Postprint: Analysis of Biological Function and Subcellular Localization of Constitutively Active CIPK9 During Pollen Tube Growth

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Abstract

Pollen tube growth in plants is a physiological process involving multiple factors that requires various signaling systems to guide plant cells to completion. Calcium ions, acting as second messengers, can participate in regulating cellular polar development by activating downstream protein kinases CIPKs through calcium sensors CBLs. In this study, CIPK9 was identified as a candidate gene; its C-terminus was fused with green fluorescent protein (GFP) and transiently expressed in tobacco pollen via biolistic bombardment to observe its subcellular localization and the phenotypes induced in pollen tubes. The results demonstrated that GFP-tagged CIPK9 localized to rapidly moving granular organelles in pollen tubes and could undergo regular movement with cytoplasmic streaming. To further investigate the biological function of CIPK9, a constitutively active CIPK9 (CACIPK9) was constructed. Compared with full-length CIPK9, CACIPK9 lacks the C-terminal regulatory domain and harbors point mutations in the activation loop of the kinase domain, thereby exhibiting unregulated, constitutively high activity. Experimental results revealed that CACIPK9 lacking the C-terminal regulatory domain displayed non-specific subcellular localization, manifesting as diffuse intracellular localization identical to the GFP control, indicating that the C-terminal regulatory domain of CIPK9 plays a crucial regulatory role in its proper localization in pollen tubes. Moreover, overexpression of CACIPK9 could induce a depolarized growth phenotype in pollen tubes. In summary, CIPK9, as a member of the calcium signaling downstream family, participates in processes related to pollen tube polar growth and exerts certain regulatory effects on pollen tube growth.

Full Text

Subcellular Localization and Biological Functional Analysis of Constitutively Active CIPK9 in Pollen Tube Growth

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Abstract

Pollen tube growth is a physiological process involving multiple factors that requires diverse signaling systems to guide plant cell development. Calcium ions function as second messengers and participate in regulating cell polarity development by activating downstream CIPK protein kinases through calcium sensor CBLs. In this study, CIPK9 was identified as a candidate gene and fused at its C-terminus with green fluorescent protein (GFP). Transient expression in tobacco pollen via gene gun technology was used to observe its subcellular localization and induced phenotypes in pollen tubes. Results showed that GFP-labeled CIPK9 localized to rapidly moving granular organelles in pollen tubes, exhibiting regular movement with cytoplasmic streaming. To further investigate the biological function of CIPK9, a constitutively active CIPK9 (CACIPK9) was constructed. Compared with full-length CIPK9, CACIPK9 lacks the C-terminal regulatory region and contains a point mutation in the activation loop of the kinase domain, resulting in unregulated, sustained high activity. Experiments revealed that CACIPK9 lacking the C-terminal regulatory region displayed non-specific subcellular localization, showing diffuse cytoplasmic distribution identical to the GFP control, indicating that the C-terminal regulatory region of CIPK9 plays a crucial role in its proper localization in pollen tubes. Additionally, CACIPK9 overexpression induced depolarized growth phenotypes in pollen tubes. In conclusion, CIPK9, as a member of the calcium signaling downstream family, participates in pollen tube polar growth processes and plays a regulatory role in pollen tube growth.

Keywords: pollen tube, calcium ion, CBL-interacting protein kinase, polarized growth, signal transduction

Introduction

Pollen germination and tube growth represent a complex dynamic process in plants. After landing on the stigma and undergoing recognition, pollen grains germinate and extend pollen tubes that grow through the style to reach the ovule, ultimately delivering sperm cells to the embryo sac to complete fertilization. This entire process involves multiple signaling pathways between male and

female gametes, including calcium signaling, small G proteins, reactive oxygen species (ROS), and phospholipids (Kolukisaoglu et al., 2004).

Plant cell growth patterns are primarily classified into two types: diffuse growth and tip growth. Diffuse growth involves uniform cell expansion without directional preference, whereas tip growth is concentrated at a specific region of the cell (the pollen tube apex). As a classic model system for tip growth, pollen tubes are widely used to study plant cell polarity development (Yang, 2002; Fu et al., 2001). Apical growth of pollen tubes depends on dynamic rearrangement of the cytoskeleton, polar vesicle transport, and exocytosis, which collectively determine the initiation site and direction of polar growth. Multiple signaling molecules, including calcium ions, small G proteins, ROS, and phospholipids, are known to play important roles in pollen tube apical growth (Rounds & Bezanilla, 2013). Among these, calcium signals can be recognized by specific calcium sensors, triggering downstream cascade reactions (Kroeger & Geitmann, 2012; Rounds & Bezanilla, 2013).

Calcineurin B-like proteins (CBLs) represent a typical family of calcium sensors unique to plants that, together with their downstream interacting protein kinases (CIPKs), form an intricate CBL-CIPK-mediated calcium signaling network (Luan, 2009; Hepler et al., 2011). Current CBL-CIPK research has primarily focused on plant stress response mechanisms. For example, under salt stress, the CBL4-CIPK24 complex regulates the Na+/K+ antiporter SOS1 on the plasma membrane to extrude sodium ions, while the CBL10-CIPK24 complex may participate in sequestering sodium ions in the vacuole (Liu et al., 2000; Kim et al., 2007; Quan et al., 2007). Under low potassium conditions, CBL1 and CBL9 interact with CIPK23 to regulate a potassium channel (Arabidopsis K+ transporter 1, AKT1), thereby mediating potassium uptake in root hairs (Xu et al., 2006).

In recent years, the role of CBL-CIPK complexes in abiotic stress tolerance (cold, drought, and salinity) has been extensively studied, but research on CBL-CIPK function in pollen tube polar growth remains limited. This study investigates the involvement of CIPK9, a member of the calcium signaling downstream family, in pollen tube polar growth. Full-length CIPK9 and its constitutively active form were fused with GFP and transiently expressed in tobacco pollen via gene gun technology to observe subcellular localization and induced phenotypes, thereby exploring the role of CIPK9 in polar growth. These findings provide theoretical insights into the signaling networks regulating pollen tube growth.

Materials and Methods

1.1 Plant Materials and Growth Conditions *Arabidopsis thaliana* L. (Col-0) plants were cultivated in a greenhouse at 22 °C with a 16 h light/8 h dark photoperiod. Tobacco (*Nicotiana tabacum* L.) plants were grown in a greenhouse at 28 °C with a 12 h/12 h light/dark cycle.

1.2 Transient Expression Vector Construction Full-length CIPK9 cDNA (accession number U15436) was obtained from ABRC (Arabidopsis Biological Resource Center). The coding sequence was PCR-amplified using upstream primer (TCTAGAATGAGTGGAAGCAGAAGGA) containing an XbaI site and downstream primer (GGATCCCTTGCTTTTGTTCTTCA) containing a BamHI site. The amplified CIPK9 fragment was cloned into a T-vector (Promega), transformed into *E. coli* DH5 , and positive clones were selected for plasmid extraction. After restriction enzyme digestion and sequencing verification, the correct fragment was inserted into the pLAT52:GFP vector (Wu et al., 2001). For CACIPK9 construction, the C-terminal regulatory region of CIPK9 (amino acids 316-451) was removed, and a point mutation was introduced in the activation loop of the kinase domain, converting threonine at position 178 to aspartate (Guo et al., 2001; Albrecht et al., 2001). The correctly sequenced fragment was then inserted into the pLAT52:GFP vector.

1.3 Gene Gun-Mediated Transient Expression Fresh tobacco pollen was collected (from 8 flowers per transformation batch) for particle bombardment. Plasmid DNA was extracted using Plasmid Mini Kits (QIAGEN, Germany) and quantified with a UV spectrophotometer. Following established protocols for transient expression in tobacco pollen (Fu et al., 2001), 0.8 g of plasmid DNA was used per transformation. Bombarded pollen grains were cultured at 28 °C in darkness for 3-4 hours before observation by fluorescence or confocal microscopy.

1.4 Pollen Tube Phenotype Analysis Transformed pollen tubes were observed using an inverted fluorescence microscope (BX51; OLYMPUS) and photographed with a CCD camera (DP70; OLYMPUS). Images were analyzed using the measurement function in Zeiss LSM Image Browser (version 3.2) to determine pollen tube length and maximum apical diameter. Three independent transformation experiments were performed for each genotype, collecting approximately 80 pollen tubes for length and width measurements to assess depolarized growth phenotypes.

1.5 Subcellular Localization Observation Confocal laser scanning microscopy (Model LSM 510 META; Zeiss, Germany) was used to observe GFP-tagged CIPK9 (or CACIPK9) localization in pollen tubes (excitation 488 nm, emission 505-530 nm). Images were analyzed using Zeiss LSM Image Browser (version 3.2).

Results

2.1 Subcellular Localization and Overexpression Phenotype of CIPK9 Calcineurin B-like proteins (CBLs) are plant-specific calcium sensors that interact with downstream CBL-interacting protein kinases (CIPKs) (Luan et al.,

2002; Shi et al., 1999). We selected CIPK9 as a candidate gene and fused it with GFP for transient expression in tobacco pollen under the pollen-specific LAT52 promoter via gene gun technology to examine phenotypes and subcellular localization. Overexpression of CIPK9 did not cause significant changes in pollen tube polar growth (Fig. 1 [Figure 1: see original paper]B). Subcellular localization revealed that GFP alone (control) showed diffuse distribution throughout the pollen tube, whereas CIPK9-GFP localized to granular organelles that moved rapidly with cytoplasmic streaming in a "reverse fountain" pattern—moving along the pollen tube periphery to the apical plasma membrane and returning along the central axis to the base (Fig. 1A, C).

2.2 Constitutively Active CIPK9 (CACIPK9) Causes Abnormal Phenotypes and Localization To further investigate CIPK9 function in pollen tube growth, we constructed a constitutively active form of CIPK9 (CACIPK9). As shown in Fig. 2 [Figure 2: see original paper]A, full-length CIPK9 contains two major domains: a kinase domain and a regulatory domain. The regulatory domain at the C-terminus includes a highly conserved NAF motif (21 amino acids) that interacts with CBLs (Kim et al., 2000). This motif also mediates autoinhibition of CIPK kinase activity by binding to the kinase domain (Kolukisaoglu et al., 2004). Adjacent to the NAF motif is a PPI motif (protein phosphatase interaction motif) that may enable interaction between CIPKs and protein phosphatase 2C (Ohta et al., 2003). CACIPK9 retains primarily the kinase domain while removing the regulatory domain (including the autoinhibitory region). Additionally, a point mutation in the activation loop (converting threonine at position 178 to aspartate) further enhances CIPK9 kinase activity.

CACIPK9 was transiently expressed in tobacco pollen using the same strategy as full-length CIPK9 to study its phenotype and localization. CACIPK9 expression induced depolarized pollen tube growth, reducing tube length from 381.67 m (control) to 335.41 m while increasing width from 8.74 m to 10.11 m. For subcellular localization, unlike the granular organelle localization of CIPK9-GFP, CACIPK9-GFP showed non-specific diffuse distribution identical to the GFP control. This suggests that the C-terminal regulatory domain is essential for proper CIPK9 localization in pollen tubes, and that abnormal subcellular localization may affect CIPK9 function in polar growth.

Discussion

Cell polarity is a fundamental property of cell development, manifested as asymmetry in cellular structure and composition (Yang, 2008). Establishment and maintenance of plant cell polarity involve multiple regulatory factors, including calcium ions, endocytosis, and the cytoskeleton, which must be maintained at specific levels to sustain stable pollen tube tip growth (Kroeger & Geitmann, 2012).

Calcium concentration plays a crucial role in regulating pollen tube growth and guidance. The *Arabidopsis* genome contains four major calcium sensor families: calmodulin (CaM), calmodulin-like (CML), calcineurin B-like (CBL), and calcium-dependent protein kinase (CPK) (McCormack et al., 2005). CBLs are plant-specific and structurally similar to the B subunit of animal calcineurin. Their downstream effectors are Ser/Thr protein kinases (CIPKs) that together perceive and transmit calcium signals (Luan et al., 2002). In this study, CIPK9-GFP localized to granular organelles moving with cytoplasmic streaming, which based on their movement pattern likely represent vesicles of the pollen tube endomembrane system (Fig. 1A). This suggests CIPK9 may influence cell polarity by regulating the vesicle system in pollen tubes.

The C-terminus of CIPK9 contains an autoinhibitory region (NAF motif) that also serves as the binding site for upstream effectors (CBLs) (Fig. 2A). CBL binding relieves this autoinhibition, thereby activating CIPK kinase activity (Luan et al., 2002; Kim et al., 2000). Our constitutively active CACIPK9 mimicked this activation and further enhanced kinase activity through mutation of conserved structures in the kinase domain. CACIPK9 overexpression inhibited longitudinal growth while promoting lateral expansion, resulting in depolarized growth (Fig. 2B, C). Moreover, C-terminal deletion caused CACIPK9-GFP to display non-specific diffuse localization (Fig. 2B), demonstrating that the C-terminal regulatory domain is critical for proper CIPK9 localization and function in pollen tube polar growth.

Previous CBL-CIPK network research has focused primarily on stress signaling pathways (Quan et al., 2007; Cheong et al., 2007; Xu et al., 2006). This study provides new insights into CIPK9 function in pollen tube polar growth, offering novel understanding of its biological role. However, the specific nature of the organelles where CIPK9 localizes (whether vesicle systems) and the precise mechanism by which CIPK9 regulates pollen tube polarity require further investigation. Future identification of CIPK9 downstream target proteins and elucidation of their mechanisms will further reveal the important roles of the CIPK signaling network in pollen tube polar growth.

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