

## Physiological, biochemical and transcriptomic studies reveal the mechanisms promoting tiller bud growth under high temperature conditions in *japonica* rice

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### Abstract

High temperature (HT) negatively impacts the initiation and development of tiller buds in *japonica* rice, resulting in a substantial decrease in yield. However, the physiological and molecular mechanisms alleviating the inhibition of HT on the growth of tiller buds in *japonica* rice remain elusive. Hence, this study aimed to dissect the underlying mechanisms of normal tiller buds' growth under HT in *japonica* rice through comparative physiological, biochemical and transcriptomics analyses of two contrasting varieties, 'Changgeng 225' ('CG225', heat-tolerant) and 'Zhonghua 11' ('ZH11', heat-sensitive). Compared with 'ZH11', the tiller number of 'CG225' decreased less under HT stress. 'CG225' outperformance over 'ZH11' was due to higher activities of the antioxidant enzymes (SOD, CAT, POD and APX), as well as higher activities of the key enzymes involved in carbon and nitrogen metabolism (NR, GOGAT, GS, GDH, SS and SPS) in tiller buds. In addition, lower contents of the tiller-inhibiting phytohormone ABA and SLs, and higher contents of the tiller-promoting phytohormone CK in 'CG225' also promoted the growth of tiller buds. Most of the significant DEGs between 'CG225' and 'ZH11' under HT conditions were assigned to chloroplast and photosynthesis. We identified potential candidate genes, including transcription factors (EP2, B3, bHLH, MYB, NAC, and WRKY), phytohormone (auxin efflux carriers and ethylene biosynthesis genes), antioxidant system (peroxidases), and sucrose pathway-related genes. Furthermore, ectopic sucrose application significantly alleviated HT inhibitory effects on tiller bud growth of 'ZH11' plants. Our results reveal a synergistic control of heat stress by several metabolic mechanisms in *japonica* rice during tillering and provide fundamental resources for improving rice HT tolerance and production.

**Keywords:** high-temperature stress; *Japonica* rice; molecular mechanism; physiological mechanism; sucrose; tiller bud

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**Abbreviation:** ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase; CK, cytokinin; GDH, glutamate dehydrogenase; GOGAT, glutamate synthetase; GS, glutamine synthetase; NR, nitrate reductase; POD, peroxidase; ROS, reactive oxygen species; SLs, strigolactones; SOD, superoxide dismutase; SPS, sucrose phosphate synthase; SS, starch synthase.

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## Introduction

Rice (*Oryza sativa* L.) is a critical cereal crop that satisfies the staple food needs of the global population. There are two prominent *sativa* subspecies, *japonica* and *indica*, which display unique transcriptomic, metabolomic, physiological, and agronomic responses under various abiotic stress conditions (Hu *et al.*, 2014). Given its high grain quality, the demand for *japonica* rice is consistently on the rise (Shi *et al.*, 2023). However, its relatively lower tolerance to HT poses a significant hurdle in expanding its cultivation area and production compared to the *indica* variety (Lin *et al.*, 2014). Notably, HT hinders tiller bud germination, which substantially reduces effective panicles per unit area, resulting in lower yields (Xu *et al.*, 2021; Yang *et al.*, 2021). Thus, unraveling the physiological and molecular mechanisms that alleviate HT's detrimental effects on tiller bud growth could underpin the development of HT-tolerant *japonica* rice varieties.

Earlier investigations have revealed that plants' stress response leads to a surge in reactive oxygen species (ROS), which drastically restrict growth and development. Indeed, ROS damage carbohydrates, proteins, lipids, DNA, and biological membranes, eventually leading to cell death (Xia *et al.*, 2022). Moreover, they cause dysfunction or loss of chloroplasts, resulting in a significant decrease in photosynthesis efficiency and primary metabolites production (Xu *et al.*, 2023; Yin *et al.*, 2010). Fortunately, plants have evolved several regulatory methods to eliminate excess ROS and balance growth and stress (Han *et al.*, 2020; Zafari *et al.*, 2022; Wei *et al.*, 2021). ROS scavengers, mainly including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), ascorbic acid peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2) and other antioxidant enzymes, are widely present in plant cells. When the rate of ROS formation is within a controllable range, plants often increase the synthesis of antioxidant enzymes to eliminate ROS. However, when plants are subjected to irreversible stress environments, such as HT, salt, and drought stress, it can cause the ROS synthesis rate to be too fast, and the ROS scavenging system often loses its main function (Sharkey, 2005). The continuous production of ROS will ultimately lead to plants' death and decrease in production (Lin *et al.*, 2011).

Carbon and nitrogen are the two most abundant nutrient elements for all living organisms, and their metabolism is essential for every aspect of plant growth, development and stress response (Xing *et al.*, 2022). The availability of high carbohydrates (glucose and sucrose) during grain filling is an important physiological characteristic related to HT tolerance (Shao *et al.*, 2021). A higher content of soluble carbohydrates can enhance plant heat tolerance, and its content can also serve as an essential indicator of plant carbon metabolism level (Cabello *et al.*, 2014). However, HT often leads to a decrease in sucrose phosphate synthase and starch synthase activities, resulting in insufficient sucrose content and defective growth and development of plants (Luo *et al.*, 2021). In addition, nitrogen nutrition levels directly or indirectly affect plant photosynthesis, thus regulating the growth of tiller buds. During the early stages of plant growth, nitrogen metabolism is vigorous, and reducing nitrogen content can inhibit the occurrence of rice tiller (Yu *et al.*, 2021). Within a certain range, increasing the amount of nitrogen fertilizer can increase the activity of nitrogen metabolism enzymes such as nitrate reductase and glutamine synthetase in rice, thus promoting nitrogen absorption (Hou *et al.*, 2021; Luo *et al.*, 2020). Moreover, increasing the nitrogen nutrition level in plants can significantly enhance the activities of antioxidant enzymes such as SOD, POD, and CAT and reduce the content of ROS and membrane lipid peroxidation product Malondialdehyde (MDA) in plants, thus improving the adaptability of plants to stress

(Lin *et al.*, 2011). Therefore, it is worth studying how tiller bud cells adjust their carbon and nitrogen metabolism to adapt to HT stress.

The rice tiller development comprises two processes, the formation and germination of axillary buds, and is controlled by genetic and environmental factors (Andres *et al.*, 2021; Cai *et al.*, 2023). After forming, the tiller buds continue to germinate and form tillers or maintain a dormant state (Ishikawa *et al.*, 2005). Phytohormones play a vital role in determining the fate of tiller buds. The auxin/indole-3-acetic acid (Aux/IAA) is transported from the top of the plant to the tiller bud through polar transport, making the tiller bud dormant by inhibiting its growth and development (Wang *et al.*, 2015). Cytokinin (CK), unlike IAA, can promote the germination of tiller buds (Tanaka *et al.*, 2006). In contrast to CK, strigolactones (SLs) regulate the elongation of tiller buds by modulating the polar auxin transport (Crawford *et al.*, 2010). In addition, Abscisic acid (ABA) inhibits the growth of tiller buds and can coordinate with CK (Liu *et al.*, 2020). When the ratio of CK to ABA is high, the growth of tiller buds is inhibited. When the ratio of CK to ABA is low, it is conducive to the growth of tiller buds (Lei *et al.*, 2022). In addition, ABA and ethylene (ETH) synergistically regulate the dormancy of tiller buds (Ophir *et al.*, 2009). Brassinolide (BR) regulates IAA-related genes, and gibberellin (GA) inhibits the development of tiller buds (Riaz *et al.*, 2023; Yang *et al.*, 2020). However, how the dynamic changes in endogenous hormone levels under HT conditions affect the growth and development of tiller buds is not clear.

In this study, the physiological, biochemical, and molecular mechanisms of tiller buds responding to HT stress were analysed in terms of characteristics of tiller bud, antioxidant enzyme activity, carbon and nitrogen metabolism, hormone levels, and transcriptome analysis with the HT-sensitive variety 'ZH11' and the HT-tolerant variety 'CG225'.

## Materials and Methods

### *Plant materials and growing conditions*

A pot experiment was conducted utilizing two *japonica* rice varieties, including 'Changgeng 225' ('CG225', heat-tolerant) and 'Zhonghua 11' ('ZH11', heat-sensitive). The two varieties were obtained from consecutive evaluation of diverse *japonica* rice varieties under HT by our research group. Nitrogen (N) was supplied as urea (46%) fertilizer, phosphorus (P) as calcium magnesium phosphate fertilizer (12%), and potassium (K) as potassium chloride fertilizer (60%). The applied fertilizer quantities were 225 kg/ha of pure nitrogen, 90 kg/ha of phosphorus, and 120 kg/ha of potassium, respectively. Rice seedlings, nine days post-transplantation, were subjected to HT stress for 15 days inside an artificial climate chamber. This arrangement was done to compare with the control temperature (CT). The temperature treatment and duration of sunlight were analogous to actual meteorological conditions. For the HT treatment, the temperature was set as follows: 31 °C from 0:00-7:00; 32.5 °C from 7:00-9:00; 40 °C from 9:00-14:00; 36.5 °C from 14:00-16:00; and 31 °C from 16:00-24:00. For CT conditions, the plants were grown at room temperature following this schedule: 25 °C from 0:00-7:00; 26.5 °C from 7:00-9:00; 32 °C from 9:00-14:00; 30.5 °C from 14:00-16:00; and 25 °C from 16:00-24:00. Further, a relative humidity of 75% was maintained, and the light intensity was set to 1400  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{S}^{-1}$  from 6:00 am to 8:00 pm, providing 14 hours of light exposure each day. Seven days after initiating the treatments, the tiller nodes and buds from the axilla of the sixth leaf at the base of the main stem were collected. Upon collection, these samples were promptly frozen in liquid nitrogen and stored at -80°C pending further analysis.

### *Phenotype analysis*

The length of tiller buds was measured using an electronic vernier calliper, while an electric weighing balance was employed to quantify the fresh weight of rice samples. To compute cell diameter, 20 fresh axillary

buds were sampled on the second day post-HT treatment between 9:00 am and 10:00 am. The samples were subsequently fixed in a solution of 50% ethanol, acetic acid, and formaldehyde (v/v/v: 90/5/5). The paraffin section was prepared as described by Wang *et al.* (2018), and cell diameter was ascertained using Image-Pro Plus software (version 6.0.0.260). The dormancy rate of tiller buds, on the other hand, was measured after six days of HT treatment.

#### *Measurement of MDA content and H<sub>2</sub>O content*

MDA content was assessed by utilizing the thiobarbituric acid method (Dhindsa *et al.*, 1981), and H<sub>2</sub>O<sub>2</sub> content was analyzed considering the titanium sulfate (TiSO<sub>4</sub>) method (Sun *et al.*, 2014).

#### *Antioxidant enzyme activity assays*

SOD and POD activities were determined by the nitrogen blue tetrazolium (NBT) and guaiacol methods, respectively. CAT activity was measured by assessing the H<sub>2</sub>O<sub>2</sub> decomposition rate at 240 nm for 3 min (Dhindsa *et al.*, 1981). The method described by Nakano and Asada (Nakano & Asada, 1981) was used for APX activity evaluation.

#### *Osmotic regulation substance content*

Soluble protein (SP) and soluble sugar contents were measured via the Coomassie bright blue and the anthrone colorimetric methods, respectively (Lillo, 1984). The content of free amino acid (FAA) was determined following the modified method from Yemm *et al.* (Yemm *et al.*, 1955), using fluorometric analysis. The resorcinol method was employed to calculate sucrose content by measuring the absorbance (OD) value at 480 nm (Ma *et al.*, 2016).

#### *Carbon and nitrogen metabolism-related key enzyme activity assays*

Nitrate reductase (NR, EC 1.7.99.4), glutamine synthetase (GS, EC 6.3.1.2), and glutamate synthetase (GOGAT, EC 1.4.1.13) activities were determined following the described methods by Savidov *et al.* (1997) and Han *et al.* (2017), Liu *et al.* (2016), respectively. The glutamate dehydrogenase (GDH, EC 1.4.1.2) activity of NADH- was determined by Groat and Vance methods (1981). SPS (Sucrose phosphate synthase) and SS (starch synthase) activities were analysed following the methods reported by Sicher and Kremer (1984) and Zuk *et al.* (2005), respectively.

#### *Determination of phytohormone content*

The tiller buds in the sixth leaf axillary (seven days after treatment) were used to determine the content of phytohormones. Fresh samples were harvested, immediately frozen in liquid nitrogen, ground into powder (30 Hz, 1 min), and stored at -80 °C. The contents of phytohormones were determined by MetWare (<http://www.metware.cn/>) based on the AB Sciex QTRAP 6500 LC-MS/MS platform.

#### *RNA-sequencing analysis*

Total RNA from tiller bud samples, treated and frozen for seven days in the sixth leaf axillary, was used for transcriptional analysis. The RNA extraction was accomplished using the Trizol reagent kit (ThermoFisher, 15596018) according to the manufacturer's instructions. All resulting cDNA libraries underwent paired-end sequencing (PE150) on an Illumina Novaseq™ 6000 platform implemented by LC-Bio Technology CO., Ltd. (Hangzhou, China). Differentially Expressed Genes (DEGs) between groups were identified using DESeq2 software. Gene Ontology (GO, <http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/kaas>) enrichment analysis for the DEGs were performed using GO seq and KOBAS (2.0) software, respectively.

#### *Real-time quantitative PCR (RT-qPCR) analysis*

RT-qPCR analysis was carried out using previously described methods (Li *et al.*, 2019). The SYBR Green Perfect mix (TaKaRa, Dalian, China) was used for the RT-qPCR at CFX96 (BioRad). Each gene's relative expression levels were calculated according to the  $2^{-\Delta\Delta CT}$  method, using the rice *ubiquitin* (UBQ) gene as the internal reference. The primers were designed with Primer 5 and are provided in Table S3.

#### *Sucrose treatment assays*

For the sucrose treatment assays, ZH11 served as the plant material under experimental conditions similar to the main experiment. On the zeroth day of HT treatment, a 4 mM sucrose solution (500 ml) was added to the HT treatment group at approximately 8:00 am, while 500 ml of deionized water was added to the control group. The tiller count in each group was taken 15 days post-HT treatment.

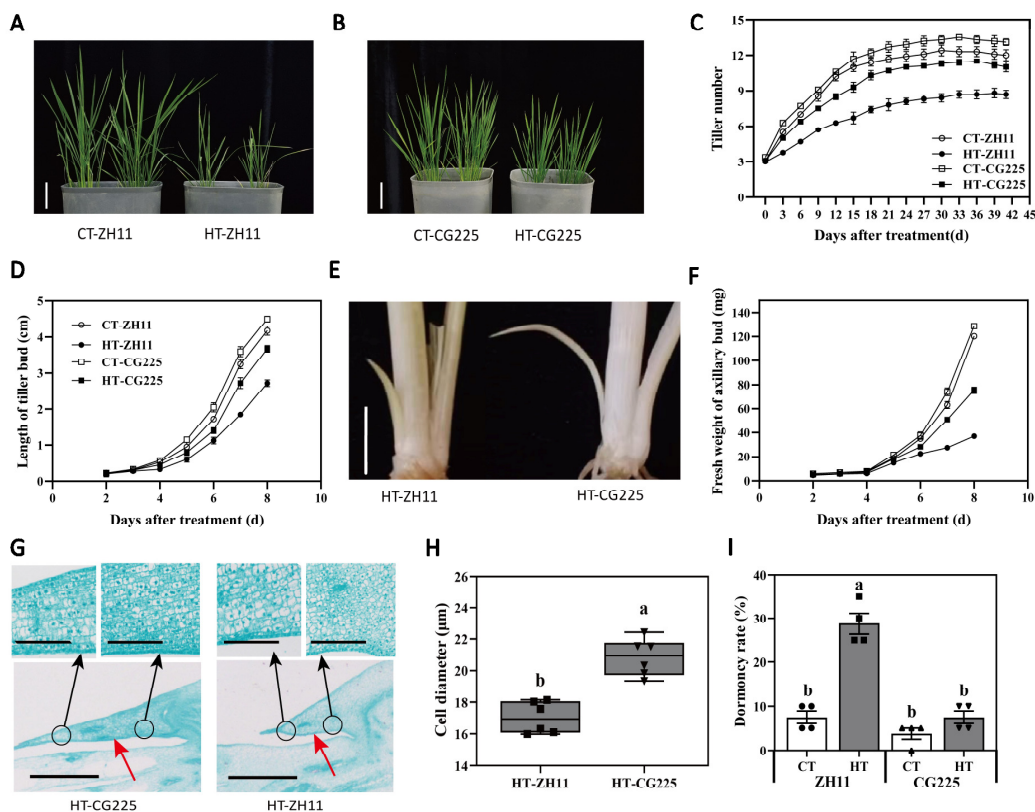
#### *Statistical analysis*

Statistical analysis was carried out using Microsoft Excel 2019, SPSS 23, GraphPad Prism 9, and R software (version 4.0.3). Duncan's multiple tests were utilized for multiple comparisons with a threshold set at  $P < 0.05$ . Heatmap construction was accomplished via TBtools software (Chen *et al.*, 2020).

## **Results**

#### *Characteristics of tiller buds in 'ZH11' and 'CG225' under HT conditions*

To reveal the growth and development characteristics of tiller buds in 'ZH11' and 'CG225' under HT conditions, we observed the tiller morphology of 'ZH11' and 'CG225'. The results showed that compared with the CT group, the growth of tillers in 'ZH11' was substantially inhibited in the HT group, while only a slight inhibition was recorded in 'CG225' (Figure 1A, B). Kinetic analysis showed that on the 15th day after HT treatment, the difference in the tiller number of 'ZH11' or 'CG225' between the CT and HT groups was the highest. The tiller number of 'ZH11' decreased by 39.09% compared with CT treatment. In contrast, that of 'CG225' only decreased by 20.51%, confirming that 'CG225' is heat-resistant (Figure 1C). To further study the development characteristics of tillers in 'ZH11' and 'CG225' under HT conditions, the dynamic changes in the length and fresh weight of tiller buds from 0 to 8 days after HT treatment were investigated. The results showed that the difference in tiller bud length of 'ZH11' or 'CG225' between the CT group and the HT group gradually increased from the 6th day after HT treatment, but there was no significant change in the first five days (Figure 1D, E). Meanwhile, the same change trend was also observed in the fresh weight of tiller buds (Figure 1F). Next, we selected representative tiller buds of 'ZH11' and 'CG225' under HT conditions and observed the morphology and size of tiller bud cells using tissue sections. We found that the diameter of cells in the tiller bud of 'CG225' was significantly higher than that of 'ZH11' (Figure 1G, H). In addition, we also examined the quantity and dormancy rate of tiller buds under both CT and HT conditions. The results revealed no variations in the formation of tiller buds between the two *japonica* rice varieties under HT stress (Table S1). However, the dormancy rate of tiller buds in the 'ZH11' variety significantly increased in the HT group compared to the CT group. In contrast, no significant change was observed in the 'CG225' variety (Figure 1I). Therefore, these results indicated that HT stress does not affect the differentiation of tiller buds but inhibits their germination and growth.



**Figure 1.** Characteristics of tiller buds in 'ZH11' and 'CG225' under HT conditions

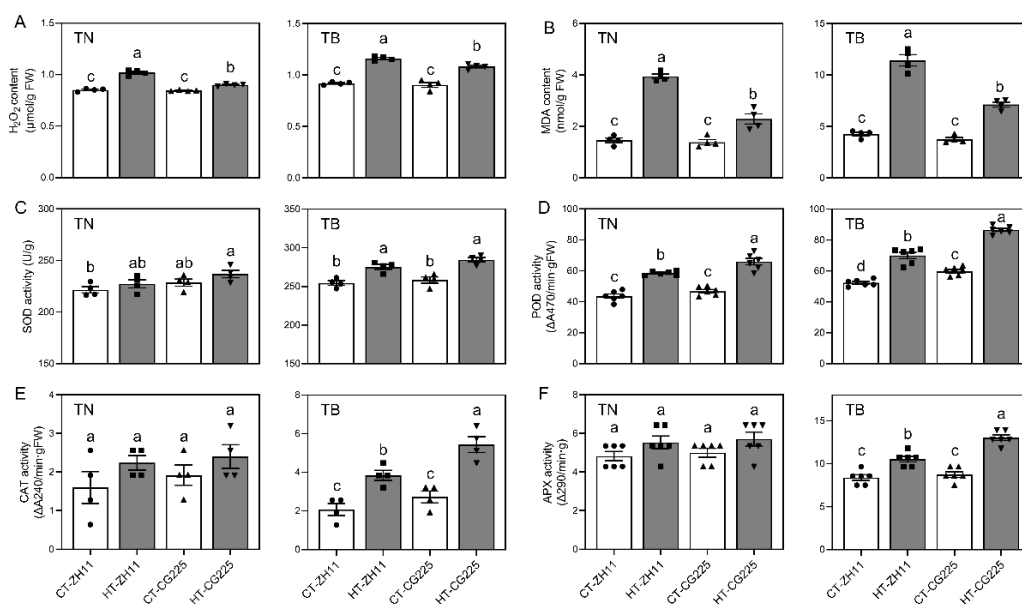
(A, B) The tiller morphology of ZH11 (A) and CG225 (B) at 15 days after CT and HT treatment. (C) Kinetic analysis of tiller number in ZH11 and CG225 plants under CT conditions and HT conditions.  $n = 10$ . (D) The dynamic changes in the length of tiller buds in ZH11 and CG225 plants from 2 to 8 days after HT treatment.  $n = 4$ . (E) The phenotype of tiller bud in ZH11 and CG225 plants at seven days after HT treatment.  $n = 4$ . (F) The dynamic changes in fresh weight of tiller bud in ZH11 and CG225 plants from 2 to 8 days after HT treatment.  $n = 4$ . (G) The cell morphology of tiller bud in ZH11 and CG225 plants at two days after HT treatment. (H) The diameter of the tiller bud cell is shown in Figure 3G.  $n = 30$ . (I) Dormancy rate of tiller bud in ZH11 and CG225 plants. The experiment was performed with four biological replicates, and each biological replicate includes 20 plants  $n = 4$ . Data are means  $\pm$  SE. The different lowercase letters indicate significant differences at the  $P < 0.05$  probability level. Bars = 10 cm (A) and (B), 1 cm (F), 100  $\mu\text{m}$  (upper line) and 1000  $\mu\text{m}$  (lower line) (G).

#### *Higher activities of antioxidant enzymes in 'CG225' accelerate the ROS scavenging*

It has been well documented that plant cells' rapid accumulation of reactive oxygen species (ROS) in response to stress has an important role in inducing signalling events. Meanwhile, the balance between ROS production and detoxification is critical for plant tolerance to stress. Therefore, it prompts us to detect the levels of  $\text{H}_2\text{O}_2$  and MDA (an indicator of the production of ROS) in node and tiller bud of 'ZH11' and 'CG225' under HT conditions. We found that the contents of  $\text{H}_2\text{O}_2$  and MDA in the HT groups were significantly higher than that of the CT groups (Figure 2A, B). In detail, the contents of  $\text{H}_2\text{O}_2$  and MDA in the node of 'ZH11' were increased by 19.64% and 171.45%, respectively, while in 'CG225', it increased by 6.81% and 65.21%, respectively (Figure 2A). The contents of  $\text{H}_2\text{O}_2$  and MDA in the tiller bud of 'ZH11' increased by 26.34% and 168.39%, respectively, while in 'CG225', it increased by 19.85% and 90.15%, respectively (Figure 2B). These results indicated that compared with 'CG225', more ROS accumulate in the nodes and tiller buds of 'ZH11' under HT conditions.

We thus examined the activities of antioxidant enzymes SOD, POD, CAT, and APX in the node and tiller bud of 'ZH11' and 'CG225', respectively. The results showed that compared with the CT groups, the

activities of SOD, CAT, and APX in the tiller node of 'ZH11' and 'CG225' were not significantly changed in the HT groups, in contrast to the activities of POD that significantly increased (Figure 2C-F). The activities of SOD, CAT, POD, and APX in the tiller buds of the HT groups were significantly increased by 8.24%, 33.30%, 84.62%, and 25.53%, respectively, in 'ZH11' (Figure 2C-F). While they increased by 10.25%, 44.66%, 100.00%, and 48.98% in 'CG225', respectively (Figure 2C-F). These results indicated that compared with 'ZH11', the higher activities of SOD, CAT, POD, and APX in tiller buds of 'CG225' may accelerate the ROS scavenging, thereby reducing the damage to tiller bud cells.



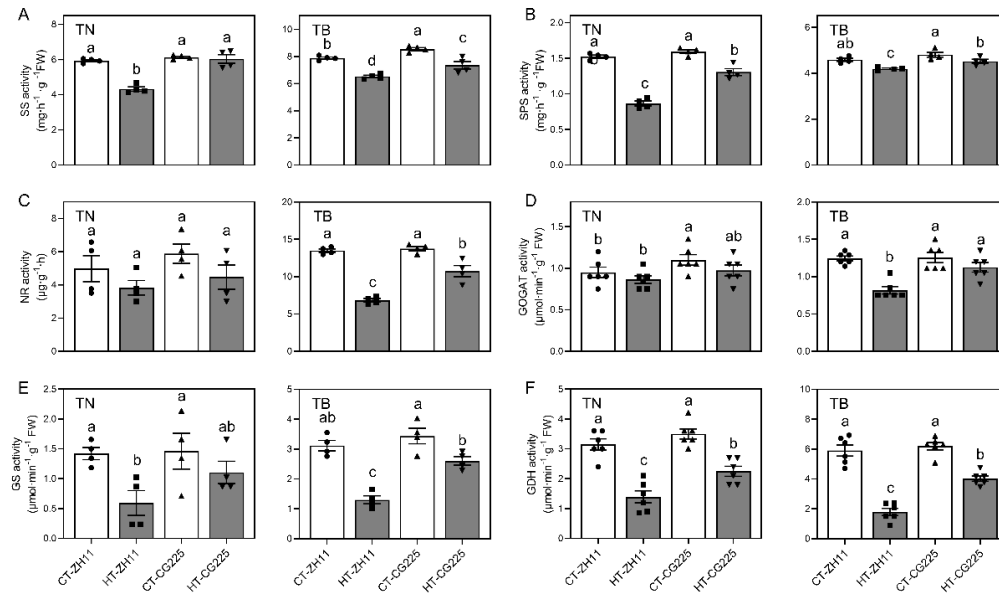
**Figure 2.** The H<sub>2</sub>O<sub>2</sub> content, MDA content, and enzyme activity of antioxidants involved ROS scavenging in the tiller node and tiller bud of 'ZH11' and 'CG225' plants under CT and HT conditions (A, B) The H<sub>2</sub>O<sub>2</sub> content and MDA content in the tiller node and tiller bud of ZH11 and CG225 plants. (C-F) The enzyme activity of SOD (C), POD (D), CAT (E), and APX (F) in the tiller node and tiller bud of ZH11 and CG225 plants. Data are means ± SE (n ≥ 4). TN, tiller node; TB, tiller bud. The different lowercase letters indicate significant differences at the P < 0.05 probability level.

#### *Higher activities of key enzymes involved in carbon and nitrogen metabolisms in 'CG225'*

Carbon and nitrogen are plants' most critical elements constituting organic compounds. To investigate how carbon and nitrogen metabolism in tiller buds is affected under HT conditions, we measured the activity of several key enzymes, including starch synthase (SS) and sucrose-phosphate synthase (SPS) involved in carbon metabolism; and nitrate reductase (NR), glutamate synthase (GOGAT), glutamine synthetase (GS), and glutamate dehydrogenase (GDH) involved in nitrogen metabolism. We found that compared with the CT groups, the activities of SS and SPS in the HT groups were decreased by 27.15% and 43.27%, respectively, in the tiller node, and by 17.27% and 8.55% in the tiller bud of 'ZH11' (Figure 3A, B). In contrast, the activities of SS and SPS in the tiller node of 'CG225' were decreased by 0% and 17.97%, respectively, and by 13.77% and 6.08% in the tiller bud (Figure 3A, B). These results might indicate that compared to 'ZH11', 'CG225' exhibits more vigorous carbon metabolism in the tiller node.

There was no significant difference in the activities of NR and GOGAT in the tiller node of 'ZH11' and 'CG225' between the CT group and the HT group. In contrast, compared with the CT group, they decreased by 49.35% and 34.35%, respectively, in the tiller bud of 'ZH11' and by 21.88% and 10.36% in 'CG225' (Figure 3C, D). In addition, the activities of GS and GDH in the node of 'ZH11' were decreased by 58.37% and

55.78%, respectively, under HT conditions and by 58.25% and 69.61%, respectively, in the tiller bud (Figure 3E, F). In contrast to ‘ZH11’, the activities of GS and GDH in the node of ‘CG225’ were decreased by 24.34% and 35.71%, respectively, under HT conditions and by 24.14% and 35.03%, respectively, in the tiller bud (Figure 3E, F). Taken together, these results suggested that the activities of key enzymes involved in nitrogen metabolism in the node and tiller bud of ‘CG225’ were significantly less inhibited than those of ‘ZH11’.

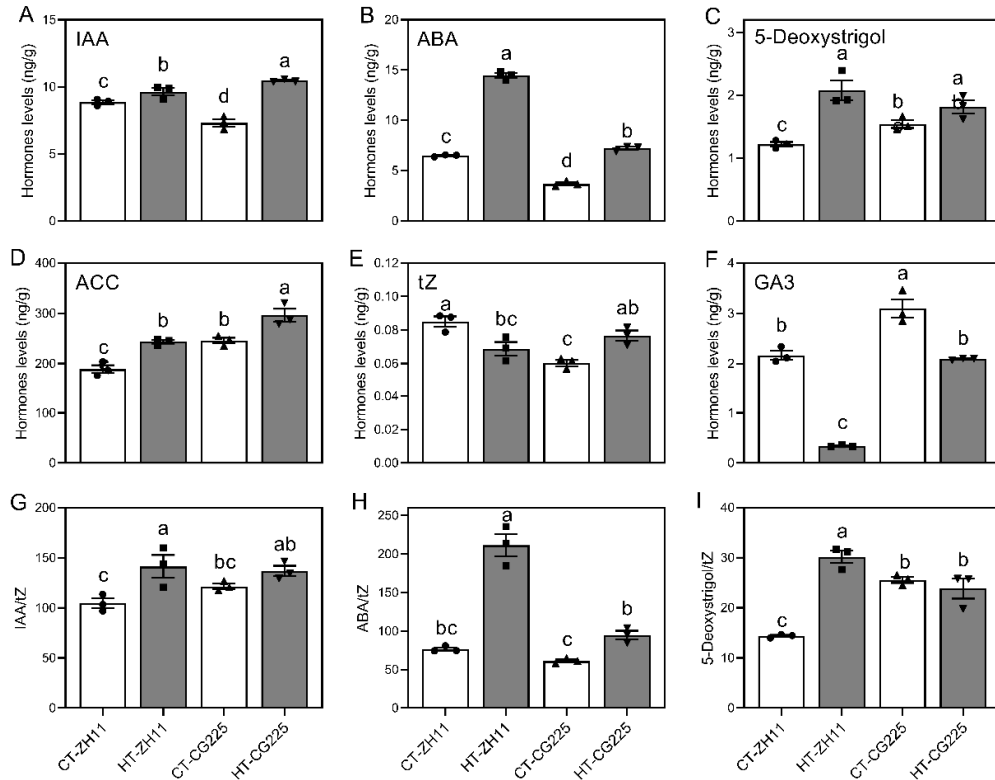


**Figure 3.** The activities of the key enzymes involved in carbon and nitrogen metabolisms in tiller node and tiller bud of ZH11 and CG225 plants under CT conditions and HT conditions (A-F) The activities of SS (A), SPS (B), NR (C), GOGAT (D), GS (E) and GDH (F) in tiller node and tiller bud of ZH11 and CG225 plants. Data are means  $\pm$  SE ( $n \geq 4$ ). TN, tiller node; TB, tiller bud. The different lowercase letters indicate significant differences at the  $P < 0.05$  probability level.

#### *Higher contents of the tiller-promoting phytohormones CK in ‘CG225’*

As phytohormones play essential roles in the initiation and development of tiller bud, we measured the content of IAA, trans-zeatin ( $\tau$ Z, a highly active CK), GA3, ABA, 2'-epi-5-deoxystrigol (epi-5DS, a native SL of rice), and 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of ethylene biosynthesis) in the buds of ‘ZH11’ and ‘CG225’ under HT conditions. We found that compared with the CT groups, the contents of IAA, ABA, SLs, and ACC in the tiller bud of ‘ZH11’ were increased by 8.86%, 122.52%, 70.22%, and 28.79%, respectively, in the HT groups (Figure 4A-D). In contrast, the contents of IAA, ABA, and ACC in tiller buds of ‘CG225’ were increased by 43.08%, 90.88%, and 20.49%, respectively, and the content of SL was not significantly changed (Figure 4A-D), indicating that ABA and SLs may be the key phytohormone that inhibit the growth of tiller bud in ‘ZH11’. The contents of  $\tau$ Z in the tiller bud of ‘ZH11’ decreased by 18.93%, while in ‘CG225’, it increased by 27.19% (Figure 4E), indicating that  $\tau$ Z may play a vital role in promoting the growth of tiller bud in ‘CG225’.





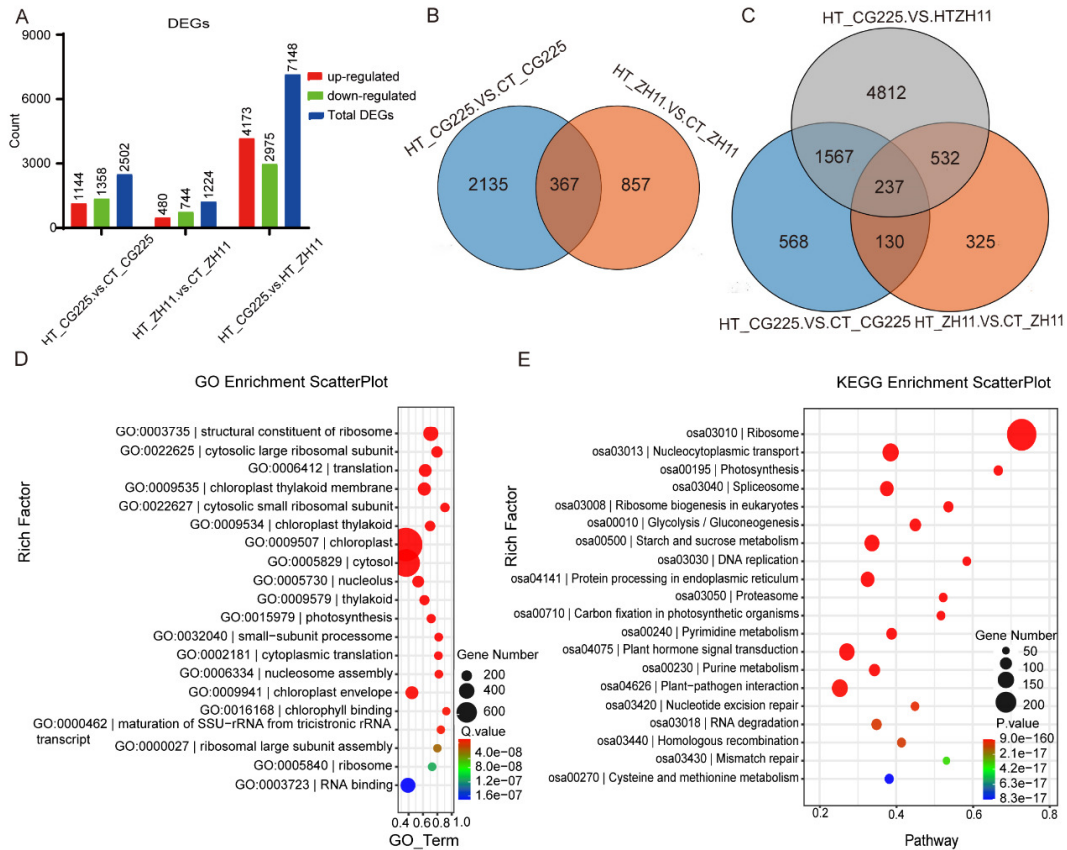
**Figure 4.** Analysis of phytohormone content in tiller bud of ZH11 and CG225 plants under CT conditions and HT conditions (A-I) The content of IAA (A), ABA (B), 5-Deoxystrigol (C), ACC (D), tZ (E), tZ (F), IAA / tZ (G), ABA / tZ (H), 5-Deoxystrigol / tZ (I) in tiller bud of ZH11 and CG225 plants. Data are means $\pm$ SE (n = 3). The different lowercase letters indicate significant differences at the  $P < 0.05$  probability level.

#### *Transcriptomics analysis of the tiller buds in 'ZH11' and 'CG225' plants*

To unveil the molecular mechanisms of HT tolerance at the tillering stage in *japonica* rice, a comparative RNA-seq analysis of the tiller buds of 'ZH11' and 'CG225' (after eight days of HT treatment) was performed. We set  $P < 0.05$  and the value of fold change ( $\log_2$ ) over 1 relative to CT groups as the cutoff threshold. In total, we detected 7178 (4173 up-regulated in 'CG225'), 2502 (1144 up-regulated), and 1224 (480 up-regulated) DEGs in pairwise comparison between HT\_CG225.vs.HT\_ZH11, HT\_CG225. vs. CT\_CG225, and HT\_ZH11.vs.CT\_ZH11, respectively (Figure 5A). Figure S1 presents the volcano plots of the DEGs, and Table S2 presents the list of DEGs between HT\_CG225.vs.HT\_ZH11. Only 367 and 237 DEGs were common to the three pairwise comparisons and two varieties, respectively (Figures 5B, C). To verify the reliability of the RNA-seq data, we randomly selected twelve DEGs for RT-qPCR analysis (Table S4). As presented in Figure S2, the expressions patterns of the twelve genes via RT-qPCR and RNA-seq in the two varieties were identical, confirming the transcriptome data is reliable.

GO annotation and enrichment analysis assigned the DEGs between 'CG225' and 'ZH11' under HT conditions mainly to the chloroplast, cytosol, ribosome, nucleus, and photosynthesis (Figure 5D). Notably, multiple chloroplast-related items have been significantly enriched, including chloroplast thylakoid membrane (GO:0009535), chloroplast thylakoid (GO:0009534), chloroplast (GO:0009537), thylakoid (GO: 0009579), photosynthesis (GO: 0015979), chloroplast envelope (GO:0009941), chlorophyll binding (GO: 0016168), which suggested that the processes in chloroplast were crucial to enhance the HT tolerance of *japonica* rice.

Meanwhile, the KEGG analysis revealed these DEGs were primarily involved in the ribosome (osa03010), nucleocytoplasmic transport (osa03013), photosynthesis (osa00195), spliceosome (osa03040), glycolysis/gluconeogenesis (osa03008), starch and sucrose metabolism (osa00500) (Figure 5E), indicating that these processes in chloroplast, cytosol, and ribosome may affect the HT tolerance process of tillering buds.



**Figure 5.** Transcriptomics analysis of the tiller bud in ZH11 and CG225 plants under CT conditions and HT conditions

(A) Number of DEGs in the pairwise comparison. (B) Venn diagram showing the number of common DEGs in the tiller bud of ZH11 and CG225 plants between CT conditions and HT conditions. (C) Venn diagram showing the number of overlapped DEGs in the three pairwise comparisons. (D, E) GO enrichment (D) and KEGG enrichment (E) results of DEGs between CG225 and ZH11 under HT conditions, respectively.

*Key transcription factor (TF), phytohormone, and antioxidant genes involved in heat stress tolerance of japonica rice at the tillering stage*

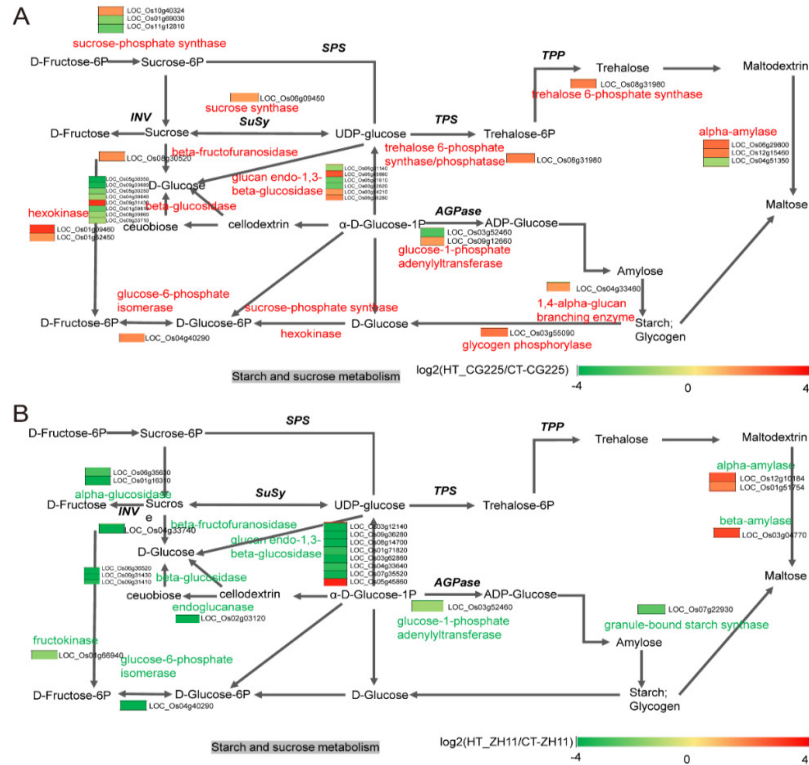
To identify potential candidate genes for improving *japonica* rice heat tolerance at the tillering stage, we screened out based on gene annotation, TF, phytohormone, antioxidant-related DEGs, and other important DEGs (up-regulated,  $\log_2FC > 2$ ) in the pairwise comparison between HT\_CG225 vs HT\_ZH11 (Figure S3). In total, 134 DEGs were filtered out, including 73 TF-related genes, 20 phytohormone-related genes, 27 antioxidant-related genes, and 14 other important genes. 17 of the TF-related genes, including two EP2 (*LOC\_Os06g44750* and *LOC\_Os04g42570*), two B3 (*LOC\_Os01g67830* and *LOC\_Os03g42420*), one bHLH (*LOC\_Os03g46860*), one MYB (*LOC\_Os01g16810*), one NAC (*LOC\_Os04g35660*), one WRKY (*LOC\_Os10g42850*), etc. were more than 10-folds ( $\log_2FC > 10$ ) up-regulated in CG225 (Figure S3A). *LOC\_Os12g04000*, *LOC\_Os05g50140*, and *LOC\_Os01g58860* (auxin efflux carrier components) and *LOC\_Os06g37590* (1-aminocyclopropane-1-carboxylate oxidase 1) were the most up-regulated

phytohormone-related genes (Figure S3B). Five peroxidase genes (*LOC\_Os04g59260*, *LOC\_Os06g35480*, *LOC\_Os03g36560*, *LOC\_Os07g48010*, and *LOC\_Os04g55740*) were the most up-regulated antioxidant enzymes (Figure S3C). Of the other DEGs, *LOC\_Os06g10310* and *LOC\_Os06g21950* encoding growth-regulating factor 2 and probable inorganic phosphate transporter, respectively, were the most significantly up-regulated (Figure S3D).

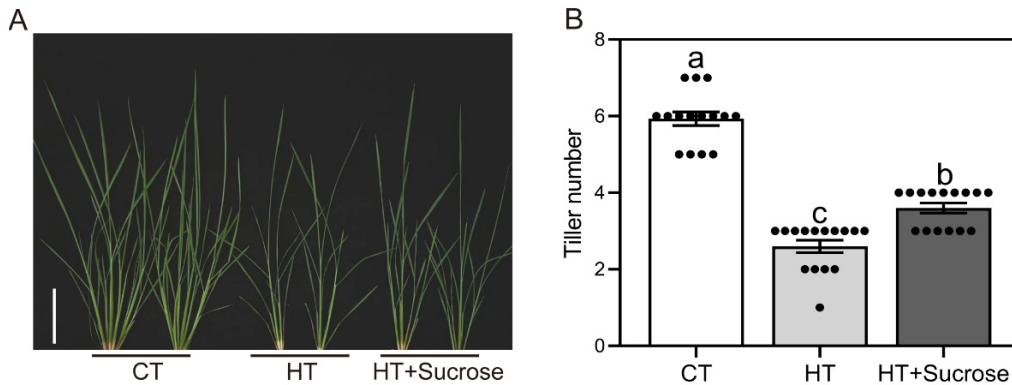
*Sucrose alleviates the inhibition of HT stress on the tiller bud growth*

As most of the significant DEGs were assigned to chloroplast and photosynthesis, the products of photosynthesis may play vital roles in enhancing *japonica* rice heat tolerance (Figure 5D). Interestingly, the genes related to the starch and sucrose metabolism pathway were enriched in the DEGs, and this pathway is the most significantly enriched in photosynthetic products (Figure 5E). Therefore, we inferred that the starch and sucrose produced in photosynthesis affect the HT tolerance of tillering buds. To verify our hypothesis, we examined the expression of genes related to the starch and sucrose metabolism pathway and found that most of the sucrose and starch biosynthetic genes were significantly up-regulated in the heat-tolerant cultivar CG225 (Figure 6A). These genes include sucrose synthase (SuSy), trehalose-6-phosphate phosphatase (TPS), 1,4-alpha-glucan branching enzyme (SBE), glucose-6-phosphate isomerase (GPI), fructokinase and so on. In contrast, most of these genes, including  $\alpha$ -glucosidase (malZ), beta-fructofuranosidase (INV), endoglucanase, fructokinase (FK), glucose-6-phosphate isomerase (PGI), etc. were down-regulated in ZH11 under HT conditions (Figure 6B).  $\alpha$ -amylase was significantly up-regulated in both varieties under HT conditions (Figure 6A, B). These results indicated that the sugar may serve as a crucial regulator to alleviate HT inhibition on the tiller bud growth.

To verify this statement, we applied 4 mM sucrose to ZH11 plants under HT conditions. We found that the ectopic sucrose treatment significantly increased the tiller number of ZH11 compared to the control (Figure 7A, B), suggesting that sucrose could be applied to alleviate the inhibition of HT stress on the growth of tiller bud.



**Figure 6.** The starch and sucrose metabolism pathway DEGs between the two varieties under heat stress (A, B) The starch and sucrose metabolism pathway DEGs in the tiller bud of CG225 (A) and ZH11 (B) plants between CT conditions and HT conditions. The legend indicates the gene expression level. The redder the colour, the higher the expression level; the blue the colour, the lower the expression level. CT, control; HT, high temperature stress. The heat map shows the genes for starch and sucrose metabolism pathways, which were identified by microarray analyses. HT\_CG225/CT\_CG225 values indicate ratios of gene expression under HT conditions (HT) relative to that under CT conditions in CG225. HT\_ZH11/CT\_ZH11 values indicate ratios of gene expression under HT conditions (HT) relative to that under CT conditions in ZH11.



**Figure 7.** Sucrose alleviates the inhibition of HT stress on the growth of the tillering bud. (A) The tiller morphology of ZH11 at 15 days after CT, HT, and HT + Sucrose treatment (A) The tiller morphology of ZH11 at 15 days after CT, HT, and HT + Sucrose treatment. (B) The tiller number of ZH11 at 15 days after CT, HT, and HT + Sucrose treatment. Data are means±SE (n = 15). The different lowercase letters indicate significant differences at the P < 0.05 probability level. Bars = 10 cm (A).

## Discussion

In order to scavenge excess ROS, the activities of antioxidant enzymes are evidently increased under stress conditions, thereby improving the cell's ability to scavenge ROS and avoiding damages to plant growth and developmental processes (Han *et al.*, 2020; Zafari *et al.*, 2022; Wei *et al.*, 2021). In this study, lower contents of ROS and MDA, and higher activities of the antioxidant enzymes (SOD, CAT, POD, and APX) in tiller buds of 'CG225' were recorded. Peroxidase family genes were the most induced in 'CG225' and recorded the highest increment of enzymatic activities. These results indicate the higher ROS scavenging ability in tiller buds contributed significantly to enhancing the HT-tolerant of 'CG225'. The accumulation of ROS under HT conditions disrupts photosynthetic electron chains and photophosphorylation and damages thylakoid membranes. In addition, it stimulates chlorophyll degradation, resulting in the loss or dysfunction of chloroplasts and decreased photosynthesis efficiency (Yin *et al.*, 2010). Consistency, most of the DEGs between the two genotypes were mainly assigned to chloroplast and its processes (Figure 5D), indicating that the photosynthesis system in the heat-sensitive variety ('ZH11') has likely been significantly damaged, in contrast to 'CG225'. To verify this hypothesis, the photosynthesis efficiency in 'ZH11' and 'CG225' leaves under different HT treatments should be measured in the future, as well as the dynamic changes in the expressions of photosynthesis and photosystem-related genes. A particular attention should be paid to peroxidase family genes for revealing their regulation mechanisms of *japonica* rice HT tolerance and identifying resources for sustainable production.

Carbon and nitrogen metabolism is essential for plant growth and development since all major cellular components, including proteins, sugar, pigments, energy carrier molecules, etc., are derived from these activities (Xing *et al.*, 2022). Compared with 'ZH11', 'CG225' showed higher activities of the key enzymes involved in carbon and nitrogen metabolism in tiller buds (Figure 3) and higher fresh weight of tiller buds under HT conditions (Figure 1F). These results show that carbon and nitrogen metabolism was induced in 'CG225' under HT, providing more abundant C and N nutrients for the tiller bud growth. Carbon and nitrogen are also important components of osmoprotectants. 'CG225' exhibited higher contents of soluble protein, free amino acid, and sucrose in tiller nodes and tiller buds under HT conditions, compared with 'ZH11' (Figure S4). The integration of these findings denotes that carbon and nitrogen metabolism may have promoted the synthesis of osmoprotectants, which in turn enabled the maintenance of cell homeostasis. Supportively, the study by Lin *et al.* revealed that increasing nitrogen nutrition levels can significantly enhance the activities of antioxidant enzymes and reduce the content of ROS and MDA in plants, leading to improved plants tolerance to stress (Lin *et al.*, 2011). Further dissection of the regulatory network of carbon and nitrogen metabolism in 'CG225' may provide key genetic resources for enhancing *japonica* rice HT tolerance during tillering and improving yield.

As a source of energy and signalling molecule, sucrose regulates numerous growth and development processes, such as root growth, branching, floral induction, yield formation, and stress response (Yoon *et al.*, 2021). In this study, many DEGs between the two varieties under HT conditions were assigned to starch and sucrose metabolic pathways (Figure 5E). Important structural genes related to these pathways were up-regulated in 'CG225', in contrast to in 'ZH11' (Figure 6). Moreover, the sucrose content in the tiller node and tiller bud is higher in 'CG225' than in 'ZH11', and ectopic sucrose treatment alleviated the inhibitory effects of HT on tiller bud growth in 'ZH11' (Figure S4 and Figure 7). These results indicate that higher content of sucrose may provide more energy for the growth of 'CG225' tiller buds. Moreover, they infer that sucrose may also serve as important signaling molecule to promote the growth of tiller buds under HT conditions by initiating and modulating synergistical stress responsive molecular mechanisms. Previous studies have shown that sucrose represses SL signaling and promotes tillering in rice (Patil *et al.*, 2022; Wang *et al.*, 2020). Consistency, SL content in tiller buds of 'CG225' was significantly decreased under HT conditions, compared with 'ZH11' (Figure 4C), suggesting that sucrose may promote tiller bud growth by inhibiting SL synthesis

and signaling pathway. Exploring the physiological and molecular mechanisms involved in sucrose signaling and regulation interactions during tiller bud growth in *japonica* rice under HT conditions is required for a thorough understanding of HT tolerance regulation mechanisms and molecular breeding perspectives. In addition, it is worth noting that sucrose could not alleviate the inhibition of high temperature on the growth of tiller buds in ‘ZH11’ completely, confirming that other important signaling pathways participate to the heat tolerance of *japonica* rice.

Besides, it has been proved that plants initiate a series of TFs phosphorylation and/or dephosphorylation under stress to enable them to bind *cis*-elements of stress-related genes and enhance tolerance (Baillio *et al.* 2019; Tian *et al.* 2022). TFs interact with phytohormone-related genes to coordinate the expression levels of specific genes that help plants conserve a particular phenotype under stress (Feng *et al.* 2018). The plant hormone signaling pathway was significantly differentially regulated between the two varieties, and many TF and phytohormones-related genes were significantly up-regulated in ‘CG225’ (Figure S3). As a result, the IAA (auxin),  $\tau$ Z (CK), ABA, and ACC (ETH) levels increased considerably in ‘CG225’ (Figure 4A, B, E, D). These results show that a coordinated hormone signal transduction is required for proper tiller formation under HT stress in *japonica* rice. A complex interaction between phytohormones to regulate tillering has been reported (Fang *et al.* 2020; Riaz *et al.* 2023; Lei *et al.* 2022; Liu *et al.* 2020; Tanaka *et al.* 2006). It is well known that auxin is transported downward from the top of the plant to inhibit axillary buds’ germination and growth (Wang *et al.* 2015). Most of the highly induced hormone-related genes in ‘CG225’ encode auxin efflux carriers (*PIN*s) (Figure S3B). *PIN*s are known to regulate the intercellular flow of auxin, limiting its export from the cytosol to the extracellular space (Ung *et al.* 2022). Therefore, we inferred that *PIN*s might promote axillary buds’ germination and development in ‘CG225’ under normal or HT conditions by modulating auxin flow. Many EP2 family genes and ethylene biosynthetic genes were also highly induced under HT stress in ‘CG225’ (Figure S3A), indicating ethylene is essential for *japonica* rice heat tolerance during tillering. Indeed, Phytohormone ethylene regulates various physiological processes, such as flowering, growth, senescence, and stress responses (Gao *et al.* 2022; Dubois *et al.*, 2018). Further studies are needed to identify major candidate genes related to HT tolerance in rice during tillering and explore their regulatory networks.

## Conclusions

The physiological and molecular mechanisms of HT tolerance and sensitivity of *japonica* rice at the tillering stage were analysed by various physiological, biochemical, and comparative transcriptomic analyses. The HT stress at the tillering stage inhibited the tiller bud growth and development of tillers, which resulted in a significant reduction in tiller number, especially for ‘ZH11’ (heat-sensitive). The tolerance to HT stress during tillering is associated with high induction of the antioxidant enzymes’ activities, plant hormone signal transduction, transcription factors, phenylpropanoid biosynthesis, starch and sucrose metabolism, and the maintenance of chloroplast ultrastructure and photosynthesis. Particularly, sucrose and starch metabolism were crucial for the normal growth of tiller buds under HT conditions. Key potential candidate genes were identified for further studies, including transcription factors, phytohormone-related genes, antioxidant system-related genes, and sucrose and starch pathway-related genes. Notably, we found that ectopic sucrose treatment could be an efficient approach to improve *japonica* rice under HT conditions. Our findings show that improving antioxidant enzymes’ activity, carbon and nitrogen metabolisms and phytohormone levels could promote tiller bud growth in *japonica* rice under HT conditions. Moreover, they offer important resources for the sustainable improvement of *japonica* rice HT tolerance and production.

### Authors' Contributions

Conceptualization, X.T. and Z.W.; methodology, S.F.; software, X.T.; validation, H.Q., H.C., A. R. and M.T. Aslam.; formal analysis, Y.W.; investigation, X.T.; resources, Z.W.; data curation, X.T.; writing—original draft preparation, X.T.; writing—review and editing, Y.W.; visualization, Y.W.; supervision, Z.W.; project administration, Y.W.; funding acquisition, Z.W. All authors read and approved the final manuscript.

### Ethical approval (for researches involving animals or humans)

Not applicable.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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