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Integrative physiological and transcriptomic analyses reveal divergent chilling tolerance mechanisms in contrasting zoysiagrass cultivars and protective effects of exogenous chitosan

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Abstract

Background Chilling stress is one of the major factors affecting the green period and turf quality in zoysiagrass (*Zoysia* spp.). However, the physiological and molecular mechanisms of differential chilling tolerance among zoysiagrass cultivars remain largely unexplored.

Results The objectives of this study were to elucidate differences of chilling tolerance between the two cultivars with different chilling tolerance through integrating physiological and transcriptomic analyses, and to investigate protective effects of exogenous chitosan on the sensitive cultivar. The results showed that the chilling-tolerant cultivar ‘Nannong NT1’ suffered less injury than the sensitive ‘Nannong LG’ under chilling stress, as evidenced by lower electrolyte leakage, reduced reactive oxygen species (ROS) accumulation, and higher survival rate. Transcriptomic analyses further identified that ‘Nannong NT1’ specifically up-regulated carbohydrate and lipid metabolism, while concurrently down-regulating photosynthesis and carbon fixation. In contrast, ‘Nannong LG’ activated carotenoid and flavonoid biosynthesis, and repressed amino acid metabolism and protein export. Additionally, foliar application of chitosan (50 mg·L⁻¹) significantly alleviated chilling injury in ‘Nannong LG’ by enhancing chlorophyll content, relative water content, osmoprotectant accumulation, and survival rate, while reducing electrolyte leakage and ROS accumulation. At the transcriptional level, chitosan specifically modulated the expression of several critical differentially expressed genes, such as *ZsNAC2*, *ZsNAC3*, *ZsLecRLK1*, *ZsLecRLK2*, and *ZsBAM*.

Conclusions These findings demonstrate that differential chilling tolerance was attributed to the divergent regulatory and metabolic strategies. Exogenous chitosan offers a complementary approach to enhance chilling tolerance in the sensitive cultivar in zoysiagrass.

Key words: Zoysiagrass; Cold Stress; Chitosan; RNA-Seq; Key Pathway

1. Background

Cold stress is one of the major abiotic constraints limiting the geographical distribution and impairing the growth and development of various plant species [1, 2]. Depending on temperature thresholds, it is classified as chilling (0-15°C) or freezing (<0°C) stress [3]. Chilling stress induces multiple phenotypic and physiological alterations, including leaf chlorosis and wilting, increased membrane permeability, photosynthetic suppression, and growth arrest [4, 5]. To cope with chilling stress, plants activate protective responses such as maintaining photosynthetic activity, accumulating compatible osmolytes, and scavenging reactive oxygen species (ROS) [6-8]. However, the physiological and molecular mechanisms underlying chilling tolerance vary considerably across species and genotypes [9, 10], and remain poorly understood in many economically important turfgrasses.

Among these turfgrasses, zoysiagrass (*Zoysia* spp.) is a warm-season, low-input turfgrass widely used on golf courses, sports fields, and lawns [11]. Although it exhibits broad tolerance to drought, salinity, disease, and wear, it is particularly sensitive to low temperatures [12, 13]. This sensitivity shortens the green period and restricts its widespread cultivation in transitional and temperate regions [14]. To elucidate the molecular mechanisms of cold tolerance in zoysiagrass, previous studies have primarily employed transcriptomic profiling of individual cultivars. Early transcriptomic analysis revealed that chilling stress down-regulated genes involved in photosynthesis and substance transport, and up-regulated genes associated with proline and starch metabolism, ROS scavenging, and DREB-mediated signaling in *Z. japonica* cv. 'Meyer' [15]. More recently, a combination of ATAC-seq and RNA-seq profiling in *Z. japonica* cv. 'Compadre' identified 80 candidate genes associated with low-temperature tolerance [16]. Nevertheless, these findings pertained only to specific genotypes, whereas zoysiagrass accessions and cultivars exhibited considerable variation in cold tolerance [17]. For instance, accessions originating from higher latitudes exhibited greater cold tolerance than those from lower latitudes [18]. The cold tolerance of *Z. japonica* biotype 'YueNong No. 9' was superior to that of 'LanYin-3', which was associated with improved anthocyanin biosynthesis, transport, and accumulation at the transcriptional level [19]. However, the molecular basis

underlying differential cold tolerance among zoysiagrass cultivars, particularly the transcriptional regulation of key metabolic pathways, remains underexplored.

As a complementary approach to genetic improvement, exogenous biostimulants have been widely used to enhance stress tolerance in various plant species [20, 21]. Among these, chitosan, a natural biopolymer derived from the deacetylation of chitin, is valued for its non-toxic, biodegradable, and environmentally friendly properties [22]. It has been applied in agriculture as both a plant immune elicitor and a growth promoter. Foliar application of chitosan significantly alleviated the adverse effects of abiotic stresses (drought, salinity, and heat) in several plant species [23-26]. Chitosan has also been reported to mitigate cold stress. The mitigation of cold injury by chitosan oligosaccharide in tea plants (*Camellia sinensis*) was attributed to improved photosynthesis and carbon metabolism [27]. Exogenous chitosan oligosaccharide enhanced chilling tolerance in cucumber (*Cucumis sativus*) seedlings by modulating the expression of genes involved in various pathways, such as carbohydrate metabolism and plant hormone signal transduction [28]. However, whether chitosan can effectively alleviate chilling injury in sensitive turfgrasses, and the transcriptional mechanisms underlying such protection, remain unexplored.

Therefore, in this study, two complementary strategies were employed: (i) physiological and comparative transcriptomic analyses of the two *Z. sinica* cultivars, chilling-tolerant cultivar ‘Nannong NT1’ and the chilling-sensitive cultivar ‘Nannong LG’, to identify cultivar-specific candidate genes and pathways, and (ii) exogenous chitosan application to the sensitive cultivar to assess its protective effects under chilling stress. This study provides a transcriptomic resource for understanding chilling tolerance in zoysiagrass, identifies candidate pathways and genes for genetic improvement, and offers a basis for the application of chitosan to enhance chilling tolerance in sensitive cultivars of turfgrass species.

2. Materials and methods

2.1 Experimental design and treatments

Three independent experiments were conducted to (i) compare the chilling tolerance

between two cultivars, (ii) determine the optimal chitosan concentration, and (iii) evaluate the protective effects of chitosan on the chilling-sensitive cultivar of zoysiagrass.

2.1.1 Experiment 1: Comparison of chilling tolerance between ‘Nannong NT1’ and ‘Nannong LG’

Two zoysiagrass (*Z. sinica*) cultivars with superior turf quality, ‘Nannong NT1’ (chilling-tolerant) and ‘Nannong LG’ (chilling-sensitive), were obtained from the germplasm nursery of Turf Experiment Station of Nanjing Agricultural University (Nanjing, China). Sods of both cultivars were transplanted into pots (12 cm in height and 15 cm in diameter) filled with a 3:1 (v/v) mixture of peat soil and vermiculite. During the establishment stage, plants were cultivated in a greenhouse for 2 months under conditions of 30/25°C (day/night), 60% relative humidity, a 14-h photoperiod, and a photosynthetic photon flux density of 800 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ at the canopy level. Plants were watered once every three days, mowed to maintain a canopy height of 6-7 cm, and fertilized once a week with water-soluble fertilizer (Scotts Miracle-Gro Company, USA). Prior to treatments, plants were transferred into a growth chamber (XBQH-1, Jinan Xubang, Jinan, Shandong, China) with the same environmental parameters as those in the greenhouse for a 7-day pre-acclimation period.

After the pre-acclimation period, plants of both cultivars, ‘Nannong NT1’ and ‘Nannong LG’, were divided into four treatments (NT1-control, NT1-chilling, LG-control, and LG-chilling) with five biological replicates for each treatment. NT1-control and LG-control plants were maintained under normal temperature conditions (30/25°C, day/night), whereas NT1-chilling and LG-chilling plants were subjected to chilling stress (4/2°C, day/night) in two growth chambers, respectively. All other environmental parameters (relative humidity and photoperiod) were kept consistent between the two chambers. During the treatments, pots were randomly relocated each day to reduce the influence of potential environmental effects.

Leaf samples were collected after 48 h of treatments and were delivered to Novogene Co., Ltd. (Beijing, China) for RNA-seq, with three biological replicates per treatment. After 42 d of chilling stress, physiological parameters were measured, including leaf firing percentage, electrolyte leakage, relative water content, osmoprotectants (soluble sugar and proline) content,

and H₂O₂ and O₂⁻ accumulation. The chilling-treated plants were then transferred back to normal conditions (30/25°C) for 14 d of recovery, after which survival rate was evaluated.

2.1.2 Experiment 2: Optimal chitosan concentration for alleviating chilling stress of chilling-sensitive cultivar 'Nannong LG'

The sods of the chilling-sensitive cultivar 'Nannong LG' were collected and grown in a greenhouse. The plant cultivation method and environmental parameters of the greenhouse were the same as described for experiment 1. After one month of establishment, plants were subjected to chilling stress in combination with different chitosan concentrations in a growth chamber at 4/2°C (day/night), with 60% relative humidity and a 14-h photoperiod. The tested concentrations (0, 50, 100, 300, 400, and 500 mg·L⁻¹) were selected based on our previous study [24], with five biological replicates per treatment. Chitosan (85% deacetylation degree) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Each pot was sprayed weekly with 10 mL of the chitosan solution. Pots were arranged with sufficient spacing to avoid cross-interference between plants. After 28 d of treatments, the effects of different chitosan concentrations were assessed by measuring leaf relative water content, electrolyte leakage, and chlorophyll content.

2.1.3 Experiment 3: Effects of exogenous chitosan on the chilling-sensitive cultivar 'Nannong LG' under chilling stress

Based on the results of experiment 2, the optimal chitosan concentration was selected for further investigation of its protective effects on the chilling-sensitive cultivar 'Nannong LG' under chilling stress. After two months of cultivation, plants were assigned to four treatments (control-H₂O, control-chitosan, chilling-H₂O, and chilling-chitosan) with five replicates per treatment in growth chambers. Control-H₂O and control-chitosan plants were sprayed weekly with 10 mL of deionized water and chitosan, respectively, under normal conditions (30/25°C, day/night). Chilling-H₂O and chilling-chitosan plants were similarly treated weekly with 10 mL of deionized water and chitosan, respectively, under chilling conditions (4/2°C, day/night). Two growth chambers were set with 60% relative humidity and a 14-h photoperiod.

Leaf samples of three biological replicates per treatment from the chilling-H₂O and chilling-chitosan treatments were collected after 48 h of treatments, and sent to Novogene Co., Ltd. (Beijing, China) for RNA-seq analysis. Additionally, chlorophyll content, leaf firing percentage, electrolyte leakage, relative water content, osmoprotectant (soluble sugar and proline) content, and ROS (H₂O₂ and O₂⁻) accumulation were assessed after 42 d of treatments. Chilling-plants were subsequently returned to non-stressed temperature (30/25°C) for a 28-day recovery period, and then survival rates were recorded.

2.2 Measurement of morphological and physiological parameters

2.2.1 Morphological assessment of chilling injury

To evaluate visible chilling injury and post-recovery survival, leaf firing percentage and survival rate were assessed as described below. Leaf firing percentage was visually assessed throughout the treatment period on a 0-100% scale, where 0% indicates no visible leaf injury and a completely green canopy, and 100% indicates complete leaf browning or death [29]. For survival rate assessment, after recovery under normal conditions, surviving plants that resumed growth or maintained their green color were counted visually. Survival rate was calculated as the percentage of surviving plants relative to the total number of treated plants [30].

2.2.2 Determination of physiological indices

Electrolyte leakage and relative water content were measured according to the methods of Blum and Ebercon [31] and Barrs [32], respectively. For electrolyte leakage measurement, fresh leaves (0.15 g) were placed in 30 mL of ultrapure water and shaken for 24 h. The initial conductivity (C₀) was measured, and after autoclaving (121°C, 20 min) and an additional 24-h shaking, final conductivity (C₁) was recorded. Both C₀ and C₁ were determined using a conductivity meter (Orion Star A212, Thermo Scientific Inc., Waltham, MA, USA). Electrolyte leakage was calculated as C₀/C₁×100%. For relative water content determination, fresh leaves (0.15 g) were immediately weighed (FW), soaked in deionized water for 24 h to obtain turgid weight (TW), and then dried at 80°C for 72 h to obtain dry weight (DW). Relative water content

was calculated as $[(FW-DW)/(TW-DW)] \times 100\%$.

Soluble sugar and proline contents, as the major osmotic adjustment substances, were measured in dried leaf samples. Soluble sugar content was determined using the phenol-sulfuric acid method [33]. Briefly, leaf samples were collected, dried, and extracted with 80% (v/v) ethanol to obtain the supernatant. The supernatant was sequentially mixed with 23% (v/v) phenol and 98% (v/v) sulfuric acid to initiate the reaction. The absorbance was then measured at 490 nm using a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, UK). Proline content was determined following the acidified ninhydrin protocol with minor modifications [34]. Briefly, dried leaf samples were extracted in 5 mL of 3% sulfosalicylic acid and heated at 100°C for 10 min. The resulting extract was then reacted with glacial acetic acid and acid ninhydrin at 100°C for 30 min. After incubation, the reaction mixture was partitioned with toluene, and the absorbance of the organic phase was recorded at 520 nm.

Chlorophyll content was determined via the dimethyl sulfoxide (DMSO) extraction method. Fresh leaf samples (0.05 g) were immersed in 8 mL of DMSO until the tissue became colorless, indicating complete pigment extraction. The absorbance of the resulting extract was recorded at 665 nm and 649 nm against a DMSO blank. After pigment extraction, the decolorized leaf tissues were dried at 80°C and weighed to determine dry weight. Chlorophyll a and b content were computed following the DMSO-specific equations described by Wellburn [35], and total chlorophyll content was derived as the sum of the two pigments.

2.2.3 Histochemical detection of ROS accumulation

To visualize the accumulation of H_2O_2 and O_2^- , leaves were stained following the protocols documented by Thordal-Christensen et al. [36] and Dai et al. [37], respectively, with minor modification. For H_2O_2 detection, intact leaves were infiltrated with 1 $mg \cdot mL^{-1}$ 3,3-diaminobenzidine (DAB) in Tris-HCl buffer (pH 7.5) under vacuum for 5 min, and subsequently incubated on a shaker in the dark for 12 h. For O_2^- staining, leaves were immersed with 5 $mg \cdot mL^{-1}$ nitroterazolium blue chloride (NBT) for 7 h in the dark. After the staining reactions were terminated, both DAB- and NBT-treated leaves were decolorized with 95% ethanol at 80°C until the chlorophyll was completely removed.

2.3 RNA-Seq library construction and bioinformatic analysis of differentially expressed genes

The RNA-seq library was constructed by Novogene Co., Ltd. (Beijing, China) and sequenced on the Illumina NovaSeq 6000 platform (PE150). Clean reads were aligned to the *Z. japonica* reference genome (Zoysia Genome Database; <http://zoysia.kazusa.or.jp/>) using HISAT2 (v2.0.5). Specifically, an index of the reference genome was first constructed, and the paired-end clean reads were then aligned to the reference genome using HISAT2.

All bioinformatic analyses of differentially expressed genes (DEGs) were conducted in NovoMagic platform (<https://magic-plus.novogene.com/>). Principal component analysis (PCA) was performed based on FPKM values to assess inter-group separation and intra-group consistency. DEGs were identified using DESeq2 (v1.20.0) with thresholds of $|\log_2FC| \geq 1$ and $\text{padj} < 0.05$. Volcano plots were generated using the ggplot2 package in R (v3.0.3), and Venn diagrams were constructed with the VennDiagram package (v3.0.3). Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis was performed via clusterProfiler (v3.8.1) to annotate the biological functions of the identified DEGs.

2.4 Quantitative reverse transcription PCR (qRT-PCR) assays

Total RNA was extracted using the SPARKeasy Plant RNA Kit (AC0305, SparkJade, Shandong, China) and reverse-transcribed into cDNA using the ABScript Neo RT Master Mix for qPCR with gDNA Remover kit (RK20433, ABclonal, Wuhan, China). qRT-PCR assays were performed using BrightCycle Blue Universal SYBR Green qPCR Mix with UDG (RK21220, ABclonal, Wuhan, China) on a Roche LightCycler® 480 II instrument (Roche Diagnostics, Rotkreuz, Switzerland). The primers used in this study are listed in Table S1, and their specificity was verified by melting curve analysis. The amplification protocol was optimized as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. The relative expression level of each target gene was normalized to that of the reference gene Actin and calculated using the $2^{-\Delta\Delta CT}$ method [38].

Table S1. Primer sequences used in the experiment

Gene name	Gene ID	Forward primers (5'→3')	Reverse primers (5'→3')
<i>Actin</i>	GU290546	GGTGTTATGGTTGGGATGG	CAGTGAGCAGGACAGGGTG

<i>ZsFD5</i>	Zjn_sc00150.1.g00600.1.am.mk	GCAGGCGACCTACAACGTCA	GGCAGCTCGATCCCTTCCTC
<i>ZsFNR</i>	Zjn_sc00014.1.g06600.1.sm.mkhc	GAGGAGTTCCGGCGTCAAGG	GGTGGCTCCTTCGCATCCTC
<i>ZsFBA</i>	Zjn_sc00008.1.g05620.1.sm.mk	CTCCGGCGTCATCCTCTTCG	AGACCCTGGGTGGTGGTCTC
<i>ZsFBP</i>	Zjn_sc00009.1.g05360.1.sm.mkhc	TCGCACCTGCGTTCTTGTGT	CCGCAGTCGATGTTGGACGA
<i>ZsPFK</i>	Zjn_sc00006.1.g01020.1.sm.mkhc	ACGCAGGAGAAGGGACATGC	GGACCAGGCACAAAGCCAGT
<i>ZsGolS2</i>	Zjn_sc00003.1.g02320.1.sm.mkhc	TCTCGCAGGGTTGCATCGTC	CGGCGTCCAGGTACACCATC
<i>ZsLBD9</i>	Zjn_sc00014.1.g04720.1.am.mk	CGACGGTGCGTTCCTATTGC	TGCTGGTGGGACACTTCCTC
<i>ZsLBD31</i>	Zjn_sc00040.1.g03820.1.am.mk	CCTTACTTCTCGCCGCACGA	GGCACCTCCAGGAGCATCTT
<i>ZsLBD45</i>	Zjn_sc00025.1.g01900.1.sm.mk	GCAAGGGTAGGACTGAGCGT	TGATCGACCCAAGGCCACTG
<i>ZsNAC2</i>	Zjn_sc00003.1.g01710.1.sm.mkhc	AACGGCGACAACAGCATGTC	TTGCACCAAAGCAGGAACCG
<i>ZsNAC3</i>	Zjn_sc00044.1.g04960.1.sm.mk	CACCGACAAGCCCATCCAC	GACGCCTGGTCCGACTTTCT
<i>ZsLecRLK1</i>	Zjn_sc00287.1.g00030.1.am.mk	AGCTCCTCTGAGGGTAGCCA	GATCGCTGGAGCAGGACTGT
<i>ZsLecRLK2</i>	Zjn_sc00013.1.g05710.1.sm.mk	AGCTCGACACCATCCAGAGC	CCGCTGATCAGGGTCAGGTT
<i>ZsBAM</i>	Zjn_sc00001.1.g07680.1.sm.mkhc	TGGACTTGGCCCTTGTGGAG	CCAGCGTCGTCAGGCAATTC

227 2.5 Statistical analyses

228 All data were presented as means $\pm$ standard error (SE) for each physiological and
229 biochemical parameter. For multi-group comparisons, one-way analysis of variance (ANOVA)
230 was performed, followed by Duncan's multiple range test to assess differences among
231 treatments, with statistical significance of $P < 0.05$. These analyses were carried out using SPSS
232 Statistics (version 21.0; SPSS Inc., Chicago, IL, USA). For qRT-PCR data, pairwise
233 comparisons between groups were performed with Student's t-test, and significance thresholds
234 were set at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
235

3. Results

3.1 Comparative chilling tolerance of ‘Nannong NT1’ and ‘Nannong LG’

Under normal conditions, no significant difference was observed between the two cultivars. However, after 42 d of treatments, both cultivars exhibited leaf rolling and significant alterations in physiological processes relative to their respective controls (Figs. 1 and 2). ‘Nannong NT1’ suffered less injury than ‘Nannong LG’ under chilling stress, exhibiting significantly lower leaf firing percentage, electrolyte leakage, soluble sugar, and proline content by 90.0%, 81.7%, 32.2%, and 33.8%, respectively (Figs. 1B, 2A, 2B, 2D). Furthermore, the relative water content and survival rate of ‘Nannong NT1’ were 3.4- and 13.1-fold higher than those of ‘Nannong LG’ (Figs. 1C and 2C). In addition, DAB and NBT staining were also lighter in ‘Nannong NT1’ than in ‘Nannong LG’, despite increased staining intensity in both cultivars relative to their controls (Fig. 2E). Therefore, the chilling tolerance of ‘Nannong NT1’ was significantly superior to that of ‘Nannong LG’.

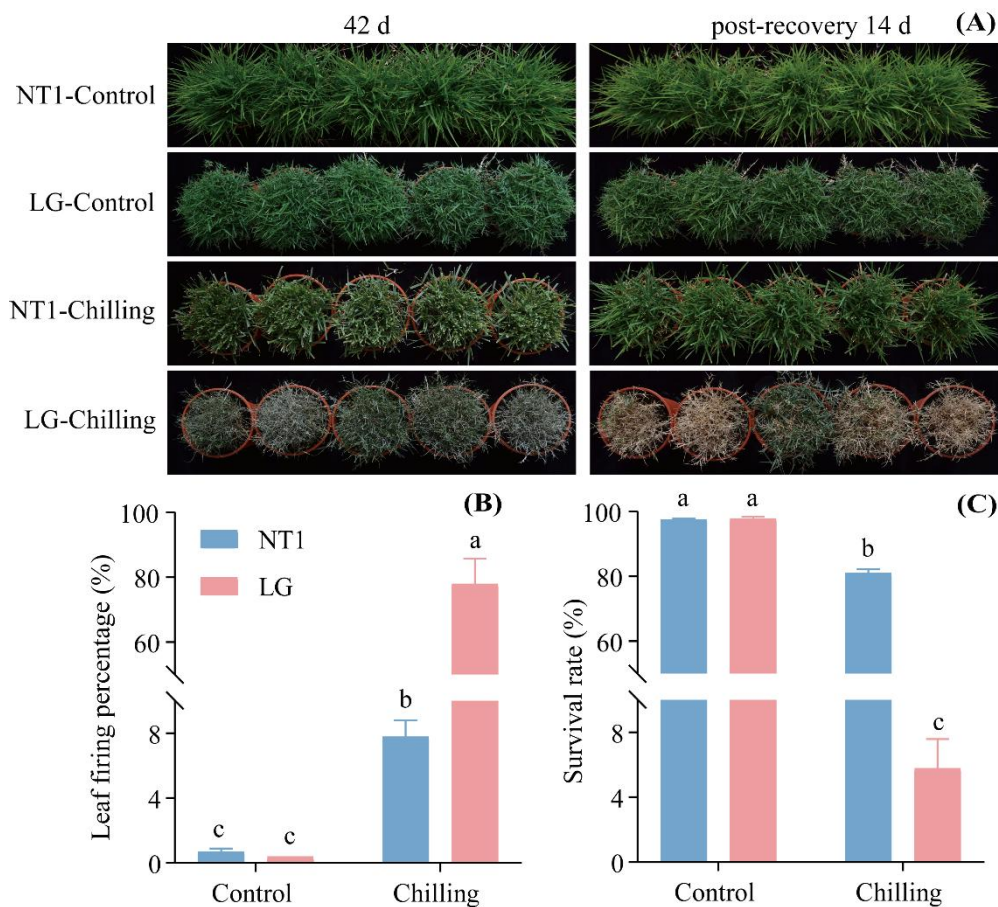


Fig. 1 The effects of chilling stress on shoot morphological parameters in two zoysiagrass cultivars. (A) shoot

phenotype under chilling stress and post-recovery treatments. (B) leaf firing percentage. (C) survival rate. NT1-control and LG-control, plants were maintained under normal temperature conditions (30/25°C, day/night). NT1-chilling and LG-chilling, plants were subjected to chilling stress (4/2°C, day/night). Different letters represent significant differences between different treatments during the experimental period ($P < 0.05$).

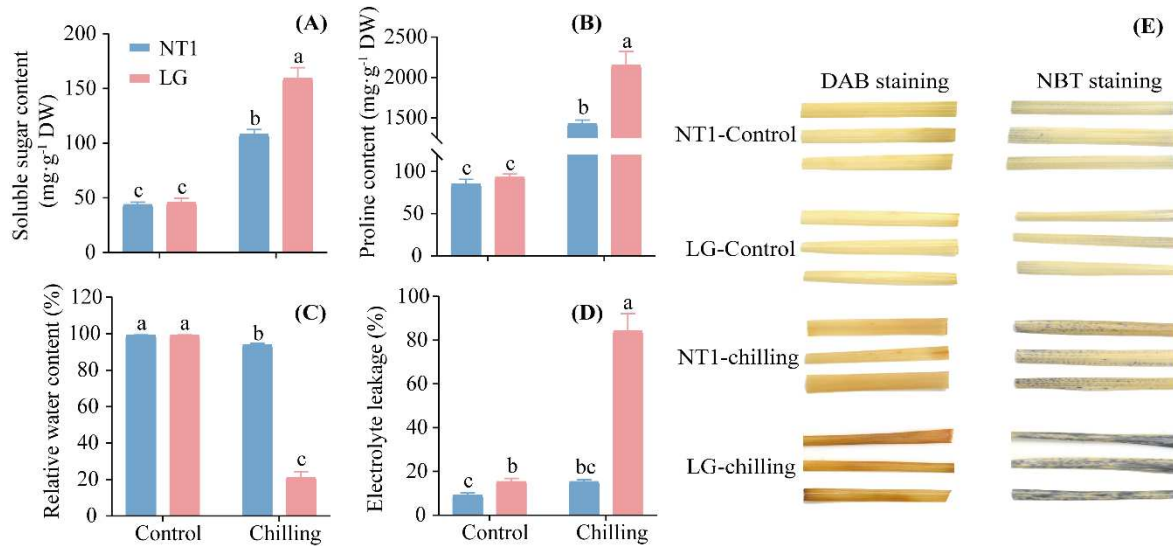


Fig. 2 The effects of chilling stress on physiological indices in two zoysiagrass cultivars. (A) soluble sugar content. (B) proline content. (C) relative water content. (D) electrolyte leakage. (E) DAB and NBT staining. NT1-control and LG-control, plants were maintained under normal temperature conditions (30/25°C, day/night). NT1-chilling and LG-chilling, plants were subjected to chilling stress (4/2°C, day/night). Different letters represent significant difference between different treatments during the experimental period ($P < 0.05$).

3.2 Transcriptome analysis of 'Nannong NT1' and 'Nannong LG' under chilling stress

To investigate the molecular basis of differential responses to chilling stress between 'Nannong NT1' and 'Nannong LG', RNA-seq on leaf samples from four treatments (NT1-control, NT1-chilling, LG-control, and LG-chilling) was performed at 48 h of treatments. In total, RNA-seq of the twelve samples generated an average of 4.61×10^7 raw reads, 6.91 Gb raw bases, 4.53×10^7 clean reads, and 6.80 Gb clean bases per sample (Table S2). PCA revealed a clear separation of samples based on both chilling stress and cultivar, and the three biological replicates within each group were tightly clustered, indicating high reproducibility of the RNA-seq data (Fig. 3A).

After treatments, 6,806 DEGs were up-regulated and 5,500 down-regulated in the NT1-control versus NT1-chilling comparison (Fig. 3C), whereas 7,362 DEGs were up-regulated and 5,100 down-regulated in the LG-control versus LG-chilling comparison (Fig. 3D). Venn diagram analysis was further performed to identify overlapping and unique DEGs between the two comparisons (Fig. 3B). Under chilling stress, 7,655 DEGs were co-regulated between the two cultivars, comprising 4,564 up-regulated and 3,091 down-regulated genes. Notably, 4,579 DEGs were exclusive to the chilling-tolerant cultivar ‘Nannong NT1’, while 4,735 DEGs were exclusive to the chilling-sensitive cultivar ‘Nannong LG’. In addition, 72 DEGs, which were up-regulated in one cultivar but down-regulated in the other, exhibited opposite regulation patterns between the two cultivars.

Table S2. Summary of reads following Illumina sequencing

sample	Raw reads	Raw bases	Clean reads	Clean bases	Q20/%	Q30/%	GC content/%
NT1_control1	47997678	7.2G	47226272	7.08G	98.55	96.12	51.91
NT1_control2	44816058	6.72G	43636978	6.55G	98.57	96.15	53.01
NT1_control3	46015626	6.9G	45244182	6.79G	98.63	96.33	50.09
NT1_chilling1	49408430	7.41G	48607656	7.29G	98.57	96.18	53.01
NT1_chilling2	48845066	7.33G	47840502	7.18G	98.56	96.15	53.09
NT1_chilling3	43011640	6.45G	42324844	6.35G	98.53	96.1	51.5
LG_control1	44985648	6.75G	44211258	6.63G	98.53	96.08	52.2
LG_control2	48309898	7.25G	47598614	7.14G	98.55	96.1	51.49
LG_control3	45741990	6.86G	45049708	6.76G	98.53	96.07	50.74
LG_chilling1	42460588	6.37G	41853034	6.28G	98.46	95.89	53.07
LG_chilling2	45299654	6.79G	44617494	6.69G	98.9	96.95	52.82
LG_chilling3	46285324	6.94G	45574582	6.84G	98.55	96.14	51.36
LG_chitosan1	45606580	6.84G	44822514	6.72G	98.82	96.71	52.88
LG_chitosan2	44435616	6.67G	43715826	6.56G	98.81	96.65	52.88
LG_chitosan3	43493746	6.52G	42832194	6.42G	98.90	96.96	51.59

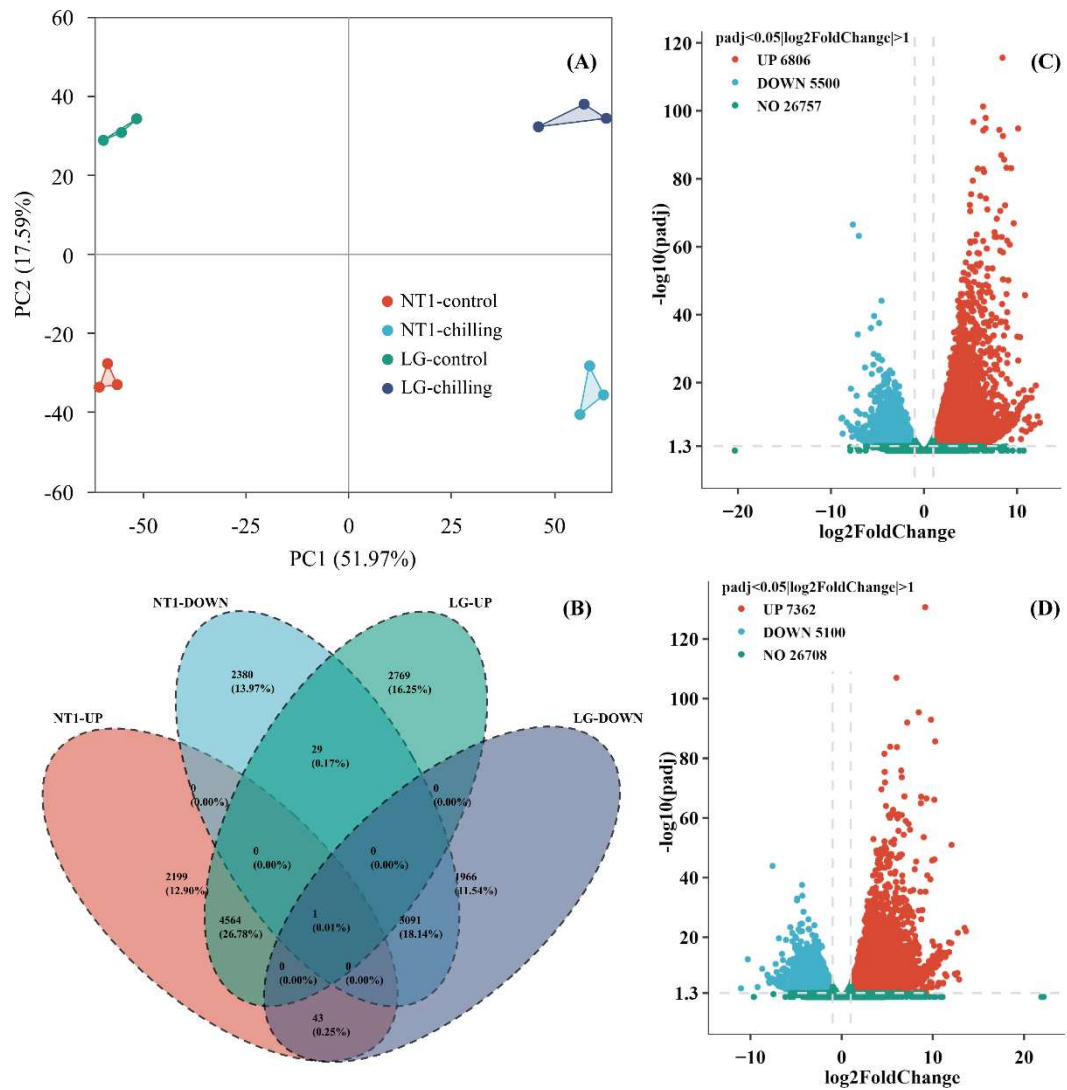


Fig. 3 Transcriptome analysis of two zoysiagrass cultivars at 48 h of treatments. (A) principal component analysis. (B) Venn diagrams of up-regulated and down-regulated DEGs in NT1-control versus NT1-chilling comparison and LG-control versus LG-chilling comparison. (C) identification of DEGs using volcano plots in NT1-control versus NT1-chilling comparison. (D) identification of DEGs using volcano plots in LG-control versus LG-chilling comparison. NT1-control and LG-control, plants were maintained under normal temperature conditions (30/25°C, day/night). NT1-chilling and LG-chilling, plants were subjected to chilling stress (4/2°C, day/night).

3.3 Comparative KEGG pathway enrichment of cultivar-specific and common pathways

To further characterize the pathway-level responses, KEGG pathway enrichment analyses were performed on the up- and down-regulated genes in both cultivars under chilling stress. In

295 the up-regulated set, the NT1 genes were uniquely enriched in galactose metabolism,
296 glycerophospholipid metabolism, glycerolipid metabolism, biosynthesis of nucleotide sugars,
297 and linoleic acid metabolism (Fig. 4C). In contrast, the LG up-regulated genes were specifically
298 associated with glyoxylate and dicarboxylate metabolism, circadian rhythm (plant), carotenoid
299 biosynthesis, fatty acid biosynthesis, and flavonoid biosynthesis (Fig. 4A). For the down-
300 regulated genes, the NT1 set was uniquely enriched in photosynthesis, carbon fixation in
301 photosynthetic organisms, porphyrin metabolism, nucleotide excision repair, and mismatch
302 repair (Fig. 4D). Meanwhile, the LG down-regulated genes were exclusively enriched in beta-
303 alanine metabolism, lysine biosynthesis, monobactam biosynthesis, isoquinoline alkaloid
304 biosynthesis, and protein export (Fig. 4B). In addition to these cultivar-specific enrichments,
305 ten enriched pathways were commonly identified in both cultivars, such as starch and sucrose
306 metabolism, plant hormone signal transduction, ribosome, and N-glycan biosynthesis.

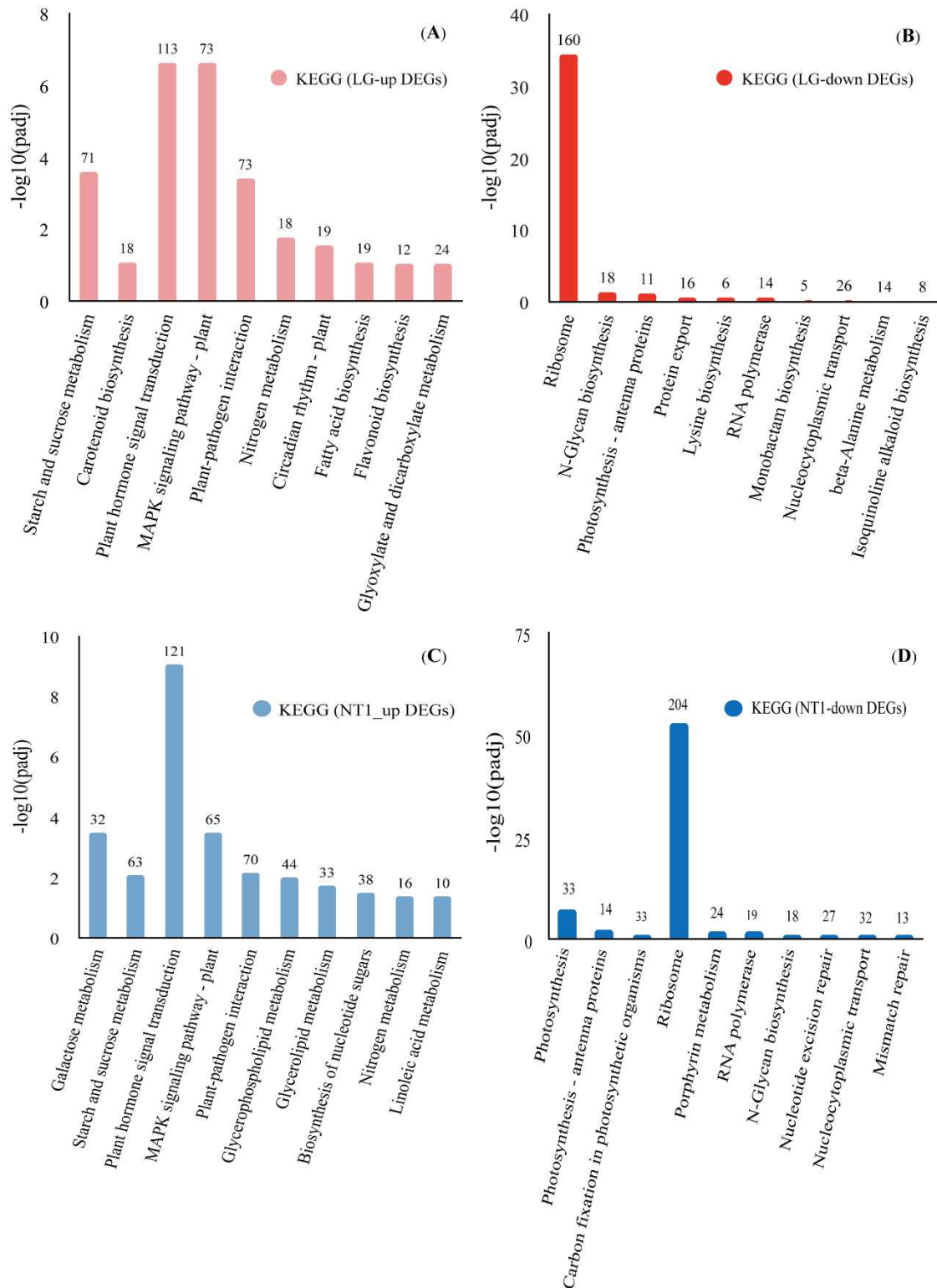


Fig. 4 KEGG pathway enrichment analyses in two zoysiagrass cultivars. (A-B) KEGG pathway enrichment analyses in up-regulated DEGs and down-regulated DEGs in LG-control versus LG-chilling comparison. (C-D) KEGG pathway enrichment analyses in up-regulated and down-regulated DEGs in NT1-control versus NT1-chilling comparison.

312

313 3.4 qRT-PCR validation of candidate DEGs from enriched pathways and transcription factor
314 families

315 To validate the reliability of the RNA-seq data, the expression patterns of nine candidate
316 DEGs were assessed via qRT-PCR. The results showed that the expression trends of these DEGs
317 were consistent between qRT-PCR and RNA-seq (Fig. 5). Six DEGs associated with three
318 significantly enriched KEGG pathways were first selected: photosynthesis, carbon fixation in
319 photosynthetic organisms, and galactose metabolism. In the photosynthesis pathway, *ZsFD5*
320 was down-regulated in both cultivars under chilling stress (Fig. 5A), whereas *ZsFNR* was
321 significantly up-regulated (Fig. 5B). For carbon fixation, both *ZsFBA* and *ZsFBP* were
322 confirmed to be down-regulated (Fig. 5C, D). By contrast, the two genes involved in galactose
323 metabolism, *ZsPFK* and *ZsGols2*, were consistently up-regulated (Fig. 5E, F). Furthermore,
324 three members of the LBD transcription factor family were selected. *ZsLBD45* was one of the
325 72 oppositely regulated genes, and exhibited a cultivar-specific response, remaining stable in
326 'Nannong NT1' but up-regulated in 'Nannong LG' (Fig. 5G). *ZsLBD9* and *ZsLBD31* showed up-
327 and down-regulation, respectively (Fig. 5H, I).

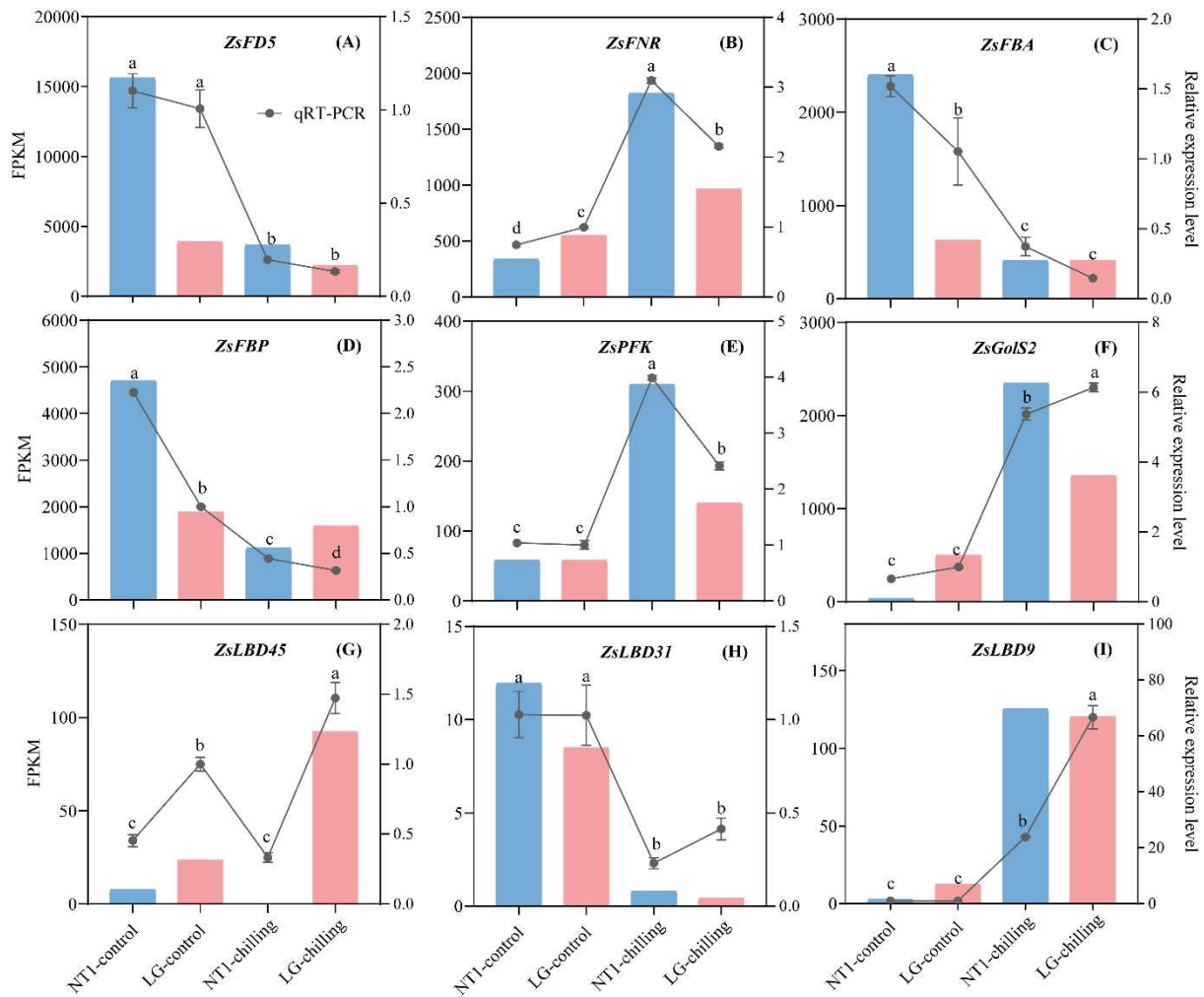


Fig. 5 Validation of selected DEGs from KEGG enriched pathways and transcription factor families via qRT-PCR in two cultivars. (A-B) *ZsFD5* and *ZsFNR*, photosynthesis pathway genes. (C-D) *ZsFBA* and *ZsFBP*, carbon fixation pathway genes. (E-F) *ZsPFK* and *ZsGolS2*, galactose metabolism pathway genes. (G-I) *ZsLBD45*, *ZsLBD31* and *ZsLBD9*, members of the LBD transcription factor family. RNA-seq (FPKM) and qRT-PCR values, representing the means of three biological replicates, are shown as bar and line graphs, respectively. NT1-control and LG-control, plants were maintained under normal temperature conditions (30/25°C, day/night). NT1-chilling and LG-chilling, plants were subjected to chilling stress (4/2°C, day/night). Different letters represent significant differences between qRT-PCR data of different treatments during the experimental period ($P < 0.05$).

3.5 Optimal chitosan concentration for enhancing chilling tolerance in chilling-sensitive cultivar

Considering the observed enrichment of photosynthesis and carbohydrate metabolism

pathways in ‘Nannong NT1’ and the known protective effects of chitosan on these processes under stress, exogenous chitosan was applied to the chilling-sensitive cultivar ‘Nannong LG’, and its effects on chilling tolerance were evaluated. At the beginning of treatments, no significant difference was observed between untreated and treated plants. After 28 d of chilling stress, ‘Nannong LG’ displayed typical chilling injury symptoms, including leaf firing and rolling (Fig. 6A). Compared with the control, four chitosan concentrations (50, 100, 300, and 400 $\text{mg}\cdot\text{L}^{-1}$) significantly increased relative water content by 149%, 106%, 99%, and 104%, respectively (Fig. 6B), and reduced electrolyte leakage by 50.9%, 45.2%, 42.6%, and 42.8%, respectively (Fig. 6C). However, only the 50 $\text{mg}\cdot\text{L}^{-1}$ chitosan treatment significantly increased chlorophyll content, by 32.4% compared with the untreated control (Fig. 6D). Therefore, 50 $\text{mg}\cdot\text{L}^{-1}$ chitosan was selected for subsequent experiments.

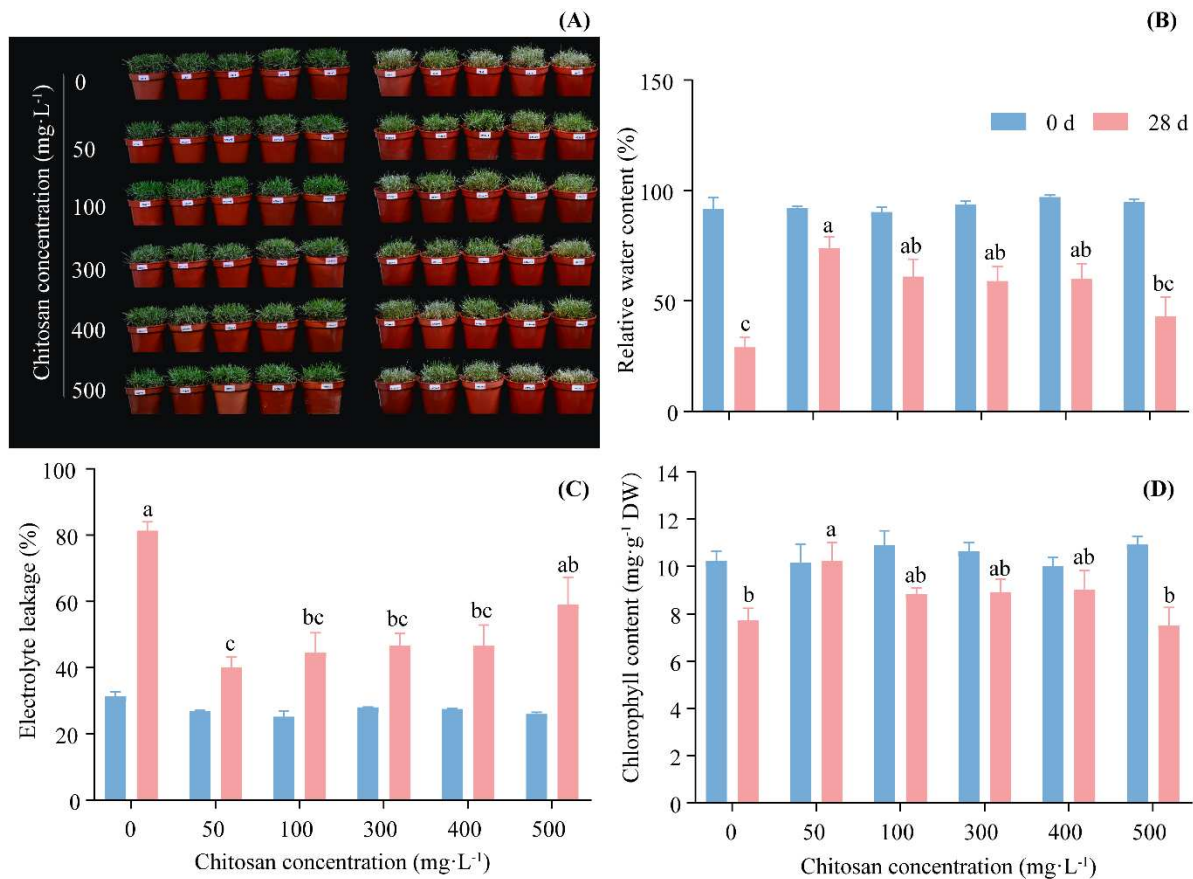


Fig. 6 The effects of different exogenous chitosan concentrations in chilling-sensitive cultivar ‘Nannong LG’ under chilling stress. (A) shoot phenotype. (B) relative water content. (C) electrolyte leakage. (D) chlorophyll content. Plants were sprayed via foliar application of 0, 50, 100, 300, 400, and 500 $\text{mg}\cdot\text{L}^{-1}$ chitosan, respectively. Different letters represent significant differences between different treatments during the

experimental period ($P < 0.05$).

3.6 Chitosan alleviates chilling injury in chilling-sensitive cultivar 'Nannong LG'

To assess the protective role of chitosan against chilling stress, 'Nannong LG' plants were treated with 50 mg·L⁻¹ chitosan or distilled water (control) under both normal and chilling conditions. Under normal conditions, no significant differences were observed between untreated and treated plants. Under chilling stress, exogenous chitosan significantly ameliorated chilling injury in 'Nannong LG', as reflected by higher chlorophyll content, relative water content, osmoprotectant accumulation, and survival rate, along with lower ROS accumulation, electrolyte leakage, and leaf firing percentage (Figs. 7 and 8).

For chlorophyll content, foliar application of chitosan significantly increased chlorophyll a content, chlorophyll a/b ratio, and total chlorophyll content by 49.2%, 21.8%, and 40.1%, respectively, compared with the control after 42 d of chilling stress (Fig. 7D, F, G). However, no significant effect on chlorophyll b content was observed (Fig. 7E). In addition, chitosan treatment significantly reduced leaf firing percentage and electrolyte leakage by 29.6% and 38.6%, respectively, and increased survival rate by 47.0% compared with the untreated control under chilling conditions (Figs. 7B, 7C, and 8C). Furthermore, chitosan application significantly enhanced soluble sugar content, proline content, and relative water content by 31.8%, 119%, and 34.2%, respectively, relative to the control under chilling stress (Fig. 8A, B, D), accompanied by reduced accumulation of H₂O₂ and O₂⁻ (Fig. 8E).

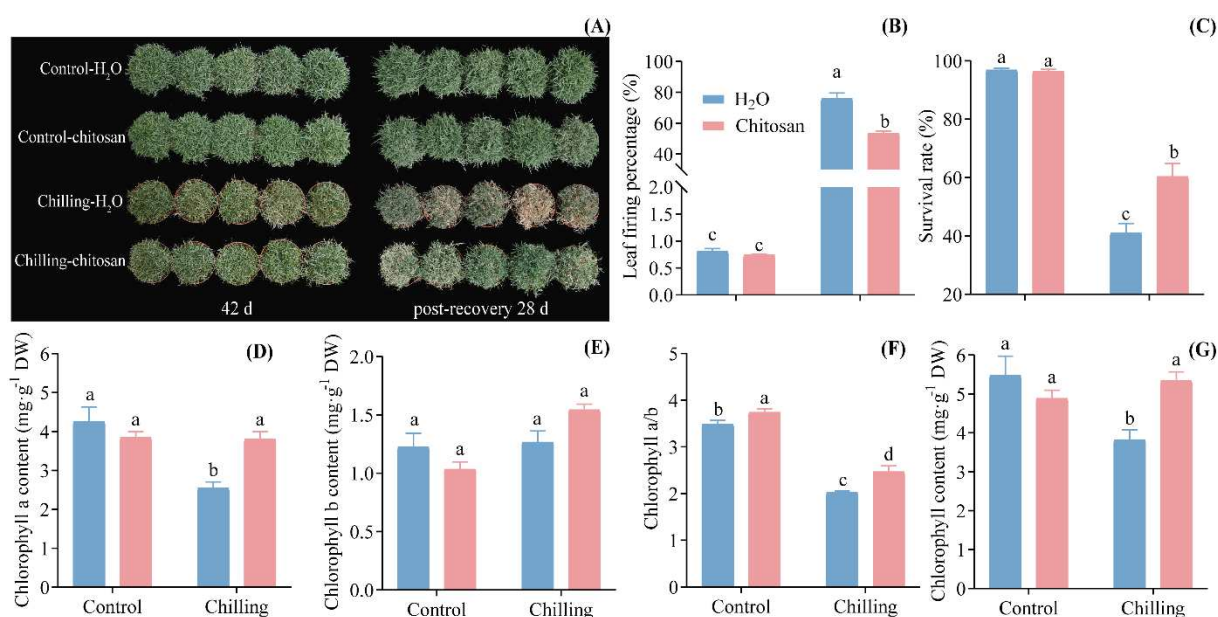


Fig. 7 The effects of exogenous chitosan concentration in chilling-sensitive cultivar 'Nannong LG' under normal condition and chilling stress. (A) shoot phenotype under chilling stress and post-recovery treatments. (B) leaf firing percentage. (C) survival rate. (D) chlorophyll a content. (E) chlorophyll b content. (F) chlorophyll a/b. (G) chlorophyll content. Control-H₂O and control-chitosan plants were sprayed with deionized water and chitosan under normal conditions (30/25°C, day/night), respectively. Chilling-H₂O and chilling-chitosan plants were similarly treated with deionized water and chitosan, under chilling conditions (4/2°C, day/night) respectively. Different letters represent significant differences between different treatments during the experimental period ($P < 0.05$).

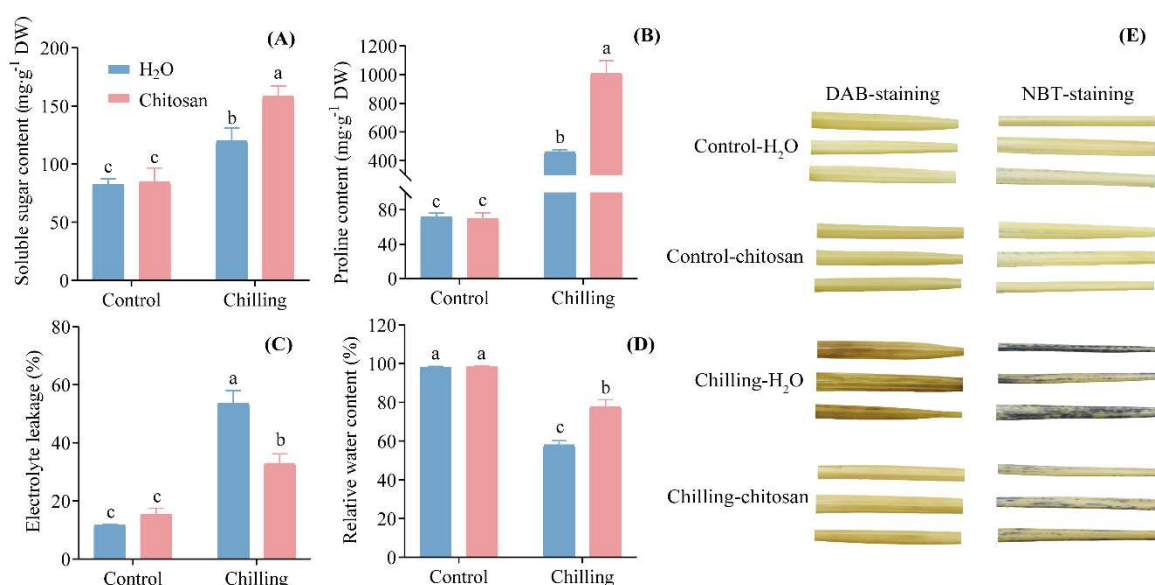


Fig. 8 The effects of exogenous chitosan concentration in chilling-sensitive cultivar 'Nannong LG' under

normal condition and chilling stress. (A) soluble sugar content. (B) proline content. (C) electrolyte leakage. (D) relative water content. (E) DAB and NBT staining. Control-H₂O and control-chitosan plants were sprayed with deionized water and chitosan under normal conditions (30/25°C, day/night), respectively. Chilling-H₂O and chilling-chitosan plants were similarly treated with deionized water and chitosan, under chilling conditions (4/2°C, day/night) respectively. Different letters represent significant differences between different treatments during the experimental period ($P < 0.05$).

3.7 Transcriptome response of 'Nannong LG' to chitosan treatment under chilling stress

To further explore transcriptional changes associated with chitosan treatment under chilling stress, transcriptomes of the LG-chilling and LG-chitosan groups were compared. This analysis identified 48 DEGs, comprising 27 up-regulated and 21 down-regulated genes (Fig. 9A). To validate candidate gene expression patterns, qRT-PCR was performed on five selected DEGs. *ZsNAC2*, *ZsNAC3*, *ZsLecRLK1*, and *ZsLecRLK2* were induced by chilling stress, and this induction was significantly attenuated following chitosan application (Fig. 9B, C, D, E). *ZsBAM* was down-regulated under chilling stress, and this down-regulation was significantly alleviated in chitosan-treated plants (Fig. 9F).

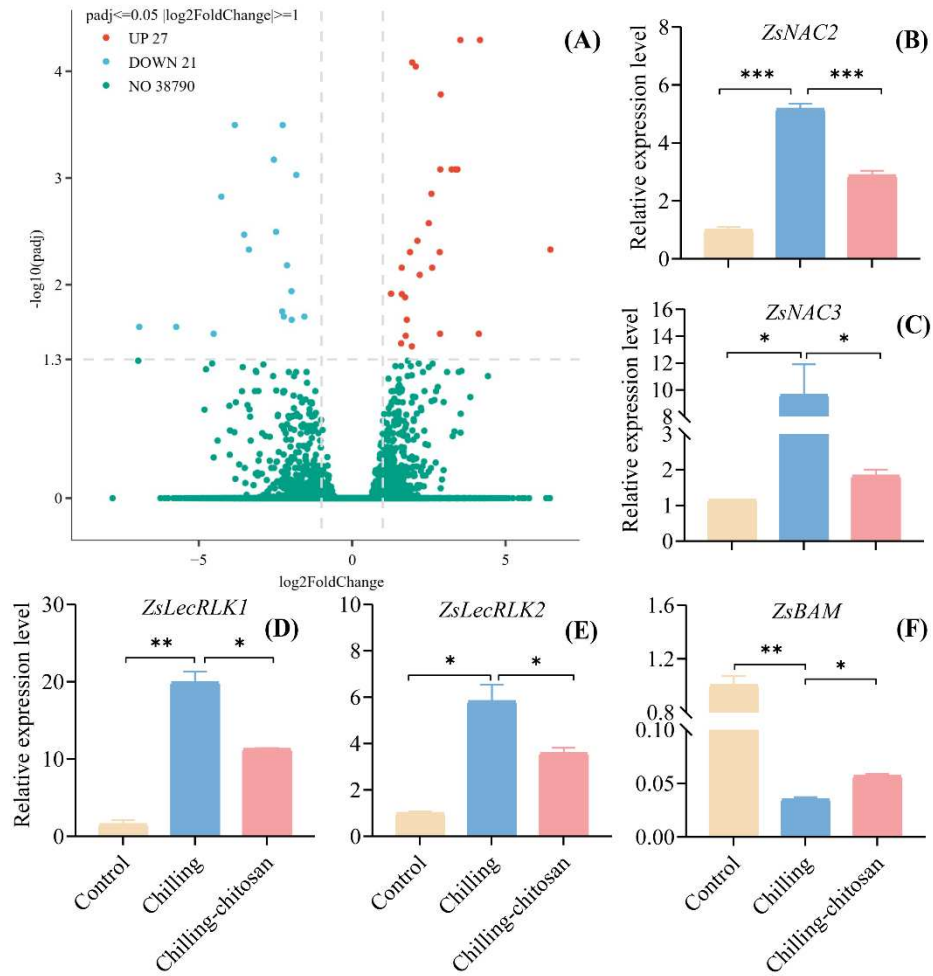


Fig. 9 Transcriptome analysis of chitosan-treated 'Nannong LG' at 48 h of the experiment. (A) volcano plots identify DEGs in LG-chitosan versus LG-chilling comparison. (B-F) qRT-PCR validation of selected genes responding to chitosan. * represents a significant difference between means under normal conditions and chilling stress. Significance level: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

Zoysiagrass is a warm-season turfgrass widely used for lawns and sports turf; however, its chilling sensitivity leads to premature autumn dormancy and growth cessation under low temperatures [11, 39]. To understand chilling tolerance mechanisms and explore mitigation strategies, this study elucidated differences of chilling tolerance between the two cultivars through integrating physiological and transcriptomic analyses, and assessed the efficacy of exogenous chitosan on the sensitive cultivar as below.

Physiologically, chilling-tolerant ‘Nannong NT1’ maintained superior membrane integrity, water status, and photosynthetic capacity relative to chilling-sensitive ‘Nannong LG’ (Figs. 1, 2). Transcriptomic analyses revealed that the cultivar-specific modulation of key metabolic pathways accounted for these differential responses to chilling stress (Figs. 3, 4). Furthermore, exogenous chitosan significantly alleviated chilling injury in ‘Nannong LG’, as evidenced by improved physiological parameters and transcriptional reprogramming in comparison with control under chilling stress (Figs. 6, 7, 8, 9).

The physiological difference between the two cultivars reflects distinct cellular protective strategies. Chilling stress disrupts cellular homeostasis through osmotic imbalance, ROS accumulation, and membrane damage [3, 40]. In tolerant genotypes, robust membrane integrity and efficient ROS scavenging collectively preserve cellular function under low temperatures, thereby conferring enhanced chilling resistance [7, 41]. For example, the chilling-tolerant cotton (*Gossypium* spp.) cultivar ‘Z1612’ displayed enhanced photosynthetic performance, and maintained cell membrane stability and effective ROS homeostasis under chilling stress [42]. Consistent with these results, ‘Nannong NT1’ exhibited lower electrolyte leakage, reduced ROS accumulation, and higher relative water content than ‘Nannong LG’ following 42 d of chilling stress, coupled with a higher post-recovery survival rate (Figs. 1, 2). Paradoxically, ‘Nannong LG’ accumulated higher levels of soluble sugars and proline under the same conditions (Fig. 2A, B). Although stress-induced accumulation of compatible osmolytes is often correlated with enhanced tolerance, some studies have suggested that it may serve as an indicator of stress severity rather than a protective mechanism [43, 44]. This interpretation is supported by our observation that the more injured ‘Nannong LG’ accumulated higher osmolytes. Collectively,

these findings indicate that ‘Nannong NT1’ suffered less chilling injury and maintained superior membrane integrity and water homeostasis under chilling stress.

Transcriptional profiling further elucidated the molecular mechanisms underlying these divergent physiological responses. Chilling stress typically triggers extensive transcriptional reprogramming in plants [16]. The differential chilling tolerance between cultivars does not appear to depend on the number of DEGs mobilized, but reflects differences in regulatory networks in this study. Although comparable numbers of DEGs were regulated in both cultivars under chilling stress with 12,462 in ‘Nannong LG’ and 12,306 in ‘Nannong NT1’, Venn analysis identified a substantial number of cultivar-specific DEGs that may serve as critical regulatory networks in these divergent responses (Fig. 3B, C, D). Among these DEGs, *ZsLBD45* remained stable in ‘Nannong NT1’ but was markedly up-regulated in ‘Nannong LG’ (Fig. 5G). *ZsLBD9* was induced in both cultivars yet exhibited higher expression in ‘Nannong LG’ (Fig. 5I). Although LBD transcription factors have traditionally been implicated in lateral organ development, accumulating evidence indicates that members of this family also function in abiotic stress responses [45]. These distinct expression patterns identify promising candidate regulators for cultivar-specific chilling tolerance and is likely to contribute to divergent metabolic reprogramming.

KEGG enrichment analysis revealed cultivar-specific metabolic reprogramming in the present study. ‘Nannong NT1’ specifically up-regulated carbohydrate and lipid metabolism pathways, including galactose metabolism, nucleotide sugar biosynthesis, glycerophospholipid, and glycerolipid metabolism (Fig. 4C). The enhanced carbohydrate metabolism may facilitate structural reinforcement and osmotic stabilization [46]. Concurrently, active remodeling of glycerophospholipid and glycerolipid metabolism further sustains membrane fluidity, that reflects a classical cold acclimation response [47]. Furthermore, ‘Nannong NT1’ exhibited protective down-regulation of photosynthesis and carbon fixation (Fig. 4D). This reflected a photoprotective response to mitigate chilling-induced photodamage and ROS generation. By contrast, ‘Nannong LG’ activated carotenoid and flavonoid biosynthesis, and repressed amino acid metabolism and protein export (Fig. 4A, B). These different changes indicated a state of metabolic dysfunction and compromised secretory capacity under chilling stress [48]. Collectively, our results suggest that ‘Nannong NT1’ adopted proactive metabolic

reprogramming and membrane preservation, whereas ‘Nannong LG’ showed stress-induced metabolic disturbances and a limited defense capacity.

Exogenous bioprotective treatments, including chitosan, can mitigate chilling injury in sensitive plants by strengthening antioxidant systems and maintaining cell structure [20, 28, 49]. In this study, foliar application of chitosan at 50 mg·L⁻¹ significantly mitigated chilling injury in ‘Nannong LG’, as evidenced by enhanced chlorophyll content, relative water content, osmoprotectant accumulation, and survival rates, coupled with reduced electrolyte leakage and ROS accumulation (Figs. 6, 7, 8). Notably, chitosan functioned through mechanisms distinct from the intrinsic tolerance of ‘Nannong NT1’. ‘Nannong NT1’ exhibited protective down-regulation of photosynthesis-related pathways and genes. By contrast, chitosan-treated ‘Nannong LG’ maintained higher chlorophyll content (Fig. 7G). These differential responses suggest that chitosan protects the photosynthetic apparatus via direct physiological stabilization rather than transcriptional repression. Similarly, the osmoprotectant response in chitosan-treated ‘Nannong LG’ contrasted with that of ‘Nannong NT1’. In ‘Nannong NT1’, the relatively lower osmolyte levels are consistent with its less severe injury and effective proactive defense. The untreated ‘Nannong LG’ passively accumulated soluble sugars and proline under severe injury. Chitosan application further increased these osmoprotectant levels in ‘Nannong LG’ while also concurrently reducing injury (Fig. 8A, B), indicating active protective modulation rather than an aggravated stress response [50, 51].

At the transcriptional level, only 48 DEGs were identified between chitosan-treated and untreated ‘Nannong LG’ under chilling stress (Fig. 9A), indicating that chitosan functions primarily through direct physiological and biochemical mechanisms rather than extensive transcriptional reprogramming. Nevertheless, chitosan can also regulate DEG responses. *ZsNAC2*, *ZsNAC3*, *ZsLecRLK1*, and *ZsLecRLK2* were induced by chilling stress, and this induction was attenuated following chitosan application (Fig. 9B, C, D, E). *ZsBAM*, encoding a β -amylase involved in starch mobilization [52], was down-regulated by chilling stress, but to a lesser extent in chitosan-treated plants (Fig. 9F). Collectively, these findings indicate that exogenous chitosan alleviated chilling injury in ‘Nannong LG’ by enhancing physiological protection and transcriptional adjustment. These mechanisms are distinct from the intrinsic genetic tolerance of ‘Nannong NT1’.

5. Conclusion

In summary, the superior chilling tolerance of ‘Nannong NT1’ was associated with lower electrolyte leakage, reduced ROS accumulation, higher relative water content, and higher post-recovery survival rate compared with ‘Nannong LG’. Transcriptomic analysis identified comparable numbers of DEGs in both cultivars, with a substantial number of cultivar-specific DEGs. In ‘Nannong NT1’, primary metabolism pathways encompassing carbohydrate metabolism and lipid metabolism were up-regulated, whereas photosynthesis and carbon fixation were protectively down-regulated. In contrast, ‘Nannong LG’ exhibited activation of carotenoid and flavonoid biosynthesis alongside repression of amino acid metabolism and protein export. Additionally, foliar application of chitosan ($50 \text{ mg} \cdot \text{L}^{-1}$) significantly alleviated chilling injury in ‘Nannong LG’ by improved chlorophyll content, relative water content, osmoprotectant accumulation, and survival rates, coupled with reduced electrolyte leakage and ROS accumulation. Chitosan specifically modulated the expression of several key DEGs, such as *ZsNAC2*, *ZsNAC3*, *ZsLecRLK1*, *ZsLecRLK2*, and *ZsBAM*. In the future, it is worthwhile to further validate the functions of candidate chilling-tolerance genes and evaluate the agronomic potential of chitosan application under field conditions.

6. Declarations

Abbreviations

ATAC-seq, assay for transposase-accessible chromatin with high-throughput sequencing

DAB, 3,3-diaminobenzidine

DEGs, differentially expressed genes

DMSO, dimethyl sulfoxide

FPKM, fragments per kilobase of exon model per million mapped fragments

KEGG, Kyoto encyclopedia of genes and genomes

NBT, nitrotetrazolium blue chloride

PCA, principal component analysis

qRT-PCR, Quantitative reverse transcription PCR

RNA-seq, RNA sequencing

ROS, reactive oxygen species

Ethics approval and consent to participate

This study does not involve human participants or animals, and therefore ethics approval and consent to participate are not applicable.

Consent for publication

All authors have read and approved the final version of the manuscript. They consent to the submission of this manuscript to the journal for peer review and potential publication.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article (and its supplementary files). Any additional data or materials are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing financial interests or personal relationships that could have influenced this work.

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Author contributions

Qiuguo Li: designed the study, performed the experiments, drafted and revised the manuscript.

Jingjin Yu: designed the study, provided resources, and revised the manuscript.

Tian Hao and Ningli Fan: assisted with the experiments and revised the manuscript.

Jiayue Liang, Ye Huang, and Binbin Liu: assisted with sample collection and experimental procedures.

Zhimin Yang: designed the study, provided resources, and supervised the whole project.

All authors have reviewed and approved the final submitted manuscript.

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References

1. Soualiou S, Duan F, Li X, Zhou W. Crop production under cold stress: An understanding of plant responses, acclimation processes, and management strategies. *Plant Physiol Bioch.* 2022; 190: 47-61.
2. Li J, Bai X, Ran F, Zhang C, Yan Y, Li P, et al. Effects of combined extreme cold and drought stress on growth, photosynthesis, and physiological characteristics of cool-season grasses. *Sci Rep.* 2024; 14(1): 116.

3. Kang H, Thomas H, Xia X, Shi H, Zhang L, Hong J, et al. An integrative overview of cold response and regulatory pathways in horticultural crops. *J Integr Plant Biol.* 2025; 67(4): 1028-1059.
4. Liu B, Su J, Fu C, Xian K, He J, Huang N. Comparative transcriptomic profiles of *Paulownia catalpifolia* under different degrees of chilling stress during the seedling stage. *BMC Genomics.* 2024; 25(1): 716.
5. Guo L, Du X, Chang J, Gong J, Chu Z, Lv J, et al. Effects of low-temperature stress at different growth stages on rice physiology, pollen viability and yield in China's cold region. *PLOS One.* 2025; 20(8): e0329441.
6. Li Z, Huang C, Han L. Differential regulations of antioxidant metabolism and cold-responsive genes in three bermudagrass genotypes under chilling and freezing stress. *Int J Mol Sci.* 2023; 24(18): 14070.
7. Vives-Peris V, Vicente-Valls J, Rodriguez-Azorin L, Gomez-Cadenas A, Lopez-Climent MF. Physiological and biochemical adaptive strategies in citrus plants facing cold stress. *Plant Growth Regul.* 2025; 105(5): 1575-1590.
8. Feng Y, Meng L, Fu X, Zhang X, Bi H, Ai X. *CsZAT10*-mediated nitric oxide signalling pathway is involved in melatonin-induced chilling tolerance in cucumber. *Plant Cell Environ.* 2026; 49(3): 1313-1329.
9. Haupt J, Glowacka K. Chilling- and dark-regulated photoprotection in *Miscanthus*, an economically important C₄ grass. *Commun Biol.* 2024; 7(1): 1660.
10. Zheng S, Li Z, You Y, Cao J, Luo W, Qian Q, et al. Natural variations in the promoter of chilling tolerance divergence 8 contribute to the functional divergence in the chilling tolerance of rice. *Plant Cell Environ.* 2025; 48(11): 8374-8387.
11. Patton A, Schwartz B, Kenworthy K. Zoysiagrass (*Zoysia* spp.) history, utilization, and improvement in the united states: A review. *Crop Sci.* 2017; 57(S1): 37-72.
12. Wang W, Shao A, Xu X, Fan S, Fu J. Comparative genomics reveals the molecular mechanism of salt adaptation for zoysiagrasses. *BMC Plant Biol.* 2022; 22(1): 355.

13. Wu Z, He L, Wang C, Liang C, Li H, Zhong D, et al. Unveiling unique alternative splicing responses to low temperature in *Zoysia japonica* through ZjRTD1.0, a high-quality reference transcript dataset. *Physiol Plantarum*. 2024; 176(2): e14280.
14. Loch DS, Ebina M, Choi JS, Han L. Ecological implications of species, distribution, and adaptation for management and use of zoysiagrasses. *ITSRJ*. 2017; 13(1): 11-25.
15. Wei S, Du Z, Gao F, Ke X, Li J, Liu J, et al. Global transcriptome profiles of ‘Meyer’ zoysiagrass in response to cold stress. *PLOS One*. 2015; 10(6): e131153.
16. Shen L, Qi Z, Dai X, Ai Y, Chen J, Chao Y, et al. Chromosome-scale genome assembly of *Zoysia japonica* uncovers cold tolerance candidate genes. *Sci Data*. 2025; 12(1): 571.
17. Patton A, Reicher Z. Zoysiagrass species and genotypes differ in their winter injury and freeze tolerance. *Crop Sci*. 2007; 47(4): 1619-1627.
18. Li S, Yang Y, Zhang Q, Liu N, Xu Q, Hu L. Differential physiological and metabolic response to low temperature in two zoysiagrass genotypes native to high and low latitude. *PLOS One*. 2018; 13(6): e198885.
19. Jin H, Jiang M, Yang J, Wu Z, Ma L, Wang C, et al. A survey of enhanced cold tolerance and low-temperature-induced anthocyanin accumulation in a novel *Zoysia japonica* biotype. *Plants*. 2022; 11(3): 429.
20. Lu H, Wang M, Zhou S, Chen K, Wang L, Yi Z, et al. Chitosan oligosaccharides mitigate flooding stress damage in rice by affecting antioxidants, osmoregulation, and hormones. *Antioxidants*. 2024; 13(5): 521.
21. Huang X, Song S, Zhou M, Ma G, Hu Z, Chen L. CdJACG1 integrates jasmonic acid signaling and CBF pathways to confer cold tolerance in bermudagrass (*Cynodon dactylon*). *Plant Physiol*. 2026; 200(3): kiag129.
22. Riseh R, Hassanisaadi M, Vatankhah M, Babaki S, Barka E. Chitosan as a potential natural compound to manage plant diseases. *Int J Biol Macromol*. 2022; 220: 998-1009.
23. Huang C, Tian Y, Zhang B, Hassan M, Li Z, Zhu Y. Chitosan (CTS) alleviates heat-induced

leaf senescence in creeping bentgrass by regulating chlorophyll metabolism, antioxidant defense, and the heat shock pathway. *Molecules*. 2021; 26(17): 5337.

24. Li Q, Li R, He F, Yang Z, Yu J. Growth and physiological effects of chitosan on heat tolerance in creeping bentgrass (*Agrostis stolonifera*). *Grass Res*. 2022; 2: 6.

25. Ahmed S, Ahmed SF, Biswas A, Sultana A, Issak M. Salicylic acid and chitosan mitigate high temperature stress of rice via growth improvement, physio-biochemical adjustments and enhanced antioxidant activity. *Plant Stress*. 2024; 11: 100343.

26. Soostani S, Ranjbar M, Memarian A, Mohammadi M, Yaghini Z. Investigating the effect of chitosan on the expression of *P5CS*, *PIP*, and *PAL* genes in rapeseed (*Brassica napus* L.) under salt stress. *BMC Plant Biol*. 2025; 25(1): 215.

27. Li Y, Zhang Q, Ou L, Ji D, Liu T, Lan R, et al. Response to the cold stress signaling of the tea plant (*Camellia sinensis*) elicited by chitosan oligosaccharide. *Agronomy-basel*. 2020; 10(6): 915.

28. Tan C, Li N, Wang Y, Yu X, Yang L, Cao R, et al. Integrated physiological and transcriptomic analyses revealed improved cold tolerance in cucumber (*Cucumis sativus* L.) by exogenous chitosan oligosaccharide. *Int J Mol Sci*. 2023; 24(7): 6202.

29. Severmutlu S, Mutlu N, Gurbuz E, Gulsen O, Hocagil M, Karaguzel O, et al. Drought resistance of warm-season turfgrasses grown in mediterranean region of turkey. *Horttechnology*. 2011; 21(6): 726-736.

30. Zhang Q, Chen Q, Wang S, Hong Y, Wang Z. Rice and cold stress: Methods for its evaluation and summary of cold tolerance-related quantitative trait loci. *Rice*. 2014; 7: 24.

31. Blum A, Ebercon A. Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Sci*. 1981; 21(1): 43-47.

32. Barrs H, Weatherley P. A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Aust J Biol Sci*. 1962; 15(3): 413.

33. Yu J, Li M, Li Q, Wang R, Li R, Yang Z. Reallocation of soluble sugars and IAA regulation

in association with enhanced stolon growth by elevated CO₂ in creeping bentgrass. *Plants*. 2022; 11(11): 1500.

34. Bates L, Waldren R, Teare I. Rapid determination of free proline for water-stress studies. *Plant and Soil*. 1973; 39: 205-207.

35. Wellburn A. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J Plant Physiol*. 1994; 144(3): 307-313.

36. Thordal-Christensen H, Zhang Z, Wei Y, Collinge D. Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J*. 1997; 11(6): 1187-1194.

37. Dai M, Huang R, Han Y, Zhang Z, Chen Y, Shi H, et al. A novel salt responsive *PvHAK16* negatively regulates salt tolerance in transgenic *Arabidopsis thaliana*. *Environ Exp Bot*. 2022; 194: 104689.

38. Schmittgen T, Livak K. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*. 2008; 3: 1101-1108.

39. Zhang J, Li L, Zhang Z, Liu Y, Mao J, Li J, et al. A single-base deletion in *ZjSGR* confers the *STAY-GREEN* phenotype and the enhanced stress tolerance in *Zoysia japonica* under dark and low temperature stresses. *Ind Crop Prod*. 2026; 241: 122836.

40. Ji L, Zhang Z, Liu S, Zhao L, Li Q, Xiao B, et al. The OsTIL1 lipocalin protects cell membranes from reactive oxygen species damage and maintains the 18:3-containing glycerolipid biosynthesis under cold stress in rice. *Plant J*. 2024; 117(1): 72-91.

41. Zhang H, Jiang C, Lei J, Dong J, Ren J, Shi X, et al. Comparative physiological and transcriptomic analyses reveal key regulatory networks and potential hub genes controlling peanut chilling tolerance. *Genomics*. 2022; 114(2): 110285.

42. Tang S, Xian Y, Wang F, Luo C, Song W, Xie S, et al. Comparative transcriptome analysis of leaves during early stages of chilling stress in two different chilling-tolerant brown-fiber cotton cultivars. *PLOS One*. 2021; 16(2): e0246801.

43. Chen X, Chen Z, Fiorentino A, Kuess M, Tharayil N, Kumar R, et al. MicroRNA169 integrates multiple factors to modulate plant growth and abiotic stress responses. *Plant Biotechnol J*. 2024; 22(9): 2541-2557.
44. Konrad C, Spormann S, Nadais P, Martins M, Soares C, Fidalgo F. Highway to stress: Proline dynamics in tomato plants under osmotic disturbances. *Plant Physiol Bioch*. 2026; 237: 111496.
45. Rong M, Gao S, Wen D, Xu Y, Wei J. The LOB domain protein, a novel transcription factor with multiple functions: A review. *Plant Physiol Bioch*. 2024; 214: 108922.
46. Yuxi Z, Dan Y, Chunying L, Shupeng G. Dynamic of carbohydrate metabolism and the related genes highlights PPP pathway activation during chilling induced bud dormancy release in tree peony (*Paeonia suffruticosa*). *Sci Hortic*. 2018; 242: 36-43.
47. Liu M, Wu X, Li M, Li S, Xiong T, Li C, et al. Multi-omics analysis reveals the physiological and molecular response to cold stress in different spring wheat cultivars at the booting stage. *Front Plant Sci*. 2025; 16: 1594676.
48. Ghimire K, Serba D, Amundsen K, Wu Y, Barnaby J. Decoding chilling temperature adaptation in bermudagrass: Temporal transcriptomic and metabolomic dynamics in tolerant and sensitive genotypes. *Crop Sci*. 2026; 66(3): e70317.
49. Song L, Tan Z, Zhang W, Li Q, Jiang Z, Shen S, et al. Exogenous melatonin improves the chilling tolerance and preharvest fruit shelf life in eggplant by affecting ROS- and senescence-related processes. *Hortic Plant J*. 2023; 9(3): 523-540.
50. Kebbeh M, Dong J, Huan C, Shen S, Liu Y, Zheng X. Melatonin treatment alleviates chilling injury in mango fruit 'Keitt' by modulating proline metabolism under chilling stress. *J Integr Agr*. 2023; 22(3): 935-944.
51. Wang Y, Zhang M, Wu C, Chen C, Meng L, Zhang G, et al. SIWRKY51 regulates proline content to enhance chilling tolerance in tomato. *Plant Cell Environ*. 2024; 47(12): 5104-5114.
52. Shi C, Li P, Li L, Qi Z, Lu T, Shi J, et al. Characterization and functional analysis of the

