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Two putative calcium-dependent protein kinases are involved in the regulation of sugarcane defense genes

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Abstract

Sugarcane is a primary sugar crop and an important source of bioenergy. Pathogens are the major factors affecting sugarcane yield and sugar content. However, the mechanisms of sugarcane defense regulation remain largely unknown, and research on prospective genetic targets for modification is scarce. As the main class of calcium sensors, calcium-dependent protein kinases (CDPKs/CPKs) play a crucial role in the immune regulatory network. Using sugarcane genomic data, we identified 229 putative ScCDPKs in primordial specie *Saccharum officinarum*. Searching the putative CDPKs in sugarcane cultivars from National Center for Biotechnology Information (NCBI), 12 putative ScCDPKs in cultivars were identified. Phylogenetic analysis revealed evolutionary relationships among these CDPKs in sugarcane cultivars and those in *S. officinarum*, *Arabidopsis thaliana*, and rice. Truncation mutants of ScCDPKs were introduced into the reporter system and examined for activity in inducing *Sugarcane Pathogenesis Related Protein 1* (*ScPR1*). The results showed that truncated ScCDPK1 and ScCDPK8 induce higher expression of *ScPR1* than full-length ScCDPK1 and ScCDPK8. Additionally, transient expression of truncated ScCDPK1 and ScCDPK8 exhibit stronger activity in sugarcane protoplasts for activation of *ScPR1* and *sugarcane Serine Protease Inhibitor* (*ScSPI*). These results demonstrate that ScCDPK1 and ScCDPK8 possess auto-inhibitory activity. The findings of this study provide a basis for an in-depth study of the sugarcane CDPK gene family and lay the foundation for further genetic improvement.

Keywords Calcium-dependent protein kinase, Sugarcane, Defense regulation, *ScPR1*

Background

Plants have evolved sophisticated signaling pathways in response to abiotic and biotic stresses in the natural environment (Hepler 2005). Within many pathways, calcium serves as an important secondary messenger in cellular signal transduction (Sanders et al. 2002). External environmental stimuli lead to a transient cytoplasmic increase in Ca^{2+}. Cytoplasmic Ca^{2+} signals are detected by several types of calcium-sensing proteins, which usually contain a conserved helix-loop-helix EF-hand motif that directly binds Ca^{2+} to the sensors. Three major classes of calcium-sensing proteins have been characterized in plants, including Calmodulin (CaM/CAM) and CaM-like (CML) proteins, calcium-dependent protein

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kinases (CDPKs/CPKs), calcineurin B-like (CBL) proteins, and CBL-interacting protein kinases (CIPKs) (Luan et al. 2002; Bouché et al. 2005; McCormack et al. 2005; Kashir et al. 2013). Different sensors decode specific calcium signatures and transduce them into downstream effects, including oxidative burst, cell death, stomatal movements, hormonal signaling, and gene expression (Martín and Busconi 2000; Harmon et al. 2001; Luan 2009; Yip Delormel and Boudsocq 2019).

CDPKs are Ser/Thr protein kinases widely distributed in plants and certain types of protists (Cheng et al. 2002). CDPK harbors four typical domains, a variable N-terminal variable domain (VNTD), a serine/threonine kinase domain, an auto-inhibitory junction domain (AID), and a C-terminal regulatory calmodulin-like domain (CaM-LD) (Yip Delormel and Boudsocq 2019). The VNTD is involved in identifying diverse substrates and plays a crucial role in subcellular localization owing to its myristoylation and palmitoylation sites (Martín and Busconi 2000). The catalytic Ser/Thr kinase domain is highly conserved, contains an ATP-binding site, and exhibits extensive homology across species (Yoo and Harmon 1996). The AID domain is adjacent to the Ser/Thr kinase domain and forms a helical structure that interacts with the kinase domain to block its active site (Sanders et al. 1999). Previous studies have shown that AID mutations (simultaneous deletion of AID and CaM-LD) produce a constitutively active kinase independent of Ca^{2+} (Yoo and Harmon 1996; Tian et al. 2020). CaM-LDs are composed of one to four EF-hand motifs for Ca^{2+} binding, and thus, the interaction between CaM-LD and AID induces conformational changes in CDPK, which exposes the active site of the kinase domain, leading to the phosphorylation of substrates. (Wernimont et al. 2011).

In plants, a rapid and transient increase in cytoplasmic Ca^{2+} concentration is one of the earliest immune responses to pathogen-associated molecular pattern (PAMP) perception. By monitoring changes in cytoplasmic Ca^{2+} concentrations, CDPKs translate cytoplasmic Ca^{2+} levels into downstream signaling cascades through the kinase-induced phosphorylation of various substrates (Zielinski 1998). In *Arabidopsis thaliana*, AtCDPK4/5/6/11 has been identified as a Ca^{2+} sensor that directly phosphorylates the NADPH oxidase AtRBOHD, as well as the specific transcription factors WRKY48 and WRKY28 to regulate the immune signaling (Dubiella et al. 2013). AtCDPK28 negatively regulates PAMP-induced signaling and resistance to *Pseudomonas syringae* pv. *tomato* by phosphorylating PLANT U-BOX 25 (PUB25) and PUB26, which controls BOTRYTIS-INDUCED KINASE 1(BIK1) accumulation (Kadota et al. 2014; Monaghan et al. 2014). TOUCH 3 (TCH3) relieves the auto-inhibition of AtCDPK5 to elicit PAMP-induced CBP60g phosphorylation (Sun et al. 2022a). Similarly, a previous study showed that the rice AtBIK1 ortholog OsRLCK176 is phosphorylated by OsCDPK4, resulting in the degradation of OsRLCK176 and restricted plant defenses (Wang et al. 2018). Furthermore, mutual phosphorylation of OsMPK5 and OsCDPK4/18 regulates kinase activity, thereby coordinating plant growth and disease resistance (Li et al. 2022a).

Sugarcane (*Saccharum* spp.) is a prime sugar crop that accounts for 26% of global bioethanol production (Oz et al. 2021), whose growth and productivity are severely affected by pathogens such as *Sporisorium scitamineum*, *Fusarium sacchari*, and *Pythium arrhenomanes*. The vast majority of sugarcane main cultivars are susceptible to diseases (Yang and Mirkov 1997). Breeding disease-resistant varieties is one of the most effective measures to cope with sugarcane pathogens. However, sugarcane is a heteropolyploid crop with a complex genetic background. Therefore, breeding disease-resistant varieties using traditional crossbreeding techniques is challenging. Moreover, the diversity of pathogens and rapid evolution of pathogen virulence increase the difficulty of breeding varieties with commercial cultivation value (Bhuiyan et al. 2021). Due to the lag in deciphering the sugarcane genome, the identification of functional genes in sugarcane has proceeded slowly. To date, only the brown rust resistance gene (*BRU1*) has been a target in breeding, and biotechnological tools to improve the breeding process are lacking, resulting in insufficient research on the regulatory mechanisms of sugarcane resistance and targets for editing in the sugarcane genome (Asnaghi et al. 2004).

With the rapid progress in sequencing technology, a growing number of major crop genome sequencing techniques have made outstanding breakthroughs. For example, genome sequencing of major food crops (wheat, corn, sorghum, millet, etc.), cash crops (cotton, soybean, etc.), and horticultural crops (main vegetables, fruit trees, etc.) has been completed, including the genome of the autopolyploid sugarcane *Saccharum spontaneum*, contributing to molecular breeding and germplasm resource research (Sun et al. 2022b; Zhang et al. 2022). Although significant progress has been made in genome analysis, the functions of immune regulatory genes in the sugarcane genome remain largely unknown and research on targets that can be used for modification is scarce.

Results

Identification and phylogenetic analysis of putative CDPKs in sugarcane

S. officinarum, whose genome has been deciphered, is one of the core original varieties of the major sugarcane cultivars (Jiang et al. 2023). Searching for CDPK genes in *S. officinarum* for further study of CDPKs in the cultivar,

we performed a genome-wide analysis based on sugarcane genomes from NCBI and the sugarcane genome hub. We used the CDPK protein sequences of *Arabidopsis* and rice as queries to perform BLASTP analysis in the local sugarcane protein database. With reference to annotations from eggNOG 5.0, we eventually received a total of 229 putative CDPKs in *S. officinarum*.

The genomes of the major cultivars of sugarcane have not yet been decoded, and there are genomic differences and genetic diversity between *S. officinarum* and sugarcane cultivar. Searching for the putative CDPKs from NCBI genomic data, 12 putative CDPKs in the cultivars were identified and analyzed for their function in defense. According to the pairwise-distance between AtCDPK1 and 12 putative CDPKs, we renamed them ScCDPK1-12.

The evolutionary relationships of putative CDPKs were analyzed through a neighbor-joining (NJ) phylogenetic tree (Fig. 1). The phylogenetic tree was constructed with

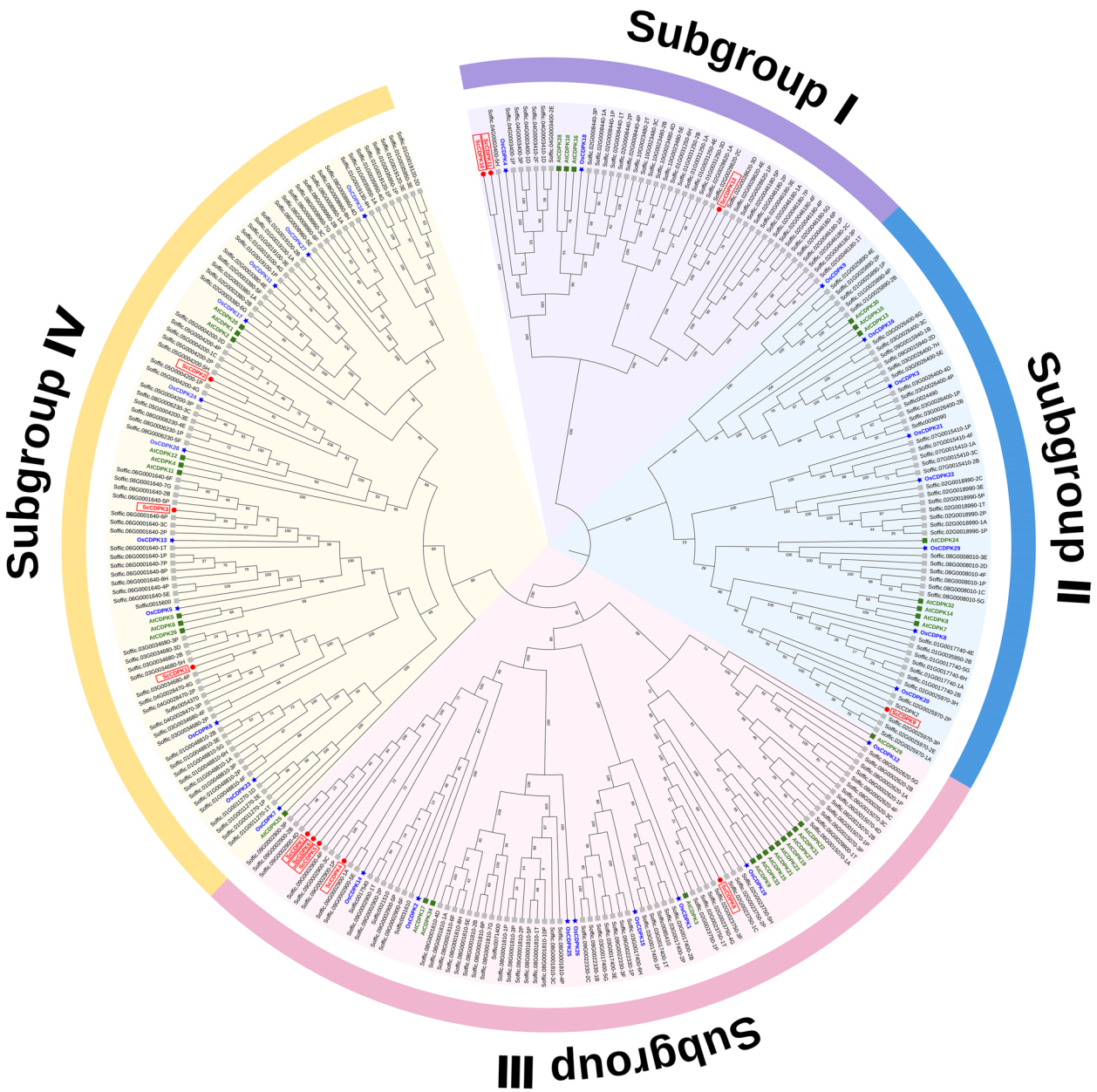


Fig. 1 Phylogenetic relationship among the calcium-dependent protein kinases (CDPK) of sugarcane cultivars, *Saccharum officinarum*, rice, and *Arabidopsis thaliana*. The phylogenetic tree was constructed based on an amino acid sequence alignment using Neighbor-Joining method with p-distance and bootstrap analysis (1000 replicates)

alignments of CDPKs from sugarcane cultivar, *S. officinarum*, *Arabidopsis*, and rice. Similar to *Arabidopsis* and rice, CDPKs in sugarcane were grouped into four subgroups, with 49, 64, 90, and 101 members in subgroup I, II, III, and IV, respectively. Among these four subgroups, subgroup IV was the largest group including 11 rice members, 10 *Arabidopsis* members, and 80 sugarcane members. Subgroup I was the smallest one with 49 members, of which 3 were from *Arabidopsis*, 2 were from rice, and 44 were from sugarcane.

ScCDPK1^{NT} and ScCDPK8^{NT} enhanced the expression of *ScPR1* in *Nicotiana benthamiana*

We analyzed the characteristics of 12 ScCDPKs. As shown in Table 1, the coding region of CDPK family members varies from 789 to 1800 bp, among which the *ScCDPK11* gene is the shortest and the *ScCDPK12* gene is the longest. The amino acids encoded by the CDPK family range from 262 to 599 aa, with a minimum isoelectric point of 5.37 (ScCDPK2) and a maximum isoelectric point of 9.01 (ScCDPK12). ScCDPKs except for ScCDPK10, ScCDPK11, and ScCDPK12, feature typical auto-inhibitory junction domains, indicating their potential for auto-inhibitory activity. In addition, most ScCDPKs have four EF-hand motifs in the CaM-LD that recognize and bind Ca^{2+}.

Since ScCDPK10, ScCDPK11, and ScCDPK12 are deficient in AID and CaM-LD, we analyzed and compared the protein structures of ScCDPK1, ScCDPK2, ScCDPK3, ScCDPK4, ScCDPK5, ScCDPK6, ScCDPK7, ScCDPK8, and ScCDPK9 with those of AtCDPK4/5/6/11, which have been associated with transcriptional reprogramming during disease resistance in *Arabidopsis*. The protein structures were predicted and annotated using UniProt and InterPro databases. As shown in Fig. 2, the 9 ScCDPKs have high similarity with AtCDPK4/5/6/11 (Fig. 2). Therefore, we selected these 9 ScCDPKs for further investigation.

After calcium influx, Ca^{2+} binding causes conformational changes in CaM-LDs, releasing the auto-inhibition of CDPK, a key link in the process through which CDPK promotes plant immunity. TCH3 promotes CDPK5 phosphorylation of CBP60 by interfering with CDPK5 auto-inhibition and positively regulates plant resistance to soil-borne fungal pathogens (Sun et al. 2022a). To obtain active ScCDPKs and elucidate their enzymatic properties, we used the major sugarcane cultivar Guitang 42 (GT42) as a template to construct genes encoding the N-terminal-truncated (NT) forms of ScCDPK1^{NT}, ScCDPK2^{NT}, ScCDPK4^{NT}, ScCDPK5^{NT}, ScCDPK6^{NT}, ScCDPK7^{NT}, ScCDPK8^{NT}, and ScCDPK9^{NT}, in which the auto-inhibitory region and CaM-LD were deleted (Fig. 3).

Pathogenesis-related protein 1 (PR-1) is an important defense protein that is activated by plants under stress conditions and is capable of enhancing plant defense. A previous study showed that the sugarcane *ScPR1* gene significantly improves tolerance to abiotic stress in sugarcane and *Arabidopsis* (Chu et al. 2022). To determine whether putative ScCDPK could induce the expression of *ScPR1*, we prepared constructs expressing each ScCDPK^{NT} driven by the 35S promoter and employed a reporter construct in which the promoter of *ScPR1* was fused to the luciferase reporter gene (*LUC*). *Agrobacteria* carrying *Pro35s:ScCDPK^{NT}-HA* and *ProScPR1:LUC* were infiltrated into *N. benthamiana* leaves. The LUC activity was measured 48 h post infiltration. Results showed that ScCDPK1^{NT} and ScCDPK8^{NT} significantly induced *ScPR1* expression, whereas ScCDPK2^{NT}, ScCDPK4^{NT}, ScCDPK5^{NT}, ScCDPK6^{NT}, ScCDPK7^{NT}, and ScCDPK9^{NT} had no obvious effect on *ScPR1* expression (Fig. 4a–f).

Table 1 Characteristics of putative CDPKs in sugarcane cultivars

Gene Name	Protein ID	Gen ID	Gene Length (bp)	Protein Length (aa)	Mv/Da (ku)	pI	Auto-inhibitory junction domain	EF-Hands
ScCDPK1	AGT16372.1	KF184826.1	1482	493	54,296.72	5.39	1	4
ScCDPK2	AYN44145.1	MG710807.1	1548	515	57,038.93	5.37	1	4
ScCDPK3	AYN44146.1	MG710808.1	1674	557	61,234.43	5.48	1	4
ScCDPK4	AGT17166.1	KF184768.1	1434	477	53,588.07	5.6	1	4
ScCDPK5	AGT15898.1	KF184680.1	1581	526	58,004.9	5.56	1	4
ScCDPK6	AGT17376.1	KF184937.1	1623	540	59,524.73	5.68	1	4
ScCDPK7	AGT16545.1	KF184964.1	1560	519	57,261.1	5.63	1	4
ScCDPK8	AYE54245.1	MG650107.1	1596	531	59,418.78	5.89	1	4
ScCDPK9	ATA66950.1	KX908136.1	1650	549	61,971.71	6.78	1	4
ScCDPK10	AGT16795.1	KF184835.1	789	262	29,543.26	7.6	0	0
ScCDPK11	AGT16817.1	KF184845.1	804	267	30,088.12	7.56	0	0
ScCDPK12	AYK39261.1	MG673947.1	1800	599	66,072.73	9.01	0	0

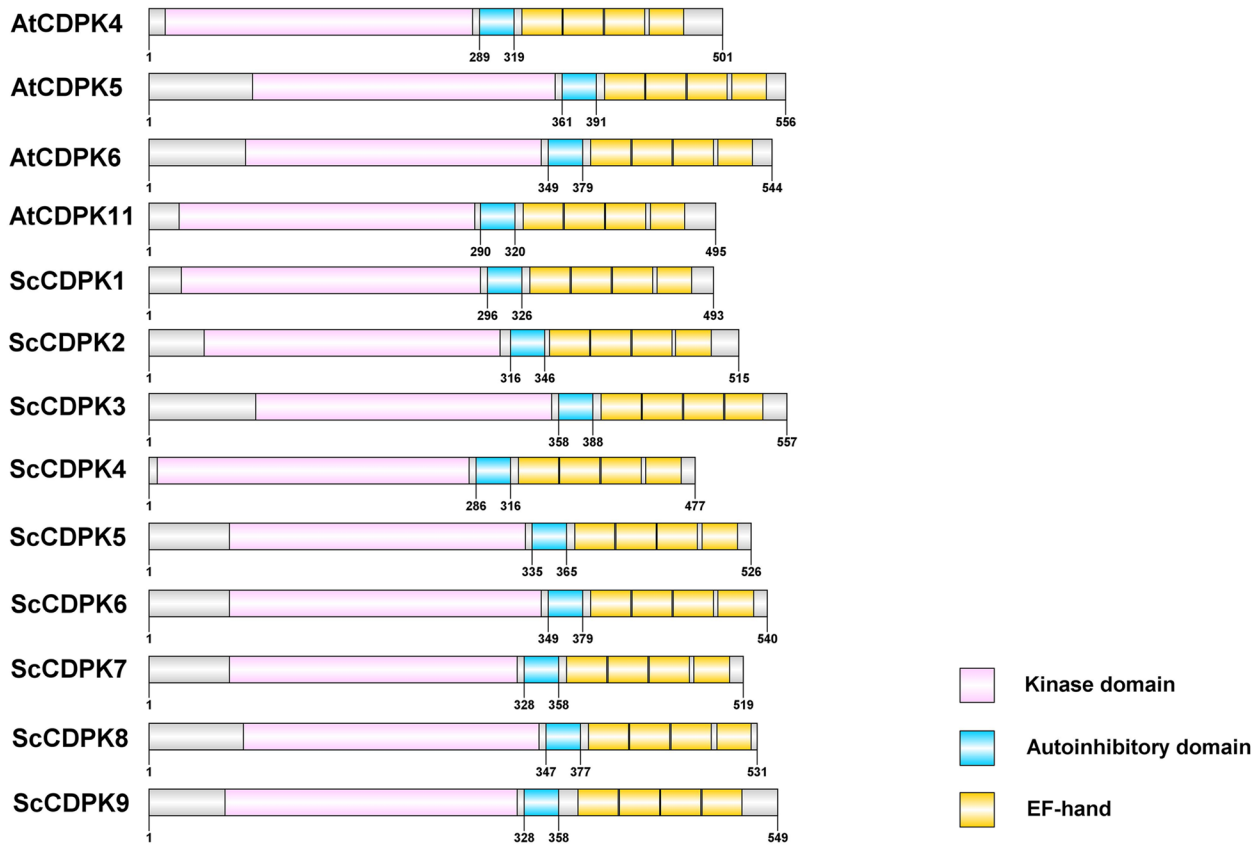


Fig. 2 Structural comparisons of AtCDPK4/5/6/11 and ScCDPKs. The sequence marked in pink, blue, and yellow represents the serine/threonine kinase domain, the auto-inhibitory junction domain, and the EF-hand, respectively

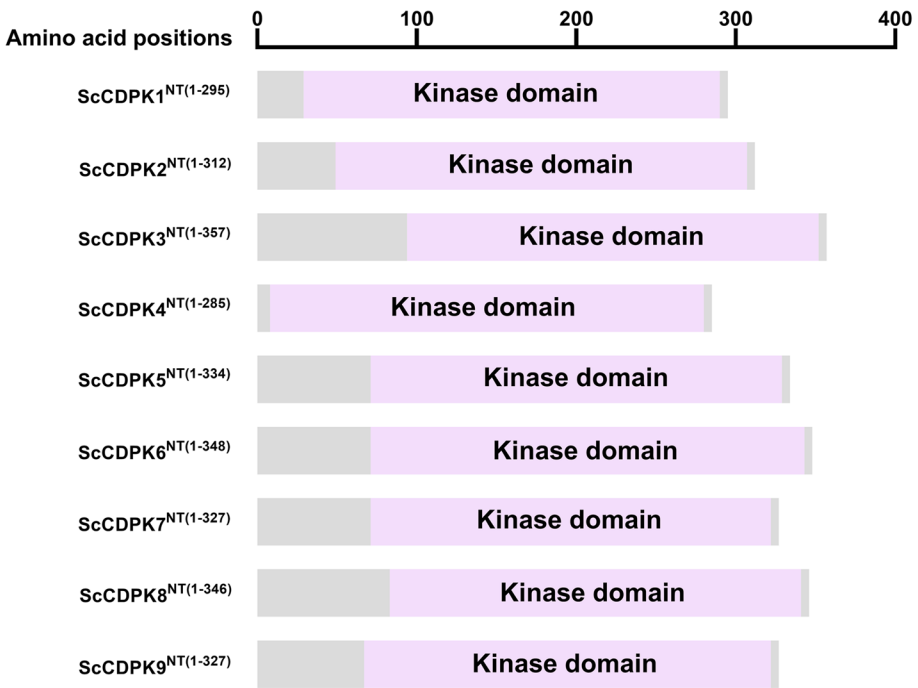


Fig. 3 Schematic diagram of N-terminal truncated (NT) ScCDPKs. The sequence marked in pink represents the serine/threonine kinase domain

ScCDPK1^{NT} and ScCDPK8^{NT} exhibited stronger activity than ScCDPK1^{FL} and ScCDPK8^{FL} in inducing *ScPR1* expression

Induction of *ScPR1* expression by ScCDPK1^{NT} and ScCDPK8^{NT} led us to investigate the auto-inhibitory activity of ScCDPKs. The full-length (FL) forms of ScCDPK1 and ScCDPK8 were constructed to determine whether ScCDPK1^{NT} and ScCDPK8^{NT} exhibited stronger activity than ScCDPK1^{FL} and ScCDPK8^{FL}. *Agrobacteria* carrying *ProScPR1:LUC* reporter were infiltrated alone or together with *Agrobacteria* carrying *Pro35s:ScCDPK1^{NT}-HA*, *Pro35s:ScCDPK8^{NT}-HA*, *Pro35s:ScCDPK1^{FL}-HA*, or *Pro35s:ScCDPK8^{FL}-HA* into *N. benthamiana* leaves. Compared to the full-length forms, truncated ScCDPK1 and ScCDPK8 dramatically increased the ability to induce *ScPR1* (Fig. 5a–d). These results indicated that ScCDPK1^{NT} and ScCDPK8^{NT} constitutively activated *ScPR1* expression in *N. benthamiana*.

ScCDPK1^{NT} and ScCDPK8^{NT} activated defense-related gene expression in sugarcane protoplasts

The ability of ScCDPK1^{NT} and ScCDPK8^{NT} to transiently activate *ScPR1* in *N. benthamiana* prompted the investigation into whether ScCDPK1^{NT} and ScCDPK8^{NT} were constitutively activated in sugarcane protoplasts. *Serine Protease Inhibitor* (*SPI*) encodes key enzymes involved in signaling pathways, and *ScSPI* is induced by *S. scitamineum* inoculation (Wu et al. 2022). Therefore, we used *ScPR1* and *ScSPI* as disease resistance-related reporter genes to investigate the auto-inhibitory activation of ScCDPK in sugarcane protoplasts. *Pro35s:ScCDPK1^{FL}-HA*, *Pro35s:ScCDPK8^{FL}*-HA, *Pro35s:ScCDPK1^{NT}-HA*, or *Pro35s:ScCDPK8^{NT}-HA* were transfected into sugarcane protoplasts. We examined *ScSPI* and *ScPR1* expression and found that truncated ScCDPK1 and ScCDPK8 significantly induced *ScPR1* and *ScSPI* expression compared to those of the full-length forms, which is consistent with our finding that truncated ScCDPK1 and ScCDPK8 activate *ScPR1* in *N. benthamiana* (Fig. 6a, b). These results demonstrate that ScCDPK1 and ScCDPK8 possess auto-inhibitory activity.

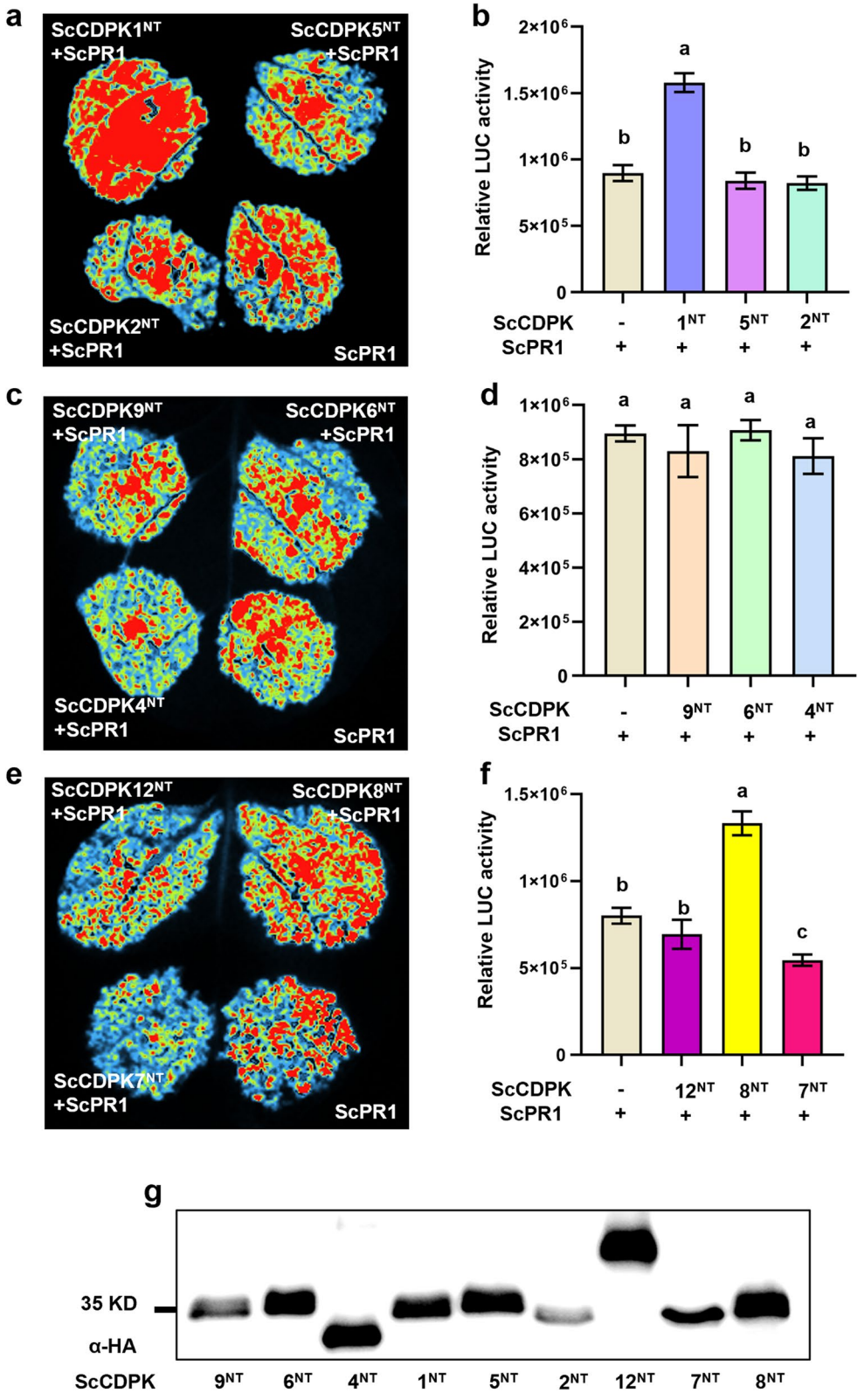
Discussion

Plants are often attacked by pathogens during their growth and development, resulting in severe consequences to their yield and quality. Ca^{2+} plays a vital role in many physiological processes, especially those related to environmental changes and stressors. As a large family of calcium-sensing proteins, CDPKs sense the rapid accumulation of cytoplasmic Ca^{2+} upon pathogen infection, then directly phosphorylate and activate the downstream components to promote the decoding of immune-related calcium signals. CDPKs have been identified in many plants, including *Arabidopsis*, tobacco, rice, wheat, and potatoes (Sun et al. 2022b). However, the abundance and function of CDPKs in sugarcane, which is the primary source of sugar, remain unknown. In this study, we found that ScCDPK1 and ScCDPK8 are involved in defense regulation. Using sugarcane genomic data, we identified 229 putative ScCDPKs in primordial specie *S. officinarum*. Searching for putative CDPKs in sugarcane cultivars from NCBI, we identified 12 putative ScCDPKs. Phylogenetic analysis revealed evolutionary relationships among these CDPKs in sugarcane cultivars and CDPKs in *S. officinarum* and *Arabidopsis*, as well as in rice. According to protein structure analysis and multiple sequence alignment, ScCDPKs have high similarity to AtCDPK4/5/6/11. Transient expression assays in *N. benthamiana* and sugarcane protoplasts showed that truncated ScCDPK1 and ScCDPK8 induce higher expression of *ScPR1* than full-length ScCDPK1 and ScCDPK8.

The major cultivated sugarcane varieties are the progeny of crosses between *S. officinarum* and *Saccharum spontaneum*, which are highly polyploid and aneuploid, respectively, with chromosomes ranging from 80 to 120 (Mohan 2016). The sugarcane genome complexity lies in the fact that its aneuploid chromosome sets contain 8–12 homologous genes, which makes sugarcane sequencing and molecular biology research challenging (Dal-Bianco et al. 2012). Although high-throughput sequencing technologies have been applied in recent years, including the combination of PacBio and high-throughput

(See figure on next page.)

Fig. 4 Truncated ScCDPK1 and ScCDPK8 significantly induced *ScPR1* expression. **a**, **c**, **e** LUC signals generated in three sets of reporter assays by expressing *ProScPR1:LUC* and *Pro35s:ScCDPK^{NT}-HA* (**a** ScCDPK1^{NT}, ScCDPK2^{NT}, and ScCDPK5^{NT}; **c** ScCDPK4^{NT}, ScCDPK6^{NT}, and ScCDPK9^{NT}; **e** ScCDPK7^{NT}, ScCDPK8^{NT}, and ScCDPK12^{NT}). **b**, **d**, **f** Quantitative analysis of LUC signals generated in the assays depicted in **a**, **c,** and **e**, respectively. **g** Protein levels of ScCDPK1^{NT}, ScCDPK2^{NT}, ScCDPK4^{NT} ScCDPK5^{NT}, ScCDPK6^{NT}, ScCDPK7^{NT}, ScCDPK8^{NT}, ScCDPK9^{NT}, and ScCDPK12^{NT} as indicated in *Nicotiana benthamiana*. As indicated, *N. benthamiana* leaves infiltrated with *Agrobacterium* strains carrying *ProScPR1:LUC* and *Pro35s:ScCDPK.^{NT}-HA* were subjected to reporter assays. Infiltrated *N. benthamiana* leaves were sliced into strips 48 h post-infiltration, and relative luminescence was determined by a microplate luminometer. Error bars indicate SD of three biological repeats. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's multiple comparisons)



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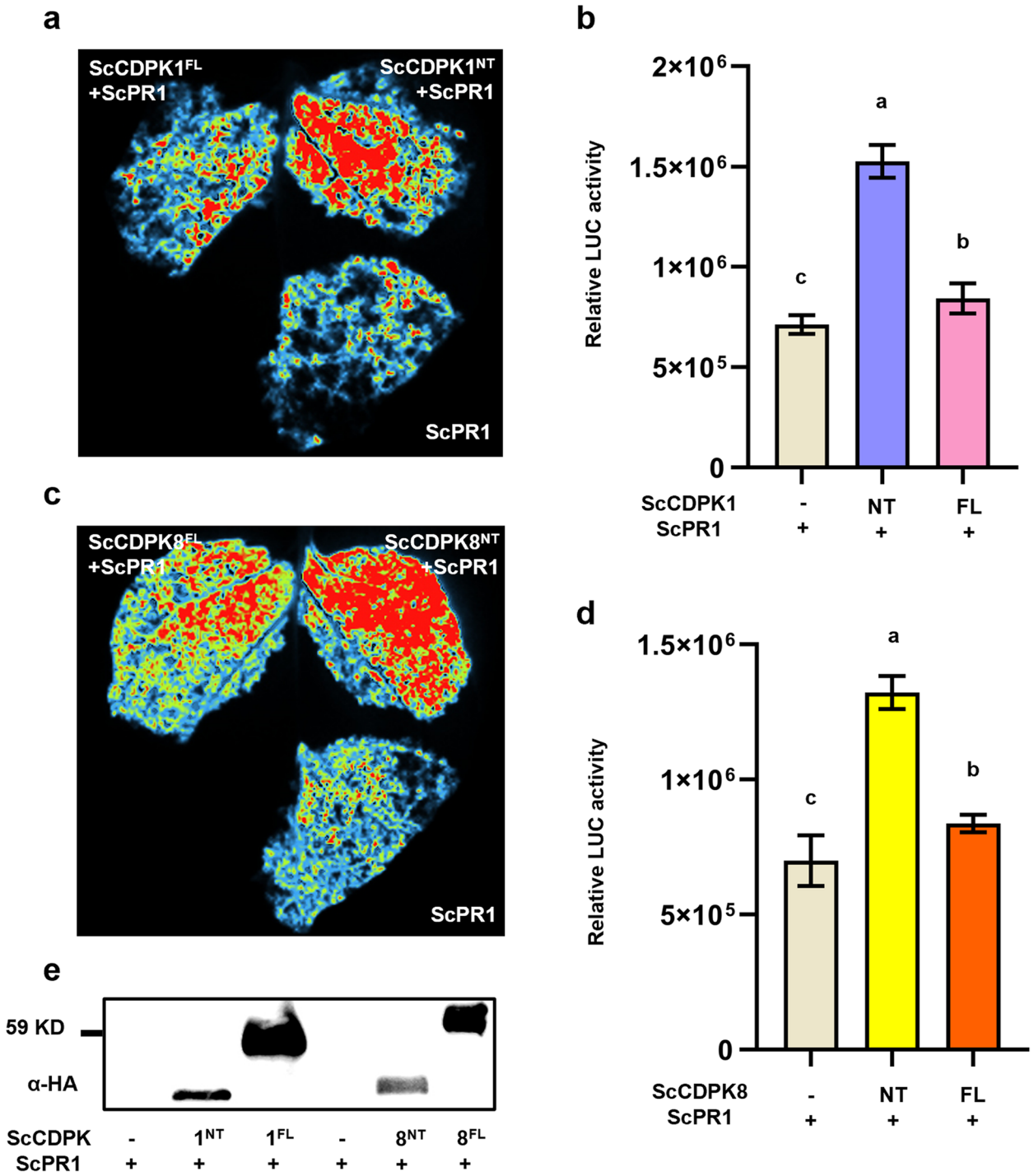

Fig. 5 Truncated ScCDPK1 and ScCDPK8 exhibited stronger activity than full-length ScCDPK1 and ScCDPK8 in inducing *ScPR1* expression. **a**, **c** LUC signals generated in three sets of reporter assays by expressing *ProScPR1:LUC* with *Pro35s:ScCDPK1*^{NT}*-HA*, *Pro35s:ScCDPK1*^{FL}*-HA*, *Pro35s:ScCDPK8*^{NT}*-HA*, or *Pro35s:ScCDPK8*^{FL}*-HA*. **b**, **d** Quantitative analysis of LUC signals generated in the assays depicted in **a** and **c**. *Agrobacteria* carrying *ProScPR1:LUC* were infiltrated alone, or together with *Agrobacteria* carrying *Pro35s:ScCDPK1*^{NT}*-HA*, *Pro35s:ScCDPK8*^{NT}*-HA*, *Pro35s:ScCDPK1*^{FL}*-HA*, or *Pro35s:ScCDPK8*^{FL}*-HA*, as indicated. **e** Protein levels of ScCDPK1^{NT}, ScCDPK1^{FL}, ScCDPK8^{NT}, and ScCDPK8.^{FL} as indicated in *N. benthamiana*. Infiltrated *N. benthamiana* leaves were sliced into strips 48 h post-infiltration, and relative luminescence was determined by a microplate luminometer. Error bars indicate SD of three biological repeats. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's multiple comparisons)

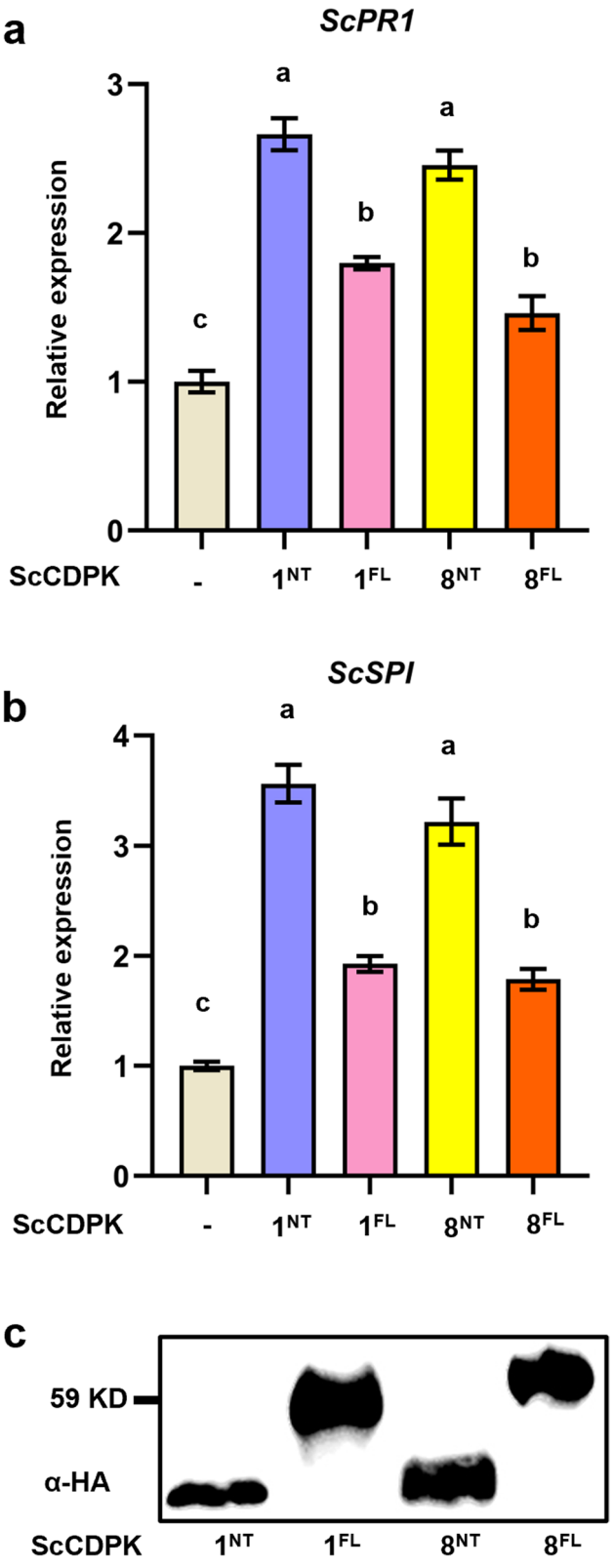

Fig. 6 Truncated ScCDPK1 and ScDPK8 induced higher expression of *ScPR1* and *ScSPI* than full-length ScCDPK1 and ScDPK8 in sugarcane protoplasts. **a** Relative levels of *ScPR1* gene transcripts in protoplasts transfected with *Pro35s:ScCDPK1*^{NT}*-HA*, *Pro35s:ScCDPK1*^{FL}-HA, *Pro35s:ScCDPK8*^{NT}*-HA*, or *Pro35s:ScCDPK8*^{FL}*-HA*. **b** Relative levels of *ScSPI* gene transcripts in protoplasts transfected with *Pro35s:ScCDPK1*^{NT}*-HA*, *Pro35s:ScCDPK1*^{FL}*-HA*, *Pro35s:ScCDPK8*^{NT}*-HA*, *or Pro35s:ScCDPK8*^{FL}*-HA*. **c** Protein levels of ScCDPK1^{NT}, ScCDPK1^{FL}, ScCDPK8^{NT}, and ScCDPK8^{FL} as indicated in sugarcane protoplasts. Protoplasts isolated from Guitang 42 were transfected with *Pro35s:ScCDPK1*^{FL}*-HA*, *Pro35s:ScCDPK8*^{FL}*-HA*, *Pro35s:ScCDPK1*^{NT}*-HA*, or *Pro35s:ScCDPK8.*^{NT}*-HA*. Total RNA was isolated from samples collected 8 h after treatments and used for cDNA synthesis followed by quantitative RT-PCR analysis of *ScPR1* and *ScSPI* transcripts with specific primers. Error bars indicate SD of three biological repeats. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's multiple comparisons)

chromosome conformation capture (Hi-C) technologies, which can perform sequencing at the chromosome level (Wang et al. 2023), the problems encountered in sugarcane sequencing cannot be completely overcome at this stage. The large number of chromosomes, similarities in morphology, and diverse modes of inheritance often lead to unbalanced increases or decreases in the number of chromosomes in sugarcane, which seriously hamper genetic research and improvement (Mohan 2016). As stable transformation of sugarcane is both difficult and time-consuming, we identified and screened ScCDPKs with auto-inhibitory activity using *ScPR1* and *ScSPI* as reporter genes in a protoplast-based transient transformation, which is a simple and versatile method for in vivo analysis of gene function (Wang et al. 2021).

Although the genomes of *S. officinarum*, *S. spontaneum*, and the diploid complex of *Erianthus rufipilus* are available (Zhang et al. 2018, 2022), there are genomic differences and genetic diversity between the main cultivars GT42 and *S. officinarum*. However, the genome assembly of GT42 is still incomplete, and the sugarcane genome has not yet been fully annotated; therefore, we may have missed genomic information in mining for the CDPKs. Furthermore, ScCDPK3 has not been cloned because of the dissimilarities between the GT42 and *S. officinarum* genomes. All of these factors contributed to the incomplete results of our analyses.

Transgenic technology is an important avenue for molecular biology research and germplasm improvement of sugarcane plants. However, sugarcane transgenic technology still has bottlenecks, such as low transformation efficiency and limited transformable genotypes (Budeguer et al. 2021). Gene editing technology can realize fixed and controllable gene modification, which avoids the uncertainty and risk of random insertion of exogenous genes and improves the precision and safety

of operations (Gao 2021). CRISPR-Cas has become one of the most advanced systems for the genome engineering of crops; this technology has rapidly expanded and applied to crops critical for food security. The discovery and utilization of functional genes are essential for gene editing to modify crops and increase resistance resources. Editing the *RESISTANCE TO BLAST1* (*RBL1*) gene, which encodes CDP-DAG synthase in rice, and the *MILDEW RESISTANCE LOCUS O* (*MLO*) gene in wheat using CRISPR-Cas has resulted in high-yield and resistance-compatible crops (Li et al. 2022b; Sha et al. 2023). *Solanum tuberosum PLASMA MEMBRANE PROTEIN 1* (*StPM1*) gene-edited potato plants, using CRISPR-Cas9 technology, showed significantly improved resistance to *Phytophthora infestans* (Bi et al. 2024).

The sugarcane genome contains numerous homologous genes with high ploidy and sequence similarities. Therefore, the development of an effective transformation method for gene modification in sugarcane remains a major technical challenge. A key requirement in the field of sugarcane genetic modification is the availability of sufficient functional genomic resources to comprehensively dissect the functional and regulatory networks of the sugarcane genome for precise gene editing strategies (Dal-Bianco et al. 2012). Genome editing is powerful in generating loss-of-function mutations of target genes and has been further developed to create gain-of-function mutations of target genes. In this study, truncated ScCDPK1 and ScCDPK8 were found to induce higher expression of the defense-related genes *ScPR1* and *ScSPI* than the full-length ScCDPK1 and ScCDPK8, indicating that the deletion of the auto-inhibitory region in ScCDPK1 and ScCDPK8 provides access to gain-of-function mutations. Therefore, removing the auto-inhibitory region of full-length ScCDPKs by genome editing may generate active ScCDPK variants. Our study suggests new targets for genome editing in sugarcane defense regulation.

Conclusions

In this study, we conducted a genome-wide analysis using the genome of *S. officinarum* to identify the CDPKs in sugarcane core primordial species. We further analyzed ScCDPK in sugarcane cultivars. As confirmed by protein structure analysis and multiple sequence alignment, ScCDPKs exhibited high similarity to those of AtCDPK4/5/6/11, which have been associated with transcriptional reprogramming during disease resistance. Transient expression assays in *N. benthamiana* and sugarcane protoplasts showed that truncated ScCDPK1 and ScCDPK8 exhibit stronger activity than full-length ScCDPK1 and ScCDPK8 for activation of defense-related genes *ScPR1* and *ScSPI*. These findings highlight the essential roles of ScCDPK1 and ScCDPK8 in defense regulation and provide novel targets for sugarcane gene editing.

Methods

Plant materials and constructs

The primer sequences used in this study are shown in Additional file 1: Table S1. Strains used in this study include *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101. *Nicotiana benthamiana* was used for transient expression assay, while sugarcane Guitang 42 (GT42) was used for protoplast preparation. To conduct the reporter assay, we fused the promoter of *ScPR1* with the firefly luciferase reporter gene (LUC) to construct the vector pCambia1300-ProScPR1:LUC-HA. The N-terminal truncated forms of *ScCDPK1*, *ScCDPK2*, *ScCDPK4*, *ScCDPK5*, *ScCDPK6*, *ScCDPK7*, *ScCDPK8*, and *ScCDPK9* coding sequences were amplified by RT-PCR from cDNA isolated from GT42 and cloned into the pCambia1300-HA vectors. The resulting constructs carry truncated ScCDPKs lacking the auto-inhibitory junction domain and the C-terminal regulatory calmodulin-like domain. The full-length of *ScCDPK1* and *ScCDPK8* were cloned into the pUC19-HA vectors to generate transient expression constructs.

Identification of CDPKs in sugarcane

Sugarcane genomes were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and sugarcane genome hub (http://sugarcane-genome.cirad.fr) (Garsmeur et al. 2018; Zhang et al. 2018, 2022). According to previous reports, we downloaded 29 CDPK protein sequences in rice and 34 CDPK protein sequences in *Arabidopsis* from NCBI and TAIR databases, respectively (Asano et al. 2005; Yip Delormel and Boudsocq 2019). The obtained sequences were subjected to a protein blast in the local protein database of sugarcane, and the results with > 70% query coverage were taken as the sugarcane CDPK gene family members. We used the eggNOG 5.0 database for protein classification and functional annotation (http://eggnogdb.embl.de/) (Huerta-Cepas et al. 2019).

Phylogenetic analysis and multiple sequence alignment

The pairwise-distance analysis of the amino acid sequences of ScCDPKs and AtCDPK1 are shown in Additional file 2: Table S2. Mega 7.0 software was used to perform muscle multiple sequence alignment of CDPKs in rice, *Arabidopsis*, and sugarcane. The phylogenetic tree was constructed by the neighbor-joining method, and the bootstrap value was set to 1000. The web-based tool iTOL (http://itol.embl.de) was used for the visualization, manipulation, and annotation of phylogenetic tree. Molecular weight (MW) and isoelectronic points (pI)

of ScCDPKs were predicted using the ExPASY PROTPARAM tool (http://web.expasy.org/protparam/).

To explore the relationship between putative ScCDPKs and AtCDPK4/5/6/11, we aligned the protein sequences with DNAMAN 9.0. To investigate the similarities between ScCDPKs and AtCDPK4/5/6/11, we clarified the structures in the protein databases UniProt (https://www.uniprot.org/) and InterPro (https://www.ebi.ac.uk/interpro/), followed by protein structure annotation via the software IBS 1.0.

Reporter assay in *Nicotiana benthamiana*

Agrobacterium strain carrying *ProScPR1:LUC-HA* was infiltrated alone or together with *Agrobacterium* carrying *Pro35s:ScCDPK^{NT}-HA* or *Pro35s:ScCDPK^{FL}-HA*, as indicated into 4-week-old *N. benthamiana* leaves. After 48 h, we sliced the infiltrated *N. benthamiana* leaves into 1 mm wide strips and measured relative luminescence with a microplate luminometer. Total proteins were separated by 8% SDS-PAGE, and ScCDPKs were detected by anti-HA immunoblot.

Transient expression in sugarcane protoplasts

Protoplasts were prepared from the leaf sheaths of 2-month-old GT42 seedlings. The protoplasts isolated were transfected with the indicated PUC-ScCDPK^{NT}-HA or PUC-ScCDPK^{FL}-HA under 40% polyethylene glycol. After 8 h of incubation, total RNA was isolated and used for cDNA synthesis, followed by quantitative RT-PCR analysis of *ScPR1* and *ScSPI* transcripts with specific primers. Total RNA was isolated with the TRIzol reagent (Invitrogen) and used for cDNA synthesis with SuperScript III FirstStrand Synthesis System for RT-qPCR (Invitrogen) following the manufacturer's instructions. The RT-qPCR was performed with the SYBR Premix Ex Taq kit (TaKaRa) following standard protocols. Total proteins were separated by 8% SDS-PAGE and ScCDPKs were detected by anti-HA immunoblot.

Abbreviations

CDPK/CPK	Calcium-dependent protein kinase
CaM/CAM	Calmodulin
CML	CaM-like
CBL	Calcineurin B-like
CIPK	CBL-interacting protein kinases
LUC	Luciferase reporter gene
VNTD	Variable N-terminal variable domain
AID	Auto-inhibitory junction domain
CaM-LD	C-terminal regulatory calmodulin-like domain
PAMP	Pathogen-associated molecular pattern
NJ	Neighbor-joining
Hi-C	High-throughput chromosome conformation capture
ScPR1	Sugarcane Pathogenesis-Related Protein 1
ScSPI	Sugarcane Serine Protease Inhibitor
NCBI	National Center for Biotechnology Information
PUB25	PLANT U-BOX 25
BIK1	BOTRYTIS-INDUCED KINASE 1
TCH3	TOUCH 3
BRU1	Brown rust resistance gene 1
RBL1	RESISTANCE TO BLAST 1
MLO	MILDEW RESISTANCE LOCUS O
StPM1	PLASMA MEMBRANE PROTEIN 1
GT42	Guitang 42
MW	Molecular weight
pI	Isoelectronic points

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00240-1.

Additional file 1: Table S1. Sequences of primers used in this study.

Additional file 2: Table S2. Pairwise-distance analysis of the amino acid sequences of ScCDPKs and AtCDPK1.

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Authors' contributions

JZ, XL, and JZ designed the experiments. JZ, JZ, XL, and LW wrote the manuscript. BL, YL, KW, YW, ZY, LS, JZ, and LY performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

Asano T, Tanaka N, Yang G, Hayashi N, Komatsu S. Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: comprehensive analysis of the *CDPKs* gene family in rice. Plant Cell Physiol. 2005;46:356–66.

Asnaghi C, Roques D, Ruffel S, Kaye C, Hoarau JY, Télismart H, et al. Targeted mapping of a sugarcane rust resistance gene (*Bru1*) using bulked segregant analysis and AFLP markers. Theor Appl Genet. 2004;108:759–64.

Bhuiyan SA, Magarey RC, McNeil MD, Aitken KS. Sugarcane smut, caused by *Sporisorium scitamineum*, a major disease of sugarcane: a contemporary review. Phytopathology. 2021;111:1905–17.

Bi W, Liu J, Li Y, He Z, Chen Y, Zhao T, et al. CRISPR/Cas9-guided editing of a novel susceptibility gene in potato improves *Phytophthora* resistance without growth penalty. Plant Biotechnol J. 2024;22:4–6.
Bouché N, Yellin A, Snedden WA, Fromm H. Plant-specific calmodulin-binding proteins. Annu Rev Plant Biol. 2005;56:435–66.
Budeguer F, Enrique R, Perera MF, Racedo J, Castagnaro AP, Noguera AS, et al. Genetic transformation of sugarcane, current status and future prospects. Front Plant Sci. 2021;12:768609.
Cheng SH, Willmann MR, Chen HC, Sheen J. Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol. 2002;129:469–85.
Chu N, Zhou J-R, Rott PC, Li J, Fu H-Y, Huang M-T, et al. *ScPR1* plays a positive role in the regulation of resistance to diverse stresses in sugarcane (*Saccharum* spp.) and *Arabidopsis thaliana*. Indust Crops Products. 2022;180:114736.
Dal-Bianco M, Carneiro MS, Hotta CT, Chapola RG, Hoffmann HP, Garcia AA, et al. Sugarcane improvement: how far can we go? Curr Opin Biotechnol. 2012;23:265–70.
Dubiella U, Seybold H, Durian G, Komander E, Lassig R, Witte CP, et al. Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. Proc Natl Acad Sci U S A. 2013;110:8744–9.
Gao C. Genome engineering for crop improvement and future agriculture. Cell. 2021;184:1621–35.
Garsmeur O, Droc G, Antonise R, Grimwood J, Potier B, Aitken K, et al. A mosaic monoploid reference sequence for the highly complex genome of sugarcane. Nat Commun. 2018;9(1):2638.
Harmon AC, Gribskov M, Gubrium E, Harper JF. The CDPK superfamily of protein kinases. New Phytol. 2001;151:175–83.
Hepler PK. Calcium: a central regulator of plant growth and development. Plant Cell. 2005;17:2142–55.
Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 2019;47:D309–14.
Jiang Q, Hua X, Shi H, Liu J, Yuan Y, Li Z, et al. Transcriptome dynamics provides insights into divergences of the photosynthesis pathway between *Saccharum officinarum* and *Saccharum spontaneum*. Plant J. 2023;113:1278–94.
Kadota Y, Sklenar J, Derbyshire P, Stransfeld L, Asai S, Ntoukakis V, et al. Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. Mol Cell. 2014;54:43–55.
Kashir J, Deguchi R, Jones C, Coward K, Stricker SA. Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. Mol Reprod Dev. 2013;80:787–815.
Li H, Zhang Y, Wu C, Bi J, Chen Y, Jiang C, et al. Fine-tuning OsCPK18/OsCPK4 activity via genome editing of phosphorylation motif improves rice yield and immunity. Plant Biotechnol J. 2022a;20:2258–71.
Li S, Lin D, Zhang Y, Deng M, Chen Y, Lv B, et al. Genome-edited powdery mildew resistance in wheat without growth penalties. Nature. 2022b;602:455–60.
Luan S. The CBL-CIPK network in plant calcium signaling. Trends Plant Sci. 2009;14:37–42.
Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W. Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell. 2002;14(Suppl):S389–400.
Martín ML, Busconi L. Membrane localization of a rice calcium-dependent protein kinase (CDPK) is mediated by myristoylation and palmitoylation. Plant J. 2000;24:429–35.
McCormack E, Tsai YC, Braam J. Handling calcium signaling: *Arabidopsis* CaMs and CMLs. Trends Plant Sci. 2005;10:383–9.
Mohan C. Genome editing in sugarcane: challenges ahead. Front Plant Sci. 2016;7:1542.
Monaghan J, Matschi S, Shorinola O, Rovenich H, Matei A, Segonzac C, et al. The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. Cell Host Microbe. 2014;16:605–15.
Oz MT, Altpeter A, Karan R, Merotto A, Altpeter F. CRISPR/Cas9-mediated multiallelic gene targeting in sugarcane confers herbicide tolerance. Front Genome Ed. 2021;3:673566.
Sanders D, Brownlee C, Harper JF. Communicating with calcium. Plant Cell. 1999;11:691–706.
Sanders D, Pelloux JRM, Brownlee C, Harper JF. Calcium at the crossroads of signaling. Plant Cell. 2002;14:S401–17.
Sha G, Sun P, Kong X, Han X, Sun Q, Fouillen L, et al. Genome editing of a rice CDP-DAG synthase confers multipathogen resistance. Nature. 2023;618:1017–23.
Sun L, Qin J, Wu X, Zhang J, Zhang J. TOUCH 3 and CALMODULIN 1/4/6 cooperate with calcium-dependent protein kinases to trigger calcium-dependent activation of CAM-BINDING PROTEIN 60-LIKE G and regulate fungal resistance in plants. Plant Cell. 2022a;34:4088–104.
Sun Y, Shang L, Zhu Q-H, Fan L, Guo L. Twenty years of plant genome sequencing: achievements and challenges. Trends Plant Sci. 2022b;27:391–401.
Tian W, Wang C, Gao Q, Li L, Luan S. Calcium spikes, waves and oscillations in plant development and biotic interactions. Nat Plants. 2020;6:750–9.
Wang J, Wang S, Hu K, Yang J, Xin X, Zhou W, et al. The kinase OsCPK4 regulates a buffering mechanism that fine-tunes innate immunity. Plant Physiol. 2018;176:1835–49.
Wang Q, Yu G, Chen Z, Han J, Hu Y, Wang K. Optimization of protoplast isolation, transformation and its application in sugarcane (*Saccharum spontaneum* L). Crop J. 2021;9:133–42.
Wang T, Duan S, Xu C, Wang Y, Zhang X, Xu X, et al. Pan-genome analysis of 13 *Malus* accessions reveals structural and sequence variations associated with fruit traits. Nat Commun. 2023;14:7377.
Wernimont AK, Amani M, Qiu W, Pizarro JC, Artz JD, Lin YH, et al. Structures of parasitic CDPK domains point to a common mechanism of activation. Proteins. 2011;79:803–20.
Wu Q, Su Y, Pan Y-B, Xu F, Zou W, Que B, et al. Genetic identification of SNP markers and candidate genes associated with sugarcane smut resistance using BSR-Seq. Front Plant Sci. 2022;13:1035266.
Yang ZN, Mirkov TE. Sequence and relationships of sugarcane mosaic and sorghum mosaic virus strains and development of RT-PCR-based RFLPs for strain discrimination. Phytopathology. 1997;87:932–9.
Yip Delormel T, Boudsocq M. Properties and functions of calcium-dependent protein kinases and their relatives in *Arabidopsis thaliana*. New Phytol. 2019;224:585–604.
Yoo BC, Harmon AC. Intramolecular binding contributes to the activation of CDPK, a protein kinase with a calmodulin-like domain. Biochemistry. 1996;35:12029–37.
Zhang J, Zhang X, Tang H, Zhang Q, Hua X, Ma X, et al. Allele-defined genome of the autopolyploid sugarcane *Saccharum spontaneum* L. Nat Genet. 2018;50:1565–73.
Zhang Q, Qi Y, Pan H, Tang H, Wang G, Hua X, et al. Genomic insights into the recent chromosome reduction of autopolyploid sugarcane *Saccharum spontaneum*. Nat Genet. 2022;54:885–96.
Zielinski RE. Calmodulin and calmodulin-binding proteins in plants. Annu Rev Plant Physiol Plant Mol Biol. 1998;49:697–725.