A Calcium Sensor-Regulated Protein Kinase, CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE19, Is Required for Pollen Tube Growth and Polarity[OPEN]

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Calcium plays an essential role in pollen tube tip growth. However, little is known concerning the molecular basis of the signaling pathways involved. Here, we identified Arabidopsis (Arabidopsis thaliana) CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE19 (CIPK19) as an important element to pollen tube growth through a functional survey for CIPK family members. The CIPK19 gene was specifically expressed in pollen grains and pollen tubes, and its overexpression induced severe loss of polarity in pollen tube growth. In the CIPK19 loss-of-function mutant, tube growth and polarity were significantly impaired, as demonstrated by both in vitro and in vivo pollen tube growth assays. Genetic analysis indicated that disruption of CIPK19 resulted in a male-specific transmission defect. Furthermore, loss of polarity induced by CIPK19 overexpression was associated with elevated cytosolic Ca²⁺ throughout the bulging tip, whereas LaCl₃, a Ca²⁺ influx blocker, rescued CIPK19 overexpression-induced growth inhibition. Our results suggest that CIPK19 may be involved in maintaining Ca²⁺ homeostasis through its potential function in the modulation of Ca²⁺ influx.

In flowering plants, fertilization is mediated by pollen tubes that extend directionally toward the ovule for sperm delivery (Krichevsky et al., 2007; Johnson, 2012). The formation of these elongated tubular structures is dependent on extreme polar growth (termed tip growth), in which cell expansion occurs exclusively in the very apical area (Yang, 2008; Rounds and Bezanilla, 2013). As this type of tip growth is amenable to genetic manipulation and cell biological analysis, the pollen tube is an excellent model system for the functional analysis of essential genes involved in polarity control and fertilization (Yang, 2008; Qin and Yang, 2011; Bloch and Yalovsky, 2013).

It is well established that Ca²⁺ plays a critical role in pollen germination and tube growth (Konrad et al., 2011; Hepler et al., 2012). A steep tip-focused Ca²⁺ gradient has been detected at the tip of elongating pollen tubes (Rathore et al., 1991; Pierson et al., 1994; Hepler, 1997). In previous studies, artificial dissipation of the Ca²⁺ gradient seriously inhibited tip growth of pollen tubes, whereas elevation of internal Ca²⁺ level induced bending of the growth axis toward the zone of higher Ca²⁺. These studies suggest that Ca²⁺ not only controls pollen tube elongation but also modulates growth orientation (Miller et al., 1992; Malho et al., 1994; Malho and Trewavas, 1996; Hepler, 1997). These Ca²⁺ signatures are perceived and relayed to downstream responses by a complex toolkit of Ca²⁺-binding proteins that function as Ca²⁺ sensors (Yang and Poovaiah, 2003; Harper et al., 2004; Dodd et al., 2010).

To date, four major Ca²⁺ sensor families have been identified in Arabidopsis (Arabidopsis thaliana), including calcium-dependent protein kinase, calmodulin (CaM), calmodulin-like (CML), and CALCINEURIN B-LIKE (CBL) proteins (Luan et al., 2002, 2009; Yang and Poovaiah, 2003; Harper et al., 2004). Calcium-dependent protein kinase family members comprise a kinase domain and a CaM-like domain in a single protein; thus, they act not only as a Ca²⁺ sensor but also as an effector, designated as sensor responders (Cheng et al., 2002). In contrast, CaM, CML, and CBL proteins do not have any enzymatic domains but transmit Ca²⁺ signals to downstream targets via Ca²⁺-dependent protein-protein interactions. Therefore, they have been designated as sensor relays (McCormack et al., 2005). While CaM and CML proteins...
interact with a diverse array of target proteins, it is generally accepted that CBLs interact specifically with a group of Ser/Thr protein kinases termed CALCIUNEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASES (CIPKs; Luan et al., 2002; Kolukisaoglu et al., 2004).

In Arabidopsis, several CBLs coupled with their target CIPKs have been demonstrated to function in the regulation of ion homeostasis and stress responses (Luan et al., 2009). Under salt stress, SALT OVERLY SENSITIVE3 (SOS3)/CBL4-SOS2/CIPK24 regulate SOS1 at the plasma membrane for Na⁺ exclusion, whereas CBL10-CIPK24 complexes appear to regulate Na⁺ sequestration at the tonoplast (Liu et al., 2000; Qiu et al., 2002; Kim et al., 2007; Quan et al., 2007). For low-K⁺ stress, CBL1 and CBL9, with 87% amino acid sequence identity, interact with CIPK23, which regulates a voltage-gated ion channel (ARABIDOPSIS K⁺ TRANSPORTER1) to mediate the uptake of K⁺ in root hairs (Li et al., 2006; Xu et al., 2006; Cheong et al., 2007). In addition, CBL1 integrates plant responses to cold, drought, salinity, and hypersmotic stresses (Albrecht et al., 2003; Cheong et al., 2003), and CBL9 is involved in abscisic acid signaling and biosynthesis during seed germination (Pandey et al., 2004). Over the past decade, the functions of CBL-CIPK complexes in abiotic stress tolerance have been studied extensively, but only limited studies focus on CBL family members in pollen tube growth. For example, CBL3 overexpression caused a defective phenotype in pollen tube growth (Zhou et al., 2009). Overexpression of CBL1 or its closest homolog CBL9 inhibited pollen germination and perturbed tube growth at high external K⁺, whereas disruption of CBL1 and CBL9 leads to a significantly reduced growth rate of pollen tubes under low-K⁺ conditions (Mähs et al., 2013). The potential roles of CIPKs in pollen tubes so far appear to be completely unknown.

In this study, we demonstrated that Arabidopsis CIPK19, a CIPK specifically expressed in pollen grains and pollen tubes, functions in pollen tube tip growth, providing a new insight into the function of the CBL-CIPK network in the control of growth polarity during pollen tube extension in fertilization.

RESULTS

Identification of CIPK19 as a Key Regulator of Pollen Tube Growth through a Genome-Wide Functional Survey

To date, 26 CIPKs have been identified in the Arabidopsis genome. We were interested in the potential function of CIPKs in pollen biology and therefore focused on those CIPK genes preferentially expressed in mature pollen grains. We reasoned that these genes are most likely involved in regulatory pathways for pollen tube growth. Based on the available microarray database (https://genevestigator.com/gv/), we focused on five CIPK family members (CIPK10–CIPK12, CIPK14, and CIPK19) that are expressed highly in mature pollen grains but not at all or weakly in other tissues. To conduct a functional survey for these CIPKs in pollen tube growth, we analyzed their overexpression phenotypes and subcellular localization patterns, as we described previously for the analysis of the CBL family (Zhou et al., 2009). For subcellular localization, the full-length CIPK coding sequences were fused to the N terminus of GFP and their expression was driven by the promoter of LYCopersicon ANther-Specific52 (LAT52), a pollen-specific gene (Twell et al., 1991; Bate et al., 1996). The resulting constructs were transiently expressed in tobacco (Nicotiana tabacum) pollen tubes via a particle bombardment-mediated transformation method (Fu et al., 2001). The pollen tubes expressing the GFP fusions were analyzed using confocal microscopy 4 h after transformation. As shown in Figure 1A, GFP alone was evenly distributed in the pollen tube cytoplasm. CIPK11-GFP was localized to granular organelles that were barely detectable in the apex region of the pollen tube, whereas the other four CIPKs (CIPK10, CIPK12, CIPK14, and CIPK19), like GFP alone, all exhibited uniform cytoplasmic localization (Fig. 1A; Supplemental Movies S1–S4).

We next investigated whether overexpression of these CIPKs affected the tip growth of pollen tubes. The length and maximum tip width of pollen tubes (from 100 transformed tubes) were measured. Except for CIPK11-GFP overexpression, which did not alter pollen tube morphology (Fig. 1B), overexpression of GFP tagged CIPK10, CIPK12, CIPK14, or CIPK19 affected the pollen tube growth to various extents (Fig. 1B). In particular, CIPK19-GFP overexpression produced the most severe depolarized growth (86% increase in tube width and 57% reduction in tube length; Fig. 1B). To avoid the potential impact of GFP fusion on the innate functionality of GFP alone was used as a control. Approximately 4 h after bombardment, localization of GFP-tagged CIPKs was analyzed using confocal microscopy. The images were captured in single optical slices. Representative images were selected from 50 to 60 samples. Bar = 5 μm. B, Quantitative analysis of pollen tube phenotypes induced by overexpression of different CIPK-GFPs. Pollen tube length and maximum tip width were measured 3.5 h after bombardment. Asterisks represent significant differences from the GFP control (P < 0.01, Student’s t test), and error bars indicate so. Data were collected from 100 tubes per experiment.
CIPKs, nonfusion constructs (LAT52:CIPKs) were co-bombarded into pollen grains with LAT52:GFP as a marker for transformation. The results indicated that overexpression of untagged CIPK genes produced a similar effect on pollen tube growth to CIPK-GFP fusions (Supplemental Fig. S1). After a functional survey of five pollen-expressed CIPKs in pollen tubes, we focused on CIPK19 for further investigation on its role in pollen tube growth.

Expression of CIPK19 Was Specifically Associated with Pollen Grains and Pollen Tubes

To further dissect the expression pattern of the CIPK19 gene, we performed reverse transcription (RT)-PCR to evaluate the expression level of CIPK19 in various tissues. As shown in Figure 2A, transcript levels of CIPK19 were only detected in flowers and strongly in pollen grains. Our observations are consistent with available microarray expression data (https://www.genevestigator.ethz.ch/). We also generated transgenic Arabidopsis plants expressing a reporter construct containing the CIPK19 promoter fused to the gene encoding GUS. Histochemical staining of tissues from these plants showed that GUS activity was detected only in pollen grains and pollen tubes (Fig. 2B). In conclusion, CIPK19 was expressed specifically in mature pollen, suggesting a specific role for CIPK19 in pollen tube growth.

Functional Analysis of CIPK19 by Expressing Its Constitutively Active Form in the Pollen Tube

To further investigate the function of CIPK19 in pollen tube growth, we produced a constitutively active (CA) form of CIPK19. CA-CIPK19 was generated by removing regulatory domains and substituting Thr-186 to Asp in the activation loop (Fig. 3A). Earlier studies have shown that an equivalent mutant of a CIPK protein exhibits increased CIPK kinase activity as compared with the wild-type protein (Albrecht et al., 2001; Guo et al., 2001). The construct containing the LAT52-driven mutant CIPK19 (CA-CIPK19) was introduced into tobacco pollen grains by particle bombardment. As shown in Figure 3, the tube growth phenotype caused by CA-CIPK19 overexpression in tobacco pollen tubes. A, Diagram of the C-terminal truncation and mutation for CIPK19 used in transient expression. The regulatory C-terminal domain is partitioned into the two interaction domains, the asparagine-alanine-phenylalanine (NAF) domain and the protein phosphatase interaction (PPI) domain. The red arrow indicates the mutation site from Thr to Asp in the activation loop. B, Representative pollen tubes transformed with CA-CIPK19. Images show pollen tubes cultured in vitro for 4 h after bombardment. A tube expressing GFP alone was used as a control. Bar = 30 μm. C, Quantitative analysis of CA-CIPK19-induced growth phenotypes. Pollen tube length and maximum tip width were measured 4 h after bombardment. Asterisks represent significant differences from the GFP control (P < 0.01, Student’s t test). Data are means ± s.e.; n = 70 to 80.
Figure 3B, overexpression of wild-type CIPK19 induced a loss of growth polarity, leading to swollen sock-like tubes. In the case of CA-CIPK19 overexpression, comparing with that of CIPK19, transformed pollen tubes exhibited more severe depolarized growth, resulting in much shorter (from 196.34 ± 31.05 to 103.57 ± 40.93 μm) but wider (from 14.12 ± 0.62 to 24.19 ± 0.95 μm) pollen tubes (Fig. 3, B and C).

Disruption of CIPK19 Causes Defects in Pollen Tube Growth in Vitro

A gain-of-function assay by transiently overexpressing CIPK19 in tobacco pollen grains provided an initial indication of its function in pollen tube growth. We next analyzed the pollen tube growth of a transfer DNA (T-DNA) insertion mutant (cipk19). The T-DNA was located in the exon of the CIPK19 gene (SALK_044735). RT-PCR analysis of the wild-type and homozygous mutant plants indicated that the expression of CIPK19 mRNA was not detectable in the mutant (Fig. 4B), confirming that the cipk19 homozygous plant is a knockout mutant. In vitro pollen tube growth assay showed that the cipk19 mutant exhibited aberrant tube morphologies, namely, partial loss of polarity and deformed growth (Fig. 4A), in contrast to the wild-type controls, which produced normal and healthy pollen tubes. Statistical analysis suggested that cipk19 pollen tubes displayed significant expansion in width (15.1 ± 0.63 μm as compared with 7.82 ± 0.31 μm of the wild type) and dramatic reduction in length (148.31 ± 41.45 μm compared with 493.24 ± 14.99 μm of the wild type; Fig. 4, C and D). This abnormal growth phenotype of the cipk19 mutant was rescued by complementation with a construct expressing the full-length cDNA of CIPK19 driven by its own promoter. No significant morphological differences were found between wild-type and complemented pollen tubes (Fig. 4). In conclusion, the growth defects of pollen tubes observed in the cipk19 mutant were derived from disrupting the expression of CIPK19, suggesting that this CIPK member plays a crucial role in the maintenance of normal pollen tube growth.

The cipk19 Mutant Exhibits Retarded Pollen Tube Growth in the Pistils

Given the in vitro morphological defects in pollen tubes caused by the disruption of CIPK19, we next performed an in vivo pollen tube growth assay in the pistils. Wild-type pistils were hand-pollinated with pollen grains, either from wild-type or homozygous cipk19 mutant plants, and subsequently stained with Aniline Blue 4, 24, or 48 h after pollination. At 4 h after

Figure 4. CIPK19 is required for normal pollen tube growth. A, Representative in vitro-germinated pollen tubes from wild-type (WT), cipk19, and complemented (COM) lines. Images show pollen tubes grown for approximately 3.5 h. Arrows indicate excess bulging on the tube surface. Bars = 30 μm. B, RT-PCR analysis of a homozygous mutant line shows the absence of CIPK19 transcripts in the T-DNA line. Amplification of Arabidopsis TUBULIN2 was used as a positive internal control. C and D, Statistical analysis of tube length and width from wild-type, cipk19, and complemented lines. Pollen tube length and maximum tip width were measured after 3.5 h of incubation. Data were collected from three individual experiments (80 tubes per experiment). Asterisks indicate significant differences from the wild type (P < 0.01, Student’s t test).

Figure 5. Growth patterns of wild-type (WT) and cipk19 mutant pollen tubes in female tissues. Wild-type pistils were pollinated with wild-type or cipk19 pollen grains and subsequently stained with Aniline Blue 4, 24, or 48 h after pollination (hap). Bars = 100 μm.
AtCIPK19 in Pollen Tube Growth

Table 1. Segregation analysis identifying non-Mendelian transmission of the T-DNA in cipk19 mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected</th>
<th>Observed</th>
<th>Transmission</th>
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<tr>
<td>Wild-type pollen × cipk19 heterozygous pistil (n = 122)</td>
<td></td>
<td></td>
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<tr>
<td>Wild type</td>
<td>61</td>
<td>63</td>
<td>51.6</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>61</td>
<td>59</td>
<td>48.4</td>
</tr>
<tr>
<td>Pollen from cipk19/CIPK19 plants × wild-type pistil (n = 104)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>52</td>
<td>74</td>
<td>71.2*</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>52</td>
<td>30</td>
<td>28.8*</td>
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Reciprocal outcrosses were performed between cipk19 heterozygous plants and wild-type plants. The offspring were genotyped, and segregation was compared with the expected Mendelian segregation (1:1). Statistical significance was determined by Pearson’s χ² test. Asterisks indicate significant differences from the segregation ratio of 1:1 (P < 0.001).

pollination, wild-type pollen grains germinated on the stigma and pollen tubes passed through the style, reaching the top of the transmitting tract. In parallel, cipk19 pollen grains also germinated, but pollen tubes were shorter and restricted within the style (Fig. 5). The differences in pollen tube growth were more striking at 24 h after pollination: wild-type pollen tubes reached the bottom of the transmitting tract, whereas cipk19 mutant pollen tubes were only halfway through the transmitting tract. Eventually, cipk19 pollen tubes fully reached the bottom at 48 h after pollination (Fig. 5).

Disruption of CIPK19 Causes a Male-Specific Transmission Defect

Despite the defect in pollen tube growth, the siliques produced by the homozygous cipk19 mutant were similar to those of wild-type plants. A comparison of silique length, seed count, and incidence of seed gaps revealed no significant differences between cipk19 mutant and wild-type plants. However, the defect of pollen tube growth observed in the cipk19 mutation suggests that the loss of CIPK19 may result in decreased pollen fitness and competitiveness in vivo. To test this hypothesis, we performed reciprocal outcrosses between cipk19 heterozygous plants and wild-type plants and genotyped the resulting progeny. When pollen grains from cipk19 heterozygous plants were used to pollinate wild-type stigmas, there were significantly fewer heterozygotes in the progeny than predicted by Mendelian genetics (Table I). By contrast, when wild-type pollen grains were used to pollinate cipk19 heterozygous stigmas, the ratio of wild type to heterozygotes was not obviously different from the expected 1:1 segregation (Table I). These results indicate that the loss of CIPK19 caused a strong male transmission defect but did not affect the female gametophyte.

To further verify this conclusion, we examined the distribution of wild-type and heterozygous seeds within the resulting siliques harvested from an outcross with pollen grains from cipk19 heterozygous plants. Prior to silique cracking, the seeds were collected and planted in the order in which they were arranged in the silique. The top-to-bottom comparison demonstrated a higher proportion of mutant heterozygotes in the top one-third region of the siliques (42.9%) compared with the bottom region (12.5%, Fig. 6). This nonrandom distribution of mutant heterozygotes suggests that slower tube growth of the mutant pollen presented a competitive disadvantage, compared with wild-type pollen, resulting in more wild-type seeds produced at the bottom of the silique. However, the cipk19 mutation does not appear to affect other processes of fertilization, as cipk19 pollen tubes reach the ovules and subsequently accomplish fertilization.

CIPK19 Is Involved in the Regulation of Ca²⁺ Fluxes

As described above, a tip-focused Ca²⁺ gradient is essential to pollen tube growth (Konrad et al., 2011; Hepler et al., 2012; Guan et al., 2013). The CBL-CIPK signaling network is considered as a Ca²⁺ sensor-responder system that recognizes Ca²⁺ signatures and translates them into the correct cellular responses (Luan, 2009; Dodd et al., 2010). Here, we speculated that Arabidopsis CIPK19, coupled with certain CBLs, have a similar function in the regulation of the Ca²⁺ response during pollen tube growth. Often as a feedback mechanism, the CBL-CIPK signaling network also may regulate Ca²⁺ mobilization in the cell. To test this possibility, we performed ratiometric Ca²⁺ imaging in tobacco pollen tubes overexpressing CIPK19-GFP. Compared with the normal tip-focused Ca²⁺ gradient in the control tube, the CIPK19-GFP overexpression tube displayed an altered Ca²⁺ gradient that extended throughout the swollen apex (Fig. 7A).

Several studies support the idea that the influx of extracellular Ca²⁺ ([Ca²⁺]o) is the primary source of the tip-focused Ca²⁺ gradient (Etter et al., 1994; Malho et al., 1995; Holdaway-Clarke et al., 1997; Dutta and Robinson, 2004). To test if tip Ca²⁺ accumulation induced by CIPK19 overexpression is derived from [Ca²⁺]o influx, we investigated the effect of LaCl₃, a known Ca²⁺ influx inhibitor through blocking plasma membrane-localized inward Ca²⁺ channels (Malho et al., 1995). As shown in Figure 7B and Supplemental Figure S2, LaCl₃ treatment

Figure 6. The distribution of wild-type and heterozygous seeds within the resulting siliques harvested from an outcross using pollen grains from cipk19 heterozygous plants and wild-type pistil. The overall percentages of wild-type and heterozygous seeds in the top, middle, and bottom sections of a silique are shown above each distinct area separated by black bars. Bar = 1 mm.
indeed rescued tobacco pollen tube elongation inhibited by CIPK19 overexpression, implying that CIPK19 overexpression caused Ca2+ overaccumulation at the tip mediated at least in part by [Ca2+]ex. To further assess the connection between CIPK19 and Ca2+ fluxes, we examined the effect of different [Ca2+]ex levels on the pollen tube growth of the cipk19 mutant. As shown in Figure 7C, the elongation of wild-type tubes showed a typical bell-shaped response to various [Ca2+]ex levels, with 5 mM being optimal. Compared with the wild-type control, the cipk19 mutant preferred a higher [Ca2+]ex level (15 mM) in order to grow normally, suggesting that the loss of CIPK19 may have inhibited calcium entry at the tip. A similar assay showed that CIPK19 overexpression in tobacco pollen tubes had a completely opposite effect: these pollen tubes preferred much lower external calcium to grow normally (Fig. 7D). These results suggest that CIPK19 may be involved in modulating Ca2+ influx at the tip of growing pollen tubes.

DISCUSSION

Calcium, as a ubiquitous second messenger, plays an essential role in pollen tube tip growth (DeFalco et al., 2010; Dodd et al., 2010). However, our understanding of how Ca2+ signals are perceived and relayed to trigger downstream responses is limited. To date, only
a few calcium signaling components have been identified and characterized in pollen tubes. In this study, we identified CIPK19 as a new component that contributes to decoding Ca\textsuperscript{2+} signals in pollen tubes.

Our functional survey of CIPK family members has identified several putative CIPKs that may play a role in the regulation of pollen tube tip growth. Overexpression of CIPK19 and its closest homolog CIPK12 caused depolarization growth of the pollen tube, resulting in dramatically reduced elongation and increased expansion (Fig. 1; Supplemental Fig. S1). Together with their similar localization and highly similar amino acid sequences, it is conceivable that CIPK19 and CIPK12 are functionally redundant for the regulation of pollen tube growth. It will be interesting to produce a double mutant of these two CIPKs in the future to test their functional relationship.

Nevertheless, the CIPK19 single knockout mutant already displayed some defects in pollen tube growth, compared with the wild type, as shown by the in vitro assay (Fig. 4). These defects include partial loss of growth polarity and deformation of the pollen tube. When tested by an in vivo growth assay, cipk19 mutant pollen were able to germinate on the surface of the stigma and penetrate the pistil, but the growth rate was much lower than that of wild-type pollen. Indeed, cipk19 pollen tubes required about twice as long (48 h after pollination) in order to fully reach the bottom of the pistil (Fig. 5). Despite the defect observed in the cipk19 mutant, the siliques produced by homozygous cipk19 mutants were similar to those of the wild type in several respects, including silique length, seed count, and organization of seeds in the silique (Supplemental Fig. S3). However, detailed analysis of F2 seeds demonstrated a reduced transmission of the cipk19 allele to the offspring, suggesting that there is a competitive disadvantage of the mutant gametophyte compared with the wild type. Taken together, the reduced growth rate of the mutant pollen tube does not prevent a self-pollinating cipk19 homozygote from producing normal siliques with viable seeds, but apparently it becomes a detrimental factor when the mutant pollen must compete with the wild type.

In this study, we noticed that either overexpression or knockout of CIPK19 would lead to partial loss of polarity in pollen tube growth. One possible explanation for this puzzling observation is this: the levels of CIPK19, like many other regulatory molecules, should be in a delicate balance, and either too much or too little activity of CIPK19 would not support normal tip growth of pollen tubes, leading to depolarization.

CIPK19 overexpression was associated with elevated Ca\textsuperscript{2+} at the apical bulged area of the pollen tube. In addition, a Ca\textsuperscript{2+} influx blocker (LaCl\textsubscript{3}) could rescue pollen tube elongation inhibited by CIPK19 overexpression (Fig. 7B; Supplemental Fig. S2). This suggests that CIPK19 may be involved in the regulation of tip Ca\textsuperscript{2+} accumulation by affecting the influx of extracellular Ca\textsuperscript{2+}. Generally, Ca\textsuperscript{2+} influx is thought to be mediated by plasma membrane Ca\textsuperscript{2+} channels at the tube apex. Constituting 20 members, the CYCLIC NUCLEOTIDE-DEGATED CHANNEL (CNGC) family forms a group of putative Ca\textsuperscript{2+} channels in pollen tubes (Jammes et al., 2011; Hedrich, 2012; Hepler et al., 2012). Plasma membrane-localized CNGC18 overexpression led to abnormal tube growth coupled with an elevation of cytosolic Ca\textsuperscript{2+} at the swelling tip, whereas CNGC18 knockout resulted in male sterility (Chang et al., 2007; Frietsch et al., 2007; Zhou et al., 2014). In addition to CNGC18, five more CNGCs (CNGC7, CNGC8–CNGC10, and CNGC16) are potentially relevant to pollen calcium fluxes because they are preferentially expressed in pollen grains and/or growing pollen tubes (Jammes et al., 2011; Hedrich, 2012). Another group of putative Ca\textsuperscript{2+} channels in pollen tubes is the GLUTAMATE RECEPTOR-LIKE (GLR) family, which contains at least six pollen-expressed members (Lam et al., 1998; Lacombe et al., 2001; Pina et al., 2005).

The Ca\textsuperscript{2+} transport activity in pollen tubes has been shown for GLR1.2 and GLR3.7 by direct electrophysiological, pharmacological, and genetic evidence (Michard et al., 2011). Thus, the identification of the CIPK19 downstream target among these putative Ca\textsuperscript{2+} channels will be an important area for future research.

It is well established that CIPK activity is regulated by one or more CBLs, a family of plant-specific Ca\textsuperscript{2+} sensors (Luan et al., 2002; Kolukisaoglu et al., 2004). CBLs and CIPKs constitute an important calcium transduction network in plants (Luan, 2009). In the future, the identification and functional characterization of CIPK19 interacting components, including its partner CBLs, will provide further insight into a CIPK19-mediated pathway regulating Ca\textsuperscript{2+} homeostasis in pollen tube growth.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia was grown at 22°C in growth chambers with a light regime of 16 h of light and 8 h of darkness. Tobacco (Nicotiana tabacum) plants were grown in a greenhouse at 28°C under a light regime of 12 h of darkness and 12 h of light.

Transient Expression in Tobacco Pollen

Full-length cDNAs for CIPKs were obtained from the Arabidopsis Biological Resource Center (ABRC). The ABRC stock numbers of CIPK cDNAs are U21917 for CIPK10 (At5g58380), U24515 for CIPK11 (At2g03560), U16129 for CIPK12 (At4g18700), U21914 for CIPK14 (At5g01820), and DQ447041 for CIPK19 (At1g43810). The PCR-amplified coding regions were cloned into pLATS2:GFP vector under the control of the LATS2 promoter for transient expression of the GFP fusion protein in pollen (Wu et al., 2003). To generate LATS2-X constructs, the GFP cDNA sequence was deleted from LATS2-X-GFP by BamHI and BglII digestion followed by self-ligation. For CA-CIPK19, the C-terminal regulatory portion (amino acids 344–483) was removed, followed by a Thr186-to-Asp point mutation in the activation loop containing conserved Asp-Phe-Gly and Ala-Pro-Glu motifs (Albrecht et al., 2001; Guo et al., 2001). For particle bombardment, all plasmids were purified using Plasmid Mini Kits (Qiagen). Mature tobacco pollen grains were collected right before each experiment and were transformed with plasmid DNA using a particle bombardment procedure described previously (Fu et al., 2001). Briefly, 0.8 µg of CIPK-GFP construct DNA was used for each bombardment in the analysis of subcellular localization and overexpression phenotype. For the co-bombardment in quantitative analysis of the overexpression phenotype, 0.8 µg of plasmid DNA for CIPKs or CA-CIPK19 and 1 µg of DNA for the GFP construct were co-bombarded. The transformed pollen grains were incubated at 28°C for 4 h before being examined with an inverted microscope (for overexpression phenotype analysis) or a confocal laser scanning microscope (for subcellular localization analysis).
Microscopy and Imaging Analysis

For the analysis of pollen tube phenotype, the images of transformed tubes were recorded through a cooled CCD camera (model DP70; Olympus) attached on an inverted microscope (model BX51; Olympus). We measured the diameter of the widest tip region and the length of the pollen tube using Zeiss LSM Image Browser software (version 3.2). Three independent experiments were performed. All the data were subjected to statistical analysis by Student’s t-test. To examine the subcellular localization of GFP signals, transformed tubes were observed with a confocal laser scanning microscope (Zeiss LSM 510 META; 488-nm excitation and 505- to 530-nm emission), and the images were analyzed by Zeiss LSM Image Browser (version 3.2) and processed with Adobe Photoshop CS5.

RT-PCR Analysis

Roots, leaves, stems, and flowers were collected from wild-type Arabidopsis separately. Twenty milligrams of pollen was harvested using a vacuum collection system as described previously (Johnson-Brousseau and McCormick, 2004). All tissue samples were frozen in liquid nitrogen, and total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from each RNA sample using the Invitrogen SuperScript III first-strand synthesis system for RT-PCR according to the kit’s instructions. PCR with primers specific to TUBULIN2 (5'-CCATATGCACTGTCGAAAAG-3' and 5'-TCCCAGTCAACGAGAAGT-3') was used as an internal standard to quantify cDNA from the different sources. PCR amplification was performed with GoTag Green (Promega) using primers specific to CIPK19 (5'-TGAATCTCCCTTCCGGGAGG-3' and 5'-AAGCCCCGTGCGCATTTCG-3'). PCR products were visualized on an agarose gel.

Analysis of Promoter Activity

A 2-kb promoter region of CIPK19 was amplified from Arabidopsis genomic DNA and subsequently cloned upstream of the GUS reporter gene in pCAMBIA1381 vector. The pCAMBIA-CIPK19promGUS construct was introduced into wild-type Arabidopsis through Agrobacterium tumefaciens strain GV3101 by the floral dip method (Clough and Bent, 1998). Seeds from transformed plants were collected, and transformants were selected on Murashige and Skoog culture medium containing 20 mg L⁻¹ hygromycin and 50 mg L⁻¹ kanamycin. Three independent lines were recovered for analysis of GUS activity as described previously (Jefferson et al., 1987).

Identification of CIPK19 T-DNA Insertion Lines

An Arabidopsis line (Columbia background) with a T-DNA insertion in CIPK19 (SALK_044735) was obtained from the ABRC. A PCR-based approach was used to identify homozygous lines. PCR genotyping was performed with primers specific to CIPK19pro-cDNA-CIPK19ter and 5'-ATACTTTTTGTGGCGG-3' as an internal standard to quantify cDNA from the different sources. PCR products were visualized on an agarose gel.

Complementation of the cipk19 Mutant

CIPK19 promoter (CIPK19prom) and CIPK19 terminator (CIPK19ter) fragments were amplified from wild-type genomic DNA. The resulting fragments were then subcloned into the pCAMBIA1300 vector, resulting in the transcriptional fusion construct CIPK19prom-CIPK19ter. The construct was introduced into homozygous cipk19 mutant plants and generated normal seedlings (Clough and Bent, 1998).

Observation of in Vitro-Cultured Pollen Tubes

Arabidopsis pollen grains were dusted from both wild-type and mutant plants and germinated on a standard germination medium as described previously (Li et al., 1999). Pollen tubes were observed 4 h after germination, and the images of pollen tubes were captured using a BX51 microscope (Olympus) equipped with a CoolSNAP HQ CCD camera (Photometrics). Pollen tube lengths and widths were measured using Image software (National Institutes of Health; http://rsb.info.nih.gov/ij/). All experiments were performed in triplicate, and 80 pollen tubes were measured in each experiment.

Aniline Blue Staining of Pollen Tubes in Pistils

Pollen tubes in pistils were stained with Aniline Blue, as described previously (Ishiguro et al., 2001). Briefly, premasculated mature wild-type flowers were pollinated with either wild-type pollen or cipk19 pollen. After 4, 24, and 48 h, pollinated pistils were incubated in fixative solution (ethanol:acetic acid [v/v] = 3:1) for at least 2 h and subsequently in 8% NaOH softening solution overnight. Samples were then stained with Aniline Blue solution in the dark and observed with an Olympus BX51 fluorescence light microscope. The UV light excitation wavelength maximum was 350 nm, and the emission spectra were collected from 440 to 612 nm.

Measurements of Cytoplasmic Ca²⁺ Concentration

Tobacco pollen tubes were microinjected with the Ca²⁺ indicator indo-1 conjugated to 10-kD dextran (Molecular Probes). Microinjection was performed using a Narishige NA1-2N micromanipulator connected to a Nikon inverted microscope (TE2000-U). Micropipettes for injection were made from borosilicate glass capillaries (model GC-1; Narishige Scientific Instruments) so that the tip diameter was approximately 1 µm. The pipette (loaded with 1 mM indo-1-dextran) tip was inserted 30 to 100 µm from the tip of the pollen tubes, and contents were gently loaded into the cytoplasm. Injected cells were allowed to recover from the microinjection for 30 min prior to ratio imaging. Approximately 10% of the pollen tubes could recover from the conditions described above. Pollen tubes that failed to regain normal growth rates were excluded from further analysis. The Ca²⁺ concentration in pollen tubes was then visualized by ratio imaging using a Zeiss LSM 410 confocal microscope. Fluorescence from the Ca²⁺ indicator was excited with the 364-nm beam of a UV laser, and emitted light was simultaneously detected at 400 to 435 nm and 480 ± 20 nm. Calcium calibration was carried out using the Calcium Calibration Buffer Kit (Molecular Probes; 0–39 µM free Ca²⁺) in the presence of 1 µM indo-1-dextran prior to ratio calculation. The ratio images were then color coded to represent different Ca²⁺ concentrations.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM125224 (AICPK10), NM128589 (AICPK11), NM117986 (AICPK12), NM120260 (AICPK14), and NM123949 (AICPK19).

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Phenotype in tobacco pollen tubes overexpressing CIPKs.

Supplemental Figure S2. Representative images of CIPK19-overexpressing tobacco pollen tubes in response to LaCl₃ treatment.

Supplemental Figure S3. Siliques from wild-type and cipk19 mutant plants.

Supplemental Movie S1. Tip growth in a tobacco pollen tube transiently expressing CIPK10-GFP.

Supplemental Movie S2. Tip growth in a tobacco pollen tube transiently expressing CIPK12-GFP.

Supplemental Movie S3. Tip growth in a tobacco pollen tube transiently expressing CIPK14-GFP.

Supplemental Movie S4. Tip growth in a tobacco pollen tube transiently expressing CIPK19-GFP.

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