Identification and Expression Profiling Analysis of the Cation/Ca\textsuperscript{2+} Exchanger (CCX) Gene Family: Overexpression of SICCX1-LIKE Regulates the Leaf Senescence in Tomato Flowering Phase

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Cation gradients in plant cellular compartments are maintained by the synergistic actions of various ion exchangers, pumps, and channels. Cation/Ca\textsuperscript{2+} exchanger (CCX) is one of the clades of the Ca\textsuperscript{2+}/cation antiporter superfamily. Here, five SICCX genes were identified in tomato. Sequence analysis indicated that SICCXs have the conserved motifs as the CCX domain. Analysis of the expression level of each member of tomato CCX gene family under cation (Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Na\textsuperscript{+}, and Ca\textsuperscript{2+}) treatment was determined by qRT-PCR. Tomato CCX demonstrated different degrees of responding to cation treatment. Changes in SICCX1-LIKE expression was induced by Mg\textsuperscript{2+} and Mn\textsuperscript{2+} treatment. Analysis of the expression of SICCX genes in different tissues demonstrated that constitutive high expression of a few genes, including SICCX1-LIKE and SICCX5, indicated their role in tomato organ growth and development. Overexpression of SICCX1-LIKE dramatically induced leaf senescence. Transcriptome analysis showed that genes related to ROS and several IAA signaling pathways were significantly downregulated, whereas ETH and ABA signaling pathway-related genes were upregulated in overexpression of SICCX1-LIKE (OE-SICCX1-LIKE) plants, compared with the wild type (WT). Moreover, overexpression of SICCX1-LIKE plants accumulated more ROS content but less Mg\textsuperscript{2+} content. Collectively, the findings of this study provide insights into the base mechanism through which CCXs regulate leaf senescence in tomato.

Keywords: CCX gene, bioinformatic analysis, expression pattern, leaf senescence, tomato

Abbreviations: NCX, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger family; NCKX, Na\textsuperscript{+}/Ca\textsuperscript{2+}, K\textsuperscript{+} exchanger family; CCX, cation/Ca\textsuperscript{2+} exchanger; CAX, H\textsuperscript{+}/cation exchanger family; CaCA, Ca\textsuperscript{2+}/cation antiporters; IAA, auxin; ETH, ethylene; ROS, reactive oxygen species; ABA, abscisic acid; GH3, Gretchen hagen 3; SOD, superoxide dismutase; POD, peroxisome dismutase; APOD, ascorbate peroxidase; ER, endoplasmic reticulum; CDS, coding sequence; NCBI, National Center for Biotechnology Information; GRAVY, grand average of hydropathicity; TM, transmembrane; DEG, differentially expressed gene; GFP, green fluorescent protein; MEME, multiple EM for motif elicitation; NJ, neighbor-joining; OE, overexpression; MT, micro-TOM.

HIGHLIGHTS
- Five SlCCX genes were identified in tomato.
- SlCCX1-LIKE was highly expressed in Mg\(^{2+}\) and Mn\(^{2+}\) treatment.
- Overexpression of SlCCX1-LIKE induced leaf senescence by increasing ROS, decreasing Mg\(^{2+}\), and hormone signaling pathways.

INTRODUCTION
Calcium (Ca\(^{2+}\)) is an essential macronutrient in plants owing to its unique role as a messenger (Hirschi, 2004). Spatiotemporal regulation of the cytosolic Ca\(^{2+}\) concentration is crucial for modulating cell responses to various internal and external stimuli (Dodd et al., 2010). Ca\(^{2+}\) influx and efflux, which are performed by transporters/channels, are regulated in a highly concerted manner, translating specific stimuli into a Ca\(^{2+}\) signal. In plants, Ca\(^{2+}\)-permeable channels, Ca\(^{2+}\)-ATPases, and Ca\(^{2+}\)/cation antiporters (CaCA) mediate Ca\(^{2+}\) fluxes and maintain cytosolic Ca\(^{2+}\) homeostasis. Functional and phylogenetic analysis demonstrated that the CaCA superfamily comprises at least five gene families: the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) family, the Na\(^{+}\)/Ca\(^{2+}\) transporter (CAX) family, the H\(^{+}\)/cation exchanger (CAX) family, the YRBG family, and the cation/calcium exchanger (CCX) family (Emery et al., 2012; Singh et al., 2014). Previous studies have suggested the evolutionary significance of Ca\(^{2+}\)/Ca\(^{2+}\) flux proteins (AtCCX1-5) reported for Arabidopsis thaliana that were previously classified as members of CAX, namely CAX7-CAX11 (Shimazaki et al., 2007). AtCCX1 regulates leaf senescence and ROS homeostasis, and knockout of AtCCX1 and AtCCX4 showed stay-green leaf phenotypes that were sensitive to Ca\(^{2+}\) deprivation (Li et al., 2016). AtCCX2 controls the dynamics of Ca\(^{2+}\) in the endoplasmic reticulum (ER), influencing plant growth in response to salt and osmotic stress (Corso et al., 2018). AtCCX3 and AtCCX4 have similar functions, because they arose from a single gene duplication (Morris et al., 2008). AtCCX3 and AtCCX4 are H\(^{+}\)/Ca\(^{2+}\) transporters that may also ferry Na\(^{+}\) and Mn\(^{2+}\) (Liu et al., 1997; Tuttle et al., 2003), while AtCCX5 might mediate K\(^{+}\) uptake and Na\(^{+}\) transport (Zhang et al., 2011). The OsCCX2 gene, a rice member of this family, encodes a protein responsible for Ca\(^{2+}\), Na\(^{+}\), Li\(^{+}\), Fe\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\) transport in certain yeast mutants (Yadav et al., 2015).

Leaf senescence is a highly integrated development stage that coordinates multidimensional alterations at the physiological and molecular levels. As a disintegrated and degenerated process, senescence is concomitant with an intensive restructuring of cells, involving the breakdown of macromolecules, such as chlorophyll, proteins, nucleic acids, and membrane lipid (Nam et al., 1980). During leaf senescence, intracellular organelles and micromolecules are dismantled and degenerated, which predominantly contributes to the source-to-sink allocation of nutrients (Noodé, 1988). The redistribution of nutrients from senescent organs to vegetative tissues is essential for plant fitness and critical for productivity and quality in crops (Lim et al., 2007). Generally, initiation of leaf senescence is age dependent and triggered by developmental cues (Woo et al., 2019).

Intrinsically, initiation of senescence is the consequence of integrated signals, including endogenous and environmental signals (Bresson et al., 2018). Developmental senescence, which is a coordinated physiological process, could be induced by the endogenous factors (Zentgraf et al., 2004). Ca\(^{2+}\) has a regulatory role in vegetative senescence and fruit ripening (Ferguson, 1984). The impairment of leaf cell Ca\(^{2+}\) uptake in leaf cells hastens senescence programming (Ma et al., 2010). Furthermore, phytohormones play both positive and negative roles in the onset of leaf senescence. Among the leaf senescence-related hormones, abscisic acid (ABA), auxin, and ethylene (ETH) can significantly accelerate the aging of leaves (Li et al., 2013). In addition to hormone responsive genes, numerous senescence-related genes, such as senescence-associated genes, have been proven to be crucial for the timing of leaf senescence (Park et al., 1998).

Calcium is an essential component involved in plant senescence signaling cascades. As the cation conducting channel, CNGC2 is involved in leaf senescence signaling. Regarding the CCX gene family, we found that the expression of SlCCX1-LIKE varies dramatically across different organs in tomato. In addition, further study showed that leaf senescence was increased in SlCCX1-LIKE-OE plants. In our experiment, SlCCX1-LIKE was selected as the candidate gene for the study through the analysis and identification of the biological information of the members of SlCCXs and the phenomenon of leaf senescence induced by SlCCX1-LIKE-OE. Hence, our findings provide further theoretical basis for the mechanism of leaf senescence, which may have important agricultural implications on yield and nutrition content as affected by CCXs.

MATERIALS AND METHODS
Identification of Members of the SlCCX Family in Tomato
To identify tomato SlCCXs, Arabidopsis CCX protein sequences were used as queries. A BLASTP search was performed to identify members of the SlCCXs in tomato against the Solanaceae Genomics Network database1. These genes were termed Solanum lycopersicum CCXs (SlCCXs which contain SlCCX1; SlCCX1-LIKE; SlCCX4; SlCCX4-LIKE; SlCCX5), and the sequences were further used as queries for the BLASTN searches, against SGN tomato whole genome scaffolds data (2.10) (International Tomato Genome Sequencing Consortium)2. The SlCCXs were further used as query sequences to search for CCXs of the other

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1 https://solgenomics.net/plantdbg.org/
2 https://solgenomics.net/tools/blast/
Solanaceae members (Supplementary Table 1), using the NCBI\textsuperscript{1} and SGN\textsuperscript{2}.

**Basic Features, Secondary Structures, and Subcellular Localization**

The molecular mass, theoretical isoelectric point, instability coefficient, and hydrophilicity index of SICCCXs were compiled by analyzing the physical and chemical properties of the amino acids using the ExPASy ProtParam tool\textsuperscript{3}. The deduced amino acid sequences of the SICCCXs were submitted to TMHMM Server v.2.0\textsuperscript{4} in FASTA format, using the default settings to predict the presence of protein domains. Structural predictions were performed using the structural functional domain of the Simple Modular Architecture Research Tool (SMART)\textsuperscript{5}. Subcellular localization was performed using the PSORTII Prediction program\textsuperscript{6}.

**Sequence Alignment, Gene Structure, and Phylogenetic Analyses**

Multiple sequence alignments for the SICCCXs were generated using ClustaW in DNAMAN 8.0 Demo sequence analysis tool, and the phylogenetic tree was constructed with the neighbor-joining algorithm in MEGA7.0 (Kumar et al., 2016). The neighbor-joining (NJ) method was applied to construct a phylogenetic tree, in which Poisson correction, pairwise deletion, and bootstrapping (1,000 replicates; random seeds) were used as the default values to evaluate the liability of the tree.

A schematic diagram of the gene structure of SICCCXs was constructed using the Gene Structure Display Server\textsuperscript{7}. To identify the conserved motifs of the SICCCX protein sequences, we used the online multiple expectation maximizations for motif elicitation (MEME) tool\textsuperscript{8}.

Sequence domains were identified through the Pfam 32.0 database collection\textsuperscript{9}. Genes that did not contain the known conserved domains and motifs of the SICCCX members were eliminated using the SMART database\textsuperscript{10} and the Conserved Domain NCBI database\textsuperscript{11} (Yue et al., 2012). Genomic information concerning the chromosome locations of the SICCCXs and the amino acid sequences of SICCCX proteins was obtained from the SGN database.

**qRT-PCR Analysis, Tomato Plant Growth, and Treatments**

Total RNA from the samples of leaves was extracted using TRIzol\textsuperscript{12} (Takara, Dalian, China). Genomic DNA contamination was removed followed by RQ1 DNase I (Promega, Madison, WI, United States). cDNA was synthesized with DNA-free RNA (2 μ). The RT-PCR for target mRNA was performed according to Liu et al. (2016). Primers are listed in Supplementary Table 2.

Tomato (S. lycopersicum “Micro-Tom”) seedlings were planted in pots containing the growth medium (nutrition soil:vermiculite:perlite = 3:2:1) and grown under a 12-h/12-h (light/ dark) photoperiod at 25°C, a relative humidity of 70–80%, and an irradiance of 300 μmol m\textsuperscript{2} s\textsuperscript{-1}. Fruits were harvested at both maturing stage (green) and ripening stage (red). Fruit pericarps were prepared with a cork borer (4 mm in diameter and 2 mm in thickness) and equilibrated for 30 min in equilibrating buffer (MES 50 mM at pH 5.5, containing 5 mM CaCl\textsubscript{2}, 5 mM MgCl\textsubscript{2}, 5 mM EDTA, 5 mM vitamin C, and 200 mM mannitol) (Télef et al., 2006; Marchler-Bauer, 2011). Mannitol was used to adjust the equal osmotic potential in the incubation system. After equilibration, disks in the control untreatment were incubated in equilibration buffer, while the other disks were incubated in equilibration buffer with 50, 100, and 200 mM CaCl\textsubscript{2}, MgCl\textsubscript{2} (Aliu et al., 2015), NaCl, or MnCl\textsubscript{2} (Xu et al., 2010) for 8 or 24 h (Jia et al., 2016) at room temperature (25°C–28°C). After being washed by double distilled water, the tissues were used immediately for assays or frozen in liquid nitrogen and kept at −80°C until use. Experiment was repeated at least three times. Each experiment consisted of three biological replicates and three technical replicates.

For H\textsubscript{2}O\textsubscript{2} treatment, the fourth and fifth detached leaves from about 35-day-old plants were incubated on 30 mM H\textsubscript{2}O\textsubscript{2} solutions for 4 days in the dark.

**Digital Expression Analysis of CCX Genes in Growth and Development**

The expression analysis of SICCCX genes were downloaded from the Tomato Functional Genomics Database\textsuperscript{14}, including sequence data from various tissues in tomato cultivar “Heinz 1706” (S. lycopersicum) and wild species LA1598 (Solanum pimpinellifolium). SICCCX genes expressed in leaves, roots, unopened flower buds, fully opened flowers, and at different stages of fruit development (1, 2, and 3 cm fruit, mature green fruit, breaker fruit, and fruit at 10 days after breaker) were summarized and used to construct a heatmap with Multiple Array Viewer software to visualize the expression profiling based on log2-transformed RPKM values.

**Generation of Transgenic Tomato Plants Overexpressed SICCCX1-LIKE**

The full-length coding sequence of SICCCX-LIKE was amplified by PCR and then inserted into a pCAMBIA3301 vector to construct overexpression vector. Harboring SICCCX-LIKE vector was transformed into Agrobacterium tumefaciens GV3301. Putative hardened transgenic plants were transferred to the greenhouse. Presence of the transgene was confirmed by PCR with kanamycin (Kana) primers. Seeds from the transformants

\textsuperscript{1}http://www.ncbi.nlm.nih.gov/
\textsuperscript{2}https://solgenomics.net/
\textsuperscript{3}http://web.expasy.org/protparam
\textsuperscript{4}http://www.cbs.dtu.dk/services/TMHMM
\textsuperscript{5}http://smart.embl-heidelberg.de
\textsuperscript{6}http://psort.hgc.jp/form2.html
\textsuperscript{7}http://gds.cbi.pku.edu.cn/
\textsuperscript{8}http://meme.sdsc.edu/meme/meme.html
\textsuperscript{9}http://pfam.janelia.org/
\textsuperscript{10}http://smart.embl-heidelberg.de/
\textsuperscript{11}http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/
\textsuperscript{12}http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi
were grown on half-strength Murashige-Skoog (MS) medium supplemented with 100 mg L\(^{-1}\) kanamycin for selection. Surviving seedlings were grown in a greenhouse to obtain the T1 generation. Four OE lines were selected for subsequent analysis.

**Determination of Chlorophyll Content and ROS Concentrations**

Tissues were homogenized in liquid nitrogen. The powdered samples were extracted with 80% acetone. Chlorophyll content was determined spectrophotometrically at wavelengths of 663 and 645 nm.

The H\(_2\)O\(_2\) and O\(_2^−\) was measured using hydrogen peroxide (H\(_2\)O\(_2\)) Content Assay Kit (Solarbio, Beijing, China) and Superoxide Anion Activity Assay Kit (Solarbio, Beijing, China) as per manufacturer’s instructions.

**Ca and Mg Determination**

Leaves were dried in an oven at 100°C for 1 h, and then 65°C for 2 days. The dried samples were weighed and digested with H\(_2\)SO\(_4\) solution at 180°C for 10 min, then the temperature was adjusted to 220°C for 30 min. The concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) were determined using Atomic Absorption Spectrophotometer 6300 (Agilent, Santa Clara, CA, United States). Experiment was repeated at least three times. Each experiment consisted of three biological replicates and three technical replicates.

**RNA-seq Library Construction and Transcriptome Analysis**

RNA extracts (three biological replicates for each of two genotypes), each containing at least 5 mg of total RNA, were sent to a private genomic laboratory, GENEWIZ, for RNA sequencing. Raw reads containing adapters, >10% of unknown nucleotides, and low-quality reads (Q-value ≤ 20; >50% of reads) were removed from the dataset. To estimate gene expression level, trimmed reads were mapped to the tomato genome CDS (ITAG release 4.0). Reads mapped to unigenes were counted and used for expression analysis.

**Subcellular Localization Assays in Tobacco Leaf Cells**

The 1.5-kb promoter sequence upstream of ATG and the SICCX1-LIKE coding region were inserted into the pCAMBIA2300-GFP vector by SacI and XbaI digestion and using the 35S promoter. Recombinant plasmids were transformed into A. tumefaciens GV3101 cells. After grown in LB medium at 28°C overnight, bacterial suspensions were infiltrated into young but fully expanded leaves of tobacco using a needleless syringe. After infiltration, plants were immediately covered with plastic bags and placed at 23°C for 48 h before bag removal. The GFP fluorescence signals were observed with a confocal laser scanning microscope (Leica SP8, Germany). The fluorescence signal was observed at an excitation wavelength of 488 or 561 nm and emission wavelength of 500–572 or 605–635 nm.

**RESULTS**

**Identification of SICCX Family Members**

In this study, we identified five SICCX genes in tomato named according to their homologous recombination in A. thaliana. Gene name, gene accession number, locus, protein sequence length, molecular weight, and isoelectric point (pI) are listed in Table 1.

We found that length of the nucleotide chain of members of the SICCX gene family was between 476 and 646 bp, with the shortest and longest being SICCX4 and SICCX4-LIKE, respectively. The molecular weight of SICCX4 was 52.42 kDa, and for the SICCX4-LIKE, it was 70.42 kDa. After analyzing the physicochemical properties of these proteins, we found that the theoretical isoelectric pI of the amino acids is between 5.73 and 8.00, which we refer as intermediate, with only the SICCX1-LIKE having a pI of 8.0. Among the identified members, the GRAVY value of SICCX5 was the highest (Table 1).

SICCX1 and SICCX1-LIKE are composed with no introns, while SICCX4 and SICCX4-LIKE have one intron. In contrast, SICCX5 has three exons and two introns. Motifs present in the five SICCX proteins were identified using their full-length protein sequence from tomato (Figure 1). A total of 10 conserved motifs were identified, and their distribution in proteins of the respective groups are presented in the phylogram in Figure 1. Motif 7 corresponds to α1 signature domain (GNGAPD), and motif 1 corresponds to α2 signature domain (G(N/D)SxGD). SICCX1, SICCX4-LIKE, and SICCX1-LIKE and SICCX5 have 10, nine, and eight motifs, respectively. Motifs 1, 2, 3, 5, and 6 were the most conserved, as shown by their presence in all SICCX protein members. However, motifs 3, 5, 7, and 9 constitute the Na\(^+/\)Ca\(^{2+}\) exchanger domain; while motifs 8, 1, 2, and 4 constitute the Na\(^+/\)Ca\(^{2+}\) exchanger. Moreover, motifs 4 and 8 were not found in SICCX5, and motif 7 was not found in SICCX4. This suggests that the SICCX4 and SICCX5 have one Na\(^+/\)Ca\(^{2+}\) exchanger domain, whereas SICCX1, SICCX1-LIKE, and SICCX4-LIKE have two Na\(^+/\)Ca\(^{2+}\) exchanger domains. In contrast, SICCX4 lacks motif 7 containing a conserved sequence within the α1 repeat regions. SICCX4 and SICCX4-LIKE motifs were similar and both lacked motif 10. This feature may affect the spatial configuration of the protein, which might affect its cation transport-related characteristics.

**Phylogenetic Analysis of SICCXs**

Using query CCX protein query sequences from tomato, we performed a genomewide analysis to identify SICCX (CCX in S. lycopersicum) members from eight Solanum genomes (Solanum pennellii, Solanum tuberosum, Nicotiana attenuata, Nicotiana tabacum, Capsicum annuum, Nicotiana sylvestris, Capsicum baccatum, Capsicum chinense). The characteristics of the databases we used in this research, as well as the number of genes, are shown in Supplementary Table 1. The nomenclature used for all CCXs from the different members of the Solanaceae family is similar to that used for SICCXs, e.g., S. tuberosum CCX (StCCX) genes, Nicotiana tomentosiformis CCX (NtCCX) genes, and N. sylvestris CCX (NsCCX) genes.
We constructed the unrooted phylogenetic tree containing 28 CCX protein sequences from various members of the Solanaceae family (five SlCCXs, two CcCCXs, four CaCCXs, three CbCCXs, three SpCCXs, four StCCXs, three NaCCXs, three NtCCXs, and one NsCCX) was constructed by aligning them with three sequences from Oryza sativa (OsCCX) and five sequences from Arabidopsis AtCCXs, using MEGA7.0 tools. These CCX sequences were categorized into three groups (Figure 2), groups 1, 2, and 3. A total of 13 sister pairs, including three SlCCX-SpCCX, two NtCCX-NaCCX, two CaCCX-CcCCX, and one each of StCCX-StCCX, OsCCX-OsCCX, AtCCX-AtCCX, CaCCX-CbCCX, StCCX-SlCCX, and NtCCX-NsCCX pairs are present in the combined phylogenetic tree. The data indicate a high homology within the Solanaceae family. The two proteins with (1–10-motif) domains (SlCCX4 and SlCCX4-LIKE) were clustered together in group 3. SlCCX1 and SlCCX1-LIKE without the 10-motif domain were listed in group 2. Group 1 consists of the SlCCX5 without the four-, eight-, and 10-motif domains.

### Analysis of the SlCCX Protein Structure

SlCCX putative protein sequences and their secondary structures are shown in Table 2 and Figure 3. In general, SlCCXs were characterized by \( \alpha \)-helices, extended chains, random coils, and \( \beta \)-turns, where the angle of \( \beta \)-turn accounted for the lowest proportion. The random curl in SlCCX4 was larger than the \( \alpha \)-helix, while the other four proteins had irregular curls that were smaller than the \( \alpha \)-helix.

Most CCXs had 10–12 transmembrane (TM) helices, with two conserved alpha repeats in the TM helices 2–3 and 7–8. The S. lycopersicum ion-exchanger sequence likely contains the \( \alpha \)-1 and \( \alpha \)-2 repeat regions, as evidenced by the presence of domains having 100% identity with the \( \alpha \)-1 signature motif GNG (A/T) PD and the \( \alpha \)-2 signature motif GNSIGD (Figure 3).

Protein modeling analysis verify the Na\(^+\)/Ca\(^{2+}\) exchanger domain in SlCCXs (Figure 4). Gene structure analysis showed that all four genes have two Na\(^+\)/Ca\(^{2+}\) exchanger and the Na\(^+\)/Ca\(^{2+}\) transporter regions, while SlCCX4 has only a conserved Na\(^+\)/Ca\(^{2+}\) exchange region.

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**TABLE 1** | CCXs gene information in tomato.

| Gene name    | Gene accession number | Locus     | Protein length (AAs) | Molecular weight (kDa) | Isoelectric point | Instable coefficient\(^a\) | The grand average of hydropathy\(^b\) |
|--------------|-----------------------|-----------|----------------------|------------------------|------------------|--------------------------|-----------------------------------|
| SlCCX1       | Solyc09g072690.1.1    | LOC101248713 | 568                  | 62.67                  | 6.31             | 37.00                    | 0.703                             |
| SlCCX1-LIKE  | Solyc07g006370        | LOC101250521 | 567                  | 62.42                  | 8.00             | 33.31                    | 0.706                             |
| SlCCX4       | Solyc02g069710.2.1    | LOC101265300 | 476                  | 52.42                  | 5.91             | 42.66                    | 0.370                             |
| SlCCX4-LIKE  | Solyc07g042000        | LOC101250709 | 646                  | 70.02                  | 5.73             | 34.73                    | 0.608                             |
| SlCCX5       | Solyc01g098800        | LOC101261070 | 555                  | 60.84                  | 6.49             | 32.67                    | 0.868                             |

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\(^a\)Instable coefficient: a protein with an instability coefficient of less than 40 is called a stable protein.

\(^b\)The grand average of hydropathicity is calculated by adding the hydropathy value for each residue and dividing by the length of the sequence.
**FIGURE 2** | Phylogenetic analysis in Solanaceae CCXs. Multiple sequence alignment of CCX proteins was done using DNAMAN, and phylogenetic tree was constructed using MEGA7.0 by the neighbor-joining method with the pairwise deletion option with Poisson correction. The number at each node represents the bootstrap value from 1,000 replicates. At, Arabidopsis thaliana; Sp, Solanaceae pennellii; St, Solanaceae tuberosum; Na, Nicotiana attenuata; Nt, Nicotiana tabacum; Ca, Capsicum annuum; Cb, Capsicum baccatum; Cc, Capsicum chinense; symbol indicates tomato SlCCX proteins.

**TABLE 2** | Secondary structures analysis of the SlCCXs.

| Protein       | α -Helix | Extended chain | Random coil | β -Turn |
|---------------|----------|----------------|-------------|---------|
| SlCCX1        | 30.99    | 31.69          | 27.64       | 9.68    |
| SlCCX1-LIKE   | 29.28    | 33.16          | 27.69       | 9.88    |
| SlCCX4        | 30.88    | 26.26          | 32.98       | 9.87    |
| SlCCX4-LIKE   | 30.65    | 30.19          | 29.10       | 10.06   |
| SlCCX5        | 39.82    | 22.88          | 27.75       | 9.55    |

Proportion is the percentage of all the parts.

**Expression Pattern of SlCCX Genes in the Tomato and Under Various Cation Treatment Conditions**

Compared with their paralogs, three genes (SlCCX1-LIKE, SlCCX4-LIKE, and SlCCX5) showed significantly higher levels of expression (compared with their two remaining paralogs) in the different organs of the tomato cultivar “Heinz1706” and the wild relative S. pimpinellifolium. SlCCX1-LIKE gene (from group 3) was highly expressed in reproductive organs, such as flowers and developing fruits, but showed reduced expression levels in the vegetative organs, such as roots and leaves. SlCCX5 expression in group 1 remained constant and high during fruit ripening. SICCX4-LIKE from group 2 was found to be preferentially expressed in roots and ripening fruits (Figure 5). To evaluate the role of SlCCX genes under various cation treatments, we determined their expression levels in the fruits of the “Micro-Tom” tomato under cation treatments. The expression of SlCCX genes was significantly affected by different cation treatments (Figure 6). At the mature green stage, the expression levels of SlCCX1-LIKE were much higher in fruits exposed to MgCl$_2$ and MnCl$_2$ than those in the control treatment, and the extent of SlCCX1-LIKE upregulation increased significantly with the increasing cation concentration and exposure time (Figure 6). Expression levels of the other SlCCX family members were similarly low in tissues exposed to different cation treatments. Compared with the control, the expression level of SlCCX1 and SlCCX4-LIKE genes were low under the different cations and exposure time treatments. SlCCX4 exhibited maximum expression after tissues were exposed to 50 mmol for 8 h. The expression of SlCCX4 increased significantly after 24 h of exposure to CaCl$_2$, compared with that after 8 h. Contrastingly, the expression level of SlCCX5 was higher at 8 h than at 24 h under NaCl and MnCl$_2$ treatments. SlCCX1-LIKE showed higher expression under the divalent cation treatments in ripening organs. Ion transport, mostly of divalent cations, may play an important role in organ developing ripening.
Overexpression of SICCCX1-LIKE Promotes Leaf Senescence in Tomato

After bioinformatics and expression analysis above, SICCCX1-LIKE was selected for further investigation. SICCCX1-LIKE was overexpressed in “Micro-Tom” tomato. SICCCX1-LIKE-OE-3, OE-4, and OE-6 plants were selected from the T1 generation plant for the transgenic stable genetic technology (Supplementary Figure 1). Thirty-day-old seedling leaves turned yellow earlier than WT (Figure 7A). To explore further the involvement of SICCCX1-LIKE-OE in ROS homeostasis, we examined the ROS tolerance of WT and OE plants to ROS by treating detached leaves from 35-day-old seedings with H$_2$O$_2$ (Figure 7B). SICCCX1-LIKE-OE leaves turned yellow at the 4 days treatment. In contrast, the WT leaves were still green after 4 days treatment of H$_2$O$_2$. Therefore, compared with WT, SICCCX1-LIKE-OE tomato seems more sensitive to H$_2$O$_2$ treatment.

Expression of Hormone-Responsive Genes and ROS-Related Gene Expression in OE Plants

We also sequenced the RNA from the fifth leaves of WT and OE-SICCCX1-LIKE plants. To validate the RNA sequencing results, 12 genes that were annotated in GenBank were selected for qRT-PCR analysis (Figure 8). The results suggest that the RNA sequencing and qRT-PCR results are similar expression tendencies, confirming that the RNA sequencing results were reliable and could be used for further analysis.

Approximately 32% of the differentially expressed genes were found to be involved in hormone processes. In total, 168 differentially expressed gene (DEGs) were classified to be involved in the hormone category, which was subclassed into the top three groups (IAA53%, ABA16%, ETH14%) (Supplementary Table 3). We also found 24 DEGs related to auxin-induced SAUR-like protein, 11 IAA family genes, and six GH3 (Gretchen Hagen 3) family protein. All these DEGs were all downregulated in OE plants. Two protein phosphatase 2C and one serine/threonine-protein kinase related to ABA signaling transduction were also upregulated in OE plants (Supplementary Table 3). Eleven genes related to the reactive oxygen scavenging enzyme system were also downregulated in OE plants. This indicates that ROS content in SICCCX1-LIKE-OE leaves might be increased. Mg$^{2+}$-related enzyme genes were also downregulated in SICCCX1-LIKE-OE leaves. The Mg$^{2+}$ signal may be influenced by SICCCX1-LIKE.

Leaf Senescence in OE Plants

Transcriptome data showed that ROS-related genes were affected in OE plants and WT; therefore, we determined their ROS content. The H$_2$O$_2$ and O$_2^-$ contents were significantly higher in SICCCX1-LIKE, compared with WT (Figures 9A, B). These
results suggest that the ROS was induced in SICCX1-LIKE-OE plants.

There were no distinguishable growth and development phenotypic differences between SICCX1-LIKE-OE and the WT. However, we observed that chlorophyll content was significantly downregulated during natural senescence in SICCX1-LIKE-OE plants (Figure 9C). The senescence-related genes were also induced in SICCX1-LIKE-OE plants (Figure 9D). Magnesium is a major component in chlorophyll and an activator of many enzymes. If calcium content declines, magnesium may replace some of the calcium. Therefore, downregulation of magnesium ions affects leaf growth and accelerates leaf aging. Mg$^{2+}$ concentration was decreased in the OE plants (Figure 10). These results indicated that SICCX1-LIKE-OE regulated the Ca$^{2+}$ and Mg$^{2+}$ concentration to accelerate the aging of leaves in the OE plants.

**Subcellular Localization of SICCX1-LIKE**

AtCCX2, a member of the *Arabidopsis* CCX subfamily, regulates Ca$^{2+}$ concentrations in the ER, while AtCCX3
and AtCCX5 are localized to the endomembrane system (Morris et al., 2008). To test CCX family localization in tomato, the SICCX1-LIKE was chosen as representative member and fused to GFP for the subcellular localization analysis (Figure 11). The transient gene expression assay showed that SICCX1-LIKE, a putative cation/Ca\textsuperscript{2+} exchanger, may be localized to the tonoplast, ER, and plasma membrane.
DISCUSSION

Recently, extensive research has focused on the regulation of cytosolic calcium concentration and its response to the internal and external stimuli, during growth and developmental processes in plants (Cai and Lytton, 2004; Hirschi, 2004; Taneja et al., 2016). A recent study analyzing the *A. thaliana* genome identified 10 CCX genes sharing high similarity with known CCXs from other species (Mäser et al., 2001). In the present study, five of the CCX genes were characterized in tomato. Their sequence and expression analyses will contribute to a better understanding of this gene family and provide potential functional characterization of the important members of the CCX family in tomato.

Functional Differences Related to SICCX Family Structure Characteristics

In this study, we identified five CCX genes in tomato. From the analysis sequence length, pI, and MW (Table 1), we verified that the CCX genes of tomato share the similarities with
FIGURE 8 | Quantitative real-time PCR validation of differentially expressed genes. (A–C) IAA response/signal-related genes; (D–F) ETH response/signal-related genes; (G–I) ABA response/signal-related genes; (J) ROS signal-related genes; (K) and (L), Mg²⁺ related genes. *Significant differences with \( P < 0.05 \) determined using a Duncan’s test compared with the control.

FIGURE 9 | The content of the \( \text{O}_2^- \) (A) and \( \text{H}_2\text{O}_2 \) (B) in WT and OE plant of 25 days seedlings. Chl content (C) and relative expression of WT and OE plants related with leaf senescence (D). SNRK 2, sucrose non-fermenting 1-related protein kinase 2; SAG15, senescence-associated gene15; PYL9, pyrabactin resistance 9. Relative expression levels of leaves senescence with OE plants compare with control (WT). The experiments were repeated three times. Error bars indicate the means ± SE of three independent replicates. Expression data of WT was normalized to 1. *Significant differences with \( P < 0.05 \) determined using a Duncan’s test compared with the control.
FIGURE 10 | The concentration of (B) Ca$^{2+}$ and (A) Mg$^{2+}$ in WT and SICX1-LIKE-OE plants of 25 days seedlings. The experiments were repeated three times. Error bars indicate the means ± SE of three independent replicates. *Significant differences with $P < 0.05$ determined using a Duncan’s test compared with the control.

FIGURE 11 | Subcellular localization of SICX1-LIKE in the leaves of Nicotiana benthamiana. (A) Tonoplast marker; (B) ER marker; (C) plasma membrane marker.

*Arabidopsis* and rice, suggesting functional similarity and an evolutionarily conserved nature. While two, four, three, three, four, one, three, and two orthologous proteins from other species of the Solanaceae family (namely *S. pennellii*, *S. tuberosum*, *N. attenuata*, *N. tabacum*, *C. annuum*, *N. sylvestris*, *C. baccatum*, and *C. chinense*, respectively) were constructed with the NJ method, dividing all CCXs into three well-supported groups (Figure 2), consistent with a previous study (Blatt, 2000). It has been demonstrated that structural divergence plays important roles in the evolution of multiple gene families, mainly through
throughout the plant and the was expressed throughout the Arabidopsis contrast, AtCCX3 shows continuous green coloration during leaf senescence. In development. Li et al. (2016) found that AtCCX1 might play a different role in the development of source-sink expression levels in root and leaves suggest that SlCCX1-LIKE during the flowering stages and fruit development but lower expression of SlCCX1-LIKE-OE plants compared with WT showed decreased expression of SlCCX1-LIKE. The SlCCX1-LIKE levels were much higher when exposed to MgCl₂ or MnCl₂. Furthermore, 8 h of exposure to NaCl and CaCl₂ slightly increased the expression levels of SlCCX1-LIKE. The expression of SlCCX4 was dramatically increased after 24 h of exposure to CaCl₂. In contrast, the expression of SlCCX4 was increased after only 8 h of exposure to Na⁺ and Mg²⁺. Collectively, expression and motif-related results demonstrate that SlCCXs participate in diverse cation homeostasis processes. OE-SlCCX1-LIKE plants showed normal growth before flowering, but leaf senescence was visible at around flowering, when the source/sink relationship changes dramatically, suggesting that the change in source/sink relationship may elevate the requirement for SlCCX1-LIKE activity to maintain proper cation balance.

Plant hormones have been extensively reported to regulate leaf senescence, with ethylene, abscisic acid, functioning as inducers and auxin as inhibitors (Gan and Amasino, 1997). Transcriptome data of SlCCX1-LIKE-OE plants compared with WT showed that most genes related to IAA, ABA, and ETH hormone signaling pathways were differentially expressed. Many SAUR genes are significantly downregulated in SlCCX1-LIKE-OE plants (Supplementary Table 3). In addition, our results showed two PP2C were downregulated. PP2Cs from inhibiting SnRK2s, which regulate the expression of phosphorylated transcription factors [ABA-responsive element-binding factors (ABFs)] to induce ABA-responsive SAGs, creating the yellowing symptoms of leaf senescence (Furihata et al., 2006). Ethylene has long been considered a key endogenous regulator of leaf senescence. Our results reveal that the expression of ethylene receptor, EIN3, ERF were upregulated in OE plants. Hence, SlCCX1-LIKE might induce the ABA and ETH signal to regulate their leaf mediation leaf senescence in tomato.

### SICCX1-LIKE Regulates Leaf Senescence by Mediating Mg²⁺ Signaling and ROS Production

Normally, up to 20% of the total Mg in plants is bound in chlorophyll (Karley and White, 2009; Cakmak and Yazici, 2010). However, this proportion is able to exceed 50% in both Mg deficiency and low light conditions (Dorenstouter et al., 1985), suggesting that prior to other biological processes, Mg²⁺...
is preferentially used for photosynthesis in chloroplasts. The step for chlorophyll synthesis is the insertion of Mg$^{2+}$ into protoporphyrin IX, which is catalyzed by Mg chelatase in an ATP-dependent reaction (Willows, 2003). Mg chelatase is a heterotrimeric enzyme composed of ChII, ChID, and ChIH in plants and which utilizes Mg$^{2+}$, ATP, and protoporphyrin IX as cofactors and substrates. Our study showed that the expression of ChII, ChID, and ChIH were all downregulated in OE plants, including Solyc10g008740 (ChII) (6.35-fold), Solyc04g015490 (ChID) (3.84-fold), and Solyc04g015750 (ChIH) (4.53-fold) (Gibson et al., 1995; Supplementary Table 4). These results suggest that the activity of Mg chelatase has been inhibited in SICCX1-LIKE-overexpressing plants. Decreased plant chlorophyll content leads to accelerated chlorosis of transgenic plants.

According to the biological phylogenetic tree analysis, SICCX1-LIKE and AtCCX1 belong to the same branch. AtCCX1 could be induced by long-term Mg$^{2+}$ deletion, and decrease of external Ca$^{2+}$ attenuated the plant growth retardation under Mg-deficient conditions (Lenz et al., 2013). In this study, the expression of SICCX1-LIKE is induced by MgCl$_2$ and MnCl$_2$. Genes related to of Mg$^{2+}$ signaling (Solyc10g008740.3 and Solyc10g077040.2) were decreased in OE plants. In plants, it had been reported that low manganese could eliminate the active oxygen and protect the membrane lipid from the hypoxia-induced damages (Li et al., 2012). Analysis of ROS content and related genes revealed that both were induced in SICCX1-LIKE-OE plants. Thus, SICCX1-LIKE maybe play a crucial role in transporting intracellular manganese to control the ROS level, affecting the leaf senescence process. In addition, ferredoxin (Fd) was recruited into the photosynthetic electron transport chain of thylakoids to mediate electron transfer from photosystem I (PSI) to a number of metabolic pathways, including NADP$^+$ reduction and carbon and nitrogen assimilation. Our results showed that the expression of Fd was all downregulated in OE plants, including Solyc11g006910 (1.88-fold), Solyc10g075160 (13.4-fold), and Solyc03g005190 (7.08-fold) (Supplementary Table 4). Nitrate is reduced to nitrite by the enzyme nitrate reductase (NR) using six moles of the reduced form of ferredoxin. The activity of transgenic NR to reduce nitrite to ammonium ions via enzyme nitrite reductase (NiR) using six moles of the reduced form of ferredoxin. The activity of transgenic NR to reduce nitrite to ammonium by obtaining electrons from Fd decreased (Guan et al., 2018). Therefore, Fd and NiR may be the key factors in regulating the process of nitrogen assimilation (Davenport et al., 2015). Our results showed that the expression of ferredoxin-nitrite reductase and ferredoxin-NAD$^+$ reductase were all downregulated in OE plants, including Solyc01g108630 (12.32-fold), Solyc10g050890 (3.3-fold), and Solyc02g083810 (7.0-fold) (Supplementary Table 4). These results suggest that the activity of nitrogen assimilation has been inhibited in SICCX1-LIKE-overexpressing plants.

The data shown here indicate that the accelerating leaf-yellowing process observed in SICCX1-LIKE-OE plants may be caused by the Mg$^{2+}$ signaling and ROS homeostasis. This emphasizes the Mg deficiency affecting plant chlorophyll synthesis, which leads to generate ROS by decreasing the concentration of chlorophyll and the subsequent photosynthetic electron transfer rate and nitrogen assimilation. Hence, the higher ROS level in OE plants may modulate a variety of senescence processes through interacting with diverse pathways including hormonal regulation. Therefore, SICCX1-LIKE regulates leaf senescence probably via Mg$^{2+}$ signaling as well as modulation of ROS homeostasis.

**SICCX1-LIKE Is Localized in the Endomembrane System**

Functional epitope tags of SICCX1-LIKE demonstrated that SICCX1-LIKE localized to the endomembrane in plants. Furthermore, SICCX1-LIKE appeared to localize to the plant vacuolar membrane, endoplasmic reticulum, and plasma membrane to function as a cation transporter (Figure 10). Whether SICCX1-LIKE is localized exclusively to the trans-Golgi network and prevacuolar compartment remains unclear. SICCX1-LIKE might have a role in a specific subset of excess cation uptake in OE plants because of high SICCX1-LIKE expression levels. Furthermore, the localization of SICCX1-LIKE to the plant vacuolar, endoplasmic reticulum, and plasma membrane, combined with its overexpression in leaves may transport different cations to regulate leaf senescence in tomato. However, the precise subcellular localization of CCX1-LIKE remains to be elucidated.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

JL designed the experiments and edited the manuscript. JL, CC, and YZ performed the experiments. JL analyzed the data. JL and XL wrote the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.683904/full#supplementary-material
REFERENCES

Aliu, S., Rusinovici, I., Fethau, S., Gashi, B., Simenovska, E., and Rozma, L. (2015). The effect of salt stress on the germination of maize (Zea mays L.) seeds and photosynthetic pigments. Acta Agric. Slov. 10, 85–94. doi: 10.14720/aas.2015.105.1.09

Blatt, M. R. (2000). Cellular signaling and volume control in stomatal movements in plants. Annu. Rev. Cell Dev. Biol. 16, 221–241. doi: 10.1146/annurev.cellbio.16.1.221

Bona, M. C., and Michelis, M. I. D. (2011). The plant Ca2+-ATPase repertoire: biochemical features and physiological functions. Plant Biol. 13, 421–430. doi: 10.1111/j.1399-3003.2010.01045.x

Bresson, J., Bieker, S., Riester, L., Doll, J., and Zentgraf, U. (2018). A guideline for leaf senescence analyses: from quantification to physiological and molecular investigations. J. Exp. Bot. 69, 769–786. doi: 10.1039/jxbcr246

Cai, X., and Lytton, J. (2004). The cation/Ca2+ exchanger super family: phylogenetic analysis and structural implications. Mol. Biol. Evol. 19, 1692–1703. doi: 10.1093/molbev/msi173

Cakmak, I., and Yatzic, A. M. (2010). Magnesium: a forgotten element in crop production. Better Crops 94, 23–25.

Corso, M., Doccula, F. G., de Melo, J. R. F., Costa, A., and Verbruggen, N. (2018). Nitrate metabolism in tobacco leaves overexpressing Arabidopsis thaliana by regulating ER and cytosolic Ca2+ dynamics in plants. A plant Ca2+/cation antiporters (CaCA): identification, characterization and expression profiling in Arabidopsis thaliana. Plant Physiol. 176, 2045–2065. doi: 10.1111/pbi.12563

Dodd, A. N., Kudla, J., and Sanders, D. (2010). The language of calcium signaling. Annu. Rev. Plant Biol. 61, 593–620.

Dorenstouter, H., Pieters, G. A., and Findenegg, G. R. (1985). Distribution of magnesium between chlorophyll and other photosynthetic functions in magnesium deficient “sun”; and “shade”; leaves of poplar. J. Plant Nutr. 8, 1089–1110. doi: 10.1080/01904168509363409

Emery, L., Whelan, S., Hirschi, K. D., and Pittman, J. K. (2012). Protein phylogenetic analysis of Ca2+/cation antiporters and insights into their evolution in plants. Front. Plant Sci. 3:1. doi: 10.3389/fpls.2012.00001

Ferguson, I. B. (1984). Calcium in plant senescence and fruit ripening. Plant Cell Environ. 7, 477–489. doi: 10.1111/j.1365-3040.1984.tb01438.x

Furukawa, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K., et al. (2006). Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc. Natl. Acad. Sci. U.S.A. 103, 1988–1993. doi: 10.1073/pnas.0505667103

Gan, S., and Amasino, R. M. (1997). Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). Plant Physiol. 113, 313–319. doi: 10.1104/pp.113.2.313

Gibson, L. D., Willows, R. D., Kannangara, D., von Wettstein, C. N., and Hunter, C. G. (1995). Magnesium-protoporphyrin chelatase of Rhodobacter sphaeroides: reconstitution of activity by combining the products of the bchA, -I, and -D genes expressed in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 92, 1941–1944. doi: 10.1073/pnas.92.6.1941

Guo, P., Chen, H., Song, C., Bouraoui, N., Wang, K., and Tian, H. L. (2018). FdC1 and leaf-type ferredoxins channel electrons from photosystem I to different downstream electron acceptors. Front. Plant Sci. 9:410. doi: 10.3389/fpls.2018.00410

Harper, J. F. (2001). Dissecting calcium oscillators in plant cells. Trends Plant Sci. 6, 395–397. doi: 10.1046/s1360-1385(01)00202-4

Hirschi, K. D. (2004). The calcuim caonundrum. Both versatile nutrient and 10.1105/tpc.113.113340

Li, Z., Wang, X. L., Chen, J. Y., Gao, J., Zhou, X., and Kusai, B. K. (2016). CCX1, a putative cation/Ca2+ exchanger, participates in regulation of reactive oxygen species homeostasis and leaf senescence. Plant Cell Physiol. 57, 2611–2619. doi: 10.1093/pcp/pct175

Liang, F., Cunningham, K. W., Harper, J. F., and Sze, H. (1997). ECA1 complements yeast mutants defective in Ca2+ pumps and encodes an endoplasmic reticulum-type Ca2+-ATPase in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A. 94, 8579–8584. doi: 10.1073/pnas.94.16.8579

Lim, P. O., Kim, H. J., and Nam, H. G. (2007). Leaf senescence. Annu. Rev. Plant Biol. 58, 115–116.

Liu, X., Xu, X., Dong, F. X., Liu, Y. D., Liu, Z. H., Shi, Z. H., et al. (2016). The role of gibberellins and auxin on the tomato cell layers in pericarp via the expression of ARFs regulation by miRNAs in fruit set. Acta Physiol. Plant. 38, 77–88.

Ma, W., Smigel, A., Walker, R. K., Moeder, W., Yoshioika, K., and Berkowitz, G. A. (2010). Leaf senescence signaling: the Ca2+-conducting Arabidopsis cyclic nucleotide gated channel2 acts through nitric oxide to repress senescence programming. Plant Physiol. 154, 733–742. doi: 10.1104/pp.110.161356

Marchler-Bauer, A. (2011). Database resources of the national center for biotechnology information. Nucleic Acid Res. 40, 13–25. doi: 10.1093/nar/gnt121

Maser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., et al. (2001). Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiol. 126, 1646–1647. doi: 10.1104/pp.126.4.1646

Morris, J., Tian, H., Park, S., Ward, J. M., and Hirschi, K. D. (2008). AtCCX3 is an Arabidopsis endomembrane H+–dependent K+ transporter. Plant Physiol. 148, 1474–1486. doi: 10.1104/pp.108.118810

Nam, H. G., Thomas, H., and Stoddart, J. L. (2008). Leaf senescence. Annu. Rev. Plant Biol. 31, 83–111.

Noode, L. D. (1988). Senescence and Aging in Plants. Cambridge, MA: Elsevier Academic Press, 526.

Park, J. H., Oh, S. A., Kim, Y. H., Woo, H. R., and Nam, H. G. (1998). Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. Plant Mol. Biol. 37, 445–454.

Shimazaki, K., Doh, M., Assmann, S. M., and Kinoshita, T. (2007). Light regulation of stomatal movement. Annu. Rev. Plant Biol. 58, 219–247. doi: 10.1146/annurev.arplant.57.032905.105434

Singh, A., Kanwar, P., Yadav, A. K., Mishra, M., Jha, S. K., Baranwal, V., et al. (2014). Genome-wide expressional and functional analysis of calcium transport elements during abiotic stress and development in rice. FEBS J. 281, 894–915. doi: 10.1111/febs.12656

Sze, H., Liang, F., Hwang, I., Curran, A. C., and Harper, J. F. (2000). Diversity and regulation of plant Ca2+ pumps: insights from expression in yeast. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 433–462. doi: 10.1146/annurev.arplant.51.1.433

Taneja, M., Tyagi, S., Sharma, S., and Upadhay, S. K. (2016). Ca2+/cation antiporters (CaCA): identification, characterization and expression profiling in bread wheat (Triticum aestivum L.). Front. Plant Sci. 7:1775. doi: 10.3389/fpls.2016.01775

Telf, N., Stammitti-Bert, L., Mortain-Bertrand, A., Maucourt, M., Carde, J. P., Rollin, D., et al. (2006). Sucrose deficiency delays lycopene accumulation in
tomato fruit pericarp discs. Plant Mol. Biol. 62, 453–469. doi: 10.1007/s11103-006-9033-y
Tuttle, M. S., Radisky, D., Li, L., and Kaplan, J. (2003). A dominant allele of PDR1 alters transition metal resistance in yeast. J. Biol. Chem. 278, 1273–1280. doi: 10.1074/jbc.M209631200
White, P. I., and Broadley, M. R. (2003). Calcium in plants. Ann. Bot. 92, 487–511.
Willows, R. D. (2003). Biosynthesis of chlorophylls from protoporphyrin IX. Nat. Prod. Rep. 20, 327–341. doi: 10.1039/b110549n
Woo, H. R., Kim, H. J., Lim, P. O., and Nam, H. G. (2019). Leaf senescence. Annu. Rev. Plant Biol. 70, 347–376.
Xu, Z. F., Jia, K., Xu, B., He, A. N., Li, J., Deng, Y., et al. (2010). Effects of MK-801, taurine and dextromethorphan on neurotoxicity caused by manganese in rats. Toxicol. Ind. Health 26, 55–60. doi: 10.1177/0748233709359275
Yadav, A. K., Shankar, A., Jha, S. K., Kanwar, P., Pandey, A., and Pandey, G. K. (2015). A rice tonoplastic calcium exchanger, OsCCX2 mediates Ca2+/cation transport in yeast. Sci. Rep. 5:17117.
Yue, H., Li, Z., and Xing, D. (2012). Roles of Arabidopsis bax inhibitor-1 in delaying methyl jasmonate-induced leaf senescence. Plant Signal. Behav. 7, 1488–1495. doi: 10.4161/psb.21776
Zentgraf, U., Jobst, J., Kolb, D., and Rentsch, D. (2004). Senescence-related gene expression profiles of rosette leaves of Arabidopsis thaliana: leaf age versus plant age. Plant Biol. 6, 178–183. doi: 10.1055/s-2004-815735
Zhang, X., Zhang, M., Takano, T., and Liu, S. (2011). Characterization of an AtCCX5 gene from Arabidopsis thaliana that involves in high-affinity K+ uptake and Na+ transport in yeast. Biochem. Biophys. Res. Commun. 414, 96–100. doi: 10.1016/j.bbrc.2011.09.030

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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