



Redefining the N-Terminal Regulatory Region of the Ca²⁺/H⁺ Antiporter CAX1 in Tomato

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Calcium (Ca²⁺) is an essential plant nutrient, and Ca²⁺/H⁺ exchangers (CAXs) regulate Ca²⁺ partitioning between subcellular compartments. AtCAX1 activity is inhibited by its N-terminal regulatory region (NRR), which was initially defined as the sequence between the first two methionines. However, the accuracy of this NRR definition and the NRR regulatory mechanism remain unclear. Here, using tomato SICAX1 as a model, we redefined the NRR of CAXs and demonstrated that our new definition is also applicable to Arabidopsis AtCAX1 and AtCAX3. The N-terminal-truncated SICAX1 (SICAX1^{Δ39}) but not the full-length SICAX1 was active in yeast, similar to Arabidopsis AtCAX1. Characterization of *slcax1* mutants generated by CRISPR-Cas9 confirmed the calcium transport ability of SICAX1. Sequence alignment between SICAX1, AtCAX1, AtCAX3, and the *Bacillus subtilis* Ca²⁺/H⁺ antiporter protein YfkE revealed that SICAX1 does not have the 2nd methionine and YfkE does not have any amino acid residues in front of the first transmembrane domain. Truncating the amino acid residues up to the first transmembrane of SICAX1 (SICAX1^{Δ66}) further increased its activity. The same truncation had a similar effect on Arabidopsis AtCAX1 and AtCAX3. Expression of full-length SICAX1 and SICAX1^{Δ66} in tomato plants confirmed the results. Our results suggest that SICAX1 is critical for Ca²⁺ homeostasis and all the amino acid residues in front of the first transmembrane domain inhibit the activity of CAXs. Our redefinition of the NRR will facilitate fine-tuning of Ca²⁺ partitioning to reduce the incidence of Ca²⁺-related physiological disorders in crops.

Keywords: calcium, CAX, NRR, tomato, CRISPR

INTRODUCTION

Calcium (Ca²⁺) is an essential macronutrient for plants and functions as a second messenger that is generated in response to different stimuli. Precise regulation of its distribution between different subcellular compartments is critical for normal plant growth, development, and adaptation to the environment. Most Ca²⁺ is stored in the cell wall, apoplast, vacuole, and endoplasmic reticulum and is maintained at extremely low levels in the cytosol

(Dodd et al., 2010). Ca^{2+} antiporters, such as $\text{Ca}^{2+}/\text{H}^{+}$ exchangers (CAXs), control the efflux of Ca^{2+} from the cytosol. This establishes a Ca^{2+} gradient between the cytosol and other compartments and maintains Ca^{2+} partitioning in plant cells (Emery et al., 2012).

Arabidopsis thaliana AtCAX1, the first identified plant CAX, was cloned based on its ability to complement the hypersensitivity of a *Saccharomyces cerevisiae* mutant with defects in vacuolar Ca^{2+} transport to high concentrations of Ca^{2+} (Hirschi et al., 1996). AtCAX1 is a high-affinity, high-capacity $\text{Ca}^{2+}/\text{H}^{+}$ antiporter. Five additional AtCAXs have been cloned from *Arabidopsis* (Shigaki et al., 2006). CAXs from other plant species have been intensively studied and the substrate range of these CAXs extends beyond Ca^{2+} (Kamiya et al., 2006; Pittman and Hirschi, 2016; Amagaya et al., 2020).

AtCAX1 activity is tightly regulated by its N-terminal regulatory region (NRR), which is 36 amino acids long (Pittman and Hirschi, 2001). The NRR was initially identified in the original AtCAX1 antiporter used in yeast complementation experiments. This antiporter is the product of a partial-length cDNA in which the N terminus is truncated, but there is a second in-frame start codon, allowing translation initiation from the methionine at the 37th amino acid position (referred to as AtCAX1^{Δ36} hereafter; Hirschi et al., 1996; Pittman and Hirschi, 2001). Heterologous expression of AtCAX1^{Δ36}, but not full-length AtCAX1 (ICAX1), complements the hypersensitivity of the yeast mutant strain to calcium, suggesting that the first 36 amino acid residues inhibit AtCAX1 activity. The region deleted in AtCAX1^{Δ36} was defined as the NRR (Pittman and Hirschi, 2001). AtCAX3 has high amino acid sequence similarity with AtCAX1, including in the NRR. Neither full-length AtCAX3 nor the 36-amino-acid N-terminal truncation of AtCAX3 (AtCAX3^{Δ36}) is as active as AtCAX1^{Δ36} in yeast expression assays. However, when longer truncations are performed, AtCAX3 is able to transport Ca^{2+} in both yeast and plants (Manohar et al., 2011). Further genetic evidence suggests that AtCAX3 functions redundantly with AtCAX1 because neither *atcax1* nor *atcax3* single mutants display an obvious phenotype, whereas the growth and development of *atcax1 atcax3* double mutants are strongly impaired (Cheng et al., 2005; Hocking et al., 2017), suggesting that AtCAX3 is an active Ca^{2+} transporter though how it is activated remains unclear.

As CAXs are important for Ca^{2+} homeostasis, tremendous effort has been made to ectopically express these genes in different economically important species, such as tomatoes, potatoes, and carrots, with the aim of improving fruit quality, abiotic stress tolerance, or shelf life, or alleviating Ca^{2+} -related physiological disorders (Park et al., 2004, 2005a,b, 2009; Kim et al., 2006; Han et al., 2009; Wu et al., 2011, 2012). However, ectopic expression of AtCAX1^{Δ36}, which is constitutively active, in certain species including tobacco and tomato resulted in strong calcium disorder symptoms such as necrosis in leaf tips or blossom-end rot (Park et al., 2005a; Wu et al., 2012). In tomato, 100% of AtCAX1^{Δ36}-expressing plants exhibited blossom-end rot due to perturbation of calcium partitioning in plant cells (Park et al., 2005a; De Freitas et al., 2011, 2012). Although bioinformatics analysis has been performed for tomato SICAX genes (Amagaya et al., 2020), their functions in Ca^{2+}

homeostasis and their roles in controlling calcium-related physiological disorders remain to be addressed.

Here, we characterized the tomato SICAX1 gene and gained insight into its regulatory mechanism of Ca^{2+} antiporters. We redefined which region constitutes the NRR and demonstrated that our new definition could also apply to *Arabidopsis* AtCAX1 and AtCAX3. These results could facilitate efforts to mitigate physiological disorders in tomato by fine-tuning Ca^{2+} homeostasis.

MATERIALS AND METHODS

Yeast Strains and Plant Materials

Yeast strain K667 (*vcx1::hisG cnb1::LEU2 pmc1::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), which lacks the vacuolar Ca^{2+} -ATPase (PMC1) and vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ antiporter (VCX1), was provided by Dr. Kendal Hirschi (Cunningham and Fink, 1996). For metal ion treatments, tomato plants (*Solanum lycopersicum* “Rubion”) were grown on 1/2 Murashige and Skoog (MS) medium for 2 weeks and then transferred onto an equal volume of solution containing the different metal ions and incubated for 16 h. The tissues were rinsed with distilled water three times and dried on tissue paper before flash freezing with liquid nitrogen. The frozen tissues were kept at -80°C before analysis.

RNA Extraction and RT-qPCR

RNA was isolated using the RNeasy Plant Kit (Zymo Research), according to the manufacturer’s instructions. RT-qPCR was performed to detect SICAX expression. First-strand cDNA was produced using the iScript™ cDNA Synthesis kit from BIO-RAD. The first-strand cDNA of 1 μl was used to amplify SICAX-specific fragments and *SIACTIN* (acc. no. TC194780) was used as a control. Specific amplification primers are shown in **Supplementary Table 1**.

Plasmid Construction and Yeast Transformation

Different versions of the SICAX (*SICAX1*, *SICAX1*^{Δ39}, *SICAX1*^{Δ58}, *SICAX1*^{Δ66}, and *SICAX1*^{Δ93}) and AtCAX (*AtCAX1*^{Δ36}, *AtCAX1*^{Δ63}, *AtCAX3*^{Δ36}, and *AtCAX3*^{Δ63}) genes were amplified from the tomato and *Arabidopsis* cDNA libraries, respectively, and cloned into the yeast expression vector *piHGpd* (Pittman et al., 2002). The first 39 and 66 amino acids of SICAX1 were randomly scrambled and codon optimized based on yeast codon preference and the DNA oligos were synthesized by Beijing Dahong Biotechnology. The *scrambled-39-SICAX1* and *scrambled-66-SICAX1* were fused with *SICAX1*^{Δ39} and *SICAX1*^{Δ66}, respectively, and cloned into the yeast expression vector *piHGpd*. The scrambled DNA sequences were shown in **Supplementary Table 2**. All PCR amplifications were performed using Phusion® High-Fidelity DNA Polymerases from New England Biolabs; the restriction sites used were XbaI and SacI. Primer sequences used in this study are shown in **Supplementary Table 3**.

Yeast cells were transformed using the standard lithium acetate method and selected on a synthetic defined medium lacking His as described (Pittman et al., 2002). For the Ca^{2+}

tolerance assays, the yeast cells were cultured on SD-His liquid medium to an OD value of 1. After series dilution, the yeast cells were cultured on solid YPD supplemented with different concentrations of CaCl₂ and grown at 30°C for 3 days (Pittman and Hirschi, 2001; Manohar et al., 2011).

Preparation of Transgenic Plants

Agrobacterium-mediated transformation was used to generate transgenic tomato plants. Different versions of *SICAX1* were fused with 2× Sterp tag II- 3x Flag tag driven by the CaMV35S promoter and cloned into the PRI101 vector (Satoh et al., 2004; Sugio et al., 2008). Primer sequences used for cloning are shown in **Supplementary Table 2**. The plasmids were introduced into *Agrobacterium* strain LBA4404 following standard protocols and then transformed into tomato (*S. lycopersicum* “Micro-Tom”) following a previously described method (Cosson et al., 2006). The transgenic lines were confirmed by genomic PCR using the following primers: *SICAX1*-PRI forward, 5'-CCA ACC ACG TCT TCA AAG CA-3' and reverse, 5'-TCC TGT AGA GTG AAA GCT GTG A-3'.

Knock-Out of *SICAX1* Using CRISPR-Cas9

CRISPR-Cas9 was used to create *slcax1* knockouts in tomato (Micro-Tom) through *Agrobacterium*-mediated transformation. We designed two guide RNAs (gRNAs) targeting the coding region of *SICAX1*. The sequence of Guide1 was ‘TCG TAG CCA TGG ACG AAC GG’ and that of Guide2 was ‘CTT CTA AAT GCA ACA TGT GG’. The two guides were cloned into the CRISPR-Cas9 vector *BGK012*, which was provided by Jiangsu Baige Gene. The vector map is shown in **Supplementary Figure 1**. The CRISPR/Cas9 construct was transferred into the *Agrobacterium* strain LBA4404 and then into tomato, as described (Li et al., 2018). The genomic regions surrounding the target sites were PCR-amplified and Sanger sequenced. All sequence information is shown in **Supplementary Table 4**.

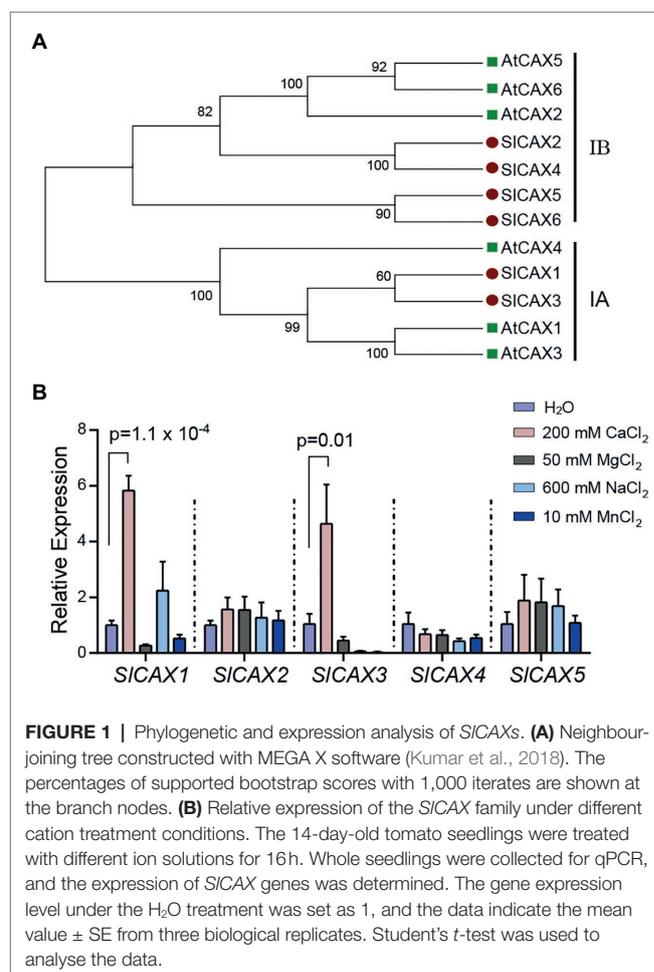
Measurement of Ca²⁺ Contents

Seeds (T2) of the *SICAX1*, *SICAX1*^{Δ66}-expressing, *SICAX1* CRISPR knockout, and wild-type plants were germinated on MS medium containing MS inorganic salts and 0.8% agar (Sigma), and the pH was adjusted to 5.8–6.0 with KOH. After 4 weeks, plants of similar size from each of the lines were harvested and rinsed with deionized water two to three times before drying with filter paper (Wu et al., 2011). The plants were further dried at 80°C for 48 h. For each sample, a total of 0.5 g (dry weight) tissue was digested with 10 ml nitric acid and 0.5 ml perchloric acid at 120°C for 1 h. The amount of Ca²⁺ present in the digested tissue was determined by inductively coupled plasma optical emission spectroscopy (Tang et al., 2015).

RESULTS

To identify SICAX proteins from tomato, we conducted a BLAST analysis of the tomato protein database in Phytozome¹

using the *Arabidopsis* AtCAX1 protein sequence as query. We identified six proteins with high similarity to *Arabidopsis* AtCAX1 (*Solyc09g005260*, *Solyc12g055750*, *Solyc06g006110*, *Solyc03g123790*, *Solyc07g056110*, and *Solyc12g011070*). Phylogenetic analysis using the Neighbour-Joining method identified two tomato proteins (*Solyc09g005260* and *Solyc06g006110*) in the same clade as AtCAX1, AtCAX3, and AtCAX4, which we named SICAX1 and SICAX3, respectively (**Figure 1A**). This clade, named the I-A clade, contains specialized Ca²⁺ transporters (Shigaki et al., 2006). SICAX2, SICAX4, SICAX5, and SICAX6 were grouped with AtCAX2, AtCAX5, and AtCAX6 in the I-B clade; proteins in this group transport multiple cations, including Ca²⁺, Cd²⁺, and Mn²⁺ (**Figure 1A**; Hirschi et al., 2000). All the tomato SICAX proteins, except SICAX6, were predicted to have 11 transmembrane (TM) domains (**Supplementary Figure 2**). Gene expression data from the Tomato eFP browser showed that *SICAX1* and *SICAX3* are mainly expressed in leaves, and *SICAX2*, *SICAX4*, and *SICAX5* are expressed in fruits, whereas *SICAX6* is only expressed in immature flowers (**Supplementary Figure 3**; Sato et al., 2012). We treated the tomato seedlings with different types of ions. Only *SICAX1* and *SICAX3* were dramatically induced



¹<https://phytozome-next.jgi.doe.gov>

by Ca^{2+} treatment, indicating their roles in Ca^{2+} transport (Figure 1B).

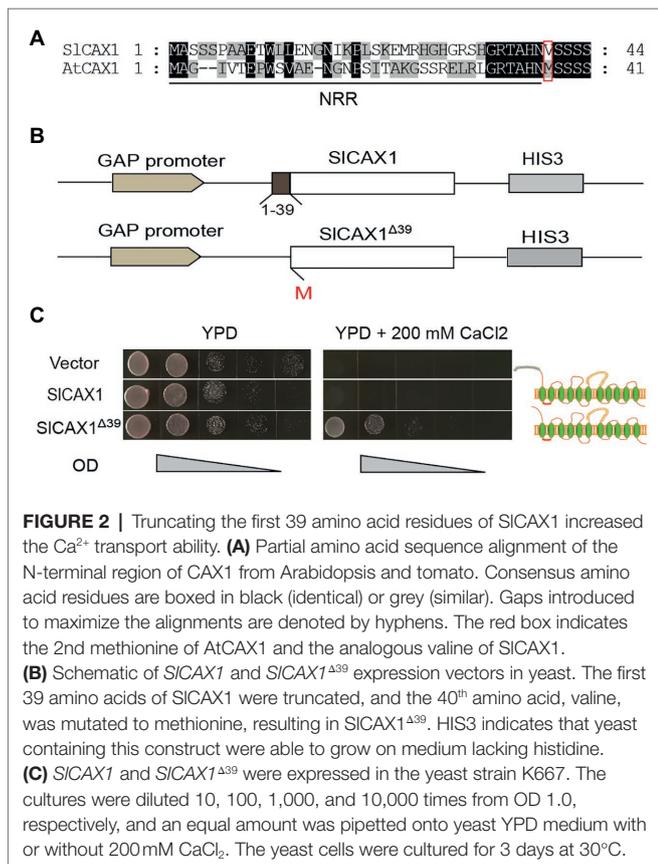
To further verify the Ca^{2+} transport ability of SICAX1, we heterologously expressed the encoding gene in the yeast K667 strain (*vcx1::hisG cnb1::LEU2 pmc1::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), which has defects in vacuolar Ca^{2+} transport (Cunningham and Fink, 1996). Expression of full-length SICAX1 failed to complement the hypersensitivity of the K667 strain to Ca^{2+} , suggesting that, similar to AtCAX1, the full-length SICAX1 is not active and an NRR may inhibit its activity (Pittman and Hirschi, 2001). Aligning the protein sequences of SICAX1 and AtCAX1 revealed that the two proteins share 79% similarity and the N-terminal sequences of these two proteins are highly conserved. However, unlike AtCAX1, there is no second methionine in the N terminus of SICAX1, and the analogous methionine at the 37th amino acid position of AtCAX1 corresponded to the valine residue at the 40th amino acid position in SICAX1 (Figure 2A). We truncated the first 39 amino acid residues of SICAX1 and changed valine to methionine to initiate translation. We named this N-terminal-truncated version SICAX1^{Δ39} (Figure 2B). Complementation analysis in yeast showed that SICAX1^{Δ39} was able to complement the hypersensitivity of the K667 strain to Ca^{2+} , suggesting that SICAX1^{Δ39} has Ca^{2+} transport ability (Figure 2C).

To confirm the Ca^{2+} transport activity of SICAX1, we used CRISPR-Cas9 to knock out this gene. We obtained different

deletions, including 37-bp and 4-bp deletions with premature stop codons predicted to result in null alleles (Figures 3A,B). The *slcax1* mutants were smaller than the wild-type plants but were able to complete the lifecycle (Figures 3C,D), and the plant height and number of flowers of *slcax1* mutants were significantly lower than those of wild-type (Figure 3E). Quantification of SICAX3 expression revealed that SICAX3 was upregulated in the *slcax1* mutants, supporting the idea of genetic redundancy between SICAX1 and SICAX3 (Figure 3F). Measuring the Ca^{2+} concentration of the wild type and mutants showed that mutants had significantly lower Ca^{2+} content (Figure 3G), confirming the Ca^{2+} -transporting ability of SICAX1. Together, the results demonstrate that SICAX1 has a Ca^{2+} transport ability in both yeast and tomato.

The NRRs of *Arabidopsis* AtCAXs were initially defined by the sequence between the first and second methionine of AtCAX1 and AtCAX3; however, we found that this methionine residue is not conserved in tomato and other species (Figure 2A). Indeed, the *Bacillus subtilis* $\text{Ca}^{2+}/\text{H}^{+}$ antiporter protein YfkE, which shares high sequence similarity with *Arabidopsis* AtCAX1, does not even have any peptides in upstream of the first TM domain (Wu et al., 2013; Supplementary Figure 4). Therefore, we hypothesized that all the amino acid residues in upstream of the first TM domain of AtCAX1 negatively regulate its activity. To test this hypothesis, instead of truncating the first 39 amino acids, we truncated all 66 upstream amino acids of the first TM domain and named it SICAX1^{Δ66} (Figure 4A). As expected, SICAX1^{Δ66} showed higher activity than SICAX1^{Δ39} in a yeast assay (Figure 4B), suggesting that all the amino acids in upstream of the first TM domain of SICAX1 negatively regulate its Ca^{2+} transporting ability. To further validate our hypothesis, we made two extra truncations, SICAX1^{Δ58} and SICAX1^{Δ93} with the first TM domain deleted (Supplementary Figure 4). The results showed that SICAX1^{Δ66} still displayed the highest activity in the yeast assay and contrasting to AtCAX1^{Δ90}, SICAX1^{Δ93} was not active at all, suggesting that the first TM of SICAX1 is critical for its activity (Supplementary Figure 5). Together, our results indicate that all the amino acids in upstream of the first TM domain of SICAX1 negatively regulate its Ca^{2+} transporting ability.

To determine if this is also applicable to other CAXs, such as *Arabidopsis* AtCAX1 and AtCAX3, we tested the Ca^{2+} transport activity of variants with different truncations in the K667 strain. Alignment of the protein sequences of SICAX1, SICAX3, AtCAX1, and AtCAX3 revealed that the sequences in upstream of the first TM domain were conserved between AtCAX1, AtCAX3 and SICAX1, but not SICAX3 (Figure 5A). Therefore, we truncated the first 63 amino acid residues before the first TM domains of AtCAX1 and AtCAX3 to create AtCAX1^{Δ63} and AtCAX3^{Δ63}, respectively, and evaluated whether this truncation increased the activity of these proteins. Similar to SICAX1^{Δ66}, both AtCAX1^{Δ63} and AtCAX3^{Δ63} showed higher activity than AtCAX1^{Δ36} and AtCAX3^{Δ36}, respectively (Figures 5B,C). The difference was particularly significant for AtCAX3 because, as indicated above, truncating the initial NRR



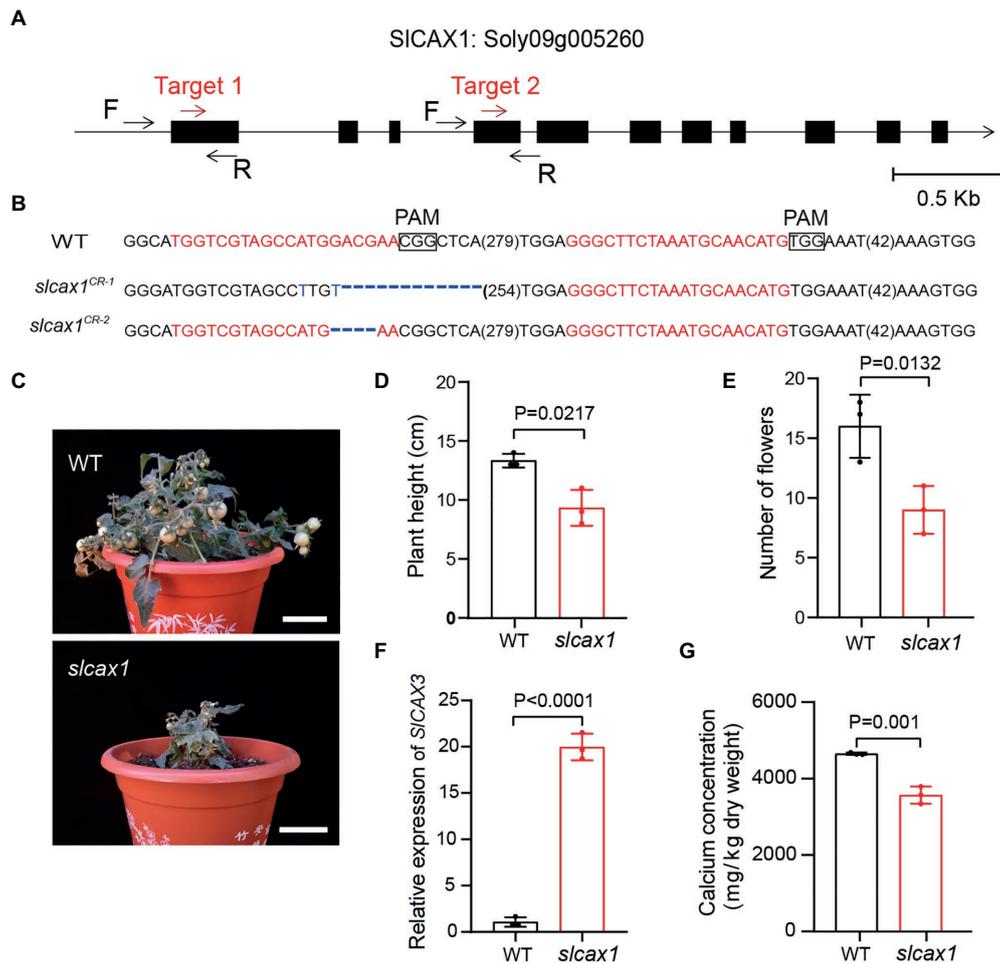


FIGURE 3 | CRISPR/Cas9-engineered mutations of *SICAX1* resulted in low Ca^{2+} concentrations in tomatoes. **(A)** The first exon of *SICAX1* was targeted by CRISPR-Cas9 using two single guide RNAs (sgRNA; target 1 and target 2; red arrows). Black arrows indicate forward (F) and reverse (R) primers used for PCR genotyping and sequencing. **(B)** Sequences of *slcax1* alleles were identified from two independent T1 transgenic plants. sgRNA targets and protospacer adjacent motif sequences are in red and bold font, respectively; deletions are indicated by blue dashes; and base mutations are shown in blue. The sequence gap length is shown in parentheses. **(C)** Phenotypes of the wild type and *slcax1* mutants. **(D)** Plant height of wild type and *slcax1* mutants grown for 2 months. **(E)** The number of flowers of wild type and *slcax1* mutants. The pictures, plant height, and flower number data were collected from 2-month-old plants. **(F)** Analysis of *SICAX3* expression by RT-qPCR. Wild-type and *slcax1* mutant leaves were harvested 2 weeks after germination for RNA extraction from three biological replicates. **(G)** Shoot calcium content in the wild type and *slcax1* mutants. The wild type and mutants were grown on MS medium for 1 month, and then the shoots were harvested for calcium content measurement ($n=3$). The Student's *t*-test was used to analyze the data for **(D-G)**. Error bars represent standard error of the mean.

domain of AtCAX3 does not activate this antiporter (Shigaki and Hirschi, 2000). Together, our results suggest that CAXs can be completely activated after truncating all of the amino acid residues before the first TM domain, thus our results redefine the NRR of CAXs.

To confirm that *SICAX1*^{Δ66} has enhanced Ca^{2+} transport ability *in planta*, we expressed *SICAX1* and *SICAX1*^{Δ66} driven by the CaMV35S promoter in tomato plants and quantified the Ca^{2+} content (Figure 6A). We confirmed the expression using PCR (Supplementary Figure 6). Overexpression of full-length *SICAX1* did not enhance plant Ca^{2+} content, consistent with its non-active nature. By contrast, overexpression of *SICAX1*^{Δ66} significantly increased plant Ca^{2+} content, indicating that truncating the amino acid residues

upstream of the first TM domain can activate *SICAX1* *in vivo* (Figure 6B). Together, the results from the yeast assays and the *in planta* data demonstrate that the region constituting the NRR of CAXs should be re-defined.

DISCUSSION

In this study, we confirmed the Ca^{2+} transporting ability of *SICAX1* using knockout mutants generated by CRISPR-Cas9 and redefined the region constituting the NRR of CAX proteins. Initially, the NRR was defined as the region between the first and second methionine of the antiporter, because both *Arabidopsis* AtCAX1 and AtCAX3 have a second

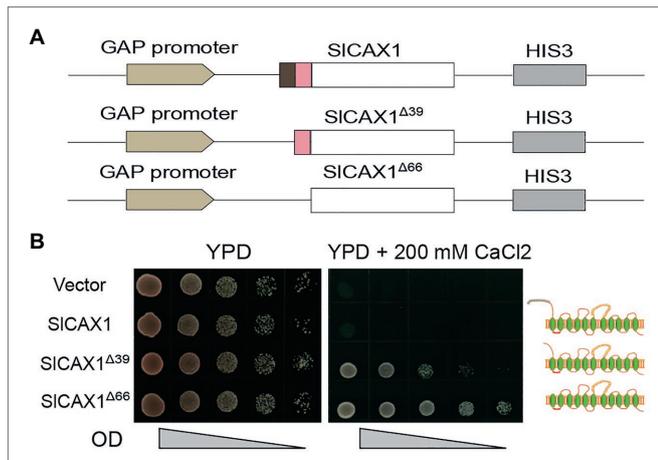


FIGURE 4 | Amino acid residues before the first TM domain of SICAX1 negatively regulate the Ca²⁺ transporting ability of the antiporter. **(A)** Schematics of the vectors used to express different truncated versions of SICAX1. **(B)** SICAX1, SICAX1^{Δ39} and SICAX1^{Δ66} were expressed in yeast strain K667. The cultures were diluted 10, 100, 1,000, and 10,000 times from OD 1.0, respectively, and an equal amount of sample was pipetted onto YPD medium with or without 200mM CaCl₂. The yeast cells were cultured at 30°C for 3 days.

methionine at the 36th amino acid position, and truncating this region increased the activity of AtCAX1 (Pittman and Hirschi, 2001). However, amino acid sequence alignment showed that the SICAX from tomato do not have a second in-frame start codon, and some CAXs do not even have any amino acid residues in front of the first TM domain, promoting us to re-evaluate the NRR region. By testing different variants of CAXs, we established that the entire region upstream of the first TM domain inhibits CAX activity. The work by Manohar et al. also revealed that AtCAX3 with N-terminal truncation of 57 amino acids and more has the higher activity than the full length and 36-amino-acid truncation of AtCAX3 (AtCAX3^{Δ36}; Manohar et al., 2011). However, how this region inhibits CAX activity remains elusive. Previous studies showed that AtCAX1 and AtCAX3 interact with each other (Cheng et al., 2005; Hocking et al., 2017), and structural analyses of the Ca²⁺ antiporter YfkE and ScVCX1 suggest that CAXs form oligomer (Waight et al., 2013; Wu et al., 2013). Therefore, it is possible that plant CAXs also form oligomeric complex. Interestingly, NRR has a high probability to consist of an intrinsically

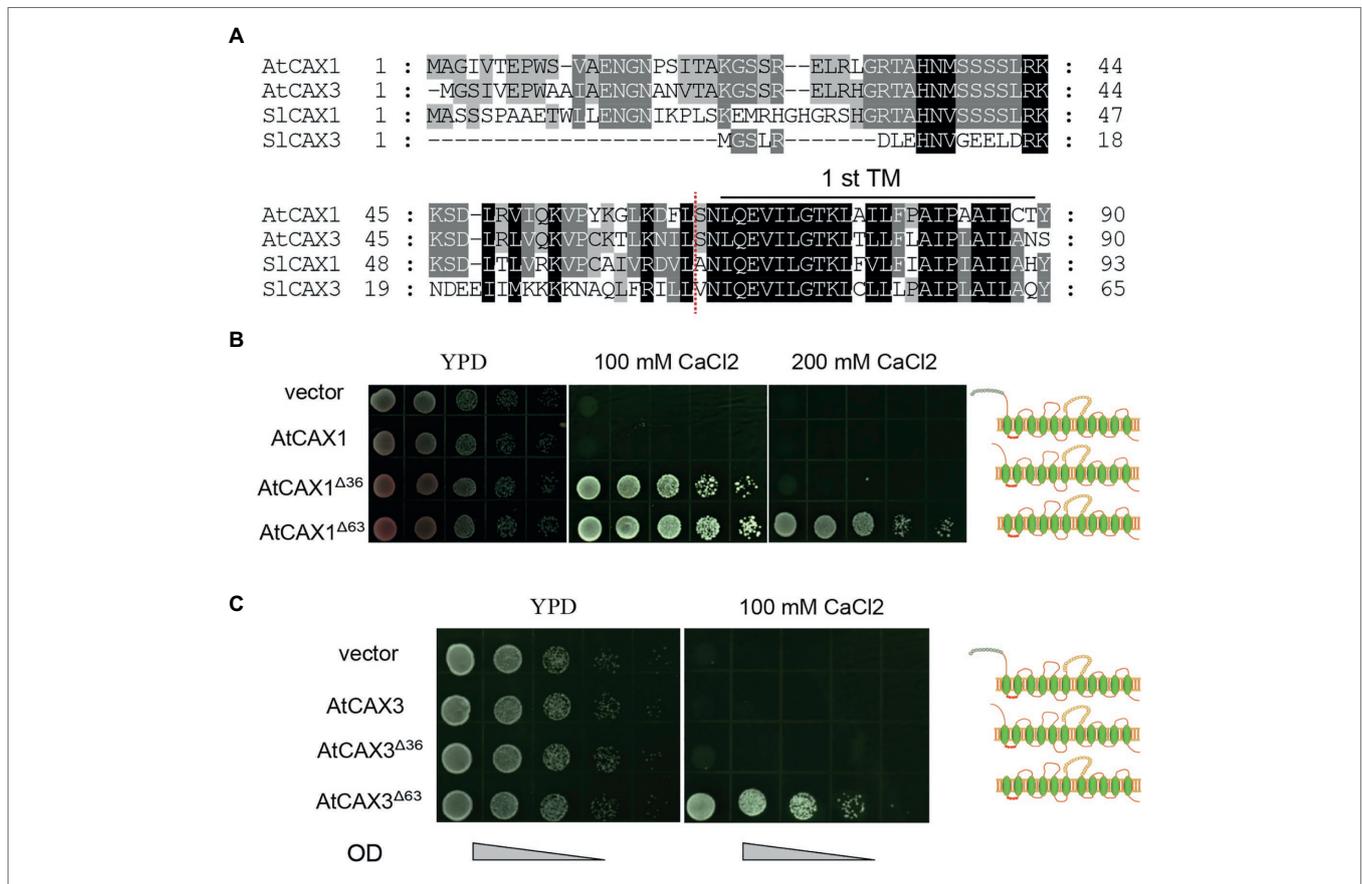
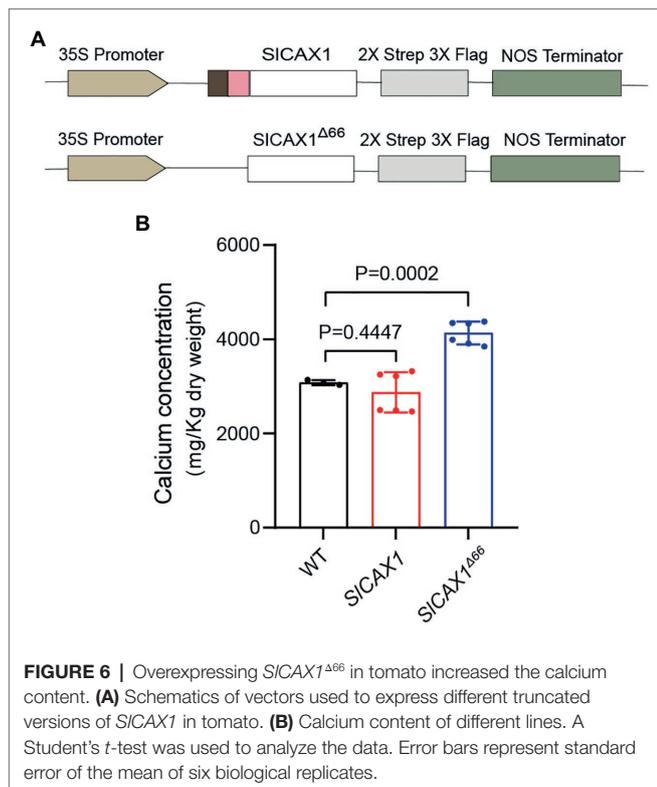


FIGURE 5 | Truncating the amino acids before the first TM further increases the activity of CAX1 and CAX3. **(A)** Alignment of deduced amino acid sequences of polypeptides encoded by Arabidopsis CAX1 and CAX3 and tomato SICAX1 and SICAX3 (partial). Consensus amino acid residues are boxed in black (identical) or grey (similar). Gaps introduced to maximize the alignments are denoted by hyphens. The amino acids residues before the red dotted lines are deleted to form AtCAX1^{Δ63} and AtCAX3^{Δ63}. **(B,C)** Truncated AtCAX1 and AtCAX3 were expressed in yeast mutant strain K667, respectively. The cultures were diluted 10, 100, 1,000, and 10,000 times from OD 1.0, respectively, and an equal amount of sample was pipetted onto yeast YPD medium with or without CaCl₂. The yeast cells were cultured at 30°C for 3 days.



disordered region (IDR), as predicted by IUPRED3,² and scrambling of the first 39 or 66 amino acids of *SICAX1* did not fully unleash its activity (**Supplementary Figure 7**), suggesting that the sequence of NRR does not need stringently specific to be functional. We speculate that the NRR of CAX consisting of IDR may fold back and loosely binds to its own or other CAXs' Ca²⁺ binding sites in the complex to inhibit the Ca²⁺ transport ability. The NRR of AtCAX1 can also be phosphorylated by CBL-INTERACTING PROTEIN KINASE24 (CIPK24), and AtCAX1 is activated upon the phosphorylation (Cheng et al., 2004), indicating that activation of the CAX complex relies on other proteins. The low complexity region in the NRR consists of four serine amino acids. It is possible that phosphorylation of NRR at these amino acids could affect the interaction of NRR with the Ca²⁺ binding sites of CAX proteins and also affects the CAX activity by increasing the accessibility to Ca²⁺. However, a detailed mechanism will not be elucidated until the structure of the CAX complex is determined. Given recent advances in the cryo-EM technique, solving the structure of this membrane complex is feasible. A complete understanding of the regulatory mechanism of plant CAX proteins will benefit efforts to improve Ca²⁺ use efficiency by crop plants.

slcax1 mutants can still complete the lifecycle, and this may be because *SICAX1* and *SICAX3* are genetically redundant. Similarly, neither the *Arabidopsis atcax1* nor *atcax3* single mutants showed strong growth defects, but the growth of

atcax1 atcax3 double mutants was severely hindered (Hocking et al., 2017). Our RT-qPCR results revealed that upregulation of *SICAX3* in *slcax1* mutants allowed for functional compensation, indicating the genetic robustness of the Ca²⁺ homeostasis pathway. Genetic robustness is also common for other biological processes, such as regulation of shoot meristem development. For instance, disruption of tomato *SICLAVATA3* resulted in upregulation of its paralogous gene *CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (SICLE9)*, and double mutants show synergistic phenotypes (Rodriguez-Leal et al., 2019). Further characterization of *slcax1 slcax3* double mutants would help dissect the functions of *SICAX1* and *SICAX3*.

Here, we used tomato, an economically important crop, as a model and determined the regulatory mechanism of CAX proteins. This study will facilitate the manipulation of Ca²⁺ antiporters in efforts to fine-tune Ca²⁺ homeostasis and thereby reduce the incidence of Ca²⁺-related physiological disorders and improve the fruit quality of crops.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BH, YT, SL, JS, XW, and TK performed the experiments. BH, YT, and QW designed research. JW, XC, SP, and QW wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.938839/full#supplementary-material>

²https://iupred.elte.hu/help_new

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