

NpSCI2A, a Potato Inhibitor I Protein from a Wild Sugarcane Relative, Negatively Regulates Drought Tolerance by Targeting Subtilases

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Abstract

Background

Drought severely limits sugarcane productivity. The wild relative of sugarcane, *Narenga porphyrocoma*, is highly drought-tolerant and represents a valuable source of stress-resistance genes. We identified *NpSCI2A*, encoding a s potato inhibitor I that is significantly downregulated under prolonged drought.

Results

Overexpression of *NpSCI2A* in rice markedly increased drought sensitivity, as evidenced by more severe leaf wilting, reduced antioxidant enzyme activities, elevated H₂O₂ and MDA levels, and lower survival rates. Transcriptome profiling revealed that *NpSCI2A* overexpression together with drought additively suppressed chloroplast- and photosynthesis-associated genes. Through co-expression analysis and protein–protein interaction assays, we identified two subtilases, SBT1.2 and SBT1.8, as direct physical interactors of *NpSCI2A*. Interestingly, SBT1.2 and SBT1.8 transcript levels were paradoxically upregulated in *NpSCI2A*-overexpressing plants under drought, suggesting a compensatory feedback response to impaired protease activity.

Conclusions

These findings establish *NpSCI2A* as a negative regulator of drought tolerance that acts by inhibiting SBT proteases, thereby disrupting chloroplast protein homeostasis and exacerbating oxidative damage. Our study uncovers a functional link between protease inhibitor-mediated proteolytic regulation and chloroplast function, offering new insights into plant drought adaptation and a potential target for the molecular breeding of stress-tolerant sugarcane.

1. Introduction

Sugarcane (*Saccharum* spp.) is a major crop for global sugar and bioethanol production, and its yield and quality are highly sensitive to drought stress [1, 2]. Drought suppresses germination, tillering, and stalk elongation, and markedly reduces photosynthetic efficiency and sucrose accumulation, making it one of the most significant abiotic stresses limiting sugarcane productivity [3–6]. With the increasing frequency of extreme drought events driven by climate change, the development of drought-tolerant sugarcane varieties has become an urgent necessity. However, the narrow genetic base, large and complex genome, and long conventional breeding cycles have made drought resistance breeding a long-standing challenge in modern sugarcane [7–10]. Therefore, the mining and utilization of drought-resistance genes from wild relatives represent a promising approach to address this bottleneck [11, 12].

Narenga porphyrocoma is a diploid wild relative closely related to sugarcane. Naturally distributed in arid and barren habitats, it has evolved drought, disease, and cold tolerance, strong ratooning ability, and adaptation to infertile soils [13, 14]. Compared with the highly polyploid and complex genome of cultivated sugarcane, *N. porphyrocoma* has a relatively simple genome (~ 1.8 Gb, 69.12% repetitive sequences) and a small chromosome number ($2n = 30$) [15, 16]. Hybrids between *N. porphyrocoma* and sugarcane show markedly enhanced drought tolerance, indicating its value as a germplasm for studying drought resistance and mining stress-related genes [16–18]. A recently assembled high-quality genome, together with drought transcriptome profiling, has enabled the preliminary identification of key drought-responsive gene families, including aquaporins, LEA proteins, and heat shock proteins, and the systematic mapping of ABA signaling and response genes (e.g., PYL, PP2C, MAPK, LEA) across the 15 chromosomes, providing direct targets for marker-assisted selection [16]. However, the identification of drought-resistance genes still depends largely on bioinformatic prediction, and functional validation and regulatory network analysis remain limited. Therefore, elucidating the molecular mechanisms of drought resistance in *N. porphyrocoma* and cloning key functional genes will help broaden the genetic base of sugarcane, overcome breeding bottlenecks, and facilitate the development of drought-tolerant cultivars.

Notably, plant protease inhibitors contribute to drought-resistance regulation. Subtilisin-chymotrypsin inhibitor-2A (SCI-2A), a member of the potato inhibitor I family (MEROPS I13) of serine protease inhibitors, inhibits both subtilisin and chymotrypsin [19]. Genes in this family are regulated by hormones such as jasmonic acid (JA) and abscisic acid (ABA) and participate in both biotic and abiotic stress responses [20]. Upon insect herbivory or pathogen infection, SCI-2A is induced and enhances plant resistance by inhibiting insect midgut digestive proteases and fungal alkaline serine proteases [21–23]. Members of this family also respond to abiotic stresses, including drought and salinity [24, 25]. In rice, 17 putative members have been identified, including two functionally validated genes, *OCPI1* and *OCPI2* [26]. They share a stress-inducible bidirectional promoter and are both ABA-inducible [24]. Their functions, however, differ: overexpression of *OCPI1* enhances drought tolerance, whereas *OCPI2* mainly responds to salt, cold, and mechanical wounding [24, 27]. The Potato inhibitor I family is therefore large and functionally diverse, with several members contributing to drought resistance. Nevertheless, the functions of this family in sugarcane and its wild relatives (such as *N. porphyrocoma*) remain unreported, and the downstream regulatory mechanisms in drought resistance are still unclear. Understanding the drought-resistance functions and regulatory mechanisms of this family in sugarcane will facilitate the genetic improvement of drought tolerance in this crop.

In this study, weighted gene co-expression network analysis (WGCNA) of transcriptome data from drought-stressed *Narenga porphyrocoma* identified a core gene, *NpSCI2A*, significantly down-regulated under drought stress. Heterologous overexpression of *NpSCI2A* in rice significantly reduced drought tolerance compared with wild-type plants, indicating that *NpSCI2A* negatively regulates rice drought resistance. Transcriptome profiling of the overexpression lines showed enrichment of differentially expressed genes in chloroplast function and photosynthesis-related pathways, which were globally down-regulated. To identify downstream targets of *NpSCI2A*, we performed co-immunoprecipitation (CO-IP) together with co-expression analysis (Pearson's $r > 0.9$), and identified 14 potential target proteins of

NpSCI2A. Among these, two subtilase family members, SBT1.2 and SBT1.8, exhibited particularly strong co-expression ($r > 0.98$). and molecular docking and yeast two-hybrid assays confirmed physical interactions between NpSCI-2A and STB1.2 and STB1.8 proteins. Our findings provide a functional gene (*NpSCI2A*) and its interacting targets (SBT1.2/1.8) that could be manipulated via gene editing or marker-assisted selection to improve drought tolerance in sugarcane cultivars.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

N. porphyrocoma plants were obtained from the Guangxi Academy of Agricultural Sciences. Transgenic rice plants overexpressing *NpSCI2A* were generated by *Agrobacterium*-mediated transformation of *japonica* cv. Zhonghua 11 (*Oryza sativa* L. ssp. *japonica* cv. Zhonghua 11) and were designated as SCI2AOE. Rice plants were grown under a 16-h light/8-h dark photoperiod at 30°C (day)/25°C (night).

2.2 physiological and biochemical assays

Superoxide dismutase (SOD) activity was measured using an NBT-based assay kit (Geruisi, Suzhou, China). Peroxidase (POD) activity was determined using a guaiacol-based assay kit (Geruisi, Suzhou, China). Catalase (CAT) activity was measured using a visible colorimetric assay kit (Geruisi, Suzhou, China). Malondialdehyde (MDA) content was determined using a thiobarbituric acid (TBA)-based assay kit (Geruisi, Suzhou, China). Hydrogen peroxide (H_2O_2) content was measured using a titanium sulfate-based assay kit (Geruisi, Suzhou, China). All assays were performed according to the manufacturer's instructions. All experiments were performed with at least three biological replicates, and data are presented as means $\pm$ standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 9.0. Leaf relative water content (RWC) was measured gravimetrically and calculated as $RWC (\%) = (FW - DW) / (TW - DW) \times 100$, where FW is fresh weight, TW is turgid weight after 4 h hydration in distilled water, and DW is dry weight after oven-drying at 80°C to constant weight. Survival rate was assessed after 10 days of drought stress followed by 3 day of re-watering and expressed as the percentage of surviving plants.

2.3 Transcriptome sequencing and data analysis

Total RNA was extracted from rice leaves using Trizol reagent (Invitrogen). After assessing concentration and integrity with a Nanodrop spectrophotometer and an Agilent 2100 Bioanalyzer, strand-specific cDNA libraries were constructed and subjected to paired-end 150-bp sequencing on an Illumina NovaSeq 6000 platform. Raw reads were filtered by fastp to remove low-quality sequences, and clean reads were aligned to the reference genome using HISAT2. Gene expression levels were quantified as FPKM by StringTie. Differentially expressed genes (DEGs) were identified with DESeq2 ($|\log_2(\text{fold change})| \geq 1$ and $FDR < 0.05$). To identify genes synergistically regulated by *NpSCI2A* overexpression and drought stress, we intersected two comparisons—SCI2AOE drought vs. wild-type drought, and SCI2AOE drought vs. SCI2AOE well-watered—to obtain common DEGs. Based on these common DEGs, GO and

KEGG enrichment analyses were performed using clusterProfiler (significance threshold $P < 0.01$). Pearson correlation coefficients between *NpSCI2A* and other genes were calculated to construct a co-expression network ($|r| > 0.9$).

2.4 Co-immunoprecipitation and mass spectrometry

Total protein was extracted from leaves of *NpSCI2A*-GFP and GFP transgenic rice plants using a plant total protein extraction kit (Coolaber, Beijing, China; catalog no. PTE001). The supernatant was incubated with anti-GFP magnetic beads (Abmart, Shanghai, China; catalog no. A10023T) at 4°C for 1 h with gentle rotation. After washing, the bound proteins were eluted with elution buffer, separated by SDS-PAGE, and detected by western blotting with an anti-GFP antibody (Abmart). A wild-type rice sample processed in parallel served as the negative control. All experiments were performed with three independent biological replicates. The eluted proteins were digested with trypsin and analyzed by LC-MS/MS on a Thermo Fisher Q Exactive HF mass spectrometer coupled to an Easy-nLC system. Acquired MS/MS spectra were searched against the rice protein database (RAP-DB, IRGSP-1.0) using MaxQuant, with peptide and protein false discovery rates (FDR) set to less than 1%.

2.5 Yeast two-hybrid assay

The coding sequence of *NpSCI2A* was cloned into pGADT7 (AD-*NpSCI2A*), and the coding sequences of *SBT1.2* and *SBT1.8* were cloned into pGBKT7 (BD-*SBT1.2* and BD-*SBT1.8*). The respective AD and BD plasmid pairs were co-transformed into yeast strain Y2HGold. Transformants were selected on SD/–Leu/–Trp medium, and single colonies were serially diluted and spotted onto SD/–Leu/–Trp/–His/–Ade medium. Plates were incubated at 30°C for 3–5 d. The combination of pGADT7-T and pGBKT7-53 served as the positive control, and co-transformation with empty pGADT7 and pGBKT7 vectors served as the negative control. All experiments were performed with three independent replicates. Primers are presented in Table S5.

3. Result

1. *NpSCI2A*, a drought-downregulated potato inhibitor I gene, is a hub gene in a co-expression module under prolonged drought stress

To identify key drought-tolerance genes in *N. porphyrocoma*, we performed weighted gene co-expression network analysis (WGCNA) on a previously published transcriptomic dataset from this species under drought stress [16]. One co-expression module exhibited the strongest association with prolonged drought treatment, within which a gene annotated as a subtilisin-chymotrypsin inhibitor 2A-like protein occupied a hub position and was designated *NpSCI2A* (Fig. 1A). RT-qPCR confirmed that *NpSCI2A* transcript levels were significantly down-regulated under long-term drought, consistent with the RNA-seq data (Fig. 1B). To characterize *NpSCI2A*, we first analyzed its evolutionary conservation and structural features. Multiple sequence alignment and phylogenetic analysis revealed that *NpSCI2A* is highly conserved among grasses, sharing the highest sequence similarity with its orthologs in maize and sorghum (Fig. S1A, B). Subsequently, 3D modeling showed that *NpSCI2A* adopts the canonical potato

inhibitor I fold, a disulfide-free α/β structure with an α -helix packed against a four-stranded mixed β -sheet, consistent with its annotation as a serine protease inhibitor (Fig. 1C). To preliminarily assess whether it is responsive to drought stress, we analyzed its promoter region. The results showed that the promoter harbors an enrichment of cis-acting elements responsive to abscisic acid, jasmonic acid, and gibberellin, as well as binding motifs for WRKY, bZIP, and NAC transcription factors (Fig. 1D). Furthermore, Pearson correlation analysis revealed that the expression of several WRKY and NAC transcription factors within the same drought-responsive module was significantly correlated with *NpSCI2A* expression (Fig. 1E). Collectively, these findings indicate that *NpSCI2A* is a drought-repressed candidate gene encoding a protease inhibitor, which likely functions downstream of a complex regulatory network mediated by multiple hormones and transcription factors, and may contribute to plant adaptation under prolonged water deficit.

3.2 *NpSCI2A* negatively regulates drought tolerance in rice

To examine the function of *NpSCI2A* in drought tolerance, we developed homozygous T2 transgenic rice lines overexpressing *NpSCI2A* (*SCI2AOE*). RT-qPCR confirmed high-level expression of *NpSCI2A* in *SCI2AOE* lines, confirming successful generation of the overexpression lines (Fig. 2A). To assess drought tolerance, three independent *NpSCI2A*-overexpressing rice lines (*SCI2AOE*-4, -5, and -6) with the highest transgene expression were selected and subjected to drought stress, with wild-type (WT) plants serving as controls. *SCI2AOE* plants displayed pronounced leaf wilting and rolling, while wild-type plants showed only mild wilting and maintained better overall growth on day 3 of drought stress (Fig. 2B). Physiological analyses revealed that the activities of the antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were significantly reduced in *SCI2AOE* lines compared with wild-type plants (Fig. 2C–E). By contrast, *SCI2AOE* lines accumulated higher levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) (Fig. 2F–G), and their leaf relative water content was significantly reduced (Fig. 2H). These results indicate that ectopic expression of *SCI2A* compromises antioxidant capacity, exacerbates oxidative damage, and reduces water retention in rice under drought stress. Following re-watering after a 10-day drought treatment, the survival rate of *SCI2AOE* seedlings was significantly lower than that of wild-type plants (Fig. 2I). Collectively, these findings demonstrate that *NpSCI2A* overexpression reduces drought tolerance in rice, affirming its role as a negative regulator of drought response, consistent with its down-regulation under drought conditions.

3.3 *NpSCI2A* overexpression suppresses chloroplast function and photosynthesis-related pathways

To elucidate the molecular mechanism underlying the reduced drought tolerance of *SCI2A*-overexpressing rice, transcriptome profiling was performed on *SCI2AOE* and wild-type plants under drought stress (DS_*SCI2AOE* vs DS_WT) and on *SCI2AOE* plants under drought versus control conditions (DS_*SCI2AOE* vs CK_*SCI2AOE*). Differential expression analysis identified 4,073 up-regulated and 3,210 down-regulated genes in the DS_*SCI2AOE* vs DS_WT comparison, and 4,539 up-regulated and 2,983

down-regulated genes in the DS_ *SCI2A*OE vs CK_ *SCI2A*OE comparison (Fig. 3A, B, Table S1). To identify genes underlying *NpSCI2A*-dependent drought sensitivity, we intersected the two comparisons and focused on the overlapping DEGs. (Fig. 3C, Table S1). This intersection filters out basal genotypic differences and general drought responses, enriching for genes synergistically regulated by *NpSCI2A* overexpression and drought. GO enrichment analysis revealed that these DEGs were enriched in chloroplast, plastid, thylakoid membrane, and photosynthetic membrane components (Fig. 3D; Table S2, $P < 0.01$), indicating that *SCI2A* overexpression broadly impairs chloroplast integrity and the photosynthetic apparatus. Consistently, KEGG analysis showed significant enrichment in photosynthesis-related pathways (including photosynthetic proteins, antenna proteins, and the Calvin cycle), protein folding, carotenoid biosynthesis, and glyoxylate and dicarboxylate metabolism (Fig. 3E; Table S3, $P < 0.01$). Additionally, we selected five genes for validation by qRT-PCR to confirm the accuracy of the transcriptome data (Fig. 3F). Together, these results suggest that *NpSCI2A* overexpression disrupts multiple chloroplast-associated metabolic processes, which likely contributes to the enhanced oxidative damage and reduced survival of *SCI2A*OE plants under drought stress.

3.4 *NpSCI2A* directly targets SBT1.2 and SBT1.8

To identify *NpSCI2A*-interacting proteins and dissect its molecular function in drought tolerance, we performed co-immunoprecipitation (Co-IP) using transgenic rice plants overexpressing GFP-tagged *NpSCI2A* and identified 420 putative interacting proteins (Table S4). Parallel transcriptome analysis of drought-responsive DEGs revealed 196 genes co-expressed with *NpSCI2A* at a Pearson correlation coefficient > 0.9 (Table S4). Intersection of the Co-IP and co-expression datasets yielded 15 high-confidence candidates that were both physically associated with *NpSCI2A* and transcriptionally coordinated with it under drought stress (Fig. 4A; Table S4). Among these 15 candidates, two subtilase family members, SBT1.2 and SBT1.8, showed particularly strong co-expression with *NpSCI2A* (correlation coefficients > 0.98 ; Fig. 4B). To identify which of these candidates are most likely to directly interact with *NpSCI2A*, we performed molecular docking analysis. The results showed that in the *NpSCI2A*–SBT1.2 complex, SBT1.2 forms three hydrogen bonds with *NpSCI2A*, involving residues Gly280 (2.8 Å), Ser278 (2.0 Å), and Val54 (3.1 Å) of SBT1.2, along with extensive van der Waals contacts (Fig. 4B). Similarly, SBT1.8 interacts with *NpSCI2A* via two hydrogen bonds, involving residues Leu56 (2.9 Å) and Asn318 (2.8 Å) of SBT1.8, and van der Waals contacts (Fig. 4C). These results indicate that *NpSCI2A* can associate with both SBT1.2 and SBT1.8. Yeast two-hybrid assays showed that cells co-expressing *NpSCI2A* with SBT1.2 or SBT1.8 grew on selective medium and were positive for β -galactosidase activity, whereas the negative control showed no growth (Fig. 4D). Together, these results confirm direct physical interactions between *NpSCI2A* and SBT1.2 and SBT1.8. Intriguingly, the transcript levels of SBT1.2 and SBT1.8 were paradoxically upregulated in *NpSCI2A*-overexpressing rice lines under drought stress (Fig. 4E). Given that *NpSCI2A* physically associates with these SBT proteases and likely attenuates their activity, this transcriptional upregulation may reflect a compensatory feedback mechanism that increases SBT synthesis to offset the reduced enzymatic activity.

4. Discussion

Here, we show that NpSCI2A, a serine protease inhibitor of the Potato inhibitor I family from the wild sugarcane species *N. porphyrocoma*, is significantly downregulated under drought stress, and its heterologous overexpression in rice reduces drought tolerance. This negative effect is mediated by direct inhibition of two subtilase proteases, SBT1.2 and SBT1.8, resulting in disrupted chloroplast proteostasis and consequently impaired photosynthetic function. These findings reveal a mechanism by which a Potato inhibitor I family member negatively regulates drought response through an SBT–photosynthesis module.

Under drought stress, plants remodel protein homeostasis by removing damaged proteins and processing signal peptides [28, 29]. Studies have shown that various serine protease inhibitors are transcriptionally repressed under abiotic stress, thereby releasing their cognate proteases to perform essential proteolytic functions [26, 30]. For instance, rice chymotrypsin inhibitor OCPI1, potato type I inhibitor, and peanut Bowman–Birk inhibitor AhBBI are all upregulated by drought [24, 31, 32]. In this study, the *NpSCI2A* of *N. porphyrocoma* was significantly downregulated under prolonged drought. These findings indicate that protease inhibitors can show contrasting transcriptional responses to drought, with some being induced while others are repressed. They may thus play positive or negative roles in drought adaptation, likely depending on their target proteases [30, 33]. The downregulation of *NpSCI2A* may reflect an adaptive de-repression mechanism that relieves inhibition of downstream proteases, thereby facilitating stress-associated protein turnover. Overexpression of *NpSCI2A* disrupted this process at the transcriptional level, leading to the repression of genes required for chloroplast development and photosynthetic membrane integrity and the downregulation of photosynthetic pathways such as light harvesting, the Calvin cycle, and carotenoid biosynthesis. (Fig. 3A-E). Suppression of these pathways may reduce light energy utilization and carbon assimilation, causing excess excitation energy to be converted into reactive oxygen species [34, 35]. In parallel, impaired photoprotective pathways further compromised ROS scavenging capacity, ultimately leading to H₂O₂ and MDA accumulation, decreased antioxidant enzyme activities, and increased water loss in *NpSCI2A*-overexpressing lines. [34, 36].

As a serine protease inhibitor of the Potato inhibitor I family, SCI2A participates in plant stress responses by directly inhibiting its target proteases. In this study, we identified two subtilase family members, SBT1.2 and SBT1.8, which are highly co-expressed with *NpSCI2A*, as downstream targets. Previous studies have shown that under drought stress, plant SBT family genes are significantly upregulated, mediating stress signaling, maintaining chloroplast proteostasis, and modulating stomatal development, thereby protecting the photosynthetic apparatus [37–39]. Previous studies have shown that Arabidopsis SBT3.8 positively regulates osmotic stress tolerance by cleaving PSK precursors to release bioactive signaling peptides [37]. Maize *ZmSDD1* enhances water retention and drought adaptation by reducing stomatal density [40]. Cotton (*Gossypium hirsutum*) *GhSBT27A* plays a key role in maintaining chloroplast structural integrity and stability under water-deficit conditions [41]. In our study, the *SCI2AOE* lines rice lines showed significant phenotypic, physiological, and transcriptomic alterations under drought stress compared with wild-type controls. Based on these results, we propose that *NpSCI2A* may directly inhibit

SBT1.2 and SBT1.8, thereby impairing the timely degradation and turnover of damaged photosynthetic proteins. This impairment could lead to over-reduction of the photosynthetic electron transport chain, excessive ROS accumulation, and oxidative damage. These findings suggest that the NpSCI2A–SBT1.2/1.8 module may contribute to the drought response by modulating chloroplast proteostasis. Notably, under drought stress conditions, the transcript levels of SBT1.2 and SBT1.8 were significantly upregulated in SCI2AOE lines (Fig. 4F). This regulatory pattern, in which an inhibitor suppresses the activity of target enzymes while the corresponding target genes undergo compensatory transcriptional upregulation, has been previously documented in protease–inhibitor interaction systems. For example, when herbivorous insects ingest plant protease inhibitors, they can upregulate the transcription of their own digestive protease genes to compensate for the inhibited enzyme activity, a mechanism observed in the serpin–protease regulatory network of the tobacco hornworm [42, 43]. These findings further confirm that under drought stress, *N. porphyrocoma* can actively downregulate NpSCI2A expression to relieve its inhibition of SBT family members, thereby activating SBT protease activity and maintaining chloroplast protein homeostasis as an adaptation to drought stress.

Although our work identifies NpSCI2A as a negative regulator of drought tolerance acting through SBT protease inhibition, several limitations remain. First, NpSCI2A was characterized in a heterologous rice system; its conserved function in *N. porphyrocoma* awaits validation by homologous transformation or gene editing. Second, only overexpression was examined; loss-of-function lines are needed to confirm that NpSCI2A downregulation is necessary and sufficient for drought tolerance. Third, the drought-specific substrates of SBT1.2 and SBT1.8, as well as the biochemical link between their inhibition and chloroplast dysfunction, remain unclear, warranting substrate profiling and subcellular localization studies. Finally, the enrichment of hormone- and TF-binding motifs in the NpSCI2A promoter and its co-expression with WRKY and NAC factors suggest a complex upstream regulatory network, whose dissection may offer new strategies for precision manipulation of crop drought responses

5. Conclusion

In summary, this study identifies a drought-repressed potato inhibitor I family protein, NpSCI2A, from the drought transcriptome of *N. porphyrocoma*, and demonstrates that it functions as a negative regulator of drought tolerance. By directly targeting the subtilases SBT1.2 and SBT1.8, NpSCI2A impairs chloroplast function and photosynthetic pathways, leading to exacerbated oxidative damage. These findings establish a functional link between protease inhibitor-mediated proteolytic regulation and chloroplast protein homeostasis, providing new insights into the molecular mechanisms of plant drought adaptation and offering candidate targets for the molecular breeding of stress-tolerant sugarcane.

Declarations

Competing interests

The authors declare that they have no competing interests.

Ethics and Consent to Participate declarations

Not applicable.

Consent for publication

Not applicable.

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Author Contribution

A. S.C. Li drafted the original manuscript and prepared figure 1. R.R. Wang and S.J. Huang contributed equally to this work as co-first authors; R.R. Wang conducted transgenic rice experiments and physiological and biochemical detection (figure 2), and S.J. Huang was responsible for transcriptome analysis (Figure 3, and Figure 4 A and B). Y.H. Tang performed yeast two-hybrid assays (Figure 4F). K. Zhu finished bioinformatics analysis. H.B. Li, H. Zhou, H.R. Huang and Y.Y. Gui participated in data analysis and manuscript revision. J.J. Wei and X.H. Liu served as co-corresponding authors, who conceived, designed and supervised the entire research project.

Data Availability

The raw transcriptome sequencing data have been deposited in the Genome Sequence Archive (GSA, <https://ngdc.cnbc.ac.cn/gsa/>) under the accession number subSAM180042. For other reasonable requests, the data can be obtained from the corresponding author or first author.

Materials availability

All materials used in this study are available from the corresponding author or the first author upon reasonable request.

Code availability

Not applicable.

References

1. Zan F, Zhang Y, Wu Z, Zhao J, Wu C, Zhao Y, Chen X, Zhao L, Qin W, Yao L, et al. Genetic analysis of agronomic traits in elite sugarcane (*Saccharum* spp.) germplasm. *PLoS ONE*. 2020;15(6):e0233752.
2. Verma KK, Song XP, Yadav G, Degu HD, Parvaiz A, Singh M, Huang HR, Mustafa G, Xu L, Li YR. Impact of Agroclimatic Variables on Proteogenomics in Sugar Cane (*Saccharum* spp.) Plant Productivity. *ACS Omega*. 2022;7(27):22997–3008.
3. Dlamini PJ. Drought stress tolerance mechanisms and breeding effort in sugarcane: A review of progress and constraints in South Africa. 2021.
4. Tippa-Art M, Klomsa-Ard P, Songsri P, Jongrungklang N. Effects of Intermittent Drought During Tillering and Stalk Elongation Stages on the Physiological Attributes of Diverse Sugarcane Genotypes. *Stresses* 2025, 5(1).
5. Tippayawat A, Jogloy S, Vorasoot N, Jongrungklang N, Kimbeng CA, Jifon JL, Khonghintaiong J, Songsri P. Timing and Duration of Drought Differentially Affect Growth and Yield Components Among Sugarcane Genotypes. *Plants (Basel)* 2025, 14(5).
6. Kasirajan L, Sebastiar S, Muthukumar R, Lissy Vargheese R, Elumalai K, Kamaraj K, Valiyaparambath R, Angannan S, Ayyadurai A, Raju G, et al. Unravelling drought stress adaptation in sugarcane interspecific hybrids: A multi-level analysis. *PLoS ONE*. 2025;20(12):e0338698.
7. Chai J, Xue L, Lei J, Yao W, Zhang M, Deng Z, Yu F. All nonhomologous chromosomes and rearrangements in *Saccharum officinarum* × *Saccharum spontaneum* allopolyploids identified by oligo-based painting. *Front Plant Sci*. 2023;14:1176914.
8. Verma KK, Song XP, Budeguer F, Nikpay A, Enrique R, Singh M, Zhang BQ, Wu JM, Li YR. Genetic engineering: an efficient approach to mitigating biotic and abiotic stresses in sugarcane cultivation. *Plant Signal Behav*. 2022;17(1):2108253.
9. Huang Y, Zhang Y, Zhang Q, Zhuang G, Li C, Wang B, Gao R, Xu Y, Qi Y, Hua X, et al. Multiscale pangenome graphs empower the genomic dissection of mixed-ploidy sugarcane species. *Science*. 2026;391(6785):eadx1616.
10. Wang Y, Li Y, Hua X, Zhang Z, Fan T, Yao W, Zhang M, Zhang J. Transcriptome Dynamics Underlying Magnesium Deficiency Stress in Three Founding *Saccharum* Species. *Int J Mol Sci* 2022, 23(17).
11. Valarmathi R, Mahadeva Swamy HK, Appunu C, Suresha GS, Mohanraj K, Hemaprabha G, Mahadevaiah C, Ulaganathan V. Comparative transcriptome profiling to unravel the key molecular signalling pathways and drought adaptive plasticity in shoot borne root system of sugarcane. *Sci Rep*. 2023;13(1):12853.
12. Ramanathan V, Pradhan AK, Gandham P, Chinnaswamy A, Mahadeva Swamy HK, Mohanraj K, Baisakh RR. Identification of new genetic resources for drought tolerance-related traits from the world *Erianthus* germplasm collection. *Front Plant Sci*. 2025;16:1684712.

13. Liu X, Zhang R, Ou H, Gui Y, Wei J, Zhou H, Tan H, Li Y. Comprehensive transcriptome analysis reveals genes in response to water deficit in the leaves of *Saccharum narenga* (Nees ex Steud.) Hack. *BMC Plant Biol.* 2018;18(1):250.
14. Al-Janabi SM, McClelland M, Petersen C, Sobral BW. Phylogenetic analysis of organellar DNA sequences in the Andropogoneae: Saccharinae. *Theor Appl Genet.* 1994;88(8):933–44.
15. Yu F, Zhao X, Chai J, Ding X, Li X, Huang Y, Wang X, Wu J, Zhang M, Yang Q, et al. Chromosome-specific painting unveils chromosomal fusions and distinct allopolyploid species in the *Saccharum* complex. *New Phytol.* 2022;233(4):1953–65.
16. Li H, Gui Y, Wei J, Zhu K, Zhou H, Zhang R, Huang D, Huang S, Li S, Zhang J et al. Unveiling the Genome of the Diploid Wild Sugarcane Relative *Narenga porphyrocoma* (Hance) Bor. *Int J Mol Sci* 2025, 26(13).
17. Xi-Hui L, Feng-Xue F, Rong-Hua Z, Huan-Zhong S, Rong-Zhong Y, Yi-Jing G, Hui-Ping OU, Jing-Chao L, Ting L, Wei-Xing D. Identification of Progenies from Sugarcane×*Narenga porphyrocoma*(Hance) Bor. by SSR Marker. *Southwest China J Agricultural Sci* 2012.
18. Weixing D, Yuxin H, Shan Z, Baoqing Z, Ting L, Cuifang Y, Yijing G, Gemin Z. Transmission of Parental Chromosomes in F₁, BC₁ and BC₂ Progeny Between *Saccharum* spp. and *Narenga porphyrocoma*(Hance) Bor. *Chin J Trop Crops* 2017.
19. McPhalen CA, Svendsen I, Jonassen I, James MN. Crystal and molecular structure of chymotrypsin inhibitor 2 from barley seeds in complex with subtilisin Novo. *Proc Natl Acad Sci U S A.* 1985;82(21):7242–6.
20. Casaretto JA, Zúñiga GE, Corcuera LJ. Absciscic acid and jasmonic acid affect proteinase inhibitor activities in barley leaves. *J Plant Physiol.* 2004;161(4):389–96.
21. Di Gennaro S, Ficca AG, Panichi D, Poerio E. cDNA cloning and heterologous expression of a wheat proteinase inhibitor of subtilisin and chymotrypsin (WSC1) that interferes with digestive enzymes of insect pests. *Biol Chem.* 2005;386(4):383–9.
22. Losvik A, Beste L, Mehrabi S, Jonsson L. The Protease Inhibitor CI2c Gene Induced by Bird Cherry-Oat Aphid in Barley Inhibits Green Peach Aphid Fecundity in Transgenic Arabidopsis. *Int J Mol Sci* 2017, 18(6).
23. Pekkarinen AI, Jones BL. Purification and identification of barley (*Hordeum vulgare* L.) proteins that inhibit the alkaline serine proteinases of *Fusarium culmorum*. *J Agric Food Chem.* 2003;51(6):1710–7.
24. Huang Y, Xiao B, Xiong L. Characterization of a stress responsive proteinase inhibitor gene with positive effect in improving drought resistance in rice. *Planta.* 2007;226(1):73–85.
25. Park YC, Chapagain S, Jang CS. The microtubule-associated RING finger protein 1 (OsMAR1) acts as a negative regulator for salt-stress response through the regulation of OCPI2 (*O. sativa* chymotrypsin protease inhibitor 2). *Planta* 2018, 247(4):875–886.
26. Singh A, Sahi C, Grover A. Chymotrypsin protease inhibitor gene family in rice: Genomic organization and evidence for the presence of a bidirectional promoter shared between two chymotrypsin

- protease inhibitor genes. *Gene*. 2009;428(1–2):9–19.
27. Tiwari LD, Mittal D, Chandra Mishra R, Grover A. Constitutive over-expression of rice chymotrypsin protease inhibitor gene OCPI2 results in enhanced growth, salinity and osmotic stress tolerance of the transgenic Arabidopsis plants. *Plant Physiol Biochem*. 2015;92:48–55.
 28. Dong C, Huang TC, Roberts TH. Genes Encoding Structurally Conserved Serpins in the Wheat Genome: Identification and Expression Profiles during Plant Development and Abiotic and Biotic Stress. *Int J Mol Sci* 2023, 24(3).
 29. Ferreira MM, Santos AS, Santos AS, Zugaib M, Pirovani CP. Plant Serpins: Potential Inhibitors of Serine and Cysteine Proteases with Multiple Functions. *Plants (Basel)* 2023, 12(20).
 30. Moloi SJ, Ngara R. The roles of plant proteases and protease inhibitors in drought response: a review. *Front Plant Sci*. 2023;14:1165845.
 31. Dramé KN, Passaquet C, Repellin A, Zuily-Fodil Y. Cloning, characterization and differential expression of a Bowman-Birk inhibitor during progressive water deficit and subsequent recovery in peanut (*Arachis hypogaea*) leaves. *J Plant Physiol*. 2013;170(2):225–9.
 32. Wellpott K, Jozefowicz AM, Meise P, Schum A, Seddig S, Mock HP, Winkelmann T, Bündig C. Combined nitrogen and drought stress leads to overlapping and unique proteomic responses in potato. *Planta*. 2023;257(3):58.
 33. D'Ippólito S, Rey-Burusco MF, Feingold SE, Guevara MG. Role of proteases in the response of plants to drought. *Plant Physiol Biochem*. 2021;168:1–9.
 34. Didaran F, Kordrostami M, Ghasemi-Soloklui AA, Pashkovskiy P, Kreslavski V, Kuznetsov V, Allakhverdiev SI. The mechanisms of photoinhibition and repair in plants under high light conditions and interplay with abiotic stressors. *J Photochem Photobiol B*. 2024;259:113004.
 35. Smirnoff N. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol*. 1993;125(1):27–58.
 36. Qiao M, Hong C, Jiao Y, Hou S, Gao H. Impacts of Drought on Photosynthesis in Major Food Crops and the Related Mechanisms of Plant Responses to Drought. *Plants (Basel)* 2024, 13(13).
 37. Stührwohltd N, Bühler E, Sauter M, Schaller A. Phytosulfokine (PSK) precursor processing by subtilase SBT3.8 and PSK signaling improve drought stress tolerance in Arabidopsis. *J Exp Bot*. 2021;72(9):3427–40.
 38. Wang Q, Guo Q, Guo Y, Yang J, Wang M, Duan X, Niu J, Liu S, Zhang J, Lu Y, et al. Arabidopsis subtilase SASP is involved in the regulation of ABA signaling and drought tolerance by interacting with OPEN STOMATA 1. *J Exp Bot*. 2018;69(18):4403–17.
 39. Wang Y, Chen F, Jin W, Xiao X, Xiang Z, Tang Q, Hou Z, Zhou Y, Lu Y. Characterization of drought-responsive subtilisin genes in upland rice: the role of OsSub14 in ABA signaling and ROS accumulation. *BMC Plant Biol*. 2025;25(1):1462.
 40. Liu Y, Qin L, Han L, Xiang Y, Zhao D. Overexpression of maize SDD1 (ZmSDD1) improves drought resistance in *Zea mays* L. by reducing stomatal density.

41. Dai M, Zhou N, Zhang Y, Zhang Y, Ni K, Wu Z, Liu L, Wang X, Chen Q. Genome-wide analysis of the SBT gene family involved in drought tolerance in cotton. *Front Plant Sci.* 2022;13:1097732.
42. Das IS, Shi Q, Dreischhoff S, Polle A. Divergent functions of three Kunitz trypsin inhibitor (KTI) proteins in herbivore defense in poplar. *BMC Plant Biol.* 2025;26(1):153.
43. Kuwar SS, Pauchet Y, Vogel H, Heckel DG. Adaptive regulation of digestive serine proteases in the larval midgut of *Helicoverpa armigera* in response to a plant protease inhibitor. *Insect Biochem Mol Biol.* 2015;59:18–29.

Figures

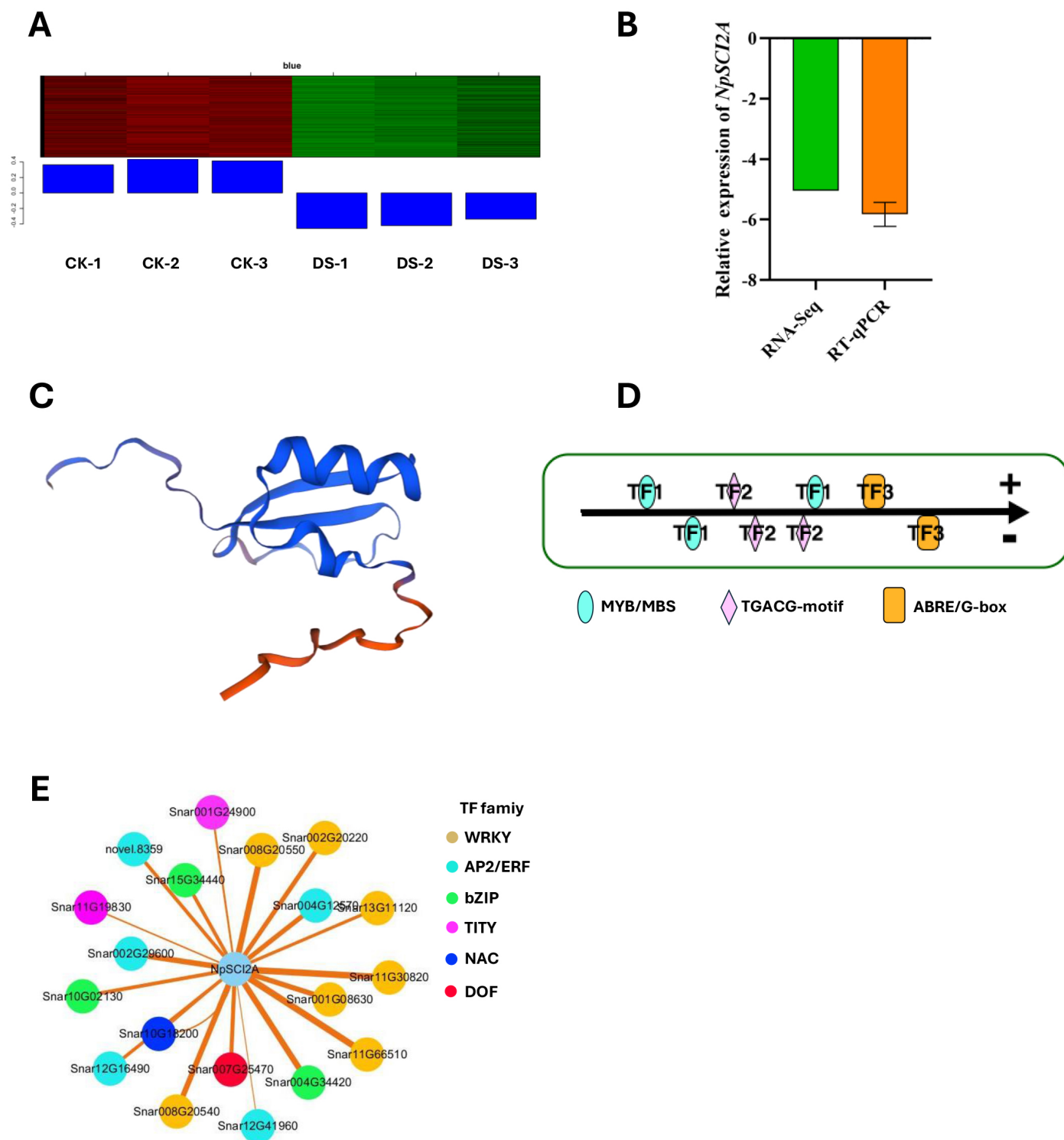


Figure 1

Molecular characterization of NpSCI2A. (A) WGCNA heatmap of the drought-induced gene module across control (CK) and drought-stressed (DS) samples. (B) Validation of NpSCI2A expression by RNA-Seq and RT-qPCR. Data are means $\pm$ SD. (C) Predicted 3D structure of the NpSCI2A protein. (D) Cis-acting elements in the 2000-bp upstream promoter of *NpSCI2A* (MYB/MBS, TGACG-motif, and ABRE/G-box). (E) Co-expression network of transcription factors (TFs) highly correlated with *NpSCI2A*.

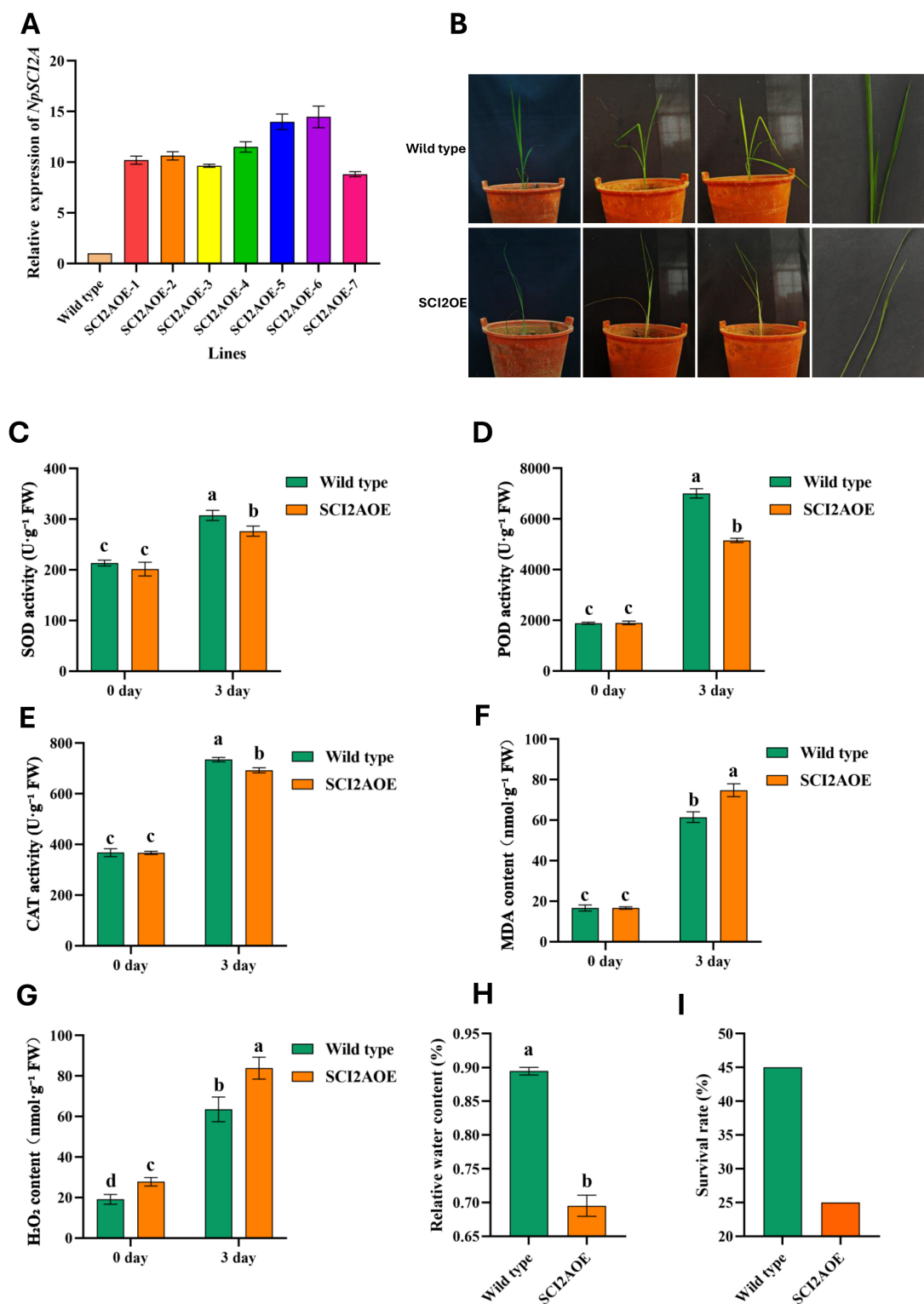


Figure 2

Overexpression of *NpSCI2A* reduces drought tolerance in rice. (A) Relative expression levels of *NpSCI2A* in wild-type (WT) and seven independent *NpSCI2A*-overexpressing (SCI2AOE) transgenic rice lines. The expression level in WT was normalized to 1. Data are presented as means $\pm$ SD ($n = 3$ biological replicates). (B) Phenotypic comparison of WT and SCI2AOE plants under drought stress. (C-E) Antioxidant enzyme activities in WT and SCI2AOE plants under drought stress. Activities of (C)

superoxide dismutase (SOD), (D) peroxidase (POD), and (E) catalase (CAT) were measured at day 0 (pre-stress) and day 3 of drought treatment.(F) Malondialdehyde (MDA) content and (G) hydrogen peroxide (H₂O₂) content were measured at day 0 and day 3 of drought treatment. (H) Relative water content of WT and SCI2AOE leaves after 3 days of drought treatment. (I) Survival rates of WT and SCI2AOE plants after 10 days of severe drought stress followed by re-watering. All physiological data are presented as means ± SD (n = 3 biological replicates). Different lowercase letters indicate significant differences between groups (p < 0.05, one-way ANOVA with Tukey's post-hoc test).

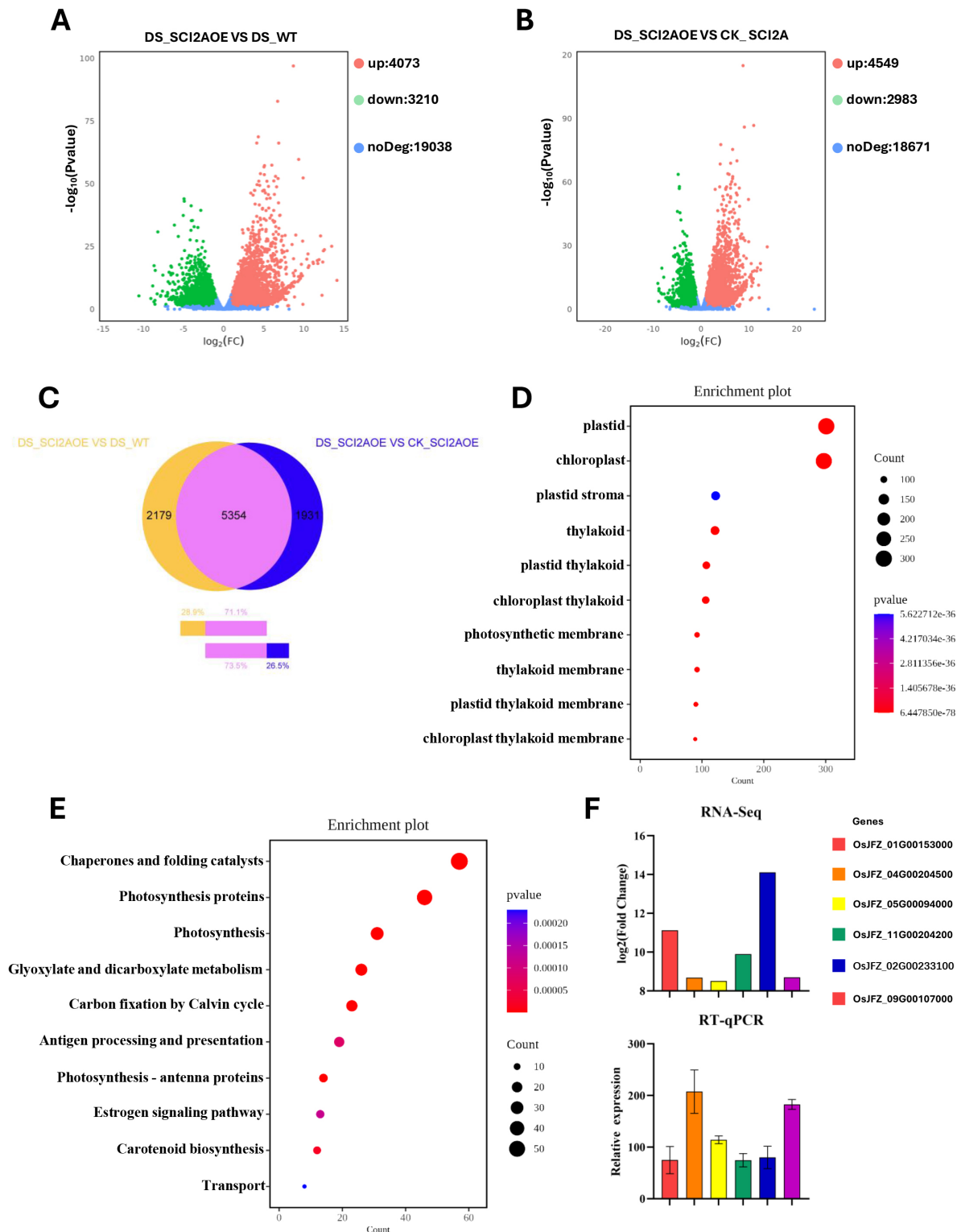


Figure 3

Transcriptomic analysis reveals global gene expression reprogramming in SCI2AOE plants under drought stress. (A-B) Volcano plots showing differentially expressed genes (DEGs) in two comparisons. (A) DEGs in *SCI2AOE* vs. wild-type (WT) plants under drought stress (DS). (B) DEGs in *SCI2AOE* plants under drought stress (DS) vs. control (CK). (C) Venn diagram showing overlapping DEGs between the two comparisons. (D-E) GO cellular component (D) and KEGG pathway (E) enrichment of core DEGs, highlighting repressed photosynthesis-related pathways in *SCI2AOE* plants under drought. (F) RT-qPCR confirming the transcriptome results.

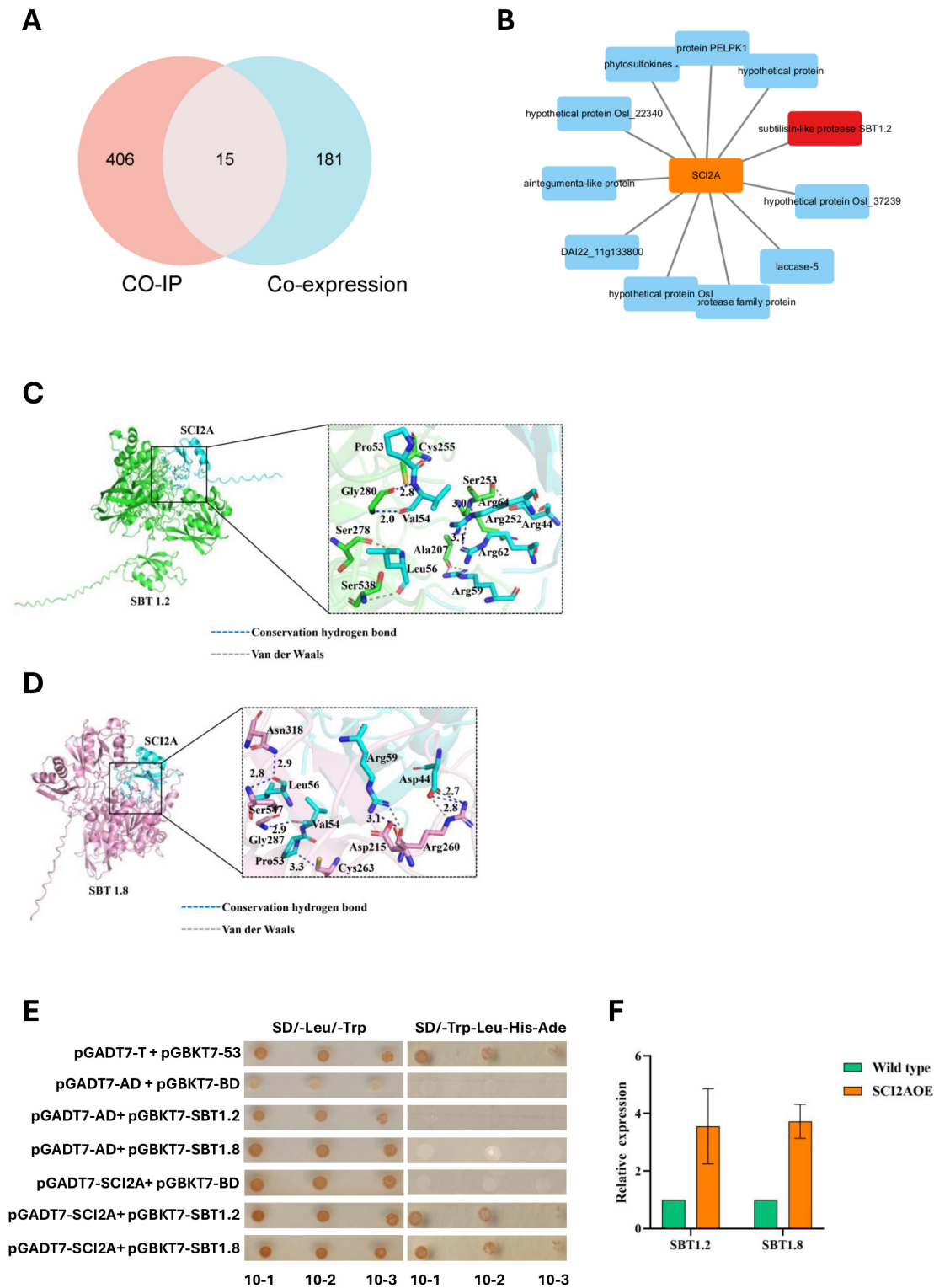


Figure 4

Identification and verification of proteins interacting with NpSCI2A. (A) Venn diagram showing the overlap of candidate proteins identified by CO-IP and co-expression analysis. (B) The top co-expressed genes with *NpSCI2A* based on Pearson correlation analysis, highlighting the subtilisin-like protease *SBT1.2*. (C-D) Molecular docking models of NpSCI2A with (C) *SBT1.2* and (D) *SBT1.8*, showing their binding interfaces and interaction forces (hydrogen bonds and van der Waals forces). (E) Yeast two-

hybrid assay confirming the physical interaction between NpSCI2A and SBT1.2/SBT1.8. (F) qRT-PCR analysis showing the expression levels of *SBT1.2* and *SBT1.8* in wild-type and SCI2AOE plants.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1DEGsofDSSCI2AOEvsCKSCI2AOEandDSSCI2AOEvsDSWT.xlsx](#)
- [TableS2GOenrichmentofoverlappingDEGs.xlsx](#)
- [TableS3KEGGenrichmentpathwayofoverlappingDEGs.xlsx](#)
- [TableS4CoIPandCoexpressionresults.xlsx](#)
- [TableS5Primerswereusedinthisstudy.xlsx](#)
- [FigureS1.jpg](#)