (Supplemental Fig. S12A). All of the single and double \textit{snrk2} mutants showed similar susceptibility to a high external Mg\(^{2+}\) concentration as that of the wild type, while the \textit{srk2d/e/i} triple mutant was significantly hypersusceptible to a high external Mg\(^{2+}\) concentration (Supplemental Fig. S12B).

We also tested whether the \textit{srk2d/e/i} and \textit{cipk26/3/9} triple mutants and the \textit{cipk26/3/9/23} quadruple mutant were hypersusceptible to high external K\(^{+}\), Na\(^{+}\) or Ca\(^{2+}\) concentrations on agar plates (Supplemental Fig. S13, A and B). The \textit{srk2d/e/i} triple mutant was specifically hypersusceptible to a high external Mg\(^{2+}\) concentration. As well as showing hypersusceptibility to a high external Mg\(^{2+}\) concentration, the \textit{cipk26/3/9} triple and \textit{cipk26/3/9/23} quadruple mutants were slightly susceptible to a
high external Ca$^{2+}$ concentration.

To reveal the genetic interactions between CIPK26/3/9/23 and SRK2D/E/I in modulating Mg$^{2+}$-susceptibility, we generated a srk2d/e/i/cipk26/3/9/23 septuple mutant. In this mutant, both the SRK2D/E/I and CIPK26/3/9/23 gene groups were disrupted. We tested the susceptibility of wild type, the srk2d/e/i triple mutant, the cipk26/3/9/23 quadruple mutant, and the srk2d/e/i/cipk26/3/9/23 septuple mutant to high external Mg$^{2+}$ concentrations (Fig. 5, D and E). The srk2d/e/i/cipk26/3/9/23 septuple mutant and the cipk26/3/9/23 quadruple mutant showed similar growth inhibition under high external Mg$^{2+}$ concentrations (10 mM or 20 mM MgCl$_2$; Fig. 5, D and E). This result suggests that SRK2D/E/I and CIPK26/3/9/23 modulate Mg$^{2+}$-susceptibility via a common pathway.

We also evaluated the activation patterns of SRK2D-sGFP proteins in response to high external Mg$^{2+}$ concentrations, using myelin basic protein as substrate. In contrast to the significant activation of SRK2D-sGFP proteins in response to ABA or mannitol treatment, the activation status of SRK2D-sGFP proteins in response to a high external Mg$^{2+}$ concentration remained comparable to that of non-treated plants (Supplemental Fig S14).

**ABA2-mediated ABA biosynthesis is important for modulating susceptibility to a high external Mg$^{2+}$ concentration**

Considering that the activities of subclass III SnRK2s are modulated by ABA (Boudsocq et al., 2004; Kobayashi et al., 2005; Furihata et al., 2006), we wondered whether ABA biosynthesis plays a role in plant growth under high external Mg$^{2+}$ concentrations. First, we tested the susceptibility of the ABA biosynthesis-deficient mutants *aba2-1* (Léon-Kloosterziel et al., 1996) and *need3-2* (Urano et al., 2009) to a high external Mg$^{2+}$ concentration. In the *aba2-1* mutant, accumulation of ABA both to basal levels under well-watered conditions and in response to drought stress is impaired (Léon-Kloosterziel et al., 1996). The *need3-2* mutant is impaired in drought-responsive ABA accumulation, but it can accumulate basal levels of ABA (Urano et al., 2009). The *aba2-1* mutant showed increased
susceptibility to a high external Mg\(^{2+}\) concentration (20 mM MgCl\(_2\)), whereas the
nced3-2 mutant did not (Fig. 6, A and B). We also tested the susceptibility of the
areb1/areb2/abf3 triple mutant to a high external Mg\(^{2+}\) concentration. This mutant
lacks three AREB/ABF transcription factors (AREB1, AREB2, and ABF3) and
shows impaired expression of many genes downstream of subclass III SnRK2 in
ABA signaling in response to osmotic stress (Yoshida et al., 2010). The susceptibility
of the areb1/areb2/abf3 triple mutant to a high external Mg\(^{2+}\) concentration (20 mM
MgCl\(_2\)) was similar to that of wild type (Fig. 6, A and B).

To further test the hypothesis that ABA biosynthesis plays an important role in
modulating Mg\(^{2+}\)-susceptibility, we tested whether two other ABA
biosynthesis-deficient mutants, aao3-4 (Seo et al., 2004) and aba3-1
(Léon-Kloosterziel et al., 1996), showed increased susceptibility to a high external
Mg\(^{2+}\) concentration (Supplemental Fig. S15, A and B). Like aba2-1, the aao3-4 and
aba3-1 mutants showed increased susceptibility to a high external Mg\(^{2+}\)
concentration (20 mM MgCl\(_2\); Supplemental Fig. S15, A and B). We further
investigated whether the hypersusceptibility of the aba2-1 mutant to a high external
Mg\(^{2+}\) concentration results from an ABA deficiency. Addition of 1 µM ABA to the
medium rescued the hypersusceptibility of the aba2-1 mutant to a high external Mg\(^{2+}\)
concentration (20 mM MgCl\(_2\)), but did not rescue the hypersensitivity of the
srk2d/e/i triple and the cipk26/3/9/23 quadruple mutants (Fig. 6, C and D). Taken
together, these results support the idea that ABA, synthesized via ABA2 plays a key
role in plant growth under high external Mg\(^{2+}\) concentrations.

**DISCUSSION**

Recent advances have furthered our understanding of the roles of protein
phosphorylation in regulating Na\(^+\) and K\(^+\) transport (Qiu et al., 2002; Li et al., 2006;
Xu et al., 2006); however, the regulatory mechanisms by which plants modulate
cellular Mg\(^{2+}\) transport and maintain Mg\(^{2+}\) homeostasis in response to changes in
external ion concentrations remain poorly understood, despite the pivotal functions
of Mg\(^{2+}\) in plant cells. Here, we reveal that two distinct families of plant-specific
protein kinases, subclass III SnRK2s (SRK2D/E/I) and CIPK26/3/9/23, modulate the susceptibility to shoot growth inhibition in response to increased external Mg$^{2+}$ concentrations (Mg$^{2+}$-susceptibility) in Arabidopsis. To date, many studies on subclass III SnRK2s have focused on their functions as positive regulators of ABA signaling in response to water-deficit stress (Mustilli et al., 2002; Kobayashi et al., 2005; Fujii and Zhu, 2009; Fujita et al., 2009). Conversely, a recent phosphoproteomic analysis identified proteins that are involved in flowering time regulation, such as MOS3 and XRN3, as possible substrates for subclass III SnRK2s (Wang et al., 2013), which was consistent with the early flowering phenotype of the srk2d/e/i triple mutant (Wang et al., 2013). This suggested that subclass III SnRK2s play diverse roles in modulating plant growth, not only under water-deficit stress conditions, but also under normal growth conditions. In this study, we revealed a novel role of subclass III SnRK2s in plant growth under high external Mg$^{2+}$ concentrations. Previous studies on CIPK26/3/9/23 have characterized the diverse and distinct functions of each of these CIPK genes (Kim et al., 2003; Li et al., 2006; Xu et al., 2006; Cheong et al., 2007; Pandey et al., 2007; Drerup et al., 2013; Kimura et al., 2013; Lyzenga et al., 2013). Besides the individual functions of CIPK26, CIPK3, CIPK9 and CIPK23, it seems that these CIPK genes also have some overlapping functions in planta, because CIPK26/3/9/23 formed a monophyletic group in the phylogenetic analysis (Supplemental Fig. S3B), and all of them could physically interact with SRK2D in planta (Fig. 2F).

In this research, we found that the cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants displayed impaired growth phenotypes, represented by small rosettes and necrotic symptoms on the leaf tips and shoot apex when grown in soil (Fig. 4, A–F). When grown in a hydroponic culture system, the growth retardation observed in the cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants was rescued by decreasing the concentration of external Mg$^{2+}$ (Fig. 4, G and H). Consistent with this observation, the impaired growth phenotype (reduced inflorescence height) of these mutants when grown in soil was partially rescued by decreasing the concentration of MgCl$_2$ (Supplemental Fig. S7, A and B). These results indicate that
CIPK26/3/9/23 play a fundamental role in plant growth even under normal external Mg$^{2+}$ concentrations. The cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants showed increased susceptibility to high external Mg$^{2+}$ concentrations on agar plates (Fig. 5, A and B). Besides these cipk mutants, the srk2d/e/i triple mutant was also hypersusceptible to a high external Mg$^{2+}$ concentration (Fig. 5, A and B). By contrast, Arabidopsis plants overexpressing CIPK26 showed significantly higher tolerance to a high external Mg$^{2+}$ concentration (Supplemental Fig. S10, A–C). In addition, the CIPK26-overexpressing lines grew well under a low external Ca$^{2+}$ concentration (Supplemental Fig. S10, D and E). Given the fact that genes closely related to CIPK26 are conserved in monocots and eudicots (Supplemental Fig. S3B), CIPK26 may be a strong candidate as a gene to improve crop growth under Mg$^{2+}$-toxic and/or Ca$^{2+}$-deficient conditions (e.g., in serpentine soils).

We considered that CIPK26, CIPK3, CIPK9 and CIPK23 could have a degree of functional redundancy in modulating Mg$^{2+}$-susceptibility (Supplemental Fig. S12A). All single and double cipk mutants, except for the cipk26/3 double mutant, showed similar susceptibility to a high external Mg$^{2+}$ concentration as that of wild type (Supplemental Fig. S12A). Conversely, the cipk26/3/9, cipk26/3/23, and cipk26/9/23 triple mutants showed increased Mg$^{2+}$-susceptibility, whereas the cipk3/9/23 triple mutant did not (Supplemental Fig. S12A). This result suggests that the functions of CIPK26, CIPK3, CIPK9 and CIPK23 in modulating Mg$^{2+}$-susceptibility overlap to some extent, and that CIPK26 plays a particularly important role in modulating Mg$^{2+}$-susceptibility. We also considered that SRK2D, SRK2E, and SRK2I could have overlapping functions in modulating Mg$^{2+}$-susceptibility (Supplemental Fig. S12B). The srk2d/e/i triple mutant was hypersusceptible to a high external Mg$^{2+}$ concentration, whereas none of the single or double T-DNA mutants were hypersusceptible (Supplemental Fig. S12B). This result suggests that SRK2D, SRK2E, and SRK2I modulate Mg$^{2+}$-susceptibility in a redundant manner.

Under a high external Mg$^{2+}$ concentration (20 mM MgCl$_2$), the srk2d/e/i and cipk26/3/9 triple mutants, and the cipk26/3/9/23 quadruple mutant, accumulated
much lower levels of Mg in their aerial parts than did the wild type (Fig. 5C), although these mutants showed greater susceptibility to a high external Mg$^{2+}$ concentration (Fig. 5, A and B). These data suggested that intracellular Mg$^{2+}$ homeostasis is disrupted in these mutants; however, it remains unclear how and where Mg$^{2+}$ homeostasis is affected. As an abundant intracellular divalent cation, Mg$^{2+}$ stabilizes phosphate compounds (e.g. DNA, RNA, and ATP) and is essential for the function of many enzymes. It also coordinates with the porphyrin ring of chlorophyll molecules. Therefore, it is essential for photosynthesis. Recent studies have revealed that several mitochondrial RNA splicing 2 (MRS2)/magnesium transport (MGT) proteins, which are structurally homologous to bacterial CorA proteins and to ALR1, ALR2, MRS2 and LPE10 in yeast, play an important role in Mg$^{2+}$ transport and plant growth under low external Mg$^{2+}$ conditions (Gebert et al., 2009; Lenz et al., 2013; Mao et al., 2014). However, it remains largely unknown how plants maintain cellular Mg$^{2+}$ homeostasis under high external Mg$^{2+}$ conditions. Taken together with a previous report that mesophyll vacuoles can accumulate up to 80 mM Mg$^{2+}$ in leaves of Arabidopsis fed with high-Mg artificial sap solutions (Conn et al., 2011), impaired Mg$^{2+}$ transport into the vacuole with a resulting increase in Mg$^{2+}$ concentration in the cytoplasm might, at least partly, explain the increased susceptibility of the srk2d/e/i and cipk26/3/9 triple mutants and the cipk26/3/9/23 quadruple mutant to high external Mg$^{2+}$ concentrations and the decreased accumulation of Mg$^{2+}$ in aerial parts. The report also showed that the disruption of MRS2-1/MGT2 or MRS2-5/MGT3, which are tonoplast-localized MRS family Mg$^{2+}$ transporters, partially impaired compartmentalization of Mg$^{2+}$ into the vacuole under a high external Mg$^{2+}$ concentration (Conn et al., 2011). In addition, the SOS2/CIPK24 protein kinase can activate the tonoplast-localized Ca$^{2+}$/H$^+$ antiporter, CAX1 (Cheng et al., 2004). Based on these reports, subclass III SnRK2s and CIPK26/3/9/23 protein kinases might target certain tonoplast-localized Mg$^{2+}$ transporters and/or channels, and modulate their activities under high external Mg$^{2+}$ concentrations to maintain the cytoplasmic Mg$^{2+}$ concentration (Fig. 7). More precisely, these protein kinases might activate certain tonoplast-localized proteins
involved in active Mg\textsuperscript{2+} transport into the vacuole, and in parallel, might inactivate certain tonoplast-localized proteins involving in Mg\textsuperscript{2+} passive transport between the cytoplasm and the vacuole. In future research, it will be important to measure the Mg contents in the cytoplasm and in various organelles, such as the vacuole, to unravel how Mg\textsuperscript{2+} homeostasis is affected in the srk2d/e/i and cipk26/3/9 triple mutants and the cipk26/3/9/23 quadruple mutant.

CIPK26 physically interacts with SRK2D \textit{in planta} (Fig. 2, A and D; Supplemental Fig. S11). CIPK26 and SRK2D are Ser/Thr protein kinases; therefore, it is possible that these two proteins could phosphorylate each other. SRK2D-MBP could not phosphorylate CIPK26\textsuperscript{K42N}-GST \textit{in vitro} (Fig. 3A), suggesting that CIPK26 is not a phosphorylation substrate for SRK2D. Conversely, CIPK26-GST was able to phosphorylate SRK2D\textsuperscript{K52N}-MBP \textit{in vitro} (Fig. 3A), suggesting that SRK2D is a potential substrate for CIPK26. The signal from trans-phosphorylation of SRK2D\textsuperscript{K52N}-MBP by CIPK26-GST was weaker than that from autophosphorylation of SRK2D-MBP (Fig. 3A). This may be because of the multiple phosphorylations of several Ser/Thr residues in the autophosphorylation of SRK2D, as is the case in the autophosphorylation of SRK2E/OST1 (Belin et al., 2006). In addition, an \textit{in vitro} phosphorylation assay showed that the regulatory domain of SRK2D (SRK2D RD) could be phosphorylated by CIPK26 (Fig. 3B). These results raised the possibility that CIPK26 affects the activity of SRK2D. We then considered whether CIPK26-GST could enhance the kinase activity of SRK2D-MBP \textit{in vitro} (Supplemental Fig. S4B; Fig. 3D). Based on a previous report that CBL1/CBL9 work together with CIPK26 in the activation of RBOHF (Drerup et al., 2013), we also considered whether the addition of CBL1/CBL9-GST with CIPK26-GST would affect the kinase activity of SRK2D-MBP (Fig. 3D). Despite the presence of CBL1/CBL9-GST, co-incubation of CIPK26-GST with SRK2D-MBP could not activate the kinase activity of SRK2D-MBP \textit{in vitro} (Fig. 3D). While we did not observe that CIPK26 activated SRK2D \textit{in vitro}, these results could not rule out the possibility that unknown factors, other than CBL1/CBL9, together with CIPK26, modulate the activity of SRK2D \textit{in vivo}. In future research, further analyses are
required to clarify whether CIPK26 is involved in modulating the activity of SRK2D in vivo, especially under high external Mg\textsuperscript{2+} concentrations. It will be important to explore novel player(s) involved in CIPK26 and SRK2D-mediated modulation of Mg\textsuperscript{2+}-susceptibility by employing co-immunoprecipitation coupled with LC-MS/MS method.

Previous reports showed that the phosphorylation of the activation loop plays a crucial role in the activation of subclass III SnRK2s (Belin et al., 2006; Umezawa et al., 2009), and a GSK3-like kinase, brassinosteroid insensitive 2 (BIN2), phosphorylates the activation loop in the kinase domain of SRK2D and activates its autophosphorylation activity and the kinase activity toward ABF2/AREB1 (Cai et al., 2014). Thus, an alternative possibility is that the phosphorylation of SRK2D by CIPK26 has functions other than activation. It has been reported that the phosphorylation of SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCaBP8)/CBL10 by SOS2/CIPK24 stabilized the SCaBP8-SOS2 interaction (Lin et al., 2009). Taken together with our finding that the regulatory domain of SRK2D is necessary and sufficient for the physical interaction with CIPK26 (Fig. 2B), the phosphorylation of the regulatory domain of SRK2D by CIPK26 might play a role in modulating the physical interactions between these protein kinases. In further research, it will be also important to identify the phosphorylation site(s) of SRK2D by CIPK26, and then to analyze mutated forms of SRK2D in which the residues phosphorylated by CIPK26 are substituted with Ala or Asp/Glu, to consider how the phosphorylation of SRK2D by CIPK26 affects the function of SRK2D in planta.

Considering that the srk2d/e/i/cipk26/3/9/23 septuple mutant and the cipk26/3/9/23 quadruple mutant show similar hypersusceptibility to high external Mg\textsuperscript{2+} concentrations (Fig. 5, D and E), subclass III SnRK2s and CIPK26/3/9/23 may modulate Mg\textsuperscript{2+}-susceptibility via a common pathway. Moreover, CIPK26 physically interacts with SRK2D under a high external Mg\textsuperscript{2+} concentration (Supplemental Fig. S11). This result provides evidence that SRK2D forms a protein complex with CIPK26 under a high external Mg\textsuperscript{2+} concentration, where subclass III SnRK2s and CIPK26/3/9/23 play an important role in modulating Mg\textsuperscript{2+}-susceptibility. Recently, it
was reported that both SRK2E/OST1 and CIPK26 are able to phosphorylate the NADPH oxidase, RBOHF (Sirichandra et al., 2009; Drerup et al., 2013; Kimura et al., 2013). The effect of the phosphorylation of RBOHF by SRK2E/OST1 is still unknown (Sirichandra et al., 2009), but it was reported that CIPK26 enhances the activity of RBOHF via its phosphorylation together with CBL1 and CBL9 in HEK293T cells (Drerup et al., 2013). These findings, together with our finding that CIPK26 interacts with SRK2D \textit{in planta} to form a protein complex under a high external Mg$^{2+}$ concentration (Supplemental Fig. S11), support the view that subclass III SnRK2s and CIPK26 cooperatively regulate activities of some downstream targets that play important roles in modulating Mg$^{2+}$-susceptibility and/or Mg$^{2+}$ transport (Fig. 7). To validate this hypothesis, it will be important to comprehensively identify downstream targets of SRK2D/E/I and CIPK26/3/9/23. This can be investigated using a phosphoproteomics approach to analyze the \textit{srk2d/e/i} and \textit{cipk26/3/9} triple mutants and the \textit{cipk26/3/9/23} quadruple mutant.

Several ABA-biosynthesis deficient mutants showed increased susceptibility to a high external Mg$^{2+}$ concentration (Supplemental Fig. S15, A and B), and the hypersusceptibility of the \textit{aba2-1} mutant to a high external Mg$^{2+}$ concentration (20 mM MgCl$_2$) was rescued by adding ABA (Fig. 6, C and D). These findings indicate that ABA, a key molecule in water-deficit stress signaling, also serves as a signaling molecule in plant growth under high external Mg$^{2+}$ conditions in Arabidopsis. Several studies have reported that ABA modulates the activities of subclass III SnRK2s (Mustilli et al., 2002; Boudsocq et al., 2004; Furihata et al., 2006; Yoshida et al., 2006) and that subclass III SnRK2s are key positive regulators of ABA signaling (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009; Umezawa et al., 2009). In addition, the \textit{srk2d/e/i} triple mutant showed increased susceptibility to a high external Mg$^{2+}$ concentration, as did \textit{aba2-1} (Fig. 6, A and B). Therefore, it is likely that ABA modulates Mg$^{2+}$-susceptibility via a subclass III SnRK2s-mediated pathway. Further research is needed to examine the involvement of PYR/PYLs/RCARs and PP2Cs in ABA-mediated modulation of Mg$^{2+}$-susceptibility (Fig. 7). In contrast to the \textit{aba2-1}, \textit{aao3-4}, and \textit{aba3-1} mutants, the \textit{nced3-2} mutant
showed a similar susceptibility to a high external Mg$^{2+}$ concentration as that of wild type (Supplemental Fig. S15, A and B), probably because of the redundant role(s) of other NCED gene(s). Considering that the nced3-2 mutant was impaired only in drought-responsive ABA accumulation, not in accumulation of basal levels of ABA (Urano et al., 2009), basal levels of ABA synthesized via other NCED(s), ABA2, and AAO3 may play a key role in modulating Mg$^{2+}$-susceptibility. This hypothesis seemed to be consistent with our observation that the activation status of SRK2D-sGFP proteins under a high external Mg$^{2+}$ concentration remained at a basal level (Supplemental Fig S14). Basal subclass III SnRK2s activity, in response to basal levels of ABA, may be necessary to modulate Mg$^{2+}$-susceptibility (Fig. 7). We observed that the susceptibility of the areb1/areb2/ahf3 triple mutant to a high Mg$^{2+}$ concentration was similar to that of the wild type (Fig. 6, A and B). This result indicated that ABA-mediated modulation of susceptibility to a high external Mg$^{2+}$ concentration is independent of osmotic stress-responsive gene expression mediated by the “SnRK2-AREB/ABF” pathways (Yoshida et al., 2014). Therefore, it is likely that subclass III SnRK2s modulate Mg$^{2+}$-susceptibility via other downstream targets (Fig. 7).

Considering that CIPK26/3/9/23 physically interacted with SRK2D (Fig. 2F), it is possible that CIPK26/3/9/23 plays a role in the ABA signaling pathway. However, our data indicated that neither the cipk26/3/9 triple mutant nor the cipk26/3/9/23 quadruple mutant exhibited any visible changes in ABA-responses, compared with those of the wild type, during the vegetative growth stage (Supplemental Fig. S6, A and B. See also Fig. 6, C and D). Consistently, the activation patterns of subclass III SnRK2s in response to ABA or mannitol treatment in the cipk26/3/9/23 quadruple mutants were similar to those in wild-type plants (Supplemental Fig. S6C). These observations suggested that CIPK26/3/9/23 are unlikely to play a key role in ABA signaling during the vegetative growth stage, and unlikely to be involved in modulating subclass III SnRK2s activity under water-deficit stress conditions. Conversely, CIPK26-overexpressors showed a slightly ABA-hypersensitive phenotype during the germination stage, and it was
suggested that CIPK26 could function as a positive regulator of plant responses to ABA (Lyzenga et al., 2013). The discrepancy between our results and the previous report could, at least partially, be explained by a difference in growth stages. Given that CIPK26 mainly localizes in the cytoplasm (Supplemental Fig. S2, B and C), whereas the localization of SRK2D is cytoplasmic and nuclear (Supplemental Fig. S2B), where it physically interacts with AREB/ABFs (Fujita et al., 2009; Yoshida et al., 2014), it is likely that SRK2D regulates AREB/ABFs in the nucleus to induce osmotic stress-responsive gene expression and other ABA-related responses, independent of CIPK26. Conversely, a previous study reported that CIPK26 localizes both to the cytoplasm and nucleus, and physically interacts with ABI5 mainly in the nucleus (Lyzenga et al., 2013). Another report showed that CIPK26 predominantly localizes in the cytoplasm and partially in the nucleus, and physically interacts with plasma membrane-localized RBOHF (Drerup et al., 2013). Further analyses are required clarify the overall functions and the downstream targets of CIPK26 and other CIPKs during ABA signaling throughout the plant’s life cycle.

Importantly, administration of ABA could not rescue the increased Mg$^{2+}$-susceptibility of the *cipk26/3/9/23* quadruple mutant (Fig. 6, C and D). This finding, together with our observation that the *cipk26/3/9/23* quadruple mutant displayed similar ABA-sensitivity to that of wild type (Supplemental Fig. S6, A and B), indicates that the increased Mg$^{2+}$-susceptibility of the *cipk26/3/9/23* quadruple mutant was not because of ABA-deficiency or disruption of ABA signaling. Taken all together, our data support the view that subclass III SnRK2s, in response to basal levels of ABA synthesized via ABA2, and AAO3, together with CIPK26/3/9/23, play a key role in plant growth under high external Mg$^{2+}$ concentrations (Fig. 7). In addition, our findings suggest that the role of subclass III SnRK2s in modulating Mg$^{2+}$-susceptibility is different from their pivotal roles in ABA signaling in response to water-deficit stress conditions, which require a considerable level of ABA accumulation, synthesized via NCED3 (Fig. 7).

Collectively, our findings provide genetic and physiological evidence that SRK2D/E/I and CIPK26/3/9/23 are required for plant growth under high external
Mg$^{2+}$ concentrations in Arabidopsis. Our results also show that ABA serves as an important signaling molecule in modulating Mg$^{2+}$-susceptibility in Arabidopsis. Our research suggests that plants modulate susceptibility to Mg$^{2+}$ via phosphorylation signaling, mediated by SRK2D/E/I and CIPK26/3/9/23. It will be challenging to understand the physiological importance of the physical interactions between CIPK26/3/9/23 and SRK2D, and to reveal the molecular mechanisms by which these two distinct protein kinases modulate susceptibility to high external Mg$^{2+}$ concentrations. In future research, it will be important to identify the downstream targets of these protein kinases that are responsible for modulating Mg$^{2+}$-susceptibility and/or Mg$^{2+}$ transport, and maintaining cellular Mg$^{2+}$ homeostasis. This will increase our understanding of how plants control cellular Mg$^{2+}$ transport and maintain cellular Mg$^{2+}$ homeostasis in response to changes in external Mg$^{2+}$ concentrations.

15 MATERIALS AND METHODS

Plant materials, growth conditions, and generation of transgenic plants

Arabidopsis thaliana (L.) Heynh ecotype Columbia (Col) was used in this study. Seeds were surface-sterilized and sown on GM agar plates. After stratification at 4 °C for 3 d in the dark, seeds were grown on GM agar plates in a growth chamber under a 16-h light/8-h dark photoperiod (40±10 µE m$^{-2}$ s$^{-1}$) at 22 °C as described previously (Kim et al., 2012). For growing plants in soil, 2-week-old plants grown on GM agar plates were transferred into a plastic pot (ø 7 cm, height 6 cm or ø 8 cm, height 6.5 cm) filled with vermiculite supplemented with a modified basal nutrient solution (BNS) (Conn et al., 2011) [in mM: 2 NH$_4$NO$_3$, 3 KNO$_3$, 0.1 CaCl$_2$, 2 KCl, 2 Ca(NO$_3$)$_2$, 2 MgSO$_4$, 0.6 KH$_2$PO$_4$, 2 NaCl with micronutrients; in µM: 50 NaFe(III)EDTA, 50 H$_3$BO$_3$, 5 MnCl$_2$, 10 ZnSO$_4$, 0.5 CuSO$_4$ and 0.1 Na$_2$MoO$_4$, pH 5.6, NaOH], and grown under a 16-h light/8-h dark photoperiod (60±10 µE m$^{-2}$ s$^{-1}$) at 22 °C. The T-DNA insertion lines cipk26 (SALK_005859C), cipk3 (SALK_137779C), cipk9 (SALK_058629), cipk23 (SALK_032341), and aao3-4 (N572361) were obtained from ABRC or the European Arabidopsis Stock Centre.
A series of multiple *cipk26, cipk3, cipk9,* and *cipk23* mutants in the Col ecotype were constructed by genetic crosses and were screened by genomic PCR using primers recommended by ABRC. The detailed procedure for construction of pGreenII-based plasmids for plant transformation is described in the Supplemental data online. Plants were transformed using *Agrobacterium tumefaciens* strain GV3101 as described previously (Kim et al., 2012).

**Co-immunoprecipitation, silver staining, and immunoblot analysis**

Proteins were extracted from 3-week-old seedlings (4 to 6 g FW) grown on GM agar plates. Samples were ground to a powder in liquid nitrogen and homogenized on ice in 3 × extraction buffer (50 mM Tris-HCl [pH 8.0], 0.2% (w/v) Triton X-100, and one tablet of complete protease inhibitor cocktail tablet/25 mL). Crude extracts were then centrifuged at 5,000 g for 10 min at 4 °C to remove cellular debris. The supernatants was passed through one layer of Miracloth (Calbiochem, La Jolla, CA, USA) and centrifuged at 20,000 g for 20 min at 4 °C. The supernatant was mixed with 100 µL µMACS Anti-GFP MicroBeads (130-091-125; Miltenyi Biotec, Japan, Tokyo, Japan) and then incubated for 30 min at 4 °C. The mixtures were applied to M columns (130-042-801; Miltenyi Biotec) placed in the magnetic field of a MiniMACS Separator to retain magnetically labeled proteins. After extensive washing of the columns with extraction buffer, immunoprecipitated protein complexes were eluted with 100 µL elution buffer (50 mM Tris HCl [pH 6.8], 50 mM DTT, 1 % SDS, 1 mM EDTA, 0.005% bromophenol blue, and 10% glycerol). The immunoprecipititates were separated by SDS-PAGE followed by either silver staining or immunoblot analysis. Silver staining was performed using the SilverQuest Silver Staining Kit (Life Technologies Corporation, Carlsbad, CA, USA). Immunoblot analyses were performed using the anti-GFP antibody (11814460001; Roche, Indianapolis, IN, USA) (1:2500) or the anti-Myc antibody (562; MBL, Nagoya, Japan) (1:1000). Signals were detected with the ECL prime Western Blotting Detection Reagent (GE Healthcare Life Sciences).
**Hydroponic culture**

Hydroponic cultivation was performed using the Araponics growing system (Araponics, http://www.araponics.com/) under a 16-h light/8-h dark photoperiod (40±10 µE m$^{-2}$ s$^{-1}$) at 22 °C. Plants were grown hydroponically in either of the following nutrient solutions: (i) a modified low-calcium solution (LCS) (Conn et al., 2011) [in mM: 2 NH$_4$NO$_3$, 5 KNO$_3$, 2 MgSO$_4$, 0.6 KH$_2$PO$_4$, 2 NaNO$_3$, with micronutrients; in µM: 50 NaFe(III)EDTA, 50 H$_3$BO$_3$, 5 MnCl$_2$, 10 ZnSO$_4$, 0.5 CuSO$_4$ and 0.1 Na$_2$MoO$_4$, pH 5.6, NaOH] supplemented with various concentrations of CaCl$_2$ as indicated; (ii) a low-magnesium solution (LMgS) [in mM: 2 NH$_4$NO$_3$, 5 KNO$_3$, 0.1 CaCl$_2$, 2 CaSO$_4$, 0.6 KH$_2$PO$_4$, 2 NaNO$_3$, with micronutrients as described above, pH 5.6, NaOH] supplemented with various concentrations of MgCl$_2$ as indicated. Nutrient solutions were constantly aerated and replaced every week.

**Physiological assays**

For Mg$^{2+}$-susceptibility assays at the seedling stage, seeds were surface-sterilized, sown on GM agar plates solidified with 1.2% (w/v) bacto-agar (BD Biosciences, San Jose, CA, USA), stratified at 4 °C for 3 d in the dark and then grown vertically in a growth room under a 16-h light/8-h dark photoperiod (60±10 µE m$^{-2}$ s$^{-1}$) at 22 °C. Four-day-old seedlings were transferred to fresh agar plates containing half-strength Murashige-Skoog (MS) medium, 1% (w/v) sucrose and 0.5 g/L of MES-KOH (pH 5.7) supplemented with various concentrations of MgCl$_2$ and solidified with 1.2% bacto-agar, and then grown vertically for an additional 14 d. Photographs were then taken and the fresh weight of each plant was determined. For K$^+$-, Na$^+$-, and Ca$^{2+}$-susceptibility assays, KCl, NaCl, and CaCl$_2$ were added to the growth medium at indicated concentrations instead of MgCl$_2$.

**In vitro phosphorylation assay**

The bacterial expression plasmids used for expression of recombinant proteins were constructed using pGEX-4T-2 (GE Healthcare Life Sciences) and pMALc2X (New England Biolabs, Beverly, MA, USA) as described in the Supplemental data. The
pGEX-4T-2- and pMALc2X-based plasmids were transformed into *Escherichia coli* strain BL21-Gold (DE3; Agilent Technologies, La Jolla, CA, USA). The GST-fused and MBP-fused proteins were bacterially expressed and affinity-purified with glutathione sepharose 4B (GE Healthcare Life Sciences) and amylose resin (New England Biolabs), respectively, according to the manufacturer’s instructions. For *in vitro* phosphorylation assays, purified proteins and/or myelin basic protein (Sigma-Aldrich Japan, Tokyo, Japan) were incubated in a total volume of 15 µl protein kinase assay buffer (50 mM Tris-HCl [pH7.5], 100 mM NaCl, 5 mM MnSO₄, 0.5 mM CaCl₂, 2 mM DTT, 10 µM ATP, and 10 µCi [γ⁻³²P]ATP) for 60 min at 30 °C. The reaction was terminated by adding 5 µl 4×SDS-PAGE sample buffer and heating samples at 95 °C for 3 min. The protein samples were then separated by SDS-PAGE and the gel was subsequently dried and exposed to a Fujifilm imaging plate (BAS-MS, GE Healthcare Life Sciences) for 1 d. Incorporation of a radioactive phosphate group was visualized by autoradiography using an FLA-5000 phosphor imager (Fujifilm, Tokyo, Japan). The protein level was analyzed by CBB staining.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Functionality of constitutively-expressed SRK2D-sGFP and visualization of immunoprecipitated proteins for LC-MS/MS analyses by silver staining.

**Supplemental Figure S2.** Expression patterns of *CIPK26* and subcellular localization of sGFP-CIPK26 in leaf epidermal cells.

**Supplemental Figure S3.** Phylogenetic analysis of CIPK family members from Arabidopsis, Rice, *S. moellendorffii*, and *P. patens*.

**Supplemental Figure S4.** Both SRK2D-MBP and CIPK26-GST are functional protein kinases.
**Supplemental Figure S5.** Growth phenotypes of *cipk26, cipk3, cipk9,* and *cipk23* single mutants and multiple mutants.

**Supplemental Figure S6.** The *cipk26/3/9* triple and *cipk26/3/9/23* quadruple mutants show similar ABA-sensitivity to that of the wild type.

**Supplemental Figure S7.** Improved growth of the *cipk26/3/9* and *cipk26/3/9/23* mutants under a low external Mg$^{2+}$ concentration in soil.

**Supplemental Figure S8.** ICP-MS analyses of Mg, Ca, K and Na concentrations in aerial parts of hydroponically grown plants.

**Supplemental Figure S9.** Complementation tests of *cipk26/3/9/23* quadruple mutant by *CIPK26*.

**Supplemental Figure S10.** *CIPK26*-overexpressing lines grow well under both high external Mg$^{2+}$ and low external Ca$^{2+}$ concentrations.

**Supplemental Figure S11.** SRK2D physically interacts with CIPK26 under a high external Mg$^{2+}$ concentration.

**Supplemental Figure S12.** Mg$^{2+}$-susceptibility of *cipk* single mutants, multiple *cipk* mutants, *snrk2* single mutants, and *snrk2* multiple mutants.

**Supplemental Figure S13.** Susceptibility of *srk2d/e/i* and *cipk26/3/9* triple mutants and *cipk26/3/9/23* quadruple mutant to high external K$^+$, Na$^+$, Mg$^{2+}$, and Ca$^{2+}$ concentrations.

**Supplemental Figure S14.** The activation status of SRK2D-sGFP under high
external Mg\(^{2+}\) concentrations, as validated by an in-gel kinase assay.

**Supplemental Figure S15.** Susceptibility of ABA biosynthesis-deficient mutants to a high external Mg\(^{2+}\) concentration.

**Table S1. Candidates of SRK2D-interacting proteins identified by LC-MS/MS analyses**

**Table S2. Primer pairs used in this study**

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**AUTHOR CONTRIBUTIONS**

J.M. (first author), Y.F., K.S., and K.Y.-S. conceived and designed the research. J.M. (first author) performed most of the experiments and analyzed the data, with help from Y.F. and T.Y. Y.F. and Y.T. contributed to the plasmid construction. H.N., Y.N., and F.T. performed the LC-MS/MS analyses and analyzed the data. T.F. and S.N.
performed the ICP-analyses. S.Y., T.I., K.M., and J.M. (14th author) contributed to the immunoprecipitation experiments, and S.K. contributed to the BiFC analyses. J.M. (14th author) performed the phylogenetic analysis. J.M. (first author), K.S., and K.Y.-S wrote the article.
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FIGURE LEGENDS

Figure 1. Identification of SRK2D-interacting proteins by co-IP coupled with LC-MS/MS analyses. (A) Western blot analysis of sGFP (left) and SRK2D-sGFP (right) after co-immunoprecipitation (co-IP). SRK2D-sGFP and associated proteins were purified from transgenic Arabidopsis plants expressing SRK2D-sGFP by co-IP using anti-GFP antibody. In control experiments, transgenic Arabidopsis plants expressing only sGFP were subjected to co-IP. Immunoprecipitates from sGFP-expressing line and SRK2D-sGFP-expressing line (line #1. See also Supplemental Fig. S1, A and B) were subjected to immunoblot analysis with the anti-GFP antibody. Stars indicate predicted sGFP and SRK2D-sGFP bands. (B) Immunoprecipitates from plants of sGFP-expressing line or SRK2D-sGFP-expressing line (line #1) treated with or without 0.8 M mannitol for 1 h were analyzed and visualized by silver staining. (C) Validation of physical interactions between SRK2D and candidate interactors by yeast two-hybrid assay. Representative growth status of yeast cells is shown on SD/-LWHA agar medium with 30 mM 3-AT from duplicate independent trials. Photographs were taken 7 d after inoculation.

Figure 2. Physical interactions between SnRK2s and CIPKs. (A) Co-IP of CIPK26 with SRK2D in Arabidopsis plants. Transgenic lines harboring both pGH-35Spro:4×myc-CIPK26 and pGK-35Spro:SRK2D-sGFP or pGK-35Spro:sGFP were subjected to co-IP using anti-GFP antibody. Immunoprecipitates were analyzed by immunoblotting with anti-GFP or anti-myc antibody. Similar results were obtained in independent experiments; representative data are shown. (B) Physical interactions between CIPK26 and SRK2D derivatives analyzed by yeast two-hybrid assay. Representative growth status of yeast cells is shown on SD/-LWHA agar media with or without 3-AT from duplicate independent trials. Photographs were taken 7 d after inoculation. (C) Yeast two-hybrid assay to validate interaction between CIPK26 and members of SnRK2s. Photographs were taken 7 d after inoculation. (D) BiFC analyses of physical interactions between SRK2D, SRK2E, or
SRK2C and CIPK26 or CIPK24 in *Nicotiana benthamiana* leaves. Transiently transformed *N. benthamiana* epidermal cells harboring indicated plasmid combinations were analyzed by confocal microscopy. YFP fluorescence and Nomarski images are shown. Scale bars = 20 µm. (E) Physical interactions between the regulatory domain of SRK2D (SRK2D RD) and CIPK26, CIPK3, CIPK9, CIPK23, or CIPK24 analyzed by yeast two-hybrid assay. Representative growth status of yeast cells is shown on SD/-LWHA agar medium with 30 mM 3-AT from duplicate independent trials. (F) BiFC visualization of interaction between SRK2D and CIPK26, CIPK3, CIPK9, and CIPK23, but not CIPK24, in *N. benthamiana* leaves. The experimental procedure was as described in (D). Scale bars = 50 µm.

**Figure 3.** CIPK26 can phosphorylate SRK2D *in vitro*. (A) Phosphorylation of SRK2D<sup>K52N</sup>-MBP by CIPK26-GST *in vitro*. *In vitro* phosphorylation assays were conducted with 200 ng SRK2D (or SRK2D<sup>K52N</sup>)-MBP, and 800 ng CIPK26 (or CIPK26<sup>K42N</sup>)-GST. Proteins were separated on 10% (w/v) SDS-polyacrylamide gel after incubation in protein kinase assay buffer containing [γ-<sup>32</sup>P]ATP. Each lane represents an independent reaction in which the indicated combinations of recombinant proteins were tested. Radioactively labeled proteins were visualized by autoradiography. Protein abundance was visualized by CBB staining. Similar results were obtained in independent experiments; representative data are shown. (B) Phosphorylation of the regulatory domain of SRK2D by CIPK26-GST *in vitro*. *In vitro* phosphorylation assays were conducted with 200 ng SRK2D KD-GST or SRK2D RD-GST and 800 ng CIPK26-GST. KD, the kinase domain; RD, the regulatory domain. (C) Effects of co-incubation of CBL1-GST or CBL9-GST with CIPK26-GST on the phosphorylation level of SRK2D<sup>K52N</sup>-MBP. *In vitro* phosphorylation assays were conducted with 200 ng SRK2D<sup>K52N</sup>-MBP, 800 ng CIPK26-GST, and 200 ng CBL1-GST or CBL9-GST. (D) Effects of co-incubation of CIPK26-GST and CBL1-GST or CBL9-GST on the SRK2D-MBP activity. The kinase activity of SRK2D-MBP toward myelin basic protein was analyzed by *in vitro* pre-incubation of SRK2D-MBP with CIPK26-GST and CBL1/CBL9-GST followed
by an in-gel kinase assay. In vitro pre-incubation was performed as described in (C), except for the absence of $[\gamma-^{32}P]ATP$ and the addition of 0.1 mM Na$_3$VO$_4$ in the protein kinase assay buffer. After pre-incubation, samples were subjected to an in-gel kinase assay. The phosphorylation reaction was performed in the protein kinase assay buffer containing $[\gamma-^{32}P]ATP$ as described in MATERIALS AND METHODS. Myelin basic protein was embedded in the gel as the substrate. Arrowheads indicate the bands corresponding to the kinase activity of SRK2D-MBP toward myelin basic protein.

Figure 4. Growth retardation of cipk26/3/9 triple mutant and cipk26/3/9/23 quadruple mutants is rescued under low external Mg$^{2+}$ concentrations. (A) Growth phenotypes of plants grown on GM agar plates for 2 weeks and then in soil for an additional 10 d. Scale bars = 1 cm. WT, the wild type. (B) Maximum rosette radius of each plant grown as described in (A). Bars indicate SD, $n = 6$. Asterisks indicate statistically significant difference compared with wild type (**$P < 0.01$, one-way ANOVA followed by a post hoc Dunnett’s multiple comparison test). Experiment was performed twice; a representative result is shown. (C) Representative images of rosette leaves of wild type, cipk26/3/9 triple mutant, and cipk26/3/9/23 quadruple mutant. (D) Growth phenotypes of plants grown on GM agar plates for 2 weeks then in soil for another 14 d. Scale bars = 1 cm. (E) Inflorescence height of each plant grown as described in (D). Bars indicate SD, $n = 6$. Asterisks indicate statistically significant difference compared with wild type as described in (B). Experiment was performed twice; a representative result is shown. (F) Representative images of shoot apexes of wild type, cipk26/3/9 triple mutant, and cipk26/3/9/23 quadruple mutant. (G) Representative images of plants of wild type, cipk26/3/9 triple mutant, and cipk26/3/9/23 quadruple mutant grown in a hydroponic culture system for 24 d. Left panel: plants grown hydroponically in modified low-calcium solution (LCS; see experimental procedures) supplemented with indicated concentrations of CaCl$_2$. Right panel: plants grown hydroponically in low-magnesium solution (LMgS; see experimental procedures) supplemented with indicated concentrations of MgCl$_2$. 

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Scale bars = 1 cm. (H) Fresh weight of aerial parts of each plant grown as described in (G). Bars indicate SD, n = 6. Columns marked with different lower-case letters represent significantly different means (P < 0.01) according to two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test. Experiment was performed twice; a representative result is shown.

Figure 5. The srk2d/e/i triple mutant shows increased susceptibility to a high external Mg$^{2+}$ concentration. (A) Growth phenotypes of wild type (WT), srk2d/e/i and cipk26/3/9 triple mutants, and cipk26/3/9/23 quadruple mutant grown under a high external Mg$^{2+}$ concentration. Photographs show representative phenotypes of 18-d-old seedlings grown vertically for 4 d on GM plates, and then for 14 d on 0.5×Murashige-Skoog medium (MS) agar plates containing indicated concentrations of MgCl$_2$. Scale bars = 1 cm. (B) Fresh weight of seedlings treated as described in (A). Bars indicate SD, n = 8. Asterisks indicate statistically significant difference (**P < 0.01, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments; representative data are shown. (C) ICP-MS analyses of Mg, Ca, K, and Na concentrations in aerial parts of plants grown vertically for 4 d on GM plates and then for 12 d on 0.5×MS agar plates with or without addition of 20 mM MgCl$_2$. White bars, control; orange bars, 20 mM MgCl$_2$. For each biological replicate, material from four plants was pooled to make one sample for ICP-MS analysis. Data represent means and SDs, n = 6. Asterisk above a bar indicates that the corresponding mean is significantly different (*P < 0.05; **P < 0.01, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test) from the mean value of wild type within each condition. (D) Susceptibility of srk2d/e/i/cipk26/3/9/23 septuple mutant to high external Mg$^{2+}$ concentrations. Plants were grown as described in (A). Scale bars = 1 cm. (E) Fresh weight of seedlings treated as described in (D). Bars indicate SD, n = 8. Similar results were obtained in independent experiments; representative data are shown.
**Figure 6.** ABA-mediated rescue of hypersusceptibility of *aba2-1* mutant to a high external Mg$^{2+}$ concentration. (A) Susceptibility to a high external Mg$^{2+}$ concentration (20 mM MgCl$_2$) in the *aba2-1, nced3-2, areb1/areb2/abf3* triple (*areb* triple) mutants. Photographs show representative phenotypes of 18-d-old seedlings grown as described in Fig. 5A. Scale bars = 1 cm. (B) Fresh weight of seedlings treated as described in (A). Bars indicate SD, *n* = 9. Asterisks indicate statistically significant difference (**P* < 0.01, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments. Representative data are shown. (C) Growth phenotypes of the wild-type, *srk2d/e/i* triple, *cibaba26/3/9/23* quadruple, and *aba2-1* mutant plants grown vertically for 4 d on GM plates and then for 14 d on 0.5×MS agar plates with or without addition of 20 or 30 mM MgCl$_2$ and 1 µM ABA. In the control experiment, an equivalent amount of ethanol (the solvent for ABA) was added to the media. Scale bars = 1 cm. (D) Fresh weight of seedlings treated as described in (C). Bars indicate SD, *n* = 8. Asterisk indicates statistically significant difference as described in (A). Similar results were obtained in independent experiments; representative data are shown.

**Figure 7.** A hypothesized schematic model of the novel roles of SRK2D/E/I and CIPK26/3/9/23 in plant growth under high external Mg$^{2+}$ concentrations in Arabidopsis. Under water-deficit stress conditions, subclass III SnRK2s (SRK2D, SRK2I, and SRK2E/OST1) are activated by high levels of ABA, and they then modulate the activities of downstream targets, including transcription factors such as AREB/ABFs and channels (e.g. SLAC1). Under high external Mg$^{2+}$ concentrations, subclass III SnRK2s, in response to basal levels of ABA, play a key role in plant growth, while interacting with CIPK26, 3, 9, and 23 (CIPK26/3/9/23). Subclass III SnRK2s and CIPK26/3/9/23 function as key modulators of the susceptibility to shoot growth inhibition in response to increased external Mg$^{2+}$ concentrations (Mg$^{2+}$-susceptibility), probably via phosphorylating, and thus modulating, downstream targets, which are assumed to modulate intracellular Mg$^{2+}$ transport, and
maintain Mg\textsuperscript{2+} homeostasis. Dashed lines indicate possible, but not confirmed, routes.

**Supplemental Figure Legends**

**Figure S1.** Functionality of constitutively-expressed SRK2D-sGFP and visualization of immunoprecipitated proteins for LC-MS/MS analyses by silver staining. (A) Photographs of plants of wild-type, sGFP control line, and SRK2D-sGFP-expressing lines (lines #1 and #2) grown for 3 weeks on germination medium (GM) agar plates. Scale bars = 1 cm. (B) Maximum rosette radius of each plant grown as described in (A). Bars indicate SD, n = 8. (C) Validation of activation patterns of SRK2D-sGFP in response to ABA or mannitol treatment by in-gel kinase assay. Proteins were extracted from SRK2D-sGFP-expressing line (line #1) treated with or without 50 µM ABA or 0.8 M mannitol at different time points (20 µg protein per lane). Myelin basic protein was used as substrate. Experiments were performed twice; a representative result is shown. (D) Extremely drought-sensitive phenotype of srk2d/e/i triple mutant was complemented by constitutive expression of SRK2D-sGFP. Plants of wild-type, srk2e/i double mutant, sGFP, or SRK2D-sGFP-expressing lines (in the srk2d/e/i triple mutant background) were grown on a GM agar plates for 3 weeks and then grown in soil for an additional 4 d before exposure to drought stress. Drought stress was imposed by withholding water for 6 to 7 d. After resuming watering for 4 d, survival rates were calculated from the number of plants that survived and continued to grow. Experiments were performed three times independently (n = 20 for each experiment). (E–G) Immunoprecipitates from whole seedlings of sGFP-expressing line or SRK2D-sGFP-expressing line (line #1) treated with or without 0.8 M mannitol for 1 h were analyzed and visualized by silver staining. Red arrows indicate protein bands detected specifically in SRK2D-sGFP samples but not in sGFP samples. (E) Immunoprecipitates from untreated whole seedlings used for first LC-MS/MS analysis. (F) Immunoprecipitates from untreated whole seedlings used for second LC-MS/MS analysis. (G)
Immunoprecipitates from whole seedlings treated with mannitol used for third LC-MS/MS analysis.

**Figure S2.** Expression patterns of CIPK26 and subcellular localization of sGFP-CIPK26 in leaf epidermal cells. (A) Histochemical localization of GUS activity in transgenic Arabidopsis line harboring pGH-CIPK26pro:GUS. 1: 1-d-old gerniating seed, 2: 8-d-old seedling, 3–5: 18-d-old seedling (3: aerial part, 4: roots, 5: true leaf). Similar staining patterns were observed in other lines; representative images are shown. (B) Co-expression of SRK2D-mCherry and sGFP-CIPK26 in N. benthamiana leaves. Transiently transformed N. benthamiana epidermal cells harboring both pGH-35Spro:SRK2D-mCherry and pGH-35Spro:sGFP-CIPK26 were analyzed by confocal microscopy. GFP fluorescence, RFP fluorescence, and Nomarski images are shown. Scale bars = 10 µm. (C) Confocal images of GFP fluorescence in leaf epidermal cells of a transgenic line harboring pGH-CIPK26pro:sGFP-CIPK26 construct. GFP fluorescence and Nomarski images are shown. Scale bars = 20 µm. Similar fluorescence patterns were observed in other lines; representative images are shown.

**Figure S3.** Phylogenetic analysis of CIPK family members from Arabidopsis, Rice, S. moellendorfii, and P. patens. (A) Alignments of sequences around kinase and NAF domains of CIPK family members in Arabidopsis, rice, S. moellendorfii (a lycophyte), and P. patens (a moss). All peptide sequences of CIPK family members from the four plants available in Phytozome database are included, except for those lacking substantial portions of either or both of the two domains. Shading indicates the conservation ratio at each site (black, 100%; mid-gray, 80%; light gray, 60%). Members of monophyletic group including CIPK26, 3, 9, and 23 are highlighted. Black lines and gray bars below alignments indicate sites used to construct phylogeny and locations of the two domains, respectively. Each sequence name is based on the corresponding transcript name in the Phytozome database. (B) Phylogenetic tree of CIPK family members in Arabidopsis, rice, S. moellendorfii,
and *P. patens*. Neighbor-joining tree was constructed based on alignments of peptide sequences described in (A). Numbers at side of each branch indicate bootstrap values (≥50%) from 1000 replicates. Scale bar indicates substitution rate per site. Monophyletic group including CIPK26, 3, 9 and 23 is highlighted.

**Figure S4.** Both SRK2D-MBP and CIPK26-GST are functional protein kinases. (A) Both recombinant SRK2D-MBP and CIPK26-GST proteins were capable of phosphorylating myelin basic protein *in vitro*. *In vitro* phosphorylation assays were conducted with 200 ng SRK2D (or SRK2D^K52N^-MBP, 800 ng CIPK26 (or CIPK26^K42N^-)GST, and 200 ng myelin basic protein. Proteins were separated on 12% (w/v) SDS-polyacrylamide gel after incubation in protein kinase assay buffer containing [γ-^32^P]ATP. Each lane represents an independent reaction in which the indicated combinations of recombinant proteins and myelin basic protein were tested. Radioactively labeled proteins were visualized by autoradiography. Protein abundance was visualized by CBB staining. (B) The effect of co-incubation of CIPK26-GST and CBL1/CBL9-GST with SRK2D-MBP on the phosphorylation level of myelin basic protein *in vitro*. *In vitro* phosphorylation assays were performed as described in (A), except for the addition of 200 ng CBL1-GST or CBL9-GST.

**Figure S5.** Growth phenotypes of *cipk26, cipk3, cipk9, and cipk23* single mutants and multiple mutants. (A) Scheme of *CIPK26/3/9/23* genes. Exons, thick lines; introns, thin lines. Position of the T-DNA insertion in each mutant is shown. (B) Expression levels of each *CIPK* gene in each mutant were determined by RT-PCR with total RNA isolated from 12-day-old seedlings of wild-type and mutants using *CIPK26-, CIPK3-, CIPK9-, CIPK23-, and TUB1*-specific primers. (C) *CIPK23* transcript levels in wild type, *cipk23* single mutant, and *cipk* multiple mutants were analyzed by quantitative RT-PCR (expression level in wild type was defined as 100%). Values are mean ± SDs of three replicate reactions. (D) Growth phenotypes of plants grown for 3 weeks on GM agar plates. Scale bars = 1 cm. (E) Maximum rosette radius of each plant grown as described in (D). Bars indicate SD, *n* = 8. (F)
Growth phenotypes of plants grown on GM agar plates for 2 weeks then in soil for another 14 d. Scale bars = 1 cm. (G) Inflorescence height of each plant grown as described in (F). Experiment was performed twice; a representative result is shown. Bars indicate SD, n = 6. (H) Seed yield of each plant (seed weight per plant) grown in soil in pots. Bars indicate SD, n = 6. Asterisks indicate statistically significant difference compared with wild type (**P < 0.01, one-way ANOVA followed by post hoc Dunnett’s multiple comparison test).

**Figure S6.** The cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants show similar ABA-sensitivity to that of the wild type. (A) ABA-sensitivity of the cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants during the vegetative growth stage. Images show representative phenotypes of 10-d-old seedlings grown vertically for 4 d on GM plates, followed by 6 d on 0.5xMS agar plates, supplemented with or without various concentrations of ABA as indicated. In the control experiment, an equivalent amount of ethanol was added to the media. Scale bars = 1 cm. (B) Quantification of relative fresh weight of seedlings treated as described in (A). The value of each genotype grown under the control condition was defined as 100%. Average fresh weight for controls were 8.3, 10.9, 8.5 and 8.9 mg for the wild-type, the srk2d/e/i triple, cipk26/3/9 triple and cipk26/3/9/23 quadruple mutants, respectively. Bars indicate SD, n = 8. Similar results were obtained in independent experiments. Representative data are shown. (C) The activation patterns of subclass III SnRK2s in response to ABA or mannitol treatment in the cipk26/3/9/23 quadruple mutant. An in-gel kinase assay was performed as described in the Supplemental Fig S1C. Myelin basic protein was used as the substrate. Arrowheads indicate the bands corresponding to subclass III SnRK2s activity.

**Figure S7.** Improved growth of the cipk26/3/9 and cipk26/3/9/23 mutants under a low external Mg^{2+} concentration in soil. (A) Growth performance of plants of wild-type, the cipk26/3/9 triple mutant, and cipk26/3/9/23 quadruple mutant in soil supplemented with LMgS containing 2 mM or 0.1 mM MgCl$_2$. Photographs show
representative phenotypes of 6-week-old seedlings grown on GM plates for 2 weeks and then in soil for an additional 4 weeks. Each pot contained 4 plants. (B) Inflorescence height of each plant grown as described in (A). Bars indicate SD, \( n = 8 \).

**Figure S8.** ICP-MS analyses of Mg, Ca, K and Na concentrations in aerial parts of hydroponically grown plants. Concentrations of Mg, Ca, K and Na in aerial parts of plants grown in modified low-calcium solution (LCS) supplemented with indicated concentrations of CaCl\(_2\) (A. See also Fig. 4G, left), or in low-magnesium solution (LMgS) supplemented with indicated concentrations of MgCl\(_2\) (B. See also Fig. 4G, right). Values are means and SDs \((n = 6)\). Asterisk above a bar indicates that corresponding mean is significantly different (*\(P < 0.05\); **\(P < 0.01\)) from mean determined for wild type (WT) at the respective CaCl\(_2\) (or MgCl\(_2\)) concentration, according to two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test.

**Figure S9.** Complementation tests of cipk26/3/9/23 quadruple mutant by CIPK26. (A) CIPK26 transcript levels in wild type (WT), cipk3/9/23 triple mutant, vector control line (in cipk26/3/9/23 quadruple mutant background), and two independent cipk26/3/9/23 quadruple mutant lines harboring CIPK26pro-CIPK26 (line #10 and #13). CIPK26 transcripts were detected by RT-PCR using total RNA extracted from 12-d-old seedlings. TUB1 transcripts were detected as a control in each experiment. (B) Growth phenotypes of plants described in (A) in soil. Plants were grown on GM agar plates for 2 weeks and then in soil for another 2 weeks. Scale bars = 1 cm. (C) Maximum rosette radius of each plant grown as described in (B). Bars indicate SD, \( n = 5 \). (D) Growth phenotypes of plants grown on GM agar plates for 2 weeks and then in soil for another 4 weeks. Scale bars = 2 cm. (E) Inflorescence height of each plant grown as described in (D). Bars indicate SD, \( n = 5 \). (F) Representative images of plants of wild type, cipk3/9/23 triple mutant, vector control line (in the cipk26/3/9/23 quadruple mutant background), and two independent cipk26/3/9/23 quadruple mutant
Figure S10. CIPK26-overexpressing lines grow well under both high external Mg\(^{2+}\) and low external Ca\(^{2+}\) concentrations. (A) CIPK26 transcript levels in wild type (WT), vector control line, and two independent CIPK26-overexpressing lines (lines #2 and #4) were analyzed by quantitative RT-PCR (expression level in wild type was defined as 1). Values are mean ± SDs of three replicate reactions. (B) Growth performance of plants of wild type, vector control line, and CIPK26-overexpressing lines under high external Mg\(^{2+}\) concentrations. Photographs show representative phenotypes of 18-d-old seedlings grown vertically for 4 d on GM plates, and then for 14 d on 0.5×Murashige-Skoog medium (MS) agar plates containing indicated concentrations of MgCl\(_2\). Scale bars = 1 cm. (C) Fresh weight of whole seedlings treated as described in (B). Bars indicate SD, \(n = 12\). Asterisks indicate statistically significant difference (**\(P < 0.01\), two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments; representative data are shown. (D) Growth performance of plants of wild type, vector control line, and CIPK26-overexpressing lines under a low external Ca\(^{2+}\) concentration. Photographs show representative phenotypes of 24-d-old seedlings grown hydroponically in the modified LCS supplemented with 0.1 mM or 2 mM CaCl\(_2\). Scale bars = 1 cm. (E) Fresh weight of aerial parts of plants treated as described in (D). Bars indicate SD, \(n = 6\). Asterisks indicate statistically significant difference (\(*P < 0.05\); **\(P < 0.01\), two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Experiment was performed twice; a representative result is shown.

Figure S11. SRK2D physically interacts with CIPK26 under a high external Mg\(^{2+}\) concentration. Co-immunoprecipitation of CIPK26 with SRK2D in Arabidopsis.
Transgenic lines harboring both pGH-35Spro:4×myc-CIPK26 and pGK-35Spro:SRK2D-sGFP or pGK-35Spro:sGFP (see Fig. 2A) were treated with 0.5×MS solution supplemented with or without 20 mM MgCl₂ for 24 h, and then subjected to co-immunoprecipitation using anti-GFP antibody. Immunoprecipitates were analyzed by immunoblotting with anti-GFP or anti-myc antibody.

**Figure S12.** Mg²⁺-susceptibility of *cipk* single mutants, multiple *cipk* mutants, *snrk2* single mutants, and *snrk2* multiple mutants. (A) Susceptibility of *cipk26, cipk3, cipk9,* and *cipk23* single mutants and multiple *cipk* mutants to a high external Mg²⁺ concentration (20 mM MgCl₂). Data represent fresh weight of seedlings grown as described in Fig. 5A. Bars indicate SD, *n* = 6. Asterisk above a bar indicates that corresponding mean is significantly different (**P < 0.01) from the mean value of wild type (WT) at the 20 mM MgCl₂ concentration, according to two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test. Similar results were obtained in independent experiments and representative data are shown. (B) Susceptibility to a high external Mg²⁺ concentration (20 mM MgCl₂) in the *srk2d, srk2e,* and *srk2i* single mutants and multiple *snrk2* mutants. Data represent fresh weight of seedlings grown as described in Fig. 5A. Bars indicate SD, *n* = 8. Asterisk above a bar indicates that corresponding mean is significantly different (**P < 0.01) from the mean value of wild type at the 20 mM MgCl₂ concentration as described in (A). Similar results were obtained in independent experiments and representative data are shown.

**Figure S13.** Susceptibility of *srk2d/e/i* and *cipk26/3/9* triple mutants and *cipk26/3/9/23* quadruple mutant to high external K⁺, Na⁺, Mg²⁺, and Ca²⁺ concentrations. (A) Growth phenotypes of wild type (WT), *srk2d/e/i* triple mutant, *cipk26/3/9* triple mutant, and *cipk26/3/9/23* quadruple mutant under high external K⁺, Na⁺, Mg²⁺ and Ca²⁺ concentrations, as indicated. Photographs show representative phenotypes of 18-d-old seedlings grown vertically for 4 d on GM plates, followed by 14 d on 0.5×MS agar plates containing 125 mM KCl, 125 mM NaCl, 20 mM MgCl₂,
or 20 mM CaCl₂. Scale bars = 1 cm. (B) Fresh weight of seedlings treated as described in (A). Bars indicate SD, $n = 8$. Asterisks indicate statistically significant difference (*$P < 0.05$; **$P < 0.01$, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments; representative data are shown.

**Figure S14.** The activation status of SRK2D-sGFP under high external Mg²⁺ concentrations, as validated by an in-gel kinase assay. Proteins were extracted from the SRK2D-sGFP-expressing line (line #1) treated with or without 50 µM ABA, 0.8 M mannitol, or 20 mM MgCl₂ at different time points (20 µg protein per lane). Myelin basic protein was used as the substrate. Lane 1; non-treated, lane 2; 50 µM ABA for 30 min, lane 3; 0.8 M mannitol for 30 min, lane 4 to 9; 20 mM MgCl₂ treatments (lane 4, for 0.5 h; lane 5, for 1 h; lane 6, for 6 h; lane 7, for 12 h; lane 8, for 24 h; lane 9, for 48 h).

**Figure S15.** Susceptibility of ABA biosynthesis-deficient mutants to a high external Mg²⁺ concentration. (A) Susceptibility of several ABA biosynthesis-deficient mutants (*nced3-2, aba2-1, aao3-4, and aba3-1*) to a high external Mg²⁺ concentration (20 mM MgCl₂). Plants were grown as described in Fig. 5A. Scale bars = 1 cm. (B) Fresh weight of seedlings treated as described in (A). Bars indicate SD, $n = 9$. Asterisks indicate statistically significant difference (**$P < 0.01$, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments. Representative data are shown.

**Table S1.** Candidates of SRK2D-interacting proteins identified by LC-MS/MS analyses

**Table S2.** Primer pairs used in this study
Figure 1. Identification of SRK2D-interacting proteins by co-IP coupled with LC-MS/MS analyses. (A) Western blot analysis of sGFP (left) and SRK2D-sGFP (right) after co-immunoprecipitation (co-IP). SRK2D-sGFP and associated proteins were purified from transgenic Arabidopsis plants expressing SRK2D-sGFP by co-IP using anti-GFP antibody. In control experiments, transgenic Arabidopsis plants expressing only sGFP were subjected to co-IP. Immune precipitates from sGFP-expressing line and SRK2D-sGFP-expressing line (line #1. See also Supplemental Fig. S1, A and B) were subjected to immunoblot analysis with the anti-GFP antibody. Stars indicate predicted sGFP and SRK2D-sGFP bands. (B) Immunoprecipitates from plants of sGFP-expressing line or SRK2D-sGFP-expressing line (line #1) treated with or without 0.8 M mannitol for 1 h were analyzed and visualized by silver staining. (C) Validation of physical interactions between SRK2D and candidate interactors by yeast two-hybrid assay. Representative growth status of yeast cells is shown on SD/-LWHA agar plates with 30 mM 3-AT from duplicate independent trials. Photographs were taken 7 d after inoculation.
Figure 2. Physical interactions between SnRK2s and CIPKs. (A) Co-IP of CIPK26 with SnRK2D in Arabidopsis plants. Transgenic lines harboring both pGH-35Spro:4×myc-CIPK26 and pGK-35Spro:SRK2D-sGFP or pGK-35Spro:sGFP were subjected to co-IP using anti-GFP antibody. Immunoprecipitates were analyzed by immunoblotting with anti-GFP or anti-myc antibody. Similar results were obtained in independent experiments; representative data are shown. (B) Physical interactions between CIPK26 and SRK2D derivatives analyzed by yeast two-hybrid assay. Representative growth status of yeast cells is shown on SD-LWHA agar media with or without 3-AT from duplicate independent trials. Photographs were taken 7 d after inoculation. (C) Yeast two-hybrid assay to validate interaction between CIPK26 and members of SnRK2s. Photographs were taken 7 d after inoculation. (D) BiFC analyses of physical interactions between SRK2D, SRK2E, or SRK2C and CIPK26 or CIPK24 in Nicotiana benthamiana leaves. Transiently transformed N. benthamiana epidermal cells harboring indicated plasmid combinations were analyzed by confocal microscopy. YFP fluorescence and Nomarski images are shown. Scale bars = 20 μm. (E) Physical interactions between the regulatory domain of SRK2D (SRK2D RD) and CIPK26, CIPK3, CIPK9, CIPK23, or CIPK24 analyzed by yeast two-hybrid assay. Representative growth status of yeast cells is shown on SD-LWHA agar media with 3-AT from duplicate independent trials. (F) BiFC visualization of interaction between SRK2D and CIPK26, CIPK3, CIPK9, and CIPK23, but not CIPK24, in N. benthamiana leaves. The experimental procedure was as described in (D). Scale bars = 50 μm.
Figure 3. CIPK26 can phosphorylate SRK2D in vitro. (A) Phosphorylation of SRK2D(K52N)-MBP by CIPK26-GST in vitro. In vitro phosphorylation assays were conducted with 200 ng SRK2D (or SRK2D(K52N))-MBP, and 800 ng CIPK26 (or CIPK26(K42N))-GST. Proteins were separated on 10% (w/v) SDS-polyacrylamide gel after incubation in protein kinase assay buffer containing [γ-32P]ATP. Each lane represents an independent reaction in which the indicated combinations of recombinant proteins were tested. Radioactively labeled proteins were visualized by autoradiography. Protein abundance was visualized by CBB staining. Similar results were obtained in independent experiments; representative data are shown. (B) Phosphorylation of the regulatory domain of SRK2D by CIPK26-GST in vitro. In vitro phosphorylation assays were conducted with 200 ng SRK2D KD-GST or SRK2D RD-GST and 800 ng CIPK26-GST. KD, the kinase domain; RD, the regulatory domain. (C) Effects of co-incubation of CBL1-GST or CBL9-GST with CIPK26-GST on the phosphorylation level of SRK2D(K52N)-MBP. In vitro phosphorylation assays were conducted with 200 ng SRK2D(K52N)-MBP, 800 ng CIPK26-GST, and 200 ng CBL1-GST or CBL9-GST. (D) Effects of co-incubation of CIPK26-GST and CBL1-GST or CBL9-GST on the SRK2D-MBP activity. The kinase activity of SRK2D-MBP toward myelin basic protein was analyzed by in vitro pre-incubation of SRK2D-MBP with CIPK26-GST and CBL1/CBL9-GST followed by an in-gel kinase assay. In vitro pre-incubation was performed as described in (C), except for the absence of [γ-32P]ATP and the addition of 0.1 mM Na3VO4 in the protein kinase assay buffer. After pre-incubation, samples were subjected an in-gel kinase assay. The phosphorylation reaction was performed in the protein kinase assay buffer containing [γ-32P]ATP as described in MATERIALS AND METHODS. Myelin basic protein was embedded in the gel as the substrate. Arrowheads indicate the bands corresponding to the kinase activity of SRK2D-MBP toward myelin basic protein.
Figure 4. Growth retardation of cikp26/3/9 triple mutant and cikp26/3/9/23 quadruple mutants is rescued under low external Mg\(^{2+}\) concentrations. (A) Growth phenotypes of plants grown on GM agar plates for 2 weeks and then in soil for an additional 10 d. Scale bars = 1 cm. WT, the wild type. (B) Maximum rosette radius of each plant grown as described in (A). Bars indicate SD, n = 6. Asterisks indicate statistically significant difference compared with wild type (**p < 0.01, one-way ANOVA followed by a post hoc Dunnett’s multiple comparison test). Experiment was performed twice; a representative result is shown. (C) Representative images of rosette leaves of wild type, cikp26/3/9 triple mutant, and cikp26/3/9/23 quadruple mutant. (D) Growth phenotypes of plants grown on GM agar plates for 2 weeks and then in soil for another 14 d. Scale bars = 1 cm. (E) Inflorescence height of each plant grown as described in (D). Bars indicate SD, n = 6. Asterisks indicate statistically significant difference compared with wild type as described in (D). Experiment was performed twice; a representative result is shown. (F) Representative images of shoot apexes of wild type, cikp26/3/9 triple mutant, and cikp26/3/9/23 quadruple mutant. (G) Representative images of plants of wild type, cikp26/3/9 triple mutant, and cikp26/3/9/23 quadruple mutant grown in a hydroponic culture system for 24 d. Left panel: plants grown hydroponically in modified low-calcium solution (LCS; see experimental procedures) supplemented with indicated concentrations of CaCl\(_2\). Right panel: plants grown hydroponically in low-magnesium solution (LMgs; see experimental procedures) supplemented with indicated concentrations of MgCl\(_2\). Scale bars = 1 cm. (H) Fresh weight of aerial parts of each plant grown as described in (G). Bars indicate SD; different lower-case letters represent significantly different means (p < 0.01) according to two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test. Experiment was performed twice; a representative result is shown.
Figure 5. The srk2d/e/i triple mutant shows increased susceptibility to a high external Mg\(^{2+}\) concentration. (A) Growth phenotypes of wild type (WT), srk2d/e/i and cikp26/3/9 triple mutants, and cikp26/3/9/23 quadruple mutant grown under a high external Mg\(^{2+}\) concentration. Photographs show representative phenotypes of 18-d-old seedlings grown vertically for 4 d on GM plates, and then for 14 d on 0.5×Murashige-Skoog medium (MS) agar plates containing indicated concentrations of MgCl\(_2\). Scale bars = 1 cm. (B) Fresh weight of seedlings treated as described in (A). Bars indicate SD, n = 8. Asterisks indicate statistically significant difference (**P < 0.01, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments; representative data are shown. (C) ICP-MS analyses of Mg, Ca, K, and Na concentrations in aerial parts of plants grown vertically for 4 d on GM plates and then for 12 d on 0.5×MS agar plates with or without addition of 20 mM MgCl\(_2\). White bars, control; orange bars, 20 mM MgCl\(_2\). For each biological replicate, material from four plants was pooled to make one sample for ICP-MS analysis. Data represent means and SDs, n = 6. Asterisk above a bar indicates that the corresponding mean is significantly different (*P < 0.05; **P < 0.01, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test) from the mean value of wild type within each condition. (D) Susceptibility of srk2d/e/i/cikp26/3/9/23 septuple mutant to high external Mg\(^{2+}\) concentration. Photographs show representative phenotypes of 18-d-old seedlings grown vertically for 4 d on GM plates, and then for 14 d on 0.5×MS agar plates containing indicated concentrations of MgCl\(_2\). Scale bars = 1 cm. (E) Fresh weight of seedlings treated as described in (D). Bars indicate SD, n = 8. Similar results were obtained in independent experiments; representative data are shown.
Figure 6. ABA-mediated rescue of hypersusceptibility of aba2-1 mutant to a high external Mg²⁺ concentration. (A) Susceptibility to a high external Mg²⁺ concentration (20 mM MgCl₂) in the aba2-1, nced3-2, areb1/areb2/abf3 triple (areb triple) mutants. Photographs show representative phenotypes of 18-d-old seedlings grown as described in Fig. 5A. Scale bars = 1 cm. (B) Fresh weight of seedlings treated as described in (A). Bars indicate SD, n = 9. Asterisks indicate statistically significant difference (**P < 0.01, two-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test). Similar results were obtained in independent experiments. Representative data are shown. (C) Growth phenotypes of the wild-type, srk2/d/e/i triple, cik26/3/9/23 quadruple, and aba2-1 mutant plants grown vertically for 4 d on GM plates and then for 14 d on 0.5×MS agar plates with or without addition of 20 or 30 mM MgCl₂ and 1 μM ABA. In the control experiment, an equivalent amount of ethanol (the solvent for ABA) was added to the media. Scale bars = 1 cm. (D) Fresh weight of seedlings treated as described in (C). Bars indicate SD, n = 9. Asterisks indicate statistically significant difference as described in (A). Similar results were obtained in independent experiments; representative data are shown.
**Figure 7.** A hypothesized schematic model of the novel roles of SRK2D/E/I and CIPK26/3/9/23 in plant growth under high external Mg\(^{2+}\) concentrations in Arabidopsis. Under water-deficit stress conditions, subclass III SnRK2s (SRK2D, SRK2I, and SRK2E/OST1) are activated by high levels of ABA, and they then modulate the activities of downstream targets including transcription factors such as AREB/ABFs and channels (e.g. SLAC1). Under high external Mg\(^{2+}\) concentrations, subclass III SnRK2s, in response to basal levels of ABA, play a key role in plant growth, while interacting with CIPK26, 3, 9, and 23 (CIPK26/3/9/23). Subclass III SnRK2s and CIPK26/3/9/23 function as key modulators of the susceptibility to shoot growth inhibition in response to increased external Mg\(^{2+}\) concentrations (Mg\(^{2+}\)-susceptibility), probably via phosphatase activity, and Ca\(^{2+}\) influx, which are assumed to modulate intracellular Mg\(^{2+}\) transport, and maintain Mg\(^{2+}\) homeostasis. Dashed lines indicate possible, but not confirmed, routes.