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ARTICLE

Overexpression of *IbSINA5* Increases Cold Tolerance through a CBF SINA-COR Mediated Module in Sweet Potato

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ABSTRACT

Seven *in absentia* (SINA) family proteins play a central role in plant growth, development and resistance to abiotic stress. However, their biological function in plant response to cold stress is still largely unknown. In this work, a *seven in absentia* gene *IbSINA5* was isolated from sweet potato. Quantitative real-time polymerase chain reaction (qRT-PCR) analyses demonstrated that *IbSINA5* was ubiquitously expressed in various tissues and organs of sweet potato, with a predominant expression in fibrous roots, and was remarkably induced by cold, drought and salt stresses. Subcellular localization assays revealed that IbSINA5-GFP fusion protein was mainly localized in cytoplasm and nucleus. Overexpression of *IbSINA5* in sweet potato led to dramatically improved resistance to cold stress in transgenic plants, which was associated with the up-regulated expression of *IbCOR* (cold-regulated) genes, increased proline production, and decreased malondialdehyde (MDA) and H_2O_2 accumulation in the leaves of transgenic plants. Furthermore, transient expression of *IbCBF3*, a C-repeat binding factor (CBF) gene, in the leaf protoplasts of wild type sweet potato plants up-regulated the expression of both *IbSINA5* and *IbCOR* genes. Our results suggest that *IbSINA5* could function as a positive regulator in the cold signaling pathway through a CBF-SINA-COR mediated module in sweet potato, and have a great potential to be used as a candidate gene for the future breeding of new plant species with improved cold resistance.

KEYWORDS

Cold stress; *IbSINA5*; seven *in absentia*; sweet potato; transgenic plant

1 Introduction

Sweet potato (*Ipomoea batatas* [L.] Lam) has been grown worldwide as a tuberous crop due to its food and commercial values, and resistance to adverse growth conditions [1–3]. However, owing to its tropical and subtropical origin, abiotic stresses such as low temperature and drought can severely affect the storage root yield of sweet potato, especially during the rapid root expansion period [4,5].

To increase the resistance of sweet potato to abiotic stresses, a number of stress related genes such as *IbCBF3*, *IbMIPS1*, *IbNAC1*, *IbOR*, *IbTPS* and *IbLEA14* have been isolated, and transgenic sweet potato

plants with improved tolerance to biotic and abiotic stresses have been generated [6–9]. Ectopic expression of rice *OCI* enhanced the resistance to stem nematode in transgenic sweet potato [10]. Constitutive expression of Arabidopsis *AtNHX1*, *HDG11* or spinach *BADH* in sweet potato increased salt and cold resistance of transgenic plants [4,5,11]. Overexpression of *IbMIPS1* or *IbCBF3* increased the resistance to stem nematode, salt, drought and cold stress of transgenic sweet potato [12,13].

SINA protein is a subfamily of the tumor necrosis factor receptor-associated factor (TRAF) super family, which function in various developmental processes of animals and plants as ubiquitin ligases. They have been characterized by an N-terminal cysteine-rich really interesting new gene (RING) domain, two zinc-finger motifs and a conserved C-terminal coiled-coil domain responsible for substrate binding and homo- or hetero-dimerization [14–17]. In plants, SINA proteins were found to function in proteasome mediated protein ubiquitination [18]. In Arabidopsis, SINAT2 interacted with RELATED TO APETALA2 (AP2) 2 (AtRAP2.2), whereas *SINAT5* worked in the degradation of NAC1 to regulate lateral root growth [19–21]. In rice, the SINA family protein OsDIS1 negatively regulated the drought tolerance in transgenic rice plants [22]. In alfalfa, ectopic expression of the dominant negative form of *SINAT5* affected the growth and nodulation of transgenic plant [23]. In tomato, overexpression of *SlSINA2* lead to altered chlorophyll level in the leaves of transgenic plants, whereas overexpression of *SlSINA5* disturbed flower development [24].

Previously, we investigated the function of Arabidopsis SINA2 (AtSINA2), a SINA protein lacking RING domain, and found that it positively regulated the drought tolerance of Arabidopsis plants [25]. Here, we demonstrate for the first time that IbSINA5, a homolog of SINA2, functions as a positively regulator in the cold signaling pathway in sweet potato.

2 Materials and Methods

2.1 Plant Materials and RNA Isolations

Sweet potato cultivars Xushu18 and Taizhong 6 were grown in greenhouse as described previously [13]. For cold, drought and salt stress treatments, three-week-old Taizhong 6 plants were kept at 4°C, or treated with 25% PEG6000 or 250 mM NaCl. The fourth fully expanded leaves counted from the tops of plants were collected at 3 h, 6 h, 12 h, 24 h and 48 h after the treatments for RNA isolation. Ten plants were used for each treatment. For *IbSINA5* expression analysis, fibrous roots, storage roots, stems, young leaves, mature leaves and senescent leaves of ten-week-old Taizhong 6 sweet potato plants were used. Total RNA was extracted as described previously [26].

2.2 Quantitative Real-Time Polymerase Chain Reaction Analysis

qRT-PCR was carried out using *IbSINA5* primers (forward: 5'-ATGTATAAAATGGAGATTGAAAGC-3'; reverse: 5'-TTAAGTGCTACAAATATTCG-3') as described previously [26]. The expression level of *IbCBF3*, *IbCOR413*, *IbCOR314* and *IbCOR27* were examined with the house keeping gene *IbTubulin* as an internal control [13].

2.3 IbSINA5 Isolation

The encoding cDNA of *IbSINA5* was cloned from sweet potato cultivar Taizhong 6 using its forward and reverse primers based on the gene information from the database [27]. The amplified cDNA fragment was cloned to the pEASY-T5 Zero Cloning Vector (Transgen, China) after sequence confirmation.

2.4 Subcellular Localization of IbSINA5

To detect the subcellular localization of IbSINA5 protein, IbSINA5 was fused in frame to the N-terminal of GFP in the pBI221:GFP vector. The resultant pBI221:IbSINA5-GFP and pBI221:GFP was transiently expressed in onion epidermal cells, respectively, as described previously [28]. The location of GFP and IbSINA5-GFP in the onion cells was observed with a confocal laser scanning microscopy (Leica TCS SP2).

2.5 Plasmid Construction and Sweet Potato Transformation

To construct the plant expression vector pCAMBIA-2301s2:IbSINA5, the full length cDNA of *IbSNIA5* was cloned into the modified pCAMBIA-1301s2 vector, driven by two copies of cauliflower mosaic virus (CaMV) 35S promoter. The resultant construct pCAMBIA-2301s2:*IbSINA5* was introduced into sweet potato cv. Xushu18 via *Agrobacterium*-mediated transformation as described previously [29].

2.6 Selection of Transgenic Sweet Potato Plants

For the molecular confirmation of transgenic plants, PCR and qRT-PCR were carried out to verify the integration and expression of *IbSNIA5*. To assess the expression levels of *IbSNIA5*, *IbCBF3* and *IbCOR* genes, total RNAs extracted from the mature leaves (the fourth leaves counted from the tops of plants) of four-week-old wild type and transgenic plants before and after cold treatment were used for qRT-PCR analyses. All gene specific primer sequences were the same as used by Jin et al. [13].

2.7 Cold Resistance Assays

For cold resistance analysis, four-week-old wild type and transgenic sweet potato plants grown in soil were kept at 4°C for 48 h and recovery at 25°C for 24 h. The phenotypes of plants before and after the treatments were photographed and the contents of proline, MDA and hydrogen peroxide (H_2O_2) in the mature leaves were measured as described previously [30,31].

2.8 Transient Transcription Dual-Luciferase Assays

To generate the reporter construct 35S::REN-ProIbSIN5::LUC, the promoter region (2001 bp) of *IbSNIA5* was inserted into pGreenII0800. To generate the effecter vector pGreenII62-SK-*IbCBF3*, *IbCBF3* were cloned into pGreenII62-SK. Sweet potato leaf protoplast preparation and transformation were carried out as described by Wang et al. [32]. Transient dual-luciferase assays in sweet potato leaf protoplast were performed as described previously [33,34].

2.9 Transient Expression of IbCBF3 in Sweet Potato Mesophyll Protoplasts

To analyze the transcription function of IbCBF3, the transcriptional factor effector pGreenII62-SK-IbCBF3 was transfected into sweet potato leaf protoplast. The transient expressions of *IbCBF3*, *IbSNIA5* and *IbCOR* genes in sweet potato leaf mesophyll protoplasts were examine by qRT-PCR as described previously [35].

2.10 Statistical Analysis

ANOVA (one-way) was used to generate every *P* value. The variability was indicated with the standard deviation (SD). *, ** and *** indicate *p*-value < 0.05, < 0.01 and < 0.001, respectively. Data are shown as mean ± standard deviation (SD) from three biological replicates each.

3 Results and Discussion

3.1 IbSNIA5 Encodes a Putative SINA Protein

Ubiquitination mediated protein degradation at different plant growth stages and in response to various abiotic stresses has been well studied [36,37]. The Arabidopsis SINAT5 (AtSINAT5) functioned as a RING type E3 ubiquitin ligase to control lateral root growth by degrading NAC1, whereas SINA2, a TRAF-like SINA family protein lacking RING domain, positively regulated the resistance to drought stress [19,25]. To explore the biological function of SINA family proteins in tuberous crops, we blast searched the sweet potato genome database using Arabidopsis *SINA2* (https://www.ipomoea-genome.org/genome_ jbrowse.html). A total number of seven *SINA2* homologues were identified. Similar to the previously reported SINA and some other TRAF-like family proteins, they all contain a conserved C-terminal TRAF-like domain [14–16]. However, among them, same as the Arabidopsis SINA2 and animal's TRAF1 proteins, IbSINA5 also lacks the RING domain (Fig. 1). Therefore, *IbSINA5* does not have E3 ligase activity required for its target protein degradation.

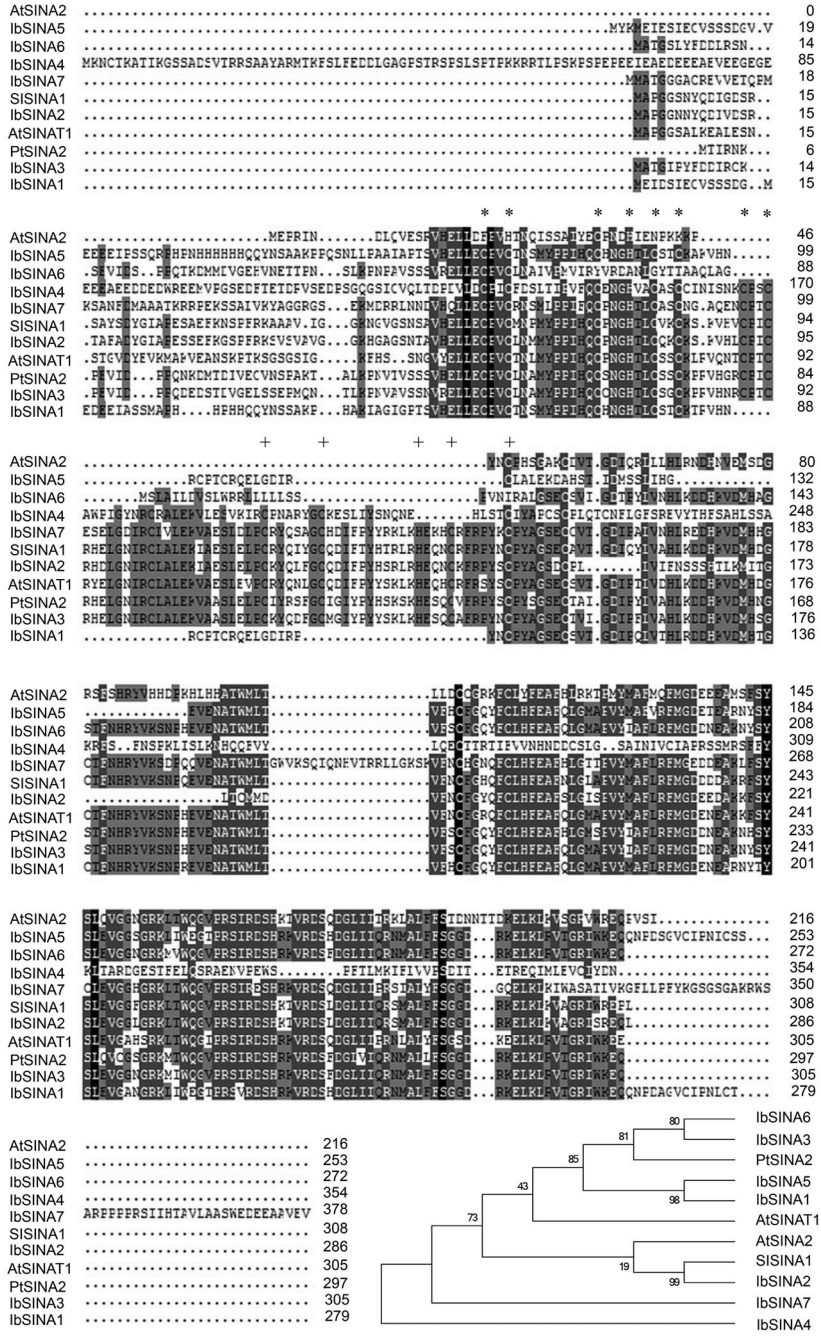

Figure 1: Sequence alignment and phylogenetic analyses of SINAs from different plant species. Comparison of *Ipomoea batatas* IbSINA5 with *Arabidopsis thaliana* AtSINAT1 (NP_181729.1), AtSINA2 (NP_187978.1), *Solanum lycopersicum* SlSINA1 (XP_004228679.1), and *Populus trichocarpa* PtSINA2 (XP_002299212.1), and phylogenetic tree of IbSINA5 homologs in different plants are shown. The gene and amino acid sequences of IbSINA1 (g919), IbSINA2 (g4297), IbSINA3 (g8848), IbSINA4 (g17091), IbSINA5 (g20660), IbSINA6 (g30553) and IbSINA7 (g58368) are extracted from the sweet potato genome database (https://www.ipomoea-genome.org/genome_ jbrowse.html). Based on their conservation levels, residues in the sequence alignment analyses are respectively highlighted in black, dark gray and light gray. The bar in phylogenetic tree represents the number of amino acid substitutions per site. Asterisk and plus signs represent the RING domain and zinc-finger motif, respectively

3.2 IbSNIA5 is Induced by Different Abiotic Stresses in Sweet Potato

To dissect the function of *IbSNIA5* in the resistance to abiotic stresses in sweet potato, its expression levels in wild type Taizhong 6 plants were examined by qRT-PCR. *IbSNIA5* was ubiquitously expressed in all the tested tissues and organs including fibrous roots, storage roots, stems, young leaves, mature leaves and senescent leaves, with a predominant expression in fibrous roots (Fig. 2A). Previously, we reported that SINA2 was strongly induced by ABA and drought [25]. We examined the expression levels of *IbSINA5* in three-week-old Taizhong 6 plants after low temperature (4°C), 25% PEG6000 or 250 mM NaCl treatments by qRT-PCR. We found that *IbSNIA5* was remarkably induced by cold, drought and salt stress, indicating its possible roles in plant response to various abiotic stresses (Figs. 2B–2D).

3.3 IbSINA5 is Mainly Localized in Nuclei and Cytoplasm

The subcellular localization of a certain protein may reflect its possible functions in the relevant biological processes it participates. To determine the subcellular localization of *IbSNIA5*, pBI221:IbSINA5-GFP and pBI221:GFP was transiently expressed in onion epidermal cells. IbSINA5-GFP fusion protein was mainly localized in nuclei and cytoplasm (Fig. 2E). Similar subcellular localization was also observed with SINA2-YFP fusion protein in Arabidopsis protoplasts [25]. The nuclei and cytoplasm localization of IbSINA5 implies that it may have a regulatory role in the expression of its upstream transcriptional factors in nuclei and its downstream functional genes in cytoplasm.

3.4 Overexpression of IbSINA5 Improves Cold Tolerance in Sweet Potato

Base on our previous study that overexpression of *SINA2* significantly augmented the resistance to drought stress in Arabidopsis plants, and the observation that *IbSINA5* was dramatically induced by cold and drought stresses, we speculated that overexpression of *IbSINA5* in sweet potato may also promote the cold resistance [25]. To verify this hypothesis, the plant expression construct pCAMBIA-2301s2:*IbSINA5* containing the coding sequence of *IbSINA5* was introduced into the genome of Xushu18 (Fig. 3A). A total number of 34 independent hygromycin resistant lines were obtained, and seven of them were randomly selected. PCR and qRT-PCR analyses confirmed the integration of *IbSINA5* in the sweet potato genome, and the overexpression of *IbSINA5* in all the tested transgenic lines (Figs. 3B and 3C).

To determine whether overexpression of *IbSINA5* would improve the resistance of transgenic sweet potato plants to cold stress, we compared their growth phenotypes with wild type plants. Three transgenic lines with different *IbSINA5* overexpression levels (L2, L5 and L7) were selected. Four-week-old wild type and transgenic plants grown in greenhouse were kept at 4°C for 48 h, then recovered at 25°C for 24 h. All transgenic plants showed less cold damage and successfully recovered after they were transferred back to room temperature, whereas wild type plants exhibited more severe cold damage and failed to recover (Fig. 4A). The damage extents were consistent with *IbSINA5* overexpression levels, as indicated by the more severe damage in transgenic line L5, which showed relatively lower *IbSINA5* overexpression level, than in transgenic lines L2 and L7, which showed relatively higher *IbSINA5* overexpression levels (Fig. 4B).

3.5 IbSINA5 Increases IbCOR Gene Expression and Oxidative Stress Tolerance in Transgenic Plants

It is well known that COR genes play a crucial role in plant response to cold stress [38]. We examined the expressions of *IbCOR413*, *IbCOR314* and *IbCOR27* in wild type and transgenic plants. As we have expected, the expression levels of *IbCOR* genes in transgenic plants were significantly higher than in wild type plants (Fig. 4C). Previous study has showed that overexpression of *IbCBF3* conferred improved tolerance to low temperature and drought stress on transgenic sweet potato, leading to decreased accumulation of MDA and H_2O_2 [13]. Similar results were also observed in transgenic sweet potato plants overexpressing *IbSINA5*. Under normal growth condition, no significant difference was observed. However, under cold stress condition, a higher proline, and lower MDA and H_2O_2 content was detected in transgenic plants (Figs. 4D–4F). The increased *IbCOR* gene expression and antioxidant ability might help explain the improved cold resistance in *IbSINA5* transgenic plants.

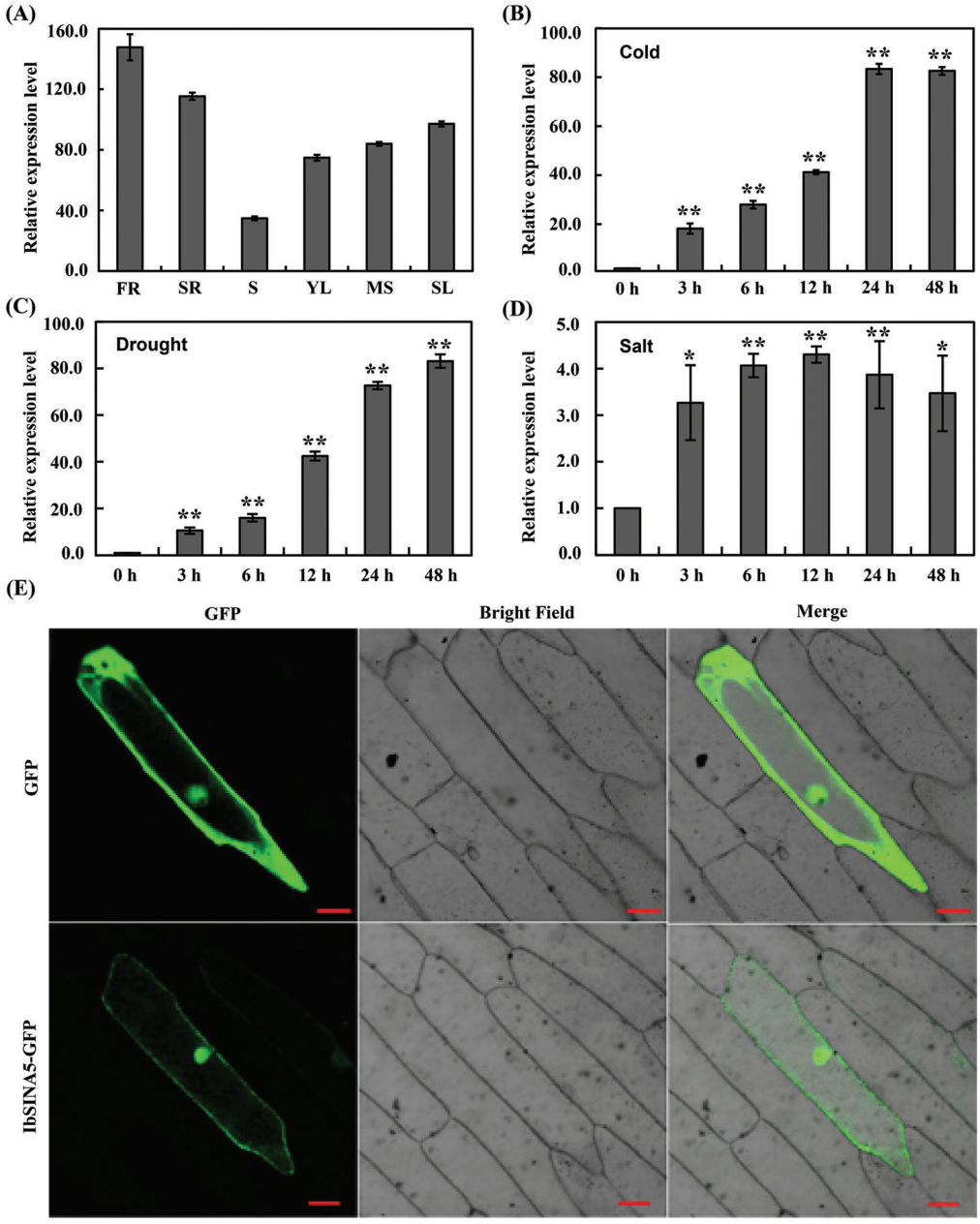

Figure 2: *IbSINA5* expression and IbSINA5 subcellular localization. (A) Expression pattern of *IbSINA5*. The transcription levels of *IbSINA5* in fibrous roots, storage roots, stems, young leaves, mature leaves and senescent leaves of ten-week-old sweet potato plants were shown. (B–D) Expression of *IbSINA5*in response to different abiotic stress treatments. Three-week-old sweet potato plants grown in greenhouse were kept at 4°C, or treated with 25% PEG6000 or 250 mM NaCl. The fourth fully expanded leaves counted from the tops of plants were collected at 3, 6, 12, 24 and 48 h after the treatments for RNA isolation. The transcription levels of *IbSINA5* were measured by qRT-PCR and normalized to *IbTubulin* expression. Values are means and standard deviations of three biological replicates. * and ** indicate p-value < 0.05 and < 0.01, respectively. (E) IbSINA5 is mainly localized in nuclei and cytoplasm. The location of GFP and IbSINA5-GFP in the onion epidermal cells was observed with a confocal laser scanning microscopy (Leica TCS SP2). Scale bars = 100 μm

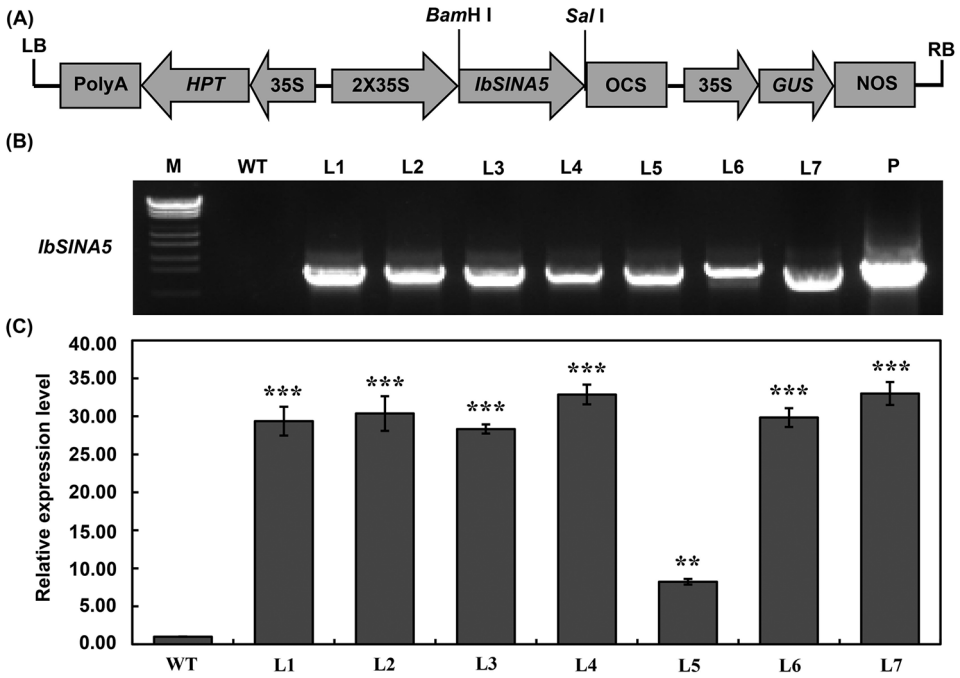

Figure 3: Molecular confirmation of *IbSINA5* transgenic plants. (A) Schematic map of pCAMBIA1301s2-*IbSINA5* construct. Expression of *IbSINA5* was driven by two copies of the cauliflower mosaic virus 35S promoter. (B) PCR analysis of *IbSINA5* integration in different transgenic lines. M, molecular marker; WT, wild type; L1-7, different transgenic lines; P, plasmid. (C) qRT-PCR analyses of *IbSINA5* in wild type and different transgenic lines. Gene expression level in WT was set to 1. Values are means and standard deviations of three biological replicates. ** and *** indicate p-value < 0.01 and < 0.001, respectively

3.6 IbSINA5 is Up-Regulated by IbCBF3 in Sweet Potato

Previous work has demonstrated that *IbCOR* genes were up-regulated by IbCBF3 [13]. We performed PlantCARE analysis with the promoter sequence of *IbSINA5*, and a dehydration-responsive element-binding/C-repeat-binding factor (DREB/CBF) binding (DRE core) element was identified (Fig. 5). To explore whether *IbSINA5* expression was regulated by CBFs in sweet potato, dual-luciferase assays were carried out. We found that IbCBF3 significantly activated the promoter of *IbSINA5*, implying that *IbSINA5* expression could be regulated by IbCBF3 (Figs. 6A and 6B). We then transiently expressed *IbCBF3* in the leaf protoplasts of wild type Xushu 18 plants. We observed that transient expression of *IbCBF3* increased the transcription of *IbSINA5*, *IbCOR413*, *IbCOR314* and *IbCOR27* (Figs. 6C and 6D).

Taken together, our results suggests that overexpression of *IbSINA5* up-regulates *IbCOR* gene expression and enhances cold resistance in transgenic sweet potato plants. IbCBF3 could be an efficient activator of *IbSINA5* to regulate the expression of *IbCOR* genes, possible in a CBF-SINA-COR module.

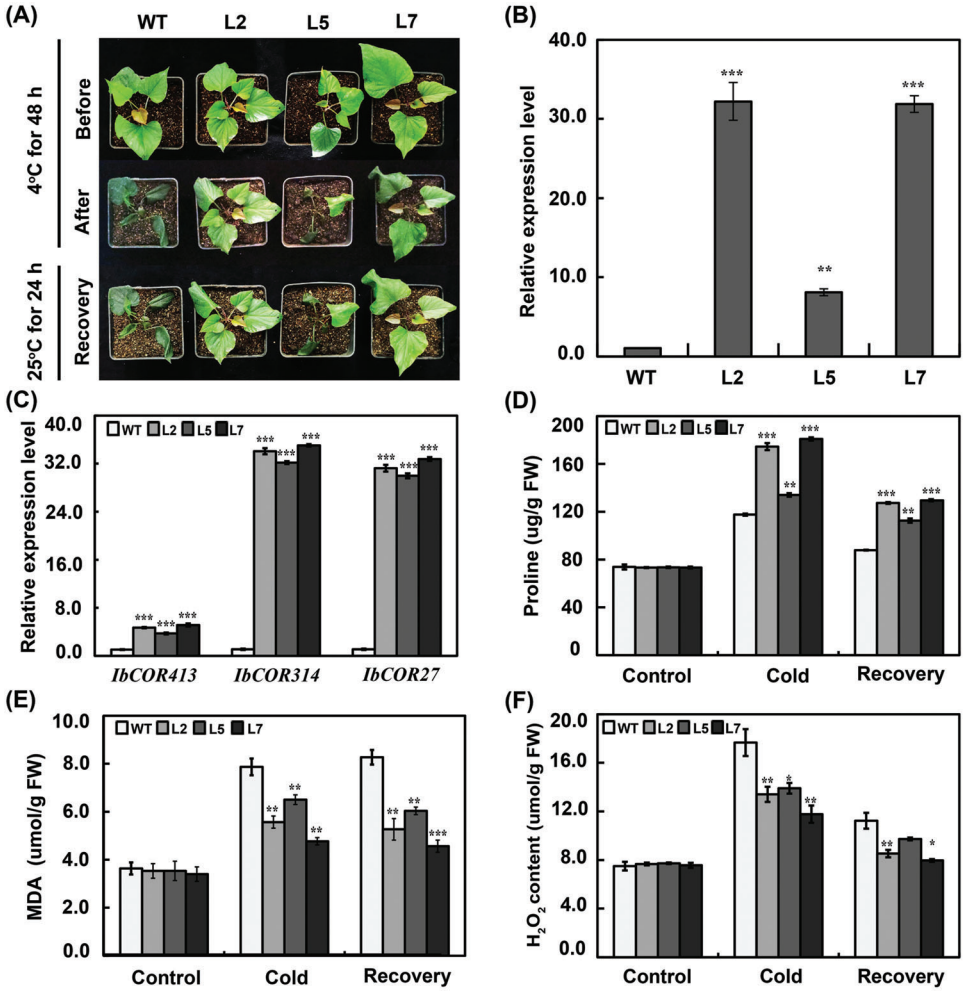

Figure 4: Cold tolerance and gene expression analyses of wild type and transgenic plants. (A) Phenotypes of sweet potato plants before and after cold stress treatment. Four-week-old wild type and transgenic were treated at 4°C for 48 h and recovered at 25°C for 24 h. Cold damage in the leaves of wild type plants was significantly severe than that of transgenic plants after 48 h cold treatment and 24 h recovery. Ten plants of wild type and each transgenic line were used for the treatment. Photos were the representatives from three replicates. (B) qRT-PCR analyses. The transcription levels of *IbSINA5* in the leaves of wild type and transgenic lines L2, L5 and L7 before cold treatments were measured by qRT-PCR. (C) *IbCOR* gene expression analyses. The transcription levels of *IbCOR413*, *IbCOR314* and *IbCOR27* in the leaves of four-week-old wild type and transgenic plants were examined by qRT-PCR. The transcript levels of these genes were normalized to *IbTubulin* expression. (D–F) Proline, MDA and H_2O_2 content analyses in the leaves of wild type and transgenic plants before and after the cold treatments. Values are means and standard deviations of three biological replicates. *, ** and *** indicate p-value < 0.05, < 0.01 and < 0.001, respectively

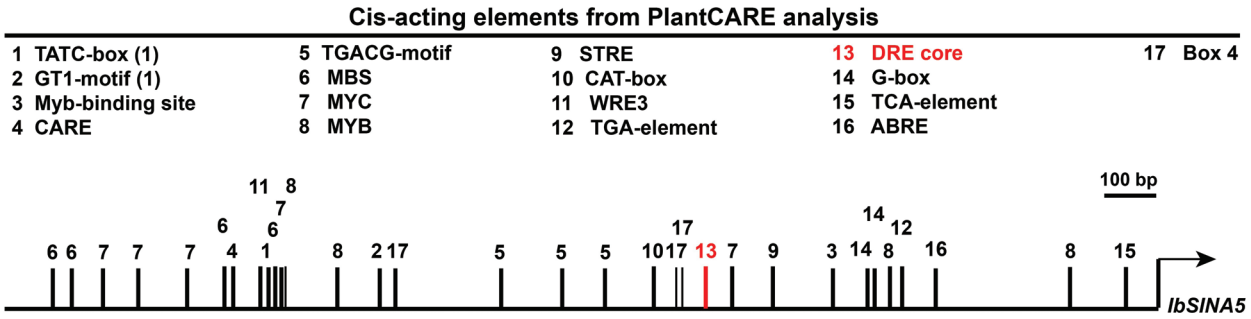

Figure 5: Plant CARE analysis. The Cis-acting elements in the promoter region of *IbSINA5* (2001 bp) were analyzed. The predicted CBF binding DRE core element was marked in red

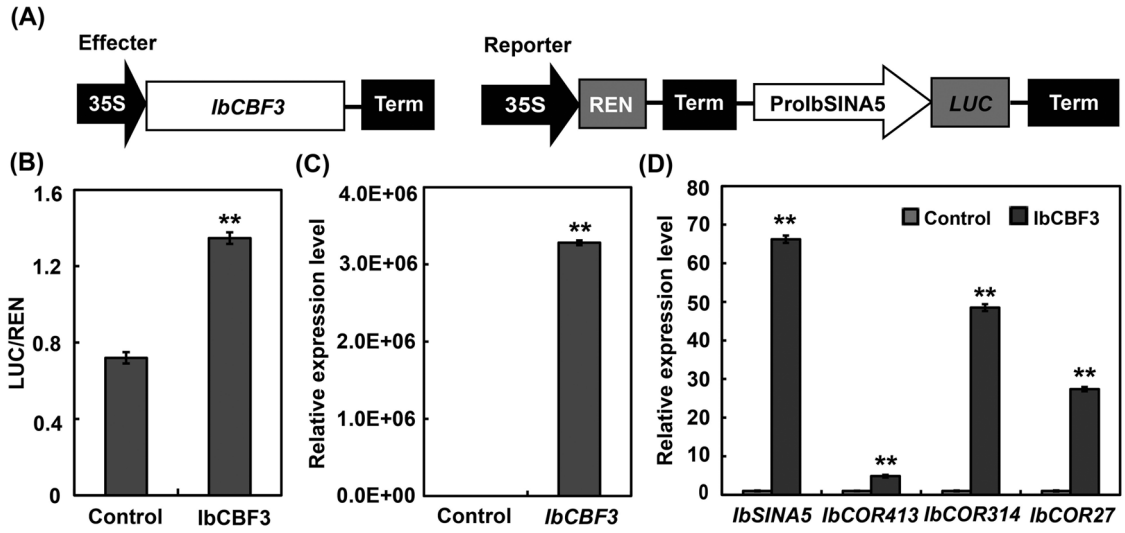

Figure 6: IbCBF3 positively regulates the expression of *IbSINA5* and *IbCOR* genes. (A) Schematic diagrams of the effector and reporter constructs. (B) IbCBF3 activates the promoter of *IbSINA5*. The control vector or pGreenII62-SK-*IbCBF3* were co-expressed with 35S::*REN*-ProIbSIN5::*LUC* in the leaf protoplasts of wild type sweet potato plants. LUC/REN ratios indicate the promoter activity of *IbSINA5* activated by IbCBF3. Control, protoplasts co-transfected with pGreenII62-SK and 35S::*REN*-ProIbSIN5::*LUC*; IbCBF3, protoplasts co-transfected with pGreenII62-SK-*IbCBF3* and 35S::*REN*-ProIbSIN5::*LUC*. (C) Expression of *IbCBF3* in the transfected sweet potato mesophyll protoplasts. (D) Expression levels of *IbSINA5* and *IbCOR* genes in the transfected protoplasts. pGreenII62-SK-*IbCBF3* was transfected into the sweet potato mesophyll protoplasts. pGreenII62-SK was used as a negative control. Total RNA was isolated from the transfected protoplasts for qRT-PCR analyses. The expression of gene in control was set to 1. Control, protoplasts transfected with pGreenII62-SK; IbCBF3, protoplasts transfected with pGreenII62-SK-*IbCBF3*. Values are means and standard deviations of three biological replicates. ** indicates p-value < 0.01

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Authors Contribution: LMW, SLZ and HXZ designed the experiments; SYL, XAL, LZZ, HQH and BL performed the experiments; ZZS and LMW analyzed the data; SYL and HXZ wrote the manuscript. All authors have read and approved the final manuscript.

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Conflicts of Interest: We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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