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The sweet potato transcription factor *IbbHLH33* enhances chilling tolerance in transgenic tobacco

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Abstract: Chilling is an important abiotic stress in plants. Sweet potato is sensitive to cold damage due to its tropical origin. In this study, we identified a basic helix-loop-helix (*bHLH*) gene, *IbbHLH33*, from our cold-tolerance-related transcriptomic data. Further analyses revealed that *IbbHLH33* encoded a nuclear protein and was most closely related to *AtbHLH33*. RT-qPCR analysis showed that *IbbHLH33* was expressed at the highest level in the roots, and its expression was strongly induced by low temperature (4 °C), H_2O_2 and abscisic acid (ABA) treatments. Transgenic tobacco plants overexpressing *IbbHLH33* were obtained by *Agrobacterium*-mediated transformation, which enhanced the chilling resistance of tobacco. At low temperatures, the proline content, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content increased significantly, while the relative conductivity decreased significantly. At the same time, the expression of proline synthesis related genes and antioxidant activity related genes increased, while the expression of ABA synthesis related genes decreased. The results showed that *IbbHLH33* is a transcription factor encoding a gene of the *bHLH* family that regulates chilling tolerance. In conclusion, these data suggest that *IbbHLH33* has the potential to improve chilling tolerance in tobacco and other plants.

Keywords: *bHLH*; cold tolerance; *Ipomoea batata* (L.) Lam.; overexpression

Sweet potato (*Ipomoea batata* (L.) Lam.) is an important root crop, which is widely grown worldwide (Bovell-Benjamin 2007; Zhang et al. 2019). Sweet potato has a high tolerance to abiotic stress, can grow in marginal land, and has high nutritional value (Bovell-Benjamin 2007). Sweet potato is used not only as food and feed but also as an important industrial raw material and new energy crop, and it plays an extremely vital role in the development of the food and biological industries (Liu 2017). As the environment changes, plant growth and development are being greatly threatened by environmental stress factors such as low temperatures (Ding et al. 2015; Jiang et al. 2020). Sweet potato originated in the tropical Americas and is vulnerable to chilling damage at low temperatures (Picha 1987). Low temperature causes discoloration and partial suppression of sweet potato root tubers, having adverse effects on storage and germination, especially during the rapid root expansion period, which severely reduces the yield and affects the planting area of sweet potato (Porter et al. 1976; Fan et al. 2012, 2015).

Weiser was the first to suggest that chilling injury might involve changes in gene expression (Weiser

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1970). To adapt to decreased temperature in the external environment, plants have evolved a complex regulatory mechanism to address chilling stress; plants in temperate regions experience low temperatures, which improves their chilling tolerance (Thomashow 1999; Shi et al. 2018). When pepper (*Capsicum annuum* L.) was subjected to cold injury, exogenous glutathione (GSH) treatment enhanced the ascorbate-glutathione cycle, which improved chilling resistance (Yao et al. 2021). The *PtrBAM1* gene of *Poncirus trifoliata* functions in chilling resistance by regulating soluble sugar levels (Peng et al. 2014). *OsGRF6* is a significant regulatory factor that balances the growth and cold tolerance of rice (*Oryza sativa* L.). Under low-temperature stress, *OsGRF6* can inhibit *OsGA2OX1*, activate *OsGA2OX1* expression, reduce active gibberellic acid (GA) levels, and enhance cold tolerance (Li et al. 2021b). In recent years, genetic engineering technology has been widely used in sweet potato to improve its stress resistance. For example, overexpression of the genes *IbBBX24*, *IbATL38*, and *IbBT4* has been shown to enhance both biological and abiotic stress resistance (Zhang et al. 2020; Zhou et al. 2020; Du et al. 2021). Genes related to chilling resistance in sweet potato have been reported as well (Jin et al. 2017; Li et al. 2021a).

The basic helix-loop-helix (bHLH) family is an ancient transcription factor family and the second largest gene family in plants (Laughon & Scott 1984). bHLH transcription factors are defined by the characteristic bHLH domain (Ferré-D'Amaré et al. 1993). BHLH motif by conservative of amino acids and two functional areas, namely the N-terminal base area and the helix-loop-helix area (Murre et al. 1989; Atchley et al. 1999). The first plant protein reported to have a bHLH domain was the Lc protein, a product of the *R* gene in maize, which is involved in the synthesis of flavonoids and anthocyanins (Ludwig et al. 1989). However, the characteristics and functions of *bHLH* transcription factors began to become clear only after the structure and function of the *PH04* gene, involved in phosphate metabolism in yeast, were elucidated (Berben et al. 1990). To date, there have been some studies on the bHLH protein in plants. The bHLH protein has diverse functions in plants, for instance, which participates in signalling (Friedrichsen et al. 2002; Oh et al. 2020) and plays a significant role in the plant stress response (Kiribuchi et al. 2004; Dong et al. 2014). Cold tolerance has also been reported to be associated with *bHLH* transcription factors. For example, *NtbHLH123* (*Nicotiana tabacum* L.) is a positive regulator of chilling resistivity that activates *NtCBF* and reactive oxygen species (ROS) to eliminate related genes and stress response genes (Zhao et al. 2018). Overexpression of the *PtrbHLH* gene enhanced the chilling resistivity of tobacco and lemon (*Citrus limon*), and a yeast one-hybrid assay demonstrated that *PtrbHLH* could bind to the E-box element in the peroxidase (POD) promoter region; these results showed that *PtrbHLH* enhanced chilling tolerance by actively regulating the removal of ROS (Huang et al. 2013). Overexpression of the *bHLH* gene in transgenic plants enhanced the resistance to cold stress, indicating that some genes of the *bHLH* family contribute to plant adaptation to harsh environments.

Although the *bHLH* transcription factor family has attracted much attention, the previous studies have focused mostly on model plants such as *Arabidopsis thaliana* and less on hexaploid sweet potato with its complex genetic background. Therefore, we identified *IbbHLH33* by transcriptome sequencing of the cold-resistant sweet potato cultivar Liaoshu 36. To verify the function of *IbbHLH33*, in this study, we overexpressed the *IbbHLH33* gene in tobacco and analysed and verified it, providing a basis for cold resistance research in sweet potato.

MATERIAL AND METHODS

Plant materials and growth conditions. The plant materials used in this study included the chilling-tolerant sweet potato cultivar Liaoshu 36, which was obtained from the Liaoning Academy of Agricultural Sciences. The tobacco cultivar Wisconsin 38 was provided by the Sweet Potato Laboratory of China Agricultural University. Liaoshu 36 was grown on Murashige and Skoog (MS) solid medium, and the culture conditions were 27 ± 1 °C with 16 h of light and 8 h of dark. The tobacco cultivar Wisconsin 38 was grown on half-strength MS solid medium, and the culture conditions were the same as those for Liaoshu 36.

Strains and plasmid vectors. *Escherichia coli* DH5α was purchased from Shenzhen Health Life Technology Co., Ltd. (Shenzhen, China). *Agrobacterium* EHA105 was provided by the Sweet Potato Laboratory of China Agricultural University. The PMD19-T vector was purchased from Bao Bioengineering Co., Ltd. (Dalian, China), and the ampicillin (AMP) resistance gene (*ampr*) was used for T/A cloning. Both the overexpression vector pCAMBIA 1300 and the subcellular localization vector pCAMBIA 1300-GFP

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were obtained from the Sweet Potato Laboratory of China Agricultural University. pCAMBIA 1300 and pCAMBIA 1300-GFP confer kanamycin (KANA) resistance and were used for positive selection.

Amplification and analysis of the coding sequence, genome sequence and promoter of *IbbHLH33*. The expressed sequence tag (EST) sequences of differentially expressed genes were screened from a chilling-resistant suppression subtractive hybridization (SSH) subtractive library constructed in our laboratory. Through NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis we identified a domain with a conserved *bHLH* structure sequence. The obtained sequences were compared with Sweetpotato GARDEN (http://sweetpotato-garden.kazusa.or.jp/index.html) to obtain the predicted *IbbHLH33* sequence. HiPer Total RNA Extraction Reagent from Mei5bio (Beijing, China) was used to extract total RNA from Liaoshu 36 leaves. A Quant cDNA Strand 1 Synthesis Kit (Beijing, China) was used for reverse transcription to obtain cDNA. *IbbHLH33* was amplified using cDNA as a template, purified, and then ligated to the pMD19-T vector. We selected five monoclones with bands amplified by PCR for sequencing analysis, and the sequencing results of these monoclones were highly consistent, thus indicating the coding sequence (CDS) of *IbbHLH33*. In addition, genomic DNA from Liaoshu 36 leaves was used as the substrate for amplification, and the genomic DNA sequence of *IbbHLH33* was obtained. The promoter sequence was obtained by using Liaoshu 36 DNA as substrate. All primers are listed in Table S1 in Electronic Supplementary Material (ESM). The amino acid sequence encoded by the *IbbHLH33* gene was deduced by Primer Premier (Ver. 5) software. NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used for domain analysis to find the conserved structure of the amino acid sequences, and ProtParam (http://web.expasy.org/protparam/) was used to predict the molecular weight and isoelectric point of the encoded protein domain. Multiple comparisons of *IbbHLH33*-homologous protein sequences were performed by DNAMAN software. The phylogenetic tree of *IbbHLH33*-homologous proteins was constructed by MEGA (Ver. 6.0) software. Using the Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) genomic DNA analysis tool, the region spanning *IbbHLH33* was analysed. Search for promoter elements on the PlantCARE web site (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Expression analysis of *IbbHLH33* in sweet potato. Total RNA was extracted from the leaves, stem segments and storage roots of 3-month-old field seedlings of Liaoshu 36 and then reverse transcribed to cDNA to analyse the expression level of *IbbHLH33* in each tissue of sweet potato. Liaoshu 36 seedlings grown in test tubes were washed and placed in 1/2 Hoagland solution for domestication for 3 days. Four-week-old plants grown *in vitro* were treated with Hoagland solution with 100 μM abscisic acid (ABA) and 200 mM hydrogen peroxide (H_2O_2) at 4 °C. After 0, 1, 3, 6, 12 and 24 h of treatment, total RNA was extracted from the leaves and reverse transcribed to cDNA. Fluorescent quantitative primers were designed according to the nonconserved CDS interval of the *IbbHLH33* gene, and the primer sequences are listed in Table S1 in ESM. The reference gene was the sweet potato actin gene (*β-actin,* GenBank, AY905538). RT-qPCR analysis was performed using a fluorescence quantitative kit from Mei5bio (Beijing, China), RR420 and a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA).

Construction of plant expression vectors. pCAMBIA 1300 and pCAMBIA1300-GFP were selected as the original vectors. *Kpn* I and *BamH* II were chosen for the double digestion technique, and the homologous recombination technique was used to link fragments to the vectors. The ligation products were transformed into *E. coli* DH5α, and monoclones were selected for sequencing. pCAMBIA1300-GFP was the same as pCAMBIA 1300 except that the termination codon had been removed. The subsequent methods for purification and recovery and for transformation of *E. coli* DH5α were the same as those used for pCAMBIA 1300. The recombinant plasmid was transformed into *Agrobacterium* EHA105 competent cells, and positive monoclones were screened out and stored for later use. All the primers are listed in Table S1 in ESM.

Subcellular localization. Protoplasts of 2-week-old Nichimoharu rice were isolated as described by Yoo et al. (2007), transfected into pCAMBIA1300-*IbbHLH33*-GFP with previously constructed plasmids, and incubated at 28 °C for 16 h. The fluorescence signal of the expressed protein was observed by fluorescence microscopy. The excitation wavelength of the green fluorescent protein (GFP) laser and 4',6-diamidino-2-phenylindole (DAPI) laser was 488 nm.

Acquisition and identification of transgenic tobacco plants. Transformation of tobacco (*Nicotiana tabacum* cv. Petit Havana) was performed following Horsch et al. (1985). Tobacco leaf discs (5 × 5 mm) without main veins were cut off in the

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middle of a superclean working table and placed on regeneration medium (MS + 1.0 mg/L, 6-BA + 0.1 mg/L NAA). The culture medium was affixed on the back and incubated at 28 °C in darkness for 2~3 days. The leaf discs were infected with *Agrobacterium* solution (at an OD600 of 0.4~0.6) for 10 min. The culture was placed on the regeneration medium in the dark at 28 °C for 2~3 days. Then, the cells were washed with sterile distilled water three times and 500 mg/L carbenicillin once and then placed on the screening medium (MS + 1.0 mg/L, 6-BA + 0.1 mg/LNAA+300 mg/L carbenicillin) to generate buds (light: 45 μmol/m^2/s, 28 °C). Subsequently, buds with a length of 1 cm were cut off and placed on MS medium to grow into complete plants. At the same time, genomic DNA was extracted from the leaves of the pseudotransgenic plants and the control plants. PCR detection was performed with vector plasmid DNA as the positive control and DNA from water and non-transgenic plants (wild type, WT) as the negative control.

Chilling resistance assays. For chilling tolerance analysis, 4-week-old WT and transgenic tobacco plants with the same status were treated at 4 °C for 48 h and allowed to recover at 25 °C for 24 h. The phenotypes of the plants were recorded before and after treatment. QRT-PCR analysis of *IbbHLH33* expression in WT and transgenic plants.

Measurement of proline content, malondialdehyde content, relative conductivity and superoxide dismutase activity. Under 4 °C treatment, the proline content, malondialdehyde (MDA) content, relative conductivity and superoxide dismutase (SOD) activity in tobacco leaves were measured according to the methods of Zhang et al. (2019) and Nanjo et al. (1999).

Expression analysis of the related genes in transgenic tobacco plants. The expression levels of stress-related genes in transgenic plants and control plants treated at 4 °C for 0 h and 48 h were analysed. The stress resistance-related genes analysed in this study included the proline synthesis-related *P5CR*, antioxidant activity-related *SOD* and *POD*, and ABA synthesis-related *ZEP*. Primers for specific gene amplification were designed using Primer Premier 5. The reference gene was the *Actin* gene from the tobacco cultivar Wisconsin 38. All the primers are listed in Table S1 in ESM.

Statistical analysis. Three biological replicates were performed for each experiment. The data are expressed as the mean ± standard deviation. Student's *t* test (double-tailed analysis) was used, and SPSS (Ver. 23.0) was used for difference analysis. Significance levels of $P < 0.05$ and $P < 0.01$ are indicated by ∗ and ∗∗, respectively.

RESULTS

Amplification and analysis of the CDS, genome sequence and promoter of *IbbHLH33*. The *IbbHLH33* CDS was 1461 bp long (Figure 1A) and encoded a 486-aa polypeptide with a molecular weight of 53.68 kDa and a *p*I of 5.21. The 2652-bp full-length DNA sequence of *IbbHLH33*, including 4 exons and 3 introns (Figure 1B), was amplified. By comparison with *bHLH* family members in *Arabidopsis thaliana*, *IbbHLH33* was found to be most closely related to AtHLH33 (Figure 1C). The *IbbHLH33* protein has a *bHLH* domain and an ACT domain at the C-terminus and belongs to the *bHLH* family. The online analysis software PlantCare was used to analyse the promoter of *IbbHLH33*. The results showed that this promoter region (~1 530 bp), containing one ABA-responsive element (ABRE), one CGTCA motif (MeJA-responsive element), and one low-temperature responsive (LTR) element, was associated with phytohormones and resistance to chilling (Table 1).

The expression of *IbbHLH33* was highest in sweet potato storage root and was induced by chilling, ABA and H_2O_2. Using the sweet potato *Actin* gene as an internal reference gene, qRT-PCR was used to analyse the expression of *IbbHLH33* in different tissues of Liaoshu 36. The results showed that *IbbHLH33* was expressed to different degrees in the storage root (SR), stems and leaves of Liaoshu 36, and the expression level in the SR of Liaoshu 36 was significantly higher than that in the leaves and stems (Figure 2A). Chilling

Table 1. Promoter elements analysis of *IbbHLH33*

Site Name	Sequence	Function
ABRE	ACGTG	cis-acting elements involved in abscisic acid reaction
CGTCA motif	CGTCA	cis-regulating element involved in MeJA reaction
LTR	CCGAAA	cis-acting elements involved in cryogenic reactions
RY element	CATGCATG	cis-regulatory elements involved in seed specific regulation

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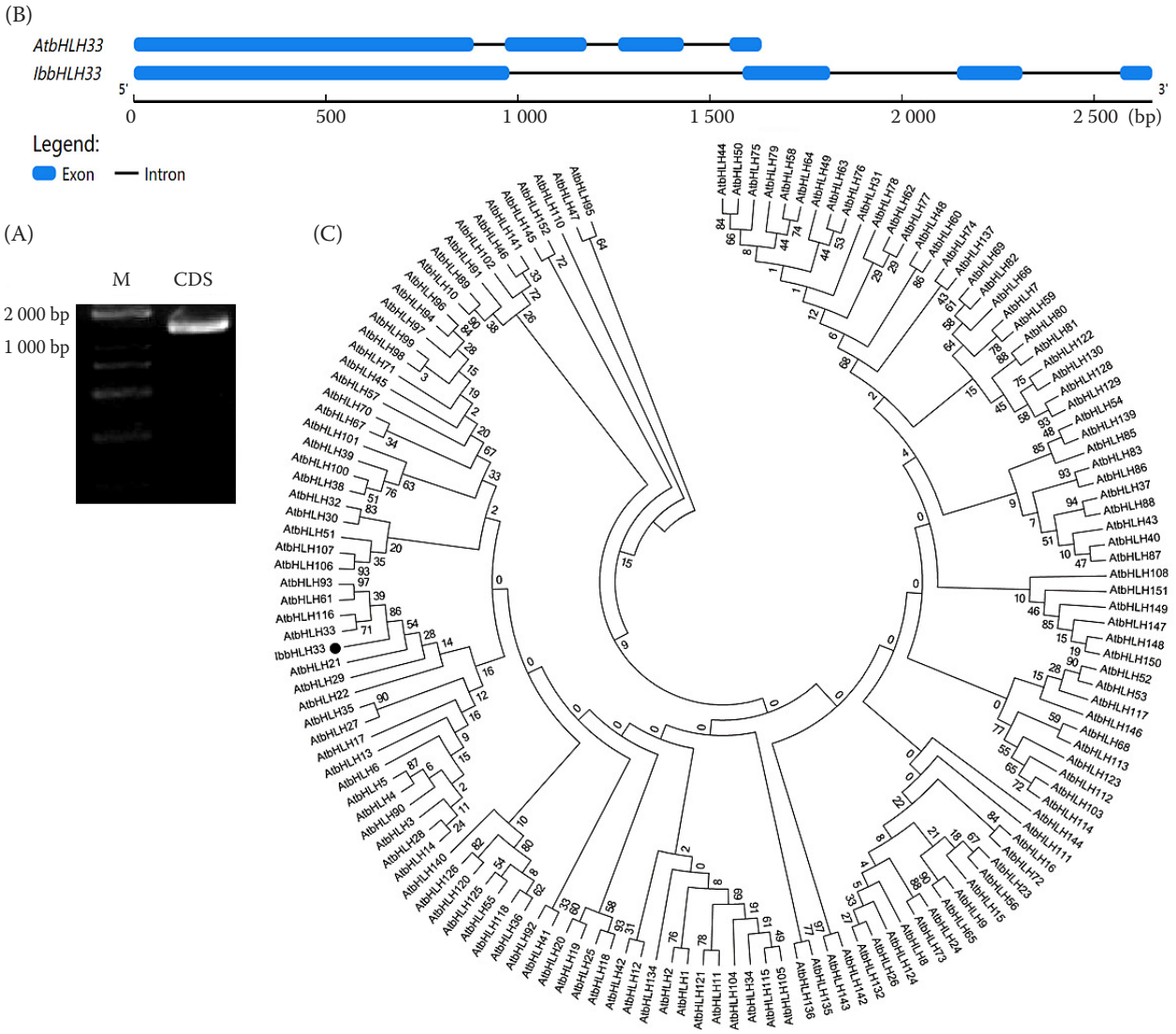

Figure 1. Sequence analysis of *IbbHLH33*: PCR amplification of *IbbHLH33* (M – marker; CDS – coding sequence) (A), genomic structures of *IbbHLH33*, boxes indicate exons, and lines indicate introns (B), phylogenetic analysis of the IbbHLH33 protein (C)

treatment (4 °C) was carried out on three-month-old seedlings of Liaoshu 36, and the results showed that *IbbHLH33* expression was strongly induced by chilling stress (Figure 2B). Under ABA treatment, genes were upregulated during 0~12 h of 4 °C stress and downregulated during 12~24 h of stress. Among them, the expression of *IbbHLH33* peaked at 12 h (10 times) (Figure 2C). Similar to the 4 °C treatment, when Liaoshu 36 was treated with 200 mM H_2O_2, the expression of *IbbHLH33* peaked at 6 h and then decreased, which indicated that *IbbHLH33* expression was induced by H_2O_2 (Figure 2D). Under the 4 °C, ABA and H_2O_2 treatments, the expression of *IbbHLH33* was induced and fluctuated simultaneously, which may be related to the spatiotemporal specificity of gene expression under stress, the regulation of the upstream gene response, and the optical density at sampling time points.

***IbbHLH33* localized to the nucleus.** The *IbbHLH33* transient expression vector 35S::*IbbHLH33*-GFP and empty vector 35S::GFP (Figure 3A) were used to transform rice protoplasts, and the distribution of the target protein in the cells was determined by measuring the GFP signal. The results showed overlap between the GFP signal and DAPI staining, indicating that *IbbHLH33* was located in the nucleus (Figure 3B). The scale bars represent 20 μm.

Acquisition of transgenic plants overexpressing *IbbHLH33*. The plant expression vector pCAMBIA

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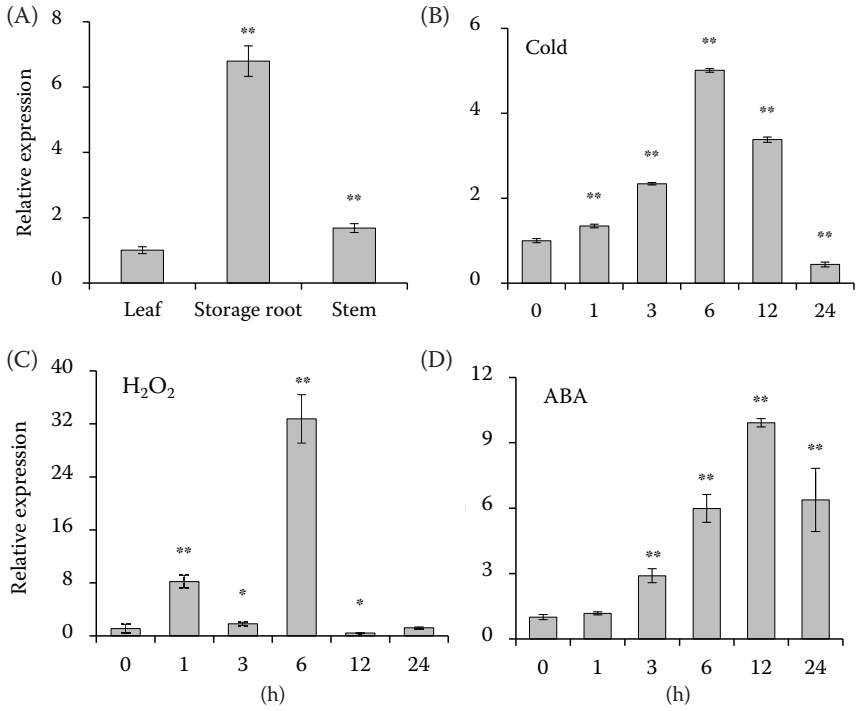

Figure 2. Expression analysis of *IbbHLH33*: transcriptional levels of *IbbHLH33* in different tissues of 3-month-old field-grown Liaoshu 36 plants (A), expression level of *IbbHLH33* in Liaoshu 36 plants under 4 °C treatment (B), expression level of *IbbHLH33* in Liaoshu 36 plants under H_2O_2 treatment (C), expression level of *IbbHLH33* in Liaoshu 36 plants under abscisic acid (ABA) treatment (D)

The data are presented as the mean ± SE (n = 3); according to Student's t test, *, **significant difference at the $P < 0.05$, 0.01 level

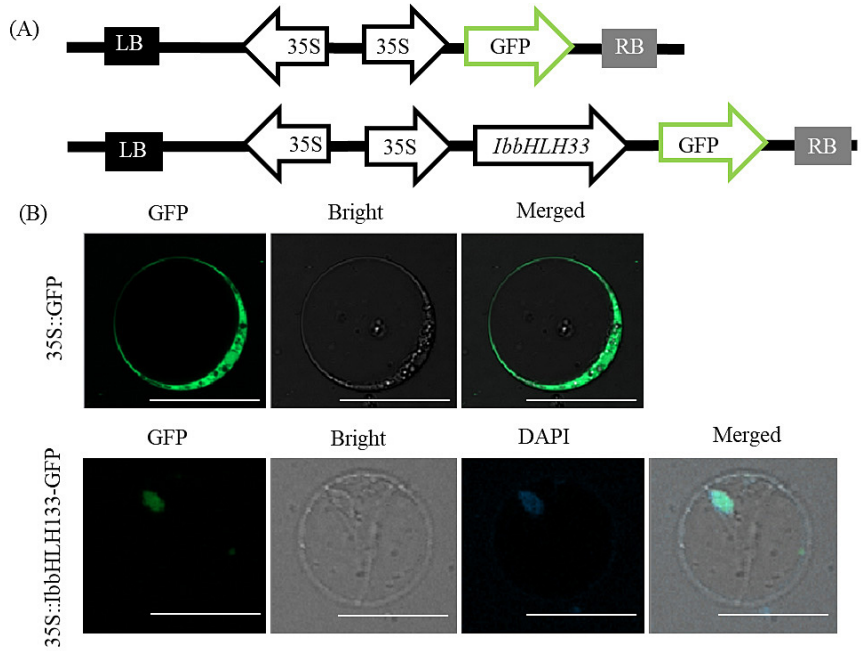

Figure 3. Subcellular localization of *IbbHLH33*: schematic diagram of the *IbbHLH333* subcellular localization vector (the empty vector is shown on top, and the constructed vector is shown on the bottom) (A), subcellular localization of the *IbbHLH33* protein in rice protoplasts (B)

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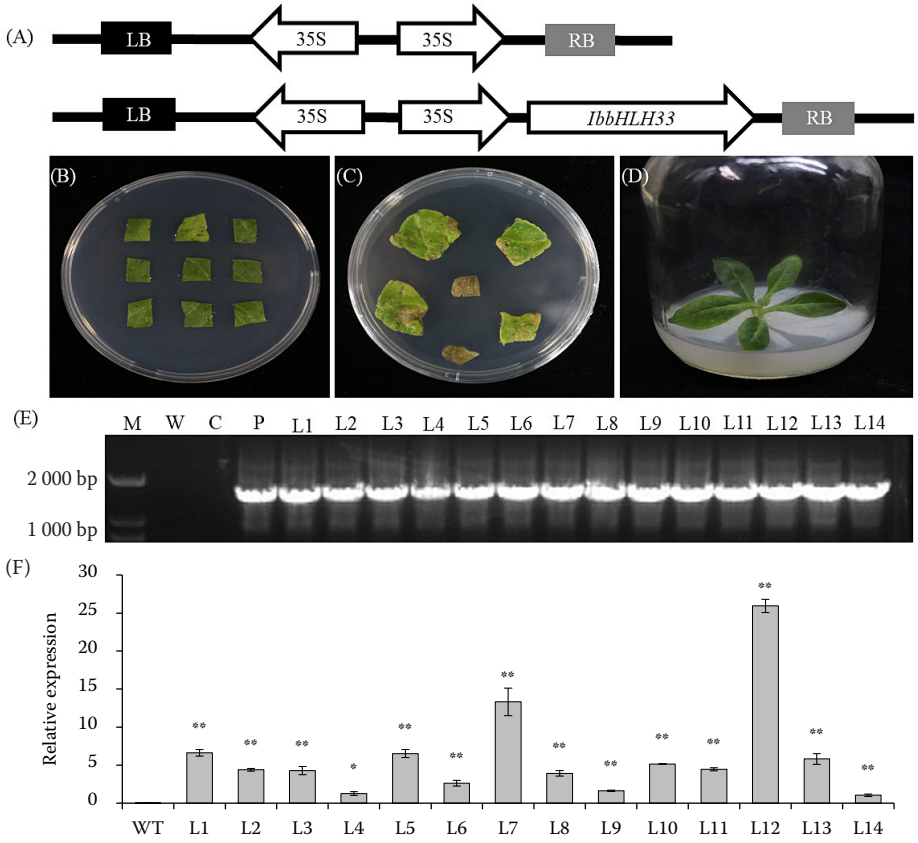

Figure 4. Acquisition of *IbbHLH33* transgenic plants: schematic diagram of the *IbbHLH33* overexpression vector, the empty vector is shown on top, and the constructed vector is shown on the bottom (A), transformation, screening and regeneration of *IbbHLH33* transgenic tobacco plants (B–D), positive identification of *IbbHLH33* pseudotransgenic tobacco plants (M – BL2000 DNA markers; W – water as a negative control; C – wild type (WT) tobacco plants; P – pCAMBIA1300-*IbbHLH33* as a positive control) (E), expression analysis of *IbbHLH33* in transgenic tobacco plants (the data are presented as the mean ± SE (n = 3); according to Student's t test, *, **significant difference at the P < 0.05, 0.01 level) (F)

1300-*IbbHLH33* was constructed (Figure 4A). The recombinant plasmid was transformed into competent *Agrobacterium tumefaciens* EHA105 cells and then into tobacco using the *Agrobacterium tumefaciens*-mediated leaf disc transformation method. After 2–3 days of dark culture, the callus was transferred to differentiation medium for light culture. After 3–5 weeks, resistant calli were formed, and the resistant buds gradually differentiated. When the resistant buds grew to approximately 1 cm, they were cut off and transferred to 1/2 MS rooting medium and finally grew into complete plants (Figure 4B–D). Genomic DNA was extracted from 30 pseudotransgenic plants, and PCR was performed with plasmids as a positive control and nontransgenic plants as a negative control. Fourteen of the 30 pseudotransgenic plants showed amplification of bands of 1 461 bp, the same size as the positive control, while the nontransgenic plants did not show amplification of this band (Figure 4E). qRT-PCR analysis showed that the expression level of *IbbHLH33* in transgenic tobacco lines L7 and L12 was significantly higher than that in other lines and the WT (Figure 4F), so the L7 and L12 strains were selected for subsequent study.

Overexpression of *IbbHLH33* in tobacco enhanced chilling tolerance. WT and transgenic lines with the same growth status were treated at 4 °C simultaneously. After 48 h of treatment, the WT showed obvious wilting, while the transgenic lines L7 and L12 showed less damage than the WT (Figure 5A). To explore the expression level of *IbbHLH33* in transgenic plants during chilling treatment, we carried out qRT-PCR analysis. *IbbHLH33* showed different expression levels before and after chilling stress

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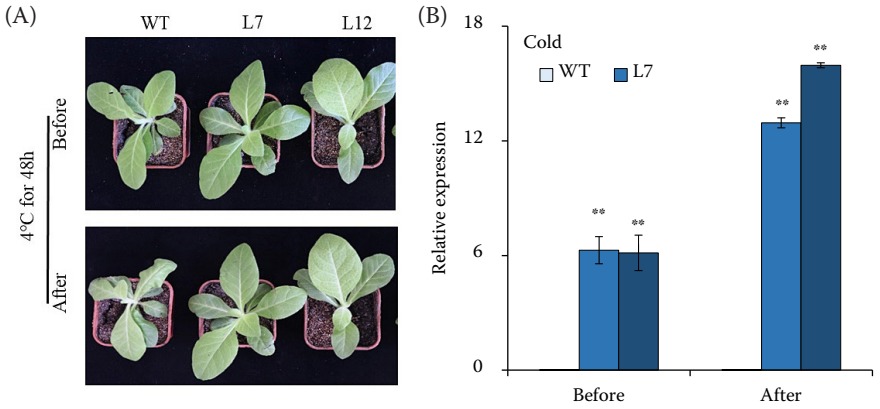

Figure 5. Chilling tolerance and gene expression analyses of wild type (WT) and transgenic plants: phenotypic changes in tobacco plants before and after chilling stress (A), expression level of *IbbHLH33* in the WT and transgenic plants before and after chilling stress (B)

The data are presented as the mean ± SE (n = 3); according to Student's t test; *, **significant difference at the $P < 0.05$, 0.01 level

(Figure 5B), which indicated that *IbbHLH33* was involved in chilling resistance-related pathways and enhanced the cold tolerance of transgenic plants.

Overexpression of *IbbHLH33* in tobacco activated the ROS-scavenging system. Before chilling treatment, there was no significant differences in proline content, SOD activity, relative conductivity or MDA content between the WT and the transgenic lines L7 and L12 (Figure 6A–D). Under chilling stress, the proline content, SOD activity and MDA content of transgenic plants were significantly higher than those before treatment (Figure 6A, B, D), while the relative conductivity was significantly lower than that in the WT (Figure 6C).

Analysis of the expression levels of *NtP5CR*, *NtSOD*, *NtPOD* and *NtZEP* in WT and transgenic plants. To further verify the differences in proline content, SOD activity, relative conductivity and MDA content between WT and transgenic plants, qRT-PCR analysis was carried out. The analysis showed

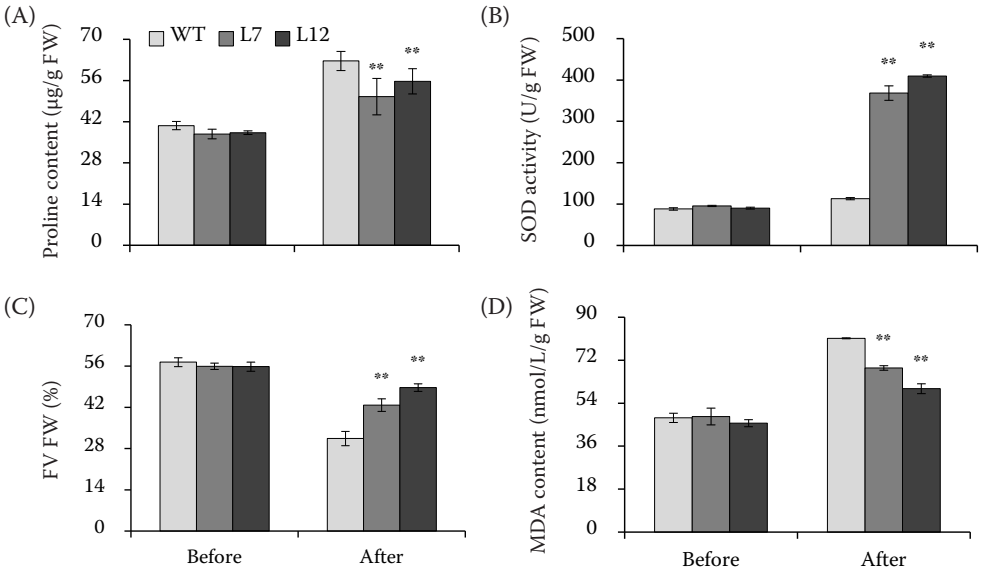

Figure 6. Analysis of the proline content (A), superoxide dismutase (SOD) activity (B), relative conductivity (C) and malondialdehyde (MDA) content (D) analyses in the leaves of wild type (WT) and transgenic plants under the chilling treatments

The data are presented as the mean ± SE (n = 3); according to Student's t test; *, **significant difference at the $P < 0.05$, 0.01 level

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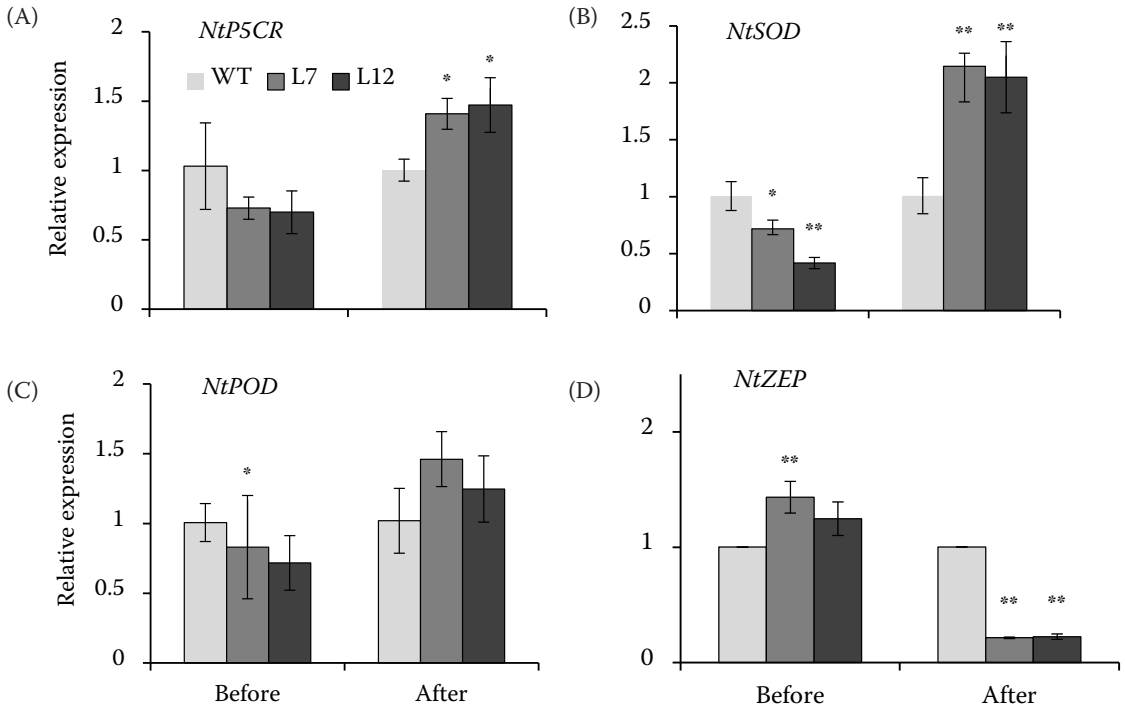

Figure 7. Analysis of *NtP5CR* (A), *NtSOD* (B), *NtPOD* (C) and *NtZEP* (D) expression in wild typw (WT) and transgenic plants before and after chilling treatment

The data are presented as the mean ± SE (n = 3); according to Student's t test; *, **significant difference at the $P < 0.05$, 0.01 level

that the expression of *NtP5CR*, *NtSOD* and *NtPOD* was up-regulated, while that of *NtZEP* was down-regulated, under chilling stress (Figure 7A–D). The results of the expression analysis were consistent with the physiological and biochemical indexes (Figure 6).

DISCUSSION

The damage caused by low temperature is mainly divided into chilling injury (above 0 °C) and (freezing injury) below 0 °C (Guy 1990). Temperature sensitivity differs among plants originating from different regions. Generally, plants originating in tropical or subtropical regions are far less resistant to low temperature than those from temperate and frigid regions.

Sweet potato originated in the tropical Americas and is vulnerable to chilling damage at low temperature (Picha 1987). Chilling injury can greatly reduce the yield, quality and geographical distribution of sweet potato (Porter et al. 1976; Fan et al. 2012, 2015). Improving the tolerance of plants to abiotic and biotic stresses can enable the maintenance of normal growth and stable yield in harsh environments (Sah et al. 2016). Therefore, we cloned the *IbbHLH33* gene from the cold-resistant sweet potato cultivar Liaoshu 36; this gene is closely related to *AtbHLH33* in *A. thaliana* (Figure 1C), so it was named *IbbHLH33*. The genomic exon and intron composition of *IbbHLH33* is similar to that of *AtbHLH33* (Figure 1B), suggesting that *IbbHLH33* is highly conserved among different species. At present, *bHLH33*-homologous genes have been reported in other species. For example, there was a significant positive correlation between *bHLH33* and *MYB6*, which are *WD40* transcription factors in *Malus hupehensis* (Pamp.) Rehder. Studies have shown that these three transcription factors can jointly regulate anthocyanin biosynthesis, revealing the spatiotemporal regulation information of anthocyanin biosynthesis (Liu et al. 2019; Han et al. 2020). The genes *bHLH33* and *MYB1-2* inhibit the accumulation of anthocyanins in sunburned apples by downregulating *MdANR* and *MdFLS* (Liu et al. 2018). *AtICE1* encodes a transcription factor of the bHLH family. Under low-temperature treatment, the expression level of the At*CBF3* gene in *ice 1* mutants was significantly reduced, which resulted in a significant decrease in the freezing resistance of the plants, and overexpression of *AtICE1* could significantly enhance the freezing resistance

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of plants (Chinnusamy et al. 2003). However, most reports about *bHLH33* have focused on the synthesis of anthocyanins, while few studies have focused on the cold resistance conferred by the bHLH family in sweet potato. In this study, we successfully overexpressed *IbbHLH33* in tobacco plants and proved that *IbbHLH33* improved the chilling tolerance and antioxidant capacity of tobacco by cold treatment (Figure 5A) and determination of related indexes (Figure 6A–D), demonstrating that *IbbHLH33* plays a crucial role in abiotic stress responses. However, during chilling treatment, we found that *IbbHLH33* appears to be induced by stress treatment in overexpressed lines (Figure 5B). Similar results can be seen in previous studies. *IbC3H18*'s expression was induced by drought, salt and methyl viologen (MV) stresses in overexpressed lines and RNA interference (RNAi) lines (Zhang et al. 2020). In transgenic *Arabidopsis thaliana* plants, *IbATL38* was up-regulated by salt stress (Du et al. 2021). It is speculated that post-transcriptional mechanisms may be involved in regulating gene expression, which needs further investigation.

The direct synthesis pathway of ABA is considered to be the main pathway for ABA synthesis in higher plants (Seo & Koshiba 2002). ABA is a crucial plant hormone that adjusts plant growth and development as well as the response to adversity (Cutler et al. 2010). This is mainly reflected in the process of leaf shedding and in plant resistance to drought, low temperature and other stresses. Exogenous ABA application can simulate the cold acclimation process of plants and increase the freezing resistance of wheat and *Arabidopsis* (Daie & Campbell 1981). However, the vital role of ABA in the plant response to low temperature remains unclear. In *Arabidopsis*, mutants of *aba1* and *aba3* (involved in ABA synthesis) exhibit a freezing-sensitive phenotype due to reduced ABA content *in vivo* (Llorente et al. 2000; Xiong et al. 2001). *ABI3*, a regulatory factor in ABA signalling, is involved in the chilling response of plants, and overexpression of *ABI3* can enhance the freezing tolerance of plants (Tamminen et al. 2001). There were ABRE-responsive element motifs in the promoter region of cold-responsive *COR* (Hu et al. 2021). In conclusion, ABA signalling is closely related to low temperature in plants; however, the underlying mechanism remains unclear. In this study, ABRE was also found in the promoter of the *IbbHLH33* (Table 1), and the *IbbHLH33* gene was induced by exogenous ABA (Figure 2D). The results of qRT-PCR analysis showed that the expression of *NtZEP*, a gene related to ABA synthesis, decreased after chilling treatment (Figure 7D), indicating that chilling treatment caused changes in ABA content in transgenic lines. However, the underlying mechanism needs further study. These results suggest that the response pathway of the *IbbHLH33* gene under chilling stress may be related to ABA.

Chilling stress can also increase the levels of ROS in plants. Excessive ROS levels can be toxic to plant cells and affect cell membrane stability and multiple enzyme activities (Chinnusamy et al. 2007; Suzuki et al. 2012). The damaging effects of ROS on plant functional molecules include membrane lipid peroxidation and the production of MDA, which is the final decomposition product of membrane lipid oxidation (Zhang et al. 2020). SOD expression was induced by stress, leading to rapid metabolization to produce O^2 and H_2O_2. The relative electrical conductivity and MDA content of plant cells are important oxidative damage indicators that can be used to measure membrane integrity and representative physiological indicators to judge the degree of chilling injury in plants. In this study, after chilling treatment, the MDA content in the three plants increased to different degrees. However, the MDA content in transgenic plants was lower than that in WT plants, and the leaf damage in transgenic plants was less than that in control (Figure 6D). There was no significant difference in SOD activity and conductivity between the WT and transgenic lines before chilling treatment, however, the conductivity in the transgenic lines was significantly lower than that in the WT

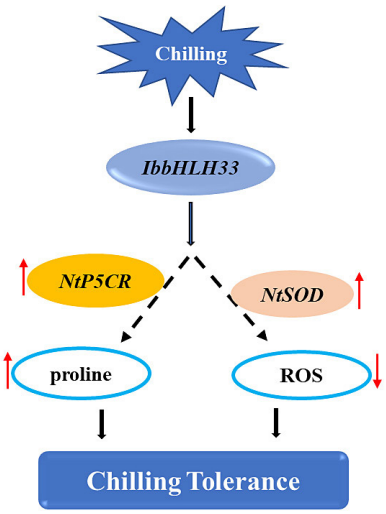

Figure 8. Regulatory response model of *IbbHLH33* under chilling stress

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under chilling treatment (Figure 6A–C). After chilling treatment, the expression of *NtSOD* and *NtPOD* increased (Figure 7B, C), which was confirmed by the enhanced activity of SOD. The results showed that the degree of damage in transgenic plants was less than that in WT plants under chilling treatment, which further indicated that the cold tolerance of the overexpression lines were enhanced (Figure 8). Proline is produced by plants in response to various types of environmental stress. It is an important protective substance synthesized by plants in response to low temperature. Proline can enhance the regulation of cell osmotic substances, maintain normal membrane and protein functions, and inhibit the production of ROS to improve plant stress resistance (Withers & King 1979; Lalk & Dorffling 1985; Yang et al. 2019). *Arabidopsis* plants were subjected to 4 °C treatment, and the proline content in the plants increased with treatment time (Kaplan et al. 2007). In *A. thaliana*, the promoter region of a class of genes contains the proline-responsive cis element ACTCAT, and the accumulation of proline at low temperature can induce the expression of these genes, thus enhancing the chilling resistance of plants (Satoh et al. 2002). In this study, the proline content under chilling treatment was significantly higher than that before chilling treatment (Figure 6A), and the expression of *NtP5CR* was also higher than that in the WT (Figure 7A), so the chilling tolerance of the transgenic lines was improved (Figure 8). An in-depth functional study of this gene will help to further clarify the function of *bHLH* transcription factors in sweet potato and provide a new candidate gene for improving the chilling tolerance of sweet potato and other important crops through genetic engineering.

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