GsCML27, a Gene Encoding a Calcium-Binding Ef-Hand Protein from Glycine soja, Plays Differential Roles in Plant Responses to Bicarbonate, Salt and Osmotic Stresses

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Abstract

Calcium, as the most widely accepted messenger, plays an important role in plant stress responses through calcium-dependent signaling pathways. The calmodulin-like family genes (CMLs) encode Ca2+ sensors and function in signaling transduction in response to environmental stimuli. However, until now, the function of plant CML proteins, especially soybean CMLs, is largely unknown. Here, we isolated a Glycine soja CML protein GsCML27, with four conserved EF-hands domains, and identified it as a calcium-binding protein through far-UV CD spectroscopy. We further found that expression of GsCML27 was induced by bicarbonate, salt and osmotic stresses. Interestingly, ectopic expression of GsCML27 in Arabidopsis enhanced plant tolerance to bicarbonate stress, but decreased the salt and osmotic tolerance during the seed germination and early growth stages. Furthermore, we found that ectopic expression of GsCML27 decreases salt tolerance through modifying both the cellular ionic (Na+, K+) content and the osmotic stress regulation. GsCML27 ectopic expression also decreased the expression levels of osmotic stress-responsive genes. Moreover, we also showed that GsCML27 localized in the whole cell, including cytoplasm, plasma membrane and nucleus in Arabidopsis protoplasts and onion epidermal cells, and displayed high expression in roots and embryos. Together, these data present evidence that GsCML27 as a Ca2+-binding EF-hand protein plays a role in plant responses to bicarbonate, salt and osmotic stresses.

Introduction

Crop growth and productivity is adversely affected by environmental challenges, such as salinity, alkalinity and osmotic stresses. Sodium bicarbonate stress, including HCO3-, CO32-, Na+ and high pH induces the disorder of intracellular pH and hypersmotic stress in plant cells, and elicits adverse effects plant on growth [1–5]. With the development of transcriptional
profiling approaches, recent studies have identified the bicarbonate responsive genes in *Tamarix androssowii* by using gene chip analysis [6], in *Glycine soja* by using transcriptome analysis [7] and in *tomato* by using iTRAQ-based analysis [8]. A handful of researches also characterized some genes involved in plant tolerance to bicarbonate stress. For example, *GsTIFY10* have been identified as a positive regulator of plant tolerance to bicarbonate stress [9], and overexpression of *H+-Ppase* improves saline-alkaline tolerance in *Arabidopsis* [10]. However, the physiological and biochemical mechanism of plant bicarbonate stress responses is still unclear.

In addition to bicarbonate stress, neutral salt and osmotic stresses also induce a wide range of complex cellular and physiological changes in plants. The signaling of salt and osmotic stresses include ionic and osmotic homeostasis signaling [11–14], as well as signaling to coordinate cell division and expansion suitable for the particular stress conditions [15, 16]. Many researches also demonstrated the crosstalk of salt stress and osmotic stress [17–19]. For example, expression of the osmotic stress-responsive genes *RD22, P5CS* and *COR47* can also be induced by salt stress [14, 20–22]. However, several studies also revealed the differences among plant responses to bicarbonate stress, neutral salt and osmotic stresses [5, 23]. Therefore, besides the common molecular basis and signal transduction pathway, it is also important to discover how plant differentially perceive and transmit the signals under these different stresses.

Calcium (Ca²⁺), as the most widely accepted second massager, plays an important role in plant stress responses [24–26], and cytoplasmic Ca²⁺ signal is recognized by Ca²⁺ sensors [27–29]. Ca²⁺ sensors can be broadly divided into four groups, including calmodulins (CaM), calmodulin-like proteins (CMLs), calcium dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs) [30, 31]. CMLs share at least 16% amino acid identity with CaMs, and are defined by the presence of two to six EF-hands motifs, and EF-hand motif is a helix-loop-helix structure that can bind a single Ca²⁺ ion [32]. There are 50 CML genes in *Arabidopsis*, and expression analysis suggests that they can be induced by various environmental stresses [33, 34]. Among them, *CML24* is identified as a salt responsive gene, and its overexpression in *Arabidopsis* lines are more tolerant to various ions including Co²⁺, Zn²⁺ and Mg²⁺ [35]. *CML18* plays a role in salinity tolerance through direct interaction with the Na⁺/H⁺ antiporter NHX1 [36]. Furthermore, *CML9* is suggested to be a negative regulator of ABA (Abscisic acid) -dependent salinity tolerance [37]. In addition, *CML37, CML38* and *CML39* also respond to a variety of environmental stimuli [38]. However, functional evidence of soybean CMLs different environmental stresses is still limited.

In this study, we isolated a CML protein GsCML27 from the salt-alkaline resistant wild soybean *Glycine soja* (07256) [39]. GsCML27 contains four conserved calcium-binding EF-hand motifs and showed Ca²⁺ binding affinity in vitro. We demonstrated the induced expression of GsCML27 in response to bicarbonate, salt and osmotic stresses. What is interesting is GsCML27 ectopic expression in *Arabidopsis* enhanced plant tolerance to bicarbonate stress, but decreased salt and osmotic tolerance.

**Results**

**Cloning and sequence analysis of GsCML27**

In our previous study [40], GsCML27 was identified as a putative bicarbonate stress (50mM NaHCO₃, pH8.5) responsive gene, by transcriptome sequencing data of the wild type soybean *Glycine soja* G07256 (S1 Fig). In this study, the full-length CDS of GsCML27 was obtained by homologous cloning with gene-specific primers designed according to the transcript sequence of *Glycine max* homolog (Glyma08g05810). GsCML27 contains an open reading frame of 543
bp that encoded a protein of 181 residues with a predicted molecular weight of 19.7 kDa and an isoelectric point of 4.32.

Protein sequence analysis showed GsCML27 contains four highly conserved calcium-binding EF-hand domains, and shared 47.8% to 61.7% sequence similarity with Arabidopsis CML family proteins (http://www.phytozome.org/) [41, 42] (Fig 1A). Among them, GsCML27 displayed the highest amino acid sequence similarity (61.7%) with AtCML27. Phylogenetic analysis showed that GsCML27 was clustered with other CML27-like proteins, such as Cicer arietinum, Fragaria vesca subsp. Vesca and Morus notabilis (Fig 1B). These results indicated that GsCML27 might be one of CML family members.

Fig 1. Sequence analysis of GsCML27. A. Multiple sequence alignment of the full-length amino acid sequences of GsCML27 with homologous CMLs from Arabidopsis. Sequences were aligned using MEGA 5.0. B. The phylogenetic relationships between GsCML27 and homologous protein kinases with the conserved amino acid sequences. The phylogenetic tree was constructed using MEGA 5.0.

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Circular dichroism (CD) spectroscopy and Subcellular localization of GsCML27

The four EF-hand motifs in GsCML27 indicated potential binding affinity to Ca^{2+}. We expressed and purified the GsCML27-His fusion protein from *E. coli* BL21. Far-UV CD spectroscopy was used to assess the impact of Ca^{2+} binding on the conformation of GsCML27. As shown in Fig 2A, the addition of CaCl_{2} to GsCML27 protein results in a slight increase in the 207–244 nm range suggesting a Ca^{2+}-induced increase in α-helical content. Deconvolution of the data predicts a 55% increase in helical content from 10% to 65%. These results suggested that GsCML27 could bind Ca^{2+} and might undergo a Ca^{2+}-dependent conformational change [43, 44].

In order to investigate the sub-cellular localization of GsCML27, the CDS region of GsCML27 was in-frame fused with the enhanced green fluorescence protein (eGFP) to generate 35S::GsCML27-eGFP. The eGFP and GsCML27-eGFP fused protein were transiently expressed in *Arabidopsis* protoplasts respectively. The green fluorescence was observed by confocal scanning. The eGFP alone was used as control and result showed that eGFP localized to the entire *Arabidopsis* protoplast cell. Confocal imaging showed that GsCML27-eGFP protein was expressed in the whole protoplast cell, including cytoplasm, plasma membrane and nucleus (Fig 2B).

To further confirm the subcellular localization of GsCML27 in protoplasts. The plasmids eGFP and GsCML27-eGFP were transiently expressed in onion epidermal cells. The fluorescence of eGFP alone appeared through the whole cell. GsCML27-eGFP was also observed in the whole protoplast cell, which is consistent with results from protoplast cells (Fig 2C). Taken together, these results suggested that GsCML27 encodes a ubiquitously expressed calcium-binding protein.

Expression profiles of GsCML27 in Glycine soja

To characterize the spatial expression pattern of GsCML27 in *Glycine soja*, the expression levels of GsCML27 in different tissues were detected by quantitative RT-PCR analysis. The results showed that GsCML27 was expressed in most of the tissues in this study. The highest expression of GsCML27 was observed in roots and embryos, and little expression was obtained in old stems (Fig 3A), indicating tissue specificity of GsCML27 expression in *Glycine soja*.

In order to investigate the potential role of GsCML27 in stress responses, we then verified the bicarbonate stress induced expression of GsCML27 in *Glycine soja*. Consistent with the RNA-seq data, the quantitative RT-PCR results confirmed that expression of GsCML27 increased and reached a maximum point at 1 h (about 70 folds) under bicarbonate stress (Fig 3B). Then GsCML27 transcripts decreased to the basal level after 3 h, indicating GsCML27 responded to bicarbonate stress at an early stage.

Considering the fact that ion poison and osmotic stress always occurred simultaneously with bicarbonate stress [45, 46], we also determined the expression profiles of GsCML27 under 200 mM NaCl (salt stress) and 350 mM mannitol (osmotic stress) treatments (Fig 3C and 3D). As expected, GsCML27 expression was also induced by both salt and osmotic stresses, and displayed similar patterns compared to bicarbonate stress. Notably, the increase of GsCML27 expression under osmotic stress (about 700 folds) was obviously greater than that under salt stress (about 14 folds). Taken together, these results indicated that GsCML27 might be involved in plant responses to bicarbonate, salt and osmotic stresses.

The opposite roles of GsCML27 in response to bicarbonate versus salt/osmotic stresses

In order to determine whether GsCML27 is involved in plant responses to bicarbonate, salt and osmotic stresses, transgenic *Arabidopsis* plants were generated by overexpressing GsCML27.
Fig 2. Circular dichroism (CD) spectroscopy assay and subcellular localization of GsCML27. A. Ca\(^{2+}\)-induced conformational changes of the GsCML27. B. Ca\(^{2+}\)-induced conformational changes of the GsCML27.
were monitored by far-UV CD spectroscopy. Spectra were collected on samples in 5 mM Tris-HCl, pH 6.9, in the presence of either 5 mM CaCl₂ or 5 mM EGTA. Results of all CD measurements are expressed as mean molar ellipticity [θ].

B. Subcellular localization of GsCML27 protein in Arabidopsis protoplasts. eGFP-tagged GsCML27 fusion protein and eGFP alone protein were transiently expressed in the protoplasts prepared from 3-week-old Arabidopsis leaves and checked the eGFP signal by using a confocal laser-scanning microscope. The eGFP or the GsCML27-eGFP fusion protein examined under fluorescent-field illumination to examine GFP fluorescence (left); Fluorescent-field illumination for chlorophyll fluorescence was used to examine chlorophyll fluorescence (middle), followed by the confocal microscopy for an overlay of GFP and chlorophyll fluorescent illumination (right). C. Subcellular localization of GsCML27 protein in onion epidermal cells. eGFP-tagged GsCML27 fusion protein and eGFP fluorescence, bright field and an overlay of bright and fluorescent illumination are shown.

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under the control of strong constitutive CaMV35S promoter. Three independent homozygous transgenic lines (#13, #15 and #33) were obtained and the transcript abundance of GsCML27 was verified by semi-quantitative RT-PCR and quantitative RT-PCR analysis (Fig 4A).

We firstly performed the plate germination assays to determine the stress tolerance of WT and GsCML27 ectopic expression Arabidopsis lines (Fig 4B). Under standard culture conditions, GsCML27 ectopic expression lines exhibited similar seed germination and early seedling growth with WT, suggesting that GsCML27 did not affect seed germination and early seedling development (Fig 4C). However, under 10 mM NaHCO₃ stress treatment, GsCML27 ectopic expression lines displayed higher seed germination rates (Fig 4C) and more seedlings with open

Fig 3. Expression profiles of GsCML27 in Glycine soja. A. Tissue specific expression of GsCML27 in Glycine soja. Total RNA was extracted from root, young stem, old stem, young leaf, old leaf, flower, embryo and episperm from 5-week-old soil-grown Glycine soja, and relative expression levels were determined by quantitative RT-PCR using GADPH as an internal control. B. Total RNA was extracted from roots of 3-week-old Glycine soja seedlings treated with 50 mM NaHCO₃, 200 mM NaCl (salt stress) and 350 mM mannitol (osmotic stress) treatments, respectively. Relative transcript levels were determined by quantitative RT-PCR and GAPDH were used as internal controls. The mean value from three fully independent biological repeats and three technical repeats is shown.
and green leaves (Fig 4D) than WT. Unexpectedly, on 1/2 MS medium containing 125 mM NaCl or 300 mM mannitol, WT exhibited better at seed germination (Fig 4C) and seedlings growth (Fig 4D), as evidenced by higher seed germination rates and more seedlings with open and green
leaves than ectopic expression lines. These results demonstrated that GsCML27 enhanced plant tolerance to bicarbonate stress, but decreased the salt and osmotic tolerance.

**GsCML27 influences plant responses to K⁺ ionic stress**

Salt stress affects plant growth and development mainly in two ways: ionic poison and osmotic stress. As described above, we have demonstrated GsCML27 participated in the osmotic regulation in plant cells, showing decreased tolerance to 300 mM mannitol treatment. Hence, we then explored whether GsCML27 responded to different ionic stresses.

As shown in S2 Fig, in the presence of different KCl concentration gradients (50, 100, 150 mM KCl), GsCML27 ectopic expression lines displayed no differences in the seed germination rates from WT. However, under 100 mM KCl treatment, ectopic expression lines showed much lower percentages of seedlings with open and green leaves on the 7th day after germination (Fig 5A). Furthermore, no significant differences in seed germination (S2 Fig) and early seedling growth (Fig 5B) were observed between WT and ectopic expression lines under different LiCl (10, 20, 30 mM) concentration gradients. These results indicated that GsCML27 regulated plant salt tolerance by modifying both cellular ionic content (mainly Na⁺, K⁺) and osmotic regulation.

**GsCML27 regulated the expression levels of osmotic stress responsive marker genes**

The analysis of some stress-inducible marker genes is a hallmark of stress adaptation in plants [47]. Considering the huge changes of GsCML27 under osmotic stress, we then examined the expression patterns of some osmotic stress induced genes, including COR47, RD22 and P5CS. As shown in Fig 6, their expression levels were obviously induced by osmotic stress. However, their expression levels were significantly down-regulated in GsCML27 ectopic expression lines compared to WT, except for P5CS at 3 h. Therefore, these results suggested that GsCML27 regulated the expression levels of osmotic stress responsive marker genes.

**Discussion**

Plants have evolved a diversity of unique proteins containing evolutionarily conserved EF-hand motifs to bind Ca²⁺ [48, 49]. Among them, CML (CAM-like) family, proteins are mostly composed of 2 to 6 EF-hands, and play important roles in plant growth, development and environmental stimuli stress responses [33]. Indeed, studies have revealed the important function of CML42 during cell branching in trichomes [50]. CML18 was involved in salinity tolerance and CML9 knockout enhanced plant tolerance to both salinity and drought stresses [36, 51]. However, most members of CMLs family are functionally uncharacterized. In this study, we isolated and characterized GsCML27, a calcium-binding EF-hand protein from Glycine soja.

Studies have revealed both GsTIFY10 and AtTIFY10 have positive function in alkaline responses [52, 53]. Overexpression of GhWRKY25 in Nicotiana benthamiana enhances plant tolerance to salt stress [54], overexpression of WRKY25 is also sufficient to increase Arabidopsis NaCl tolerance [55]. In this study, GsCML27 shared the highest amino acid similarity with AtCML27 (61.7%) among the 50 CML proteins in reference plant Arabidopsis [33]. Similar to AtCML27 and other Arabidopsis CMLs proteins, GsCML27 contains four conserved EF-hand motifs (Fig 1A). So we indicate that AtCML27 may have similar function with GsCML27. Studies have shown Arabidopsis CML8 [56] and CML24 [57] have Ca²⁺-binding ability so that they can take part in signaling transduction in response to diverse stimuli. As expected, we also verified the Ca²⁺-binding property of GsCML27 protein through the far-UV CD spectroscopy (Fig 2A).
The majority of CMLs are predicted to be located in cytosolic or nuclear in *Arabidopsis* [33]. *CML42*, a calcium sensor, localized to both cytosol and nucleus responses to spodoptera herbivory and abiotic stresses[58]. To prove the GsCML27 might correlate with the spatial pattern of Ca$^{2+}$ elevation, we observed the localization of GsCML27. As a result, we observed GsCML27 protein are localized in the whole cell, including cytoplasm, plasma membrane and nucleus (Fig 2B and 2C). So it can be assumed that GsCML27 can bind Ca$^{2+}$ and undergo conformational changes to bind target proteins in the whole cell.
Fig 6. GsCML27 ectopic expression altered expression patterns of a set of osmotic stresses signal related genes. Expression patterns of osmotic stress related genes. Relative expression levels were
A handful of research have found that CMLs expression were greatly induced by environmental stresses such as drought, salt and osmotic stress [36–38, 59]. For example, studies have revealed CML37, CML38 and CML39 temporal response to drought, salt and osmotic stresses [60], and AtCML9 played essential roles in modulating responses to salt stress [51]. In the present research, we also found the induced expression of GsCML27 under salt and osmotic stresses (Fig 3C and 3D). It is noteworthy that GsCML27 expression was also strongly induced by bicarbonate stress, as supported by the transcriptome data (S1 Fig) and quantitative RT-PCR assays (Fig 3B). Interestingly, based on the RNA-seq data, we further identified a group of genes co-expressed with GsCML27 (S2 Table). These genes were found to be involved in plant stress responses, including three alkaline responsive genes (GsJAZ2, GsTIFY10a, GsTIFY11b) [52, 61, 62] and nine salt/drought/osmotic responsive genes (for example: calmodulin binding protein 25, transcription factor MYB44, myb domain protein 78) [63–65]. Overall, these data suggested that GsCML27 may extensively regulator plant responses to environmental stresses.

As expected, we further demonstrated the important function of GsCML27 in stress responses. However, intriguingly, GsCML27 ectopic expression promoted seed germination in response to bicarbonate stress, but inhibited seed germination under salt and osmotic stresses during the plate germination assays (Fig 4). Similar phenomenon were also found for CBL1, which serves as a positive regulator of the salt and drought signaling pathways and as a negative regulator of the cold response pathway in Arabidopsis [66]. One explanation for this fact is that the specificity and complexity of GsCML27 function in different stress signaling pathways, and studies also suggest that different pathways may share common components that serve as crosstalk nodes [67].

Salinity exerts negative impact mainly by disrupting the cell ionic and osmotic equilibrium [68, 69]. The decreased tolerance under osmotic stress suggested that GsCML27 was involved in osmotic regulation under salt stress. To explore whether GsCML27 affected salt tolerance only by osmotic regulation or by both ionic and osmotic regulation, we carried out the plate germination assays under KCl and LiCl treatments (Fig 5 and S2 Fig). Our results revealed that besides osmotic regulation, GsCML27 might also affect plant tolerance by modifying intracellular Na⁺/K⁺ content [18, 70]. In line with our studies, CML24 was found to participate in plant responses to ion stress [35]. AtCML18 interacted with AtNHX1 (a vacuolar Na⁺/H⁺ antiporter) in a Ca²⁺/pH-dependent manner, and repressed the Na⁺/H⁺ exchange activity [36]. The expression levels of CML37, CML38 and CML39 also induced by osmotic stress [38]. Moreover, we showed that GsCML27 ectopic expression also down-regulated expression of osmotic responsive genes, such as P5CS, COR47, and RD22 (Fig 6). It is not known, however, how important the mechanisms under salt stress will be an important area for further studies.

Another intriguing things is that no obvious phenotypes between WT and GsCML27 ectopic expression lines was found during the root length assays at the seedling stage (data not show). One possible reason is GsCML27 mainly function at the seed germination and/or early seedlings stages. Our studies also found that GsCML27 displayed high transcript level in the wild soybean seeds, and the expression of GsCML27 significantly decreased with the seed germination and seedling growth (S3 Fig). Additionally, in Arabidopsis, AtEXP2 only confered salt and osmotic stress tolerance at seeding germination stages [71], and overexpression of AtWRKY30 also enhanced abiotic stress tolerance during early growth stages [72].
In conclusion, GsCML27 is a positive regulator of plant tolerance to bicarbonate stress, but a negative regulator of salt or osmotic stresses during early growth stages. And ectopic expression of GsCML27 may influence plant salt tolerance by ion content (Na+/K+) and mainly osmotic stress. However, for the future studies, we will focus on the exact mechanism by which GsCML27 responses to bicarbonate, salt or osmotic stress, and explore the relationship between bicarbonate and salt/osmotic stresses.

Materials and Methods

Plant materials, growth conditions and stress treatments

Seeds of *Glycine soja* (07256) were acquired from Jilin Academy of Agricultural Sciences (Changchun, China). The seeds were treated with 98% sulfuric acid for 15 min, washed with sterilized water, germinated in a dark culture room, and then grew in 1/4 Hoagland solution at 24–26°C and a light regime of 16 h light/8 h dark [52]. For gene expression analysis, the 3-week-old seedlings were transferred into 1/4 Hoagland’s solution containing either 50 mM NaHCO$_3$ (for bicarbonate treatment), or 200 mM NaCl (for salt treatment), or 350 mM mannitol (for osmotic treatment), respectively. Equal amounts of roots were sampled at 0 h, 1 h, 3 h, 6 h and 12 h time points after treatments and the samples were stored at -80°C for RNA extraction.

The *Arabidopsis thaliana* (Columbia) were grown in a greenhouse under controlled environmental conditions (21–23°C, 100 μmol photons m$^{-2}$ s$^{-1}$, 60% relative humidity, 16 h light/8 h dark cycles). For the expression analysis of osmotic responsive marker genes, the 3-day-old wild-type (WT) and GsCML27 ectopic expression transgenic (line #13) plants were harvested from 1/2 MS liquid plates containing 300 mM mannitol.

Isolation and sequence characterization of GsCML27

The CDS region of GsCML27 was obtained by using homologous cloning. Briefly, total RNA was isolated from *Glycine soja* seedling roots by using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and then the cDNA was generated using the SuperScriptTM III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Gene specific primers (FW 5′-CTGTTGAGCGATAGCAATGGC-3′ and RV 5′-CTATAAAATTCA AATCCAAAGGGCC-3′) were designed according to the corresponding gene sequence from *Glycine max* to clone the full-length CDS of GsCML27. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) to clone the full-length CDS of GsCML27. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and subjected to sequencing. Sequence alignment and phylogenetic analysis were carried out by using MEGA 5.0 [73].

GsCML27 protein expression and circular dichroism (CD) spectroscopy assay

The full-length CDS of GsCML27 was amplified by PCR with a forward primer containing a KpnI site 5′-GGGTTACCATGGCCACGAATCG-3′ and a reverse primer containing a SalI site 5′-ACGCGTCTGACATCTGTTTTTTCTCTGACATTG-3′. The PCR products were cloned into the pET-32b vector (Promega, Madison, WI), Where GsCML27 was fused to the C/N-terminus of His-tag. The recombinant plasmid was transformed into *E. coli* BL21. Protein expression was induced with 1 mM isopropyl thiogalactopyranoside (IPTG) at 37°C. Bacterial cells were harvested after IPTG induction for 6 h by centrifuging the culture at 5000 g for 8 min. The cells were resuspended in 8 ml 1/5 Native Binding Buffer (2.5 M NaCl, 250 mM NaH$_2$PO$_4$, pH 8.0), using a sonicator to broken bacteria cells, sonicate the solution on ice using six 15-second bursts at high intensity with a 20-second cooling period between each burst,
centrifuge at 12000 × g for 15 minutes to pellet the cellular debris and transfer the supernatant to a fresh tube. For recombinant protein purification, the procedure followed ProBond™ Purification System (Invitrogen, Carlsbad, CA, USA). Added 8 ml of supernatant under native conditions to a prepared Purification Column, binding 30–60 minutes and then settle the resin by low speed centrifugation (800 × g). Washed with 8 ml Native Wash Buffer (30 ml, 1 x Native Binding Buffer and 100 μl 3M Imidazole, PH 8.0) three more time, and elute the protein with 8 ml Native Elution Buffer (13.75 ml, 1 x Native Binding Buffer and 1.25 ml 3M Imidazole, PH 8.0). Collected fractions and analyzed with SDS-PAGE.

Far-UV CD spectra of GsCML27 assay was carried out according to the method described previous [50, 74, 75]. Far-UV CD spectra were acquired from 190 to 260 nm on a rapid scanning monochromator fitted with a CD module (J-815, Jasco, Japan), using a 0.1 mm path length cylindrical quartz cuvette at 25°C. Spectra were collected on samples in 5 mM Tris-HCl, pH 6.9, in the presence of either 5 mM CaCl2 or 5 mM EGTA. Results of all CD measurements are expressed as mean molar ellipticity \( \theta = \theta^0/100/(ncl) \), where \( n \) is the mean the number of amino acids of the protein, \( c \) is the protein concentration (mg/cm³), and \( l \) is the path length (cm).

**Quantitative RT-PCR**

Quantitative RT-PCR was used to analyze the spatial expression characteristic of GsCML27 soja under 50 mM NaHCO₃, or 200 mM NaCl, or 350 mM mannitol stresses in Glycine soja and expression profiles of GsCML27 and stress responsive marker genes (P5CS, COR47, and RD22) under osmotic stresses in ectopic expression lines. Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and the cDNA was generated using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR was performed by using the SYBR Green Master Mix on an ABI 7500 sequence detection system. ACTIN2 and GAPDH were used as internal controls in Arabidopsis and Glycine soja, respectively [76, 77]. cDNA quality was assessed by PCR using GADPH or ACTIN2 specific primers to exclude genomic DNA contamination. Expression levels for all genes were calculated using the 2\(^{-\Delta\Delta CT}\) method [78]. Three independent biological replicates were carried out and subjected to enable statistical analysis. Primers efficiency was assessed by quantitative PCR and primers used for quantitative RT-PCR are listed in S1 Table.

**Subcellular localization**

GsCML27-eGFP was constructed by digesting the coding region of GsCML27 with NcoI and SpeI and cloning it into the pCAMBIA-1302 vector. Briefly, the full length GsCML27 coding region was PCR amplified with the gene specific primer pair containing an NcoI site in the forward primer (5'-CATGCCATGGCCCTTTGTAAGCGAT-3') and a SpeI site in the reverse primer (5'-GGACTAGTCGGAGGATCTGTTTTTTCTTCAGGAT-3'). The PCR product was double-digested with NcoI and SpeI, and inserted into the NcoI/SpeI digested pCAMBIA-1302 vector, to generate pCAMBIA1302-GsCML27.

GsCML27-eGFP fusion protein and eGFP protein were transiently expressed in Arabidopsis protoplast cells as described [79]. Briefly, the rosette leaves of 3-week-old Arabidopsis were cut into 0.5–1 mm strips, and digested in 10 mL enzyme solution (20 mM MES (pH 5.7), 1.5% (w/v) cellulase R10, 0.4% (w/v) macerozyme R10, 0.4 M mannitol, 20 mM KCl, 10 mM CaCl₂, 5 mM beta-mercaptoethanol, 0.1% (w/v) BSA) for 3 h at room temperature in dark. The solution was filtered with a nylon mesh after diluted with an equal volume of W5 solution (2 mM MES (pH 5.7), 150 mM NaCl, 125 mM CaCl₂, 5 mM KCl). The protoplasts were centrifuged at 100 g for 2 min to pellet the protoplasts and re-suspended in W5 solution. Then, 100 μl of
protoplasts, 10 μg of plasmid DNA and 110 μl of PEG solution (40% (w/v) PEG4000, 0.2 M mannitol, 100 mM CaCl₂) were mixed completely, incubated at room temperature for 5–15 min, and wash with W5 solution twice. The transformed protoplasts were incubated for 10–16 h before checking the GFP signal by using a confocal laser-scanning microscope (SP2, Leica, Germany).

The plasmids eGFP and GsCML27-eGFP were precipitated onto gold beads to transient expression in onion epidermal cells. Localization of fluorescent protein in onion epidermal cells was observed at 488 nm using a confocal laser-scanning microscope (SP2, Leica, Germany). eGFP fluorescence and light field vision were recorded in separate channels and merged into an overlay image.

Transformation of Arabidopsis
To identify the biological function of GsCML27, the coding region of GsCML27 was cloned into the pCAMBIA330035S vector under the control of CaMV35S promoter through the USER™ cloning technique [80]. Then the resulting pCAMBIA330035S-GsCML27 vector was introduced into Agrobacterium tumefaciens strain LBA4404, and transformation of Arabidopsis thaliana was performed using the Agrobacterium tumefaciens-mediated floral-dip method [81]. Transformants were selected on 1/2 MS medium containing 25 mg L⁻¹ glufosinate ammonium, and the T₃ generation from three independent ectopic expression transgenic lines (#13, #15, and #33) were randomly chosen for further functional studies.

Phenotypic analysis of transgenic Arabidopsis under stress treatments
All Arabidopsis seeds were surfaced-sterilized as described [82]. During the seed germination and early seedling growth stage, the wild-type (WT) and ectopic expression seeds were sown on either normal 1/2 agar MS medium, or 1/2 MS medium, supplemented with either 10 mM NaHCO₃, or 125 mM NaCl, or 300 mM mannitol, or KCl (50, 100, 150 mM) or LiCl (10, 20, 30 mM). The germination rates were recorded for consecutive 6 days after sowing. On the 7th day, photos were taken to show the seedling growth performance, and the numbers of seedlings with open and green leaves were recorded.

For the root length assays, the 6-day-old WT and ectopic expression seedlings, grown on normal 1/2 MS medium, were transferred to fresh medium with 8 mM NaHCO₃, 150 mM NaCl or 350 mM mannitol. The length of seedling primary roots was measured after vertical growth after 9 days. All experiments were repeated at least three times. The numerical data was subjected to statistical analyses using EXCEL and SPSS statistical softwares.

Supporting Information
S1 Fig. Transcriptome sequencing data of GsCML27. Transcriptome sequencing data of GsCML27 in the wild type soybean Glycine soja G07256 under bicarbonate stress (50 mM NaHCO₃, pH 8.5).
(TIF)

S2 Fig. Ectopic expression of GsCML27 in Arabidopsis did not response to KCl and LiCl during the seed germination stage. A-G. Growth performance of WT and ectopic expression seedlings on 1/2 MS medium with 50, 100, 150 mM KCl or 10, 20, 30 mM LiCl. Germination was recorded daily up to 5 days. Data shown represent the means (±SE) of three independent experiments.
(TIF)
S3 Fig. Tissue specific expression of GsCML27 in Glycine soja at early growth stage. Total RNA was extracted from Glycine soja at early growth stage (0 to 10 days). Relative expression levels were determined by quantitative RT-PCR using GADPH as an internal control. All of the values represent the means of three fully independent biological replicates. (TIF)

S1 Table. Gene-specific primers used for quantitative RT-PCR assays. (DOCX)

S2 Table. Function of genes co-expression with GsCML27. (DOCX)

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Author Contributions

Conceived and designed the experiments: CC XS HD JX YZ. Performed the experiments: CC. Analyzed the data: CC YY LC. Contributed reagents/materials/analysis tools: AL BJ. Wrote the paper: CC XS DZ.

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