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The Variable Domain of a Plant Calcium-dependent Protein Kinase (CDPK) Confers Subcellular Localization and Substrate Recognition for NADPH Oxidase*

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Background: Substrate specificity of CDPKs involved in diverse physiological processes is largely unknown.
Results: The variable domain of StCDPK5 confers plasma membrane localization and ability to phosphorylate its substrate NADPH oxidase.
Conclusion: The contribution of variable domains to localization and substrate specificity of CDPKs *in vivo* is proposed.
Significance: This is the first indication of substrate discrimination of CDPKs via proper subcellular localization.

Calcium-dependent protein kinases (CDPKs) are Ca²⁺ sensors that regulate diverse biological processes in plants and apicomplexans. However, how CDPKs discriminate specific substrates *in vivo* is still largely unknown. Previously, we found that a potato StCDPK5 is dominantly localized to the plasma membrane and activates the plasma membrane NADPH oxidase (RBOH; for respiratory burst oxidase homolog) StRBOHB by direct phosphorylation of the N-terminal region. Here, we report the contribution of the StCDPK5 N-terminal variable (V) domain to activation of StRBOHB *in vivo* using heterologous expression system in *Nicotiana benthamiana*. Mutations of N-terminal myristoylation and palmitoylation sites in the V domain eliminated the predominantly plasma membrane localization and the capacity of StCDPK5 to activate StRBOHB *in vivo*. A tomato SlCDPK2, which also contains myristoylation and palmitoylation sites in its N terminus, phosphorylated StRBOHB *in vitro* but not *in vivo*. Functional domains responsible for activation and phosphorylation of StRBOHB were identified by swapping regions for each domain between StCDPK5 and SlCDPK2. The substitution of the V domain of StCDPK5 with that of SlCDPK2 abolished the activation and phosphorylation abilities of StRBOHB *in vivo* and relocalized the chimeric CDPK to the *trans*-Golgi network, as observed for SlCDPK2. Conversely, SlCDPK2 substituted with the V domain of StCDPK5 localized to the plasma membrane and activated StRBOHB. These results suggest that the V domains confer substrate specificity *in vivo* by dictating proper subcellular localization of CDPKs.

Protein kinases regulate almost all aspects of cell life through phosphorylation of Ser, Thr, and Tyr. Protein phosphorylation is known to affect protein functions such as enzymatic activity, stabilization, subcellular localization, and interactions with other biomolecules (1). The spatiotemporal control of phosphorylation is crucial to cellular processes and relies on the proper regulation of protein kinases. Misregulation of protein phosphorylation often results in negative consequences such as cancer and complex regulatory diseases. Indeed, the first oncogene to be discovered, v-src, encodes an aberrantly regulated Tyr kinase (2). The substrate specificity is believed to depend on the interaction between the active site of kinase and the amino acid sequences surrounding the phosphorylation sites of substrate (*e.g.* by way of conserved docking motifs on substrate) (3, 4). Studies on kinase substrate identification in yeast (5, 6), however, suggest that the presence of a consensus site on substrate is insufficient to account for the substrate specificity. In addition to kinase-substrate interaction, subcellular localization, differential timing, and tissue expression, and/or functional adaptors such as scaffold proteins could be determinant for the substrate *in vivo*. Although identification of *in vivo* substrates is important to understand aspects of cell life, how protein kinases discriminate specific substrates *in vivo* is still largely unknown.

Calcium-dependent protein kinases (CDPKs)² are Ser/Thr protein kinases acting as Ca²⁺ sensors that are broadly distributed in plants and some protozoans such as ciliates and apicomplexans (7, 8). CDPKs are composed of an N-terminal variable (V) domain, a protein kinase (K) domain, an autoinhibitory junction (J) domain, and a calmodulin-like (C) domain including four EF-hand motifs and are activated via conformational changes triggered by the binding of Ca²⁺ to the C domain (9,

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[2] The abbreviations used are: CDPK, calcium-dependent protein kinase; GUS, β-glucuronidase; V, variable; K, kinase; J, junction; C, calmodulin-like; RBOH, respiratory burst oxidase homolog; ROS, reactive oxygen species; TGN, *trans*-Golgi network; Rubisco, ribulose-1,5-bisphosphate carboxylase.

10). The *Arabidopsis* genome encodes 34 CDPKs (also designated as CPKs) that are clustered into four subgroups on the basis of sequence similarity (8). Although none of the CDPKs has transmembrane domains, the majority of CDPKs have potential myristoylation and palmitoylation motifs at the beginning of their N-terminal V domain that may be responsible for membrane association (8). Indeed, these lipid modifications have been shown to be required for the attachment to specific cellular membranes (11–16). Moreover, it has been demonstrated that additional CDPKs are localized to different cellular membranes, including plasma membrane, endoplasmic reticulum membrane, and peroxisome membrane (12, 17). The application of reverse genetics techniques has shown that CDPKs participate in diverse physiological processes, such as resistance to biotic and abiotic stress, hormonal signaling, and development (18). However, how CDPKs specifically recognize substrates *in vivo* remains to be elucidated.

Potato (*Solanum tuberosum*) StCDPK4 and StCDPK5 phosphorylate and activate the plasma membrane NADPH oxidase (RBOH; for respiratory burst oxidase homolog) StRBOHB, resulting in increased reactive oxygen species (ROS) production (19). ROS generated by RBOHs participate in diverse biological processes (20), including defense responses to near obligate hemibiotrophic pathogens such as *Phytophthora infestans*, but have a negative role in resistance or have a positive role in expansion of disease lesions caused by necrotrophic pathogens (21–23). Correspondingly, transgenic potato plants containing a constitutively active form of StCDPK5, StCDPK5VK, under the control of a pathogen-inducible promoter (24) showed high resistance to *P. infestans* but high susceptibility to the necrotrophic pathogen *Alternaria solani* (25, 26). Moreover, phosphorylation of N-terminal Ser-82 and Ser-97 in StRBOHB is shown to be required for the full activity when coexpressed with StCDPK5VK in *Nicotiana benthamiana* leaves (19). Phosphoproteomic analyses in *Arabidopsis* also indicate that the N-terminal region of AtRBOHD is phosphorylated *in vivo* when treated with elicitors (27, 28). AtRBOHD is synergistically activated by Ca^{2+} influx and phosphorylation in a heterologous expression system using a mammalian cell line (29). There reports suggest involvement of phosphorylation of RBOH in the activation process.

Here, we investigated *in vivo* substrate specificity between StCDPK5 and StRBOHB. Mutations of N-terminal myristoylation and palmitoylation sites in StCDPK5, which are responsible for localization at the membrane, eliminated the StCDPK5-StRBOHB interaction and StCDPK5-mediated StRBOHB phosphorylation *in vivo*. The N-terminal V domain of StCDPK5 conferred proper localization and the ability to activate StRBOHB *in vivo* to a distinct CDPK that phosphorylates StRBOHB *in vitro* but not *in vivo*. These results indicate the N-terminal V domain of StCDPK5 for proper subcellular localization and consequent discrimination of specific substrates *in vivo*.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—*N. benthamiana* plants were grown at 25 °C and 70% humidity under a 16-h photoperiod and an 8-h dark period in environmentally controlled growth cabinets.

Agroinfiltration Assay—For agroinfiltration, cDNA fragments of *CDPK* were generated by PCR and cloned into pGreen binary vector (30), in which a HA tag was added to the C-terminal end. Amino acid substitution of the constitutively active form StCDPK5CA (A344P/V345D/H351P/F352E/S353D/A354L) and SlCDPK2CA (A389P/V390D/Q396P/F397E/S398D/A399L), kinase-inactivated variants (K/M), the N-terminal acylation site-mutated variants (G2A/C5A) were introduced by PCR-based, site-directed mutagenesis. Chimeric *CDPK* (5V2KJC and 2V5KJC) were generated by In-Fusion® HD Cloning Kit (Takara Bio). The constructs of *GUS* and *StRBOHB* and mutated *StRBOHB* (S82A/S97A) were described by Asai *et al.* (22) and Kobayashi *et al.* (19), respectively. Transformation of *Agrobacterium* GV3101 by electroporation and infiltration of *Agrobacterium* suspensions were done as described by Asai *et al.* (22). Immunoprecipitation of CDPKs and StRBOHB was done by coinfiltration of *Agrobacterium* expressing *p19*, the suppressor of posttranscriptional gene silencing of tomato bushy stunt virus (31).

ROS Measurements—ROS measurements were done as described by Kobayashi *et al.* (19).

Confocal Microscopy—For subcellular localization analysis in *N. benthamiana* leaves, cDNA fragments of *CDPKs* were cloned into pK7FWG2 binary vector, which fused GFP to the C terminus of the protein (32). The constructs of fluorescent organelle markers (Golgi, peroxisomes, and endoplasmic reticulum) with mCherry were described by Nelson *et al.* (33). The construct of RFP-tagged potato Remorin 1.3 (RFP-StREM1.3) was described by Perraki *et al.* (34). The construct of RFP-tagged membrane-integral V-ATPase subunit VHA-a1 (RFP-VHA-a1) was described by Dettmer *et al.* (35) and Viotti *et al.* (36). These constructs were transiently expressed by agroinfiltration in 4- to 5-week-old *N. benthamiana* leaves. The fluorescence was observed using confocal microscopy (DM6000B/TCS SP5, Leica). GFP, RFP, and mCherry were excited by a 488, 561, and 561 nm laser and detected with bandpass 500–540, 575–630, and 575–630 nm filters, respectively.

Preparation of Protein Extracts—Total protein extracts from *N. benthamiana* leaves were prepared as described by Kobayashi *et al.* (19). Plasma membrane-rich fraction was fractionated by the aqueous two-phase partitioning method as described by Kobayashi *et al.* (19).

Antibody Production and Immunoblotting—Preparation of anti-StRBOHB N-terminal antiserum and anti-pSer-82 antibody was described previously (19, 37). HA-tagged and FLAG-tagged proteins were detected by monoclonal anti-HA antibody (clone HA-7; Sigma-Aldrich) and monoclonal anti-FLAG antibody (F3165; Sigma-Aldrich), respectively. For immunoblotting, equal amounts of proteins were separated on a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). After blocking in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk for 1 h at room temperature or overnight at 4 °C, the membranes were incubated with anti-StRBOHB, anti-pSer-82, anti-HA, or anti-FLAG antibodies diluted with TBS-T at room temperature for 1 h or at 4 °C overnight. After washing with TBS-T, the membranes were incubated with horseradish

peroxidase-conjugated anti-rabbit Ig or anti-mouse Ig antibody (GE Healthcare) diluted with TBS-T for 1 h at room temperature. The antibody-antigen complex was detected using the ECL protein gel blot detection kit (GE Healthcare) and Light-Capture equipped with a CCD camera (ATTO), and immunostained bands were analyzed by the CS Analyzer 2.1 (ATTO).

Expression and Purification of Recombinant Proteins—For recombinant proteins, cDNA fragments of *CDPK* were cloned into pET44a(+) (Novagen) and were transformed into *Escherichia coli* BL21-CodonPlus (DE3)RIPL (Stratagene). The bacteria were cultured overnight at 37 °C, transferred to 100-fold LB medium containing 50 mg/ml ampicillin, and then incubated to A_{600} of 0.6 at 37 °C. Protein synthesis was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 25 °C. Cells were collected and resuspended in the buffer (50 mM HEPES-NaOH, pH 7.5, 200 mM NaCl). NusA-fused CDPKs were extracted via sonication and purified using nickel-Sepharose 6 Fast Flow according to the manufacturer's instructions (GE Healthcare). StRBOHB N-terminal fragments were prepared as described by Kobayashi *et al.* (19).

Kinase Assay—Kinase activity of recombinant proteins was determined in 15 μl of phosphorylation buffer (20 mM HEPES-KOH, pH 7.6, 1 mM DTT, 5 mM MgCl$_2$, and 1 mM CaCl$_2$) containing 2 μg of substrate, StRBOHB N-terminal peptides, or histone IIIS (Sigma-Aldrich) and 0.25 μg of enzyme. Reactions were started by the addition of 50 μM ATP with/without 50 μCi/ml of [γ-^{32}P]ATP at 30 °C for 30 min. The reaction was stopped by adding SDS-PAGE sample loading buffer. After electrophoresis on SDS-polyacrylamide gel, the phosphorylated StRBOHB N-terminal peptides and histone IIIS were visualized by autoradiography or by immunoblotting using anti-pSer-82 antibody. The immunocomplex kinase assay was done as described by Asai *et al.* (22).

Immunoprecipitation—Protein extraction for immunoprecipitation was done as described by Schwessinger *et al.* (38). Immunoprecipitation was performed using μMACS HA-tagged protein isolation kit according to the manufacturer's instructions (Miltenyi Biotec).

RESULTS

Myristoylation and Palmitoylation in StCDPK5 Are Required for the Plasma Membrane Localization and Phosphorylation of StRBOHB in Vivo—CDPKs consist of a V domain comprising highly variable amino acid sequences, a K domain that phosphorylates a substrate, a J domain that acts as an autoinhibitor in a pseudo-substrate fashion, and a C domain, including Ca^{2+}-sensing EF-hand motifs (Fig. 1*A*). Upon elevation of cytosolic Ca^{2+} concentration, binding of Ca^{2+} to the C domain triggers a conformational change to release autoinhibition and allows enzyme activation (9, 10). A point-mutated variant of CDPK (constitutively active), which has a six-residue substitution in the J domain, acts a constitutively active kinase (19, 39). C-terminal HA-fused constitutively active variants of StCDPK5 show constitutive kinase activity, except for kinase-inactive K/M mutant (Fig. 1, *A* and *B*) (40). Ectopic coexpression of StCDPK5CA with StRBOHB but not a variant with Ser-to-Ala mutations in Ser-82 and Ser-97 (S82A/S97A) remarkably induced ROS production in leaves (Fig. 1*C*). These results indi-
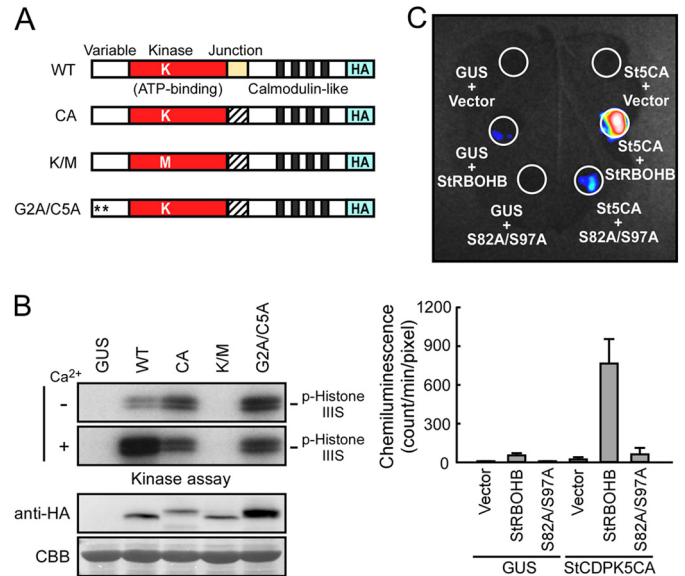

FIGURE 1. **Ser-82 and Ser-97 in StRBOHB are required for its activation by StCDPK5.** *A*, schematic structures of StCDPK5 variants. *Diagonal boxes* indicate a six-residue substitution in the J domain. *K* indicates a Lys residue for ATP binding in the K domain, and *M* indicates amino acid substitution in Lys to Met. *Asterisks* indicate point-mutated sites to Ala in predicted myristoylation and palmitoylation sites. *B*, kinase activity of StCDPK5 variants. Total proteins were prepared from *N. benthamiana* expressing HA-tagged StCDPK5 variants or GUS as a control. Immunoprecipitates using anti-HA antibody were incubated with histone IIIS and [γ-^{32}P]ATP with CaCl$_2$ (indicated as Ca^{2+} +) or EGTA (indicated as Ca^{2+} −). Phosphorylation of histone IIIS was detected by x-ray film (*top two panels*). Total protein extracts were used for immunoblot analysis with anti-HA antibody (*middle panel*). Protein loads were monitored by Coomassie Brilliant Blue (*CBB*) staining of the bands corresponding to ribulose-1,5-bisphosphate carboxylase (Rubisco) large subunit (*bottom panel*). *C*, ROS production in *N. benthamiana* leaves coexpressing HA-tagged StCDPK5CA or GUS as a control with StRBOHB or point-mutated StRBOHB (S82A/S97A). ROS production was measured 2 days after agroinfiltration by chemiluminescence mediated by L-012. Data are means ± S.D. from four experiments. *CA*, constitutively active.

cate that ROS production induced by StCDPK5CA may be attributed to the phosphorylation of Ser-82 and Ser-97 in StRBOHB.

Several CDPKs are reported to associate with various membranes, and the correct localization requires N-terminal acylation such as myristoylation and palmitoylation. StCDPK5, which is localized at the plasma membrane (25), has a Gly residue at the second position and a Cys residue at the fifth position that are predicted myristoylation and palmitoylation sites, respectively. To investigate role of myristoylation and palmitoylation in plasma membrane localization of StCDPK5, GFP-tagged StCDPK5CA (StCDPK5CA-GFP) and its variant in which Gly-2 and Cys-5 were mutated to Ala (G2A/C5A-GFP; Fig. 1*A*) were coexpressed with RFP-tagged potato StREM1.3 (RFP-StREM1.3) which is localized to plasma membrane (34) in *N. benthamiana* leaves. Similar to StRBOHB (37), StCDPK5CA-GFP was predominantly located to the plasma membrane, whereas substitution of Gly-2 and Cys-5 for Ala (G2A/C5A) led to a change in the localization to cytoplasm (Fig. 2*A*). Similarly, the amount of the G2A/C5A variant present in plasma membrane-rich fraction was extremely low compared with that of StCDPK5CA (Fig. 2*D*), whereas the amounts of StCDPK5CA and G2A/C5A in total protein extracts were comparable (Fig. 1*B*). Co-immunoprecipitation experiments
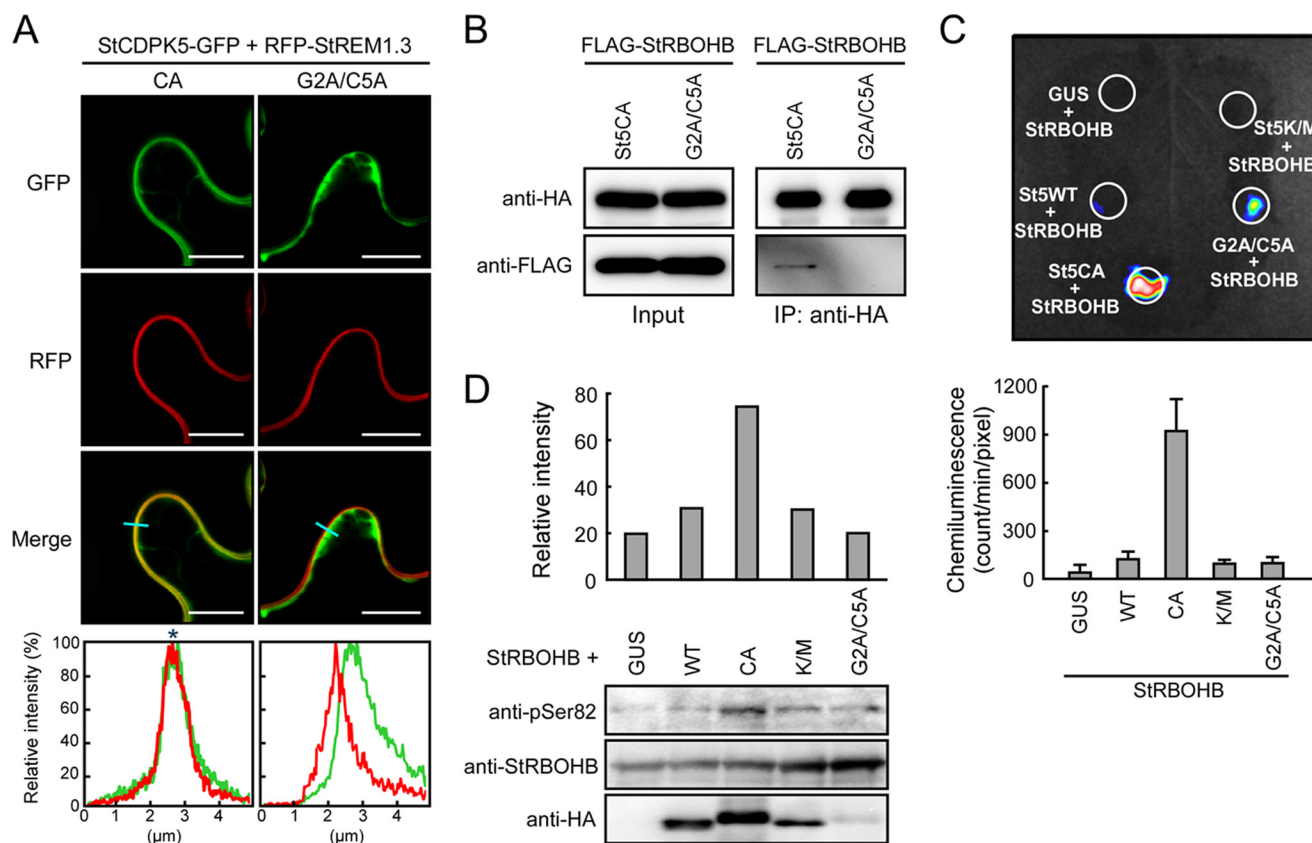
FIGURE 2. **Myristoylation and palmitoylation of StCDPK5 are required for interaction with and activation of StRBOHB.** *A*, requirement of myristoylation and palmitoylation in StCDPK5 for the localization at plasma membrane. GFP-tagged StCDPK5 variants were transiently coexpressed with RFP-StREM1.3 via agroinfiltration in *N. benthamiana*. The *upper* image is from the GFP channel, the *middle* image is from the RFP channel, and the *lower* image is the overlay of the GFP and RFP channels. Fluorescence intensity profile (GFP, *green*; RFP, *red*) across the *pale blue line* was performed using the analyzing software (Leica, *bottom*). An *asterisk* indicates the corresponding peak of fluorescence intensity. *Scale bars*, 10 μm. *B*, requirement of myristoylation and palmitoylation in StCDPK5 for interaction with StRBOHB *in vivo*. Co-immunoprecipitation was performed with extracts from *N. benthamiana* leaves coexpressing HA-tagged StCDPK5 variants with FLAG-tagged StRBOHB. MACS MicroBeads with monoclonal HA antibody was used for immunoprecipitation, and anti-HA and anti-FLAG antibodies were used to detect the related proteins in the immunoprecipitates. *C*, ROS production in *N. benthamiana* leaves coexpressing HA-tagged StCDPK5 variants or GUS as a control with StRBOHB. ROS production was measured as described in Fig. 1*C*. Data are means $\pm$ S.D. from four experiments. *D*, *in vivo* phosphorylation of Ser-82 in StRBOHB by StCDPK5 variants. StRBOHB was transiently coexpressed with HA-tagged StCDPK5 variants or GUS as a control via agroinfiltration in *N. benthamiana* leaves. Plasma membrane proteins were prepared at 2 days after agroinfiltration. The intensities of each band were quantified, and relative intensity in immunoblot analysis using anti-pSer-82 antibody to anti-StRBOHB antiserum was expressed (*top*). Plasma membrane proteins were used for immunoblot analysis with anti-HA antibody (*bottom panel*). *CA*, constitutively active.

showed the interaction between StRBOHB and StCDPK5CA, but not G2A/C5A, when coexpressed in *N. benthamiana* (Fig. 2*B*). G2A/C5A as well as kinase-inactive K/M did not provoke StRBOHB-mediated ROS production (Fig. 2*C*). Immunoblot analysis using anti-phosphopeptide antibody against peptides, including phospho-Ser-82 (pSer-82) showed that Ser-82 in StRBOHB was phosphorylated when StCDPK5CA was coexpressed but not when G2A/C5A was coexpressed (Fig. 2*D*). Although the anti-pSer-82 antibody does not recognize non-phosphopeptide (Fig. 3*D*), we detected low level phosphorylation of Ser-82 even in the β-glucuronidase control, suggesting that overexpression of StRBOHB could increase phosphorylation of Ser-82, but not to an extent that can induce detectable ROS production in our system (Fig. 2*C*). We also confirmed that G2A/C5A and StCDPK5CA, which had been expressed in *N. benthamiana* leaves and purified, had similar kinase activity (Fig. 1*B*) and that the recombinant proteins of G2A/C5A phosphorylate N-terminal fragments of StRBOHB (data not shown). These results suggest that myristoylation and palmitoylation in StCDPK5 are required for the localization at plasma membrane to interact with and phosphorylate StRBOHB *in vivo*.

Overexpression of SlCDPK2 Does Not Induce StRBOHB-dependent ROS Production in N. benthamiana—Although the N-terminal acylation of StCDPK5 appears to be important for determining a substrate *in vivo*, the mechanisms of substrate specificity in CDPKs remain to be elucidated because the majority of CDPKs (29 of 34 CPKs in *Arabidopsis*) contain predicted acylation sites (8). Based on amino acid sequences, 34 *Arabidopsis* CDPKs can be divided into four subgroups (8). Tomato (*Solanum lycopersicum*) SlCDPK2, which has predicted myristoylation and palmitoylation sites and belongs to group I as well as StCDPK5 (data not shown), is a CDPK that phosphorylates 1-aminocyclopropane-1-carboxylic acid synthase 2 for ethylene synthesis (41). To investigate how CDPKs phosphorylate a specific substrate, a potato homolog of SlCDPK2, StCDPK6, was cloned as a CDPK compared with StCDPK5. Although StCDPK6 shares 98.8% identity with SlCDPK2, recombinant proteins of SlCDPK2, but not StCDPK6, showed kinase activity (data not shown). Therefore, a constitutively active form of SlCDPK2, SlCDPK2CA, was constructed and investigated in the following experiments. Coexpression of SlCDPK2CA with StRBOHB did not induce ROS production (Fig.
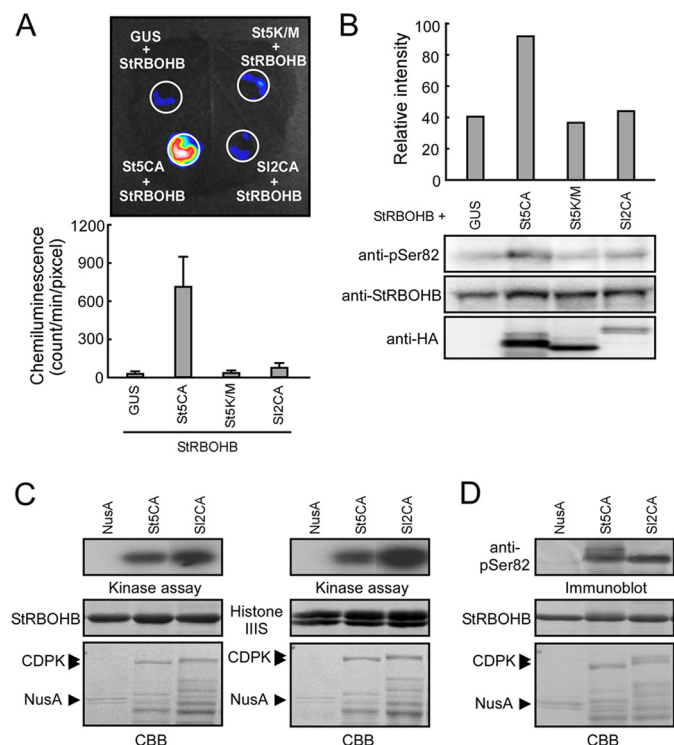
FIGURE 3. **SlCDPK2CA phosphorylates StRBOHB *in vitro* but not *in vivo*.** *A*, ROS production in *N. benthamiana* leaves coexpressing HA-tagged StCDPK5 variants, SlCDPK2CA, or GUS as a control with StRBOHB. ROS production was measured as described in Fig. 1*C*. Data are means ± S.D. from four experiments. *B*, *in vivo* phosphorylation of Ser-82 in StRBOHB by StCDPK5CA but not SlCDPK2CA. Immunoblot analysis was performed as described in Fig. 2*D*. *C*, phosphorylation of StRBOHB by recombinant StCDPK5CA and SlCDPK2CA. Purified N-terminal peptides of StRBOHB (*left*) or histone IIIS (*right*) were used as substrates for bacterially expressed NusA-fused StCDPK5CA and SlCDPK2CA. Phosphorylation of StRBOHB and histone IIIS was detected by x-ray film (*top panel*). Protein loads were monitored by Coomassie Brilliant Blue (*CBB*) staining (*middle* and *bottom panels*). Every image was taken from the same gel/autoradiogram and edited. *D*, phosphorylation of Ser-82 in StRBOHB by recombinant StCDPK5CA and SlCDPK2CA. N-terminal peptides of StRBOHB were used as substrates for NusA-fused StCDPK5CA and SlCDPK2CA. Immunoblot analysis was performed using anti-pSer82 antibody (*top panel*). Protein loads were monitored by Coomassie Brilliant Blue staining (*middle* and *bottom panels*).

3*A*). Although SlCDPK2CA expressed in *N. benthamiana* showed higher kinase activity with histone IIIS *in vitro* than StCDPK5CA (Fig. 4*B*), phosphorylation of Ser-82 in StRBOHB in plasma membrane by SlCDPK2CA was very low at a level similar to the negative controls GUS and StCDPK5K/M (Fig. 3*B*). Surprisingly, recombinant proteins of SlCDPK2CA phosphorylated N-terminal fragments of StRBOHB at the same level as StCDPK5CA (Fig. 3, *C* and *D*), suggesting that SlCDPK2 could phosphorylate StRBOHB *in vitro* but not *in vivo*.

The Variable Domain of StCDPK5 Is Required for Phosphorylation and Activation of StRBOHB in Vivo—To evaluate which domain of StCDPK5 is required for phosphorylation of StRBOHB *in vivo*, a series of chimeric CDPKs were constructed by reciprocal exchange of domains between StCDPK5CA and SlCDPK2CA. We found that a chimeric CDPK, which consists of the V domain of StCDPK5 and K, J, and C domains of SlCDPK2CA (5V2KJC; Fig. 4*A*), induced StRBOHB-mediated ROS production at the same level as StCDPK5CA (Fig. 4*C*). By contrast, ROS production was not observed in leaves that express a chimeric CDPK in which V domain of StCDPK5CA

was substituted with that of SlCDPK2 (2V5KJC; Fig. 4, *A* and *C*). Correspondingly, Ser-82 in StRBOHB was phosphorylated to the same degree as StCDPK5CA when coexpressing StRBOHB with 5V2KJC but not 2V5KJC (Fig. 4*D*). We also confirmed that 5V2KJC, 2V5KJC, and StCDPK5CA, which had been expressed in *N. benthamiana* and purified, had similar kinase activity (Fig. 4*B*). These results suggest that the V domain of StCDPK5 is the determinant for the activation of StRBOHB accompanied by phosphorylation *in vivo*.

The Subcellular Localization of CDPKs Is Determined By the Variable Domain—Although plasma membrane StRBOHB was phosphorylated and activated by StCDPK5CA but not by SlCDPK2CA *in vivo*, StRBOHB was phosphorylated by both StCDPK5CA and SlCDPK2CA *in vitro* (Fig. 3). This finding prompted us to examine the subcellular localization of SlCDPK2. The subcellular distribution of SlCDPK2CA fused to GFP at the C-terminal region (SlCDPK2CA-GFP) was distinguishable from that of StCDPK5CA-GFP in *N. benthamiana* leaves (Fig. 5*A*). The fluorescence was seen in small and spherical organelles such as peroxisomes and Golgi. To determine where SlCDPK2 is localized, SlCDPK2CA-GFP was coexpressed with a series of organelle markers tagged with mCherry (33) in *N. benthamiana* leaves. Comparison of SlCDPK2CA-GFP labeling with peroxisome-mCherry and endoplasmic reticulum-mCherry markers did not reveal any colocalized signals (data not shown). By contrast, when SlCDPK2CA-GFP was coexpressed with the *cis*-Golgi-mCherry marker (33, 42), both fluorescence signals were observed in the same small bodies but were not perfectly matched, and distinct fluorescence signals from GFP and mCherry were also observed in some vesicles (Fig. 5*B*), suggesting that SlCDPK2CA is localized to the *trans*-Golgi network (TGN). To confirm the hypothesis, SlCDPK2CA was coexpressed with RFP-tagged VHA-a1 (RFP-VHA-a1) which is localized to the TGN (35, 36). As a result, colocalization of GFP and RFP was observed (Fig. 5*B*). We also investigated the subcellular localization of chimeric CDPKs. The distribution patterns of 5V2KJC-GFP and 2V5KJC-GFP were similar to that of StCDPK5CA-GFP and SlCDPK2CA-GFP, respectively (Fig. 5*A*), suggesting that the V domains of StCDPK5 and SlCDPK2 are required for their plasma membrane and TGN subcellular localizations, respectively. Co-immunoprecipitation experiments showed that StCDPK5CA and 5V2KJC, but not SlCDPK2CA and 2V5KJC, associate with StRBOHB *in vivo* when coexpressed in *N. benthamiana* (Fig. 5*C*). The results described above suggest that plasma membrane localization of StCDPK5 via its V domain determines whether it can phosphorylate and activate StRBOHB *in vivo*.

DISCUSSION

Several studies have pointed to a crucial role of CDPK-mediated phosphorylation in regulating a diverse array of biological processes not only in plants but also in apicomplexans that cause malaria and toxoplasmosis (19, 43–48). CDPKs are encoded by a large multigene family with possible redundancy and/or diversity in their functions (7, 8). For example, AtCPK4 and AtCPK11 participate in abscisic acid responses through phosphorylation of AtABF1 and AtABF4 (44), but also have redundant roles with AtCPK1, AtCPK2, AtCPK5, and AtCPK6
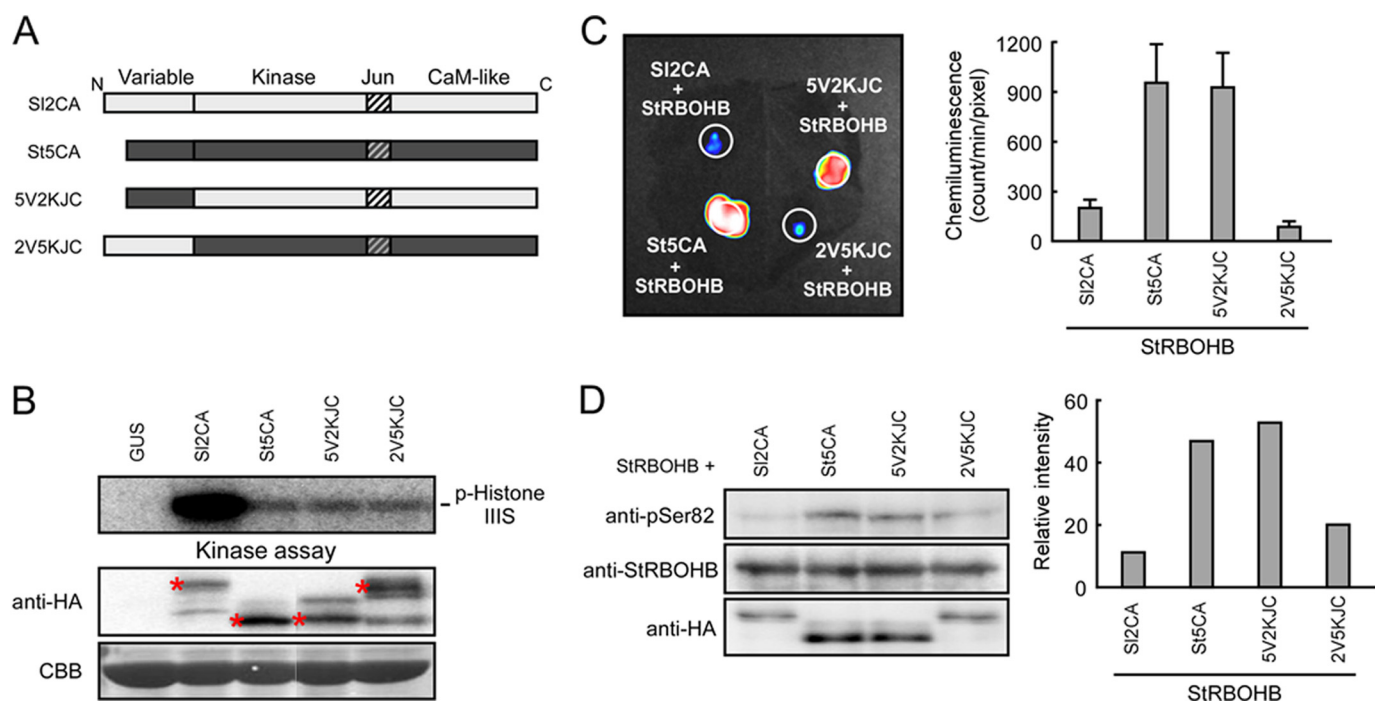
FIGURE 4. **Requirement of the V domain of StCDPK5 for phosphorylation and activation of StRBOHB *in vivo*.** *A*, schematic structures of StCDPK5, SlCDPK2, and chimeric constructs (5V2KJC and 2V5KJC). *Diagonal boxes* indicate a six-residue substitution in the J domain. *B*, kinase activity of chimeric CDPKs. The immunocomplex kinase assay and immunoblot analysis with anti-HA antibody in *N. benthamiana* leaves coexpressing HA-tagged StCDPK5CA, SlCDPK2CA, 5V2KJC, 2V5KJC, or GUS as a control with StRBOHB were done as described in Fig. 1*B*. *Asterisks* indicate the detected CDPK constructs. Every image was taken from the same gel/blot/autoradiogram and edited. *C*, ROS production in *N. benthamiana* leaves coexpressing HA-tagged StCDPK5CA, SlCDPK2CA, 5V2KJC, or 2V5KJC with StRBOHB. ROS production was measured as described in Fig. 1*C*. Data are means ± S.D. from three experiments. *D*, *In vivo* phosphorylation of Ser-82 in StRBOHB by StCDPK5CA and 5V2KJC but not SlCDPK2CA and 2V5KJC. Immunoblot analysis was performed as described in Fig. 2*D*.

in ROS production and induction of immune gene expression through phosphorylation of specific WRKY transcription factors (49, 50). Despite being implicated in regulating diverse aspects of plant and protozoan biology, little is known about how CDPKs recognize their specific target substrate *in vivo* to avoid inappropriate cross-talk. In this study, we reported that the N-terminal V domain of StCDPK5, including myristoylation and palmitoylation sites, confers its proper subcellular localization, resulting in interaction with and phosphorylation of StRBOHB *in vivo*.

The highly conserved myristoylation and palmitoylation sites in CDPKs have been reported to be required for their proper subcellular localization (11–16). Correspondingly, a StCDPK5 variant (G2A/C5A) in which the predicted myristoylation and palmitoylation sites were mutated lost its functions in dominant plasma membrane localization and activation of StRBOHB *in vivo* (Fig. 2). Myristoylation is also reported to participate in protein stability and protein-protein interactions (51, 52). We cannot rule out the possibility that G2A/C5A does not activate StRBOHB *in vivo* because the substitutions affect the stability and the interaction with StRBOHB and unknown other proteins that are responsible for the activation. However, our data indicate that the G2A/C5A accumulates in *N. benthamiana* and is still an active kinase capable of phosphorylating StRBOHB *in vitro* (Fig. 1*B*). SlCDPK2, which is localized to the TGN (Fig. 5*B*), has the predicted myristoylation and palmitoylation sites. Chimeric CDPKs 5V2KJC and 2V5KJC were localized at the plasma membrane and TGN, respectively (Fig. 5*A*), indicating requirement of V domains for correct subcellular

localization. The V domains are highly variable in length and amino acid sequence even among CDPKs from the same subgroup (8, 53). As observed for StCDPK5 and SlCDPK2, the V domains of other CDPKs may participate in their proper subcellular localization, resulting in phosphorylating the correct substrates *in vivo*.

Recent work showed that tobacco NtCDPK1 regulates the transcription factor RSG in response to gibberellins by phosphorylation of Ser-114 in RSG and that the V domain is required for interaction with RSG (53, 54). On the other hand, yeast two-hybrid approaches suggested that the K domain of AtCPK32 is necessary but not sufficient for the interaction with a substrate AtABF4, and both the N-terminal V domain and the C-terminal JC domains are required for the normal interaction (55). In this study, we showed that StCDPK5CA and 5V2KJC are localized primarily to the plasma membrane and interact with StRBOHB *in vivo*, whereas SlCDPK2CA and 2V5KJC do not interact with StRBOHB *in vivo* due to their distinct localization to the TGN (Fig. 5). Both StCDPK5CA and SlCDPK2CA seem to interact with StRBOHB *in vitro* because both recombinant proteins phosphorylated the N-terminal fragment of StRBOHB (Fig. 3, *C* and *D*). Although *in vitro* kinase analysis in a previous study has shown that StCDPK5 phosphorylates only Ser-82 and Ser-97 in the N-terminal fragment of StRBOHB (19), we cannot rule out the possibility that SlCDPK2 phosphorylates other Ser and Thr residues than Ser-82 and Ser-97 *in vivo*. According to immunoblot analysis using anti-pSer-82 antibody, SlCDPK2CA seems to phosphorylate at least Ser-82 of StRBOHB *in vitro* at the same level as StCDPK5CA (Fig. 3*D*).
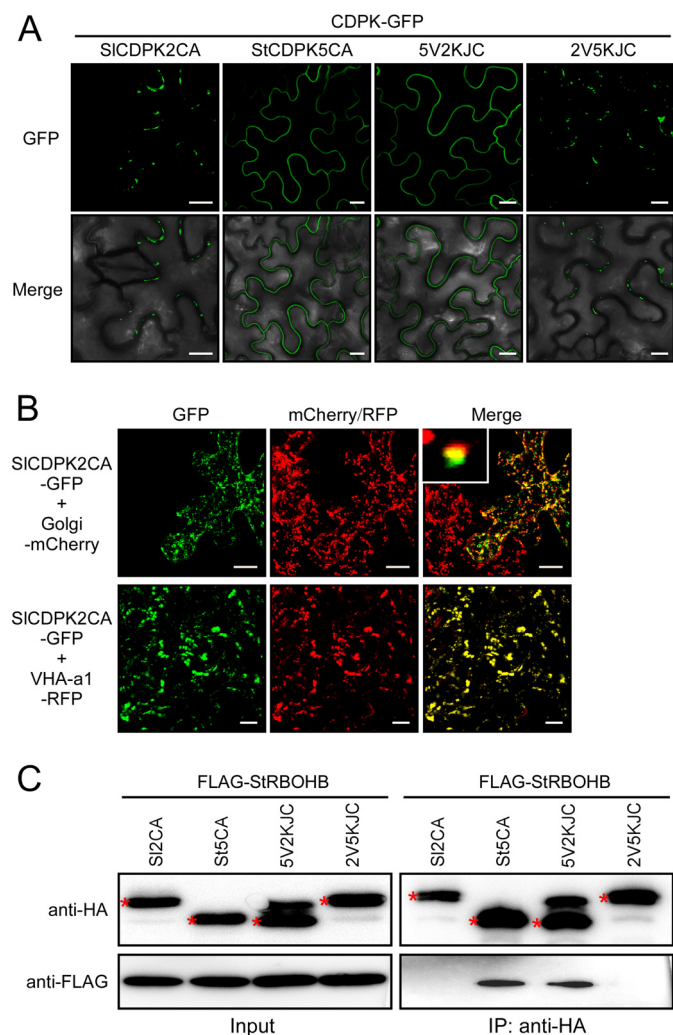
A

B

C

FIGURE 5. **The V domain of StCDPK5 is required for plasma membrane localization and interaction with StRBOHB *in vivo*.** *A*, subcellular localization of StCDPK5CA-GFP, SlCDPK2CA-GFP, 5V2KJC-GFP, and 2V5KJC-GFP. The indicated CDPK constructs were transiently expressed in *N. benthamiana* leaves. The *upper* image is from GFP channel, and the *lower* image is the overlay of differential interference contrast image and GFP channel. Images are single-plane confocal images. *Scale bars*, 10 μm. *B*, colocalization of SlCDPK2CA-GFP with Golgi markers. SlCDPK2CA-GFP was transiently coexpressed with an mCherry-tagged Golgi marker (*top*) and a RFP-tagged VHA-a1 (*bottom*) in *N. benthamiana*. The *left* image is from the GFP channel, the *middle* image is from mCherry/RFP channel, and the *right* image is the overlay of GFP and mCherry/RFP channels. Images are Z-stack confocal images. An *inset* indicates magnification of a selected single-plane confocal image. *Scale bars*, 10 μm. *C*, interaction of StCDPK5CA and 5V2KJC, but not SlCDPK2CA and 2V5KJC, with StRBOHB *in vivo*. Co-immunoprecipitation was performed with extracts from *N. benthamiana* leaves coexpressing the indicated HA-tagged CDPK constructs with FLAG-tagged StRBOHB. MACS MicroBeads with monoclonal HA antibody was used for immunoprecipitation and anti-HA, and anti-FLAG antibodies were used to detect the related proteins in the immunoprecipitates. The *asterisks* indicate the detected CDPK constructs.

Tobacco NtCDPK2, the closest homolog of SlCDPK2 (93% amino acid identity), has been shown to be involved in plant defense signaling (56, 57). Although *N. benthamiana* leaves expressing a constitutive active form of NtCDPK2 did not show any visual phenotype, the leaves responded to a mild hypo-osmotic shock, triggering ROS production (57). Correspondingly, coexpression of SlCDPK2CA with StRBOHB did not provoke ROS production under our experimental conditions

(without an abiotic stress stimulus) (Fig. 3*A*). Although the results here did not indicate the involvement of SlCDPK2 in activation of StRBOHB, SlCDPK2 might participate in ROS production via ethylene synthesis (41) or in response to other stimuli (57).

Site-directed mutagenesis indicated that phosphorylation of Ser-82 and Ser-97 in StRBOHB is required for the activation by StCDPK5 (Fig. 1*C*). Ser-82 is highly conserved among RBOHs, and Ser-97 is found in most stress-inducible RBOHs, including AtRBOHD (19), which is required for ROS production during innate immunity in *Arabidopsis* (58). Recently, Curran *et al.* (59) revealed candidate substrates for CDPKs through *in vitro* kinase reactions using peptides derived from *in vivo* mapping and *in silico* prediction strategies. Comparing phosphorylation targets of AtCPK1, AtCPK34, AtCPK10, and AtCPK16 which belong to group I, II, III, and IV, respectively (8), showed distinct and overlapped substrates among these CDPKs. A peptide derived from AtRBOHD, including a Ser residue corresponding to Ser-97 in StRBOHB, seems to be phosphorylated by recombinant AtCPK1, a close homolog of SlCDPK2, consistent with our result that SlCDPK2 phosphorylates StRBOHB *in vitro* (Fig. 3, *C* and *D*). However, SlCDPK2 did not trigger activation of StRBOHB accompanied by the phosphorylation *in vivo* (Fig. 3, *A* and *B*). By contrast, both *in vitro* and *in vivo* substrate specificities of NtCDPK1 seem to be strictly determined through recognition by the V domain, in concert with the interaction between the K domain and the phosphorylation site of the substrate RSG (53). The isoform-specific functions of CDPKs are potentially influenced by features that control the substrate specificity through kinase-substrate interaction, their subcellular localization, Ca^{2+} activation kinetics, lipid regulation, and stimulus-specific and spatiotemporal regulation of expression (9). Our results suggest that the V domains contribute substrate specificity *in vivo* by regulating the proper subcellular localization of CDPKs. Because CDPKs are involved in diverse physiological processes, cells need to coordinate multiple signaling pathways to maintain the information flow while preventing unwanted cross-talk. The amino acid sequences of the V domain in CDPKs could have been selected during evolution for the recognition of their physiological substrates through their proper subcellular localization and consequent interaction with substrates.

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