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Endophytic *Bacillus subtilis* GUCC4 Enhances Passion Fruit Seedling Tolerance to Low-Temperature Stress

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ABSTRACT

Passion fruit (*Passiflora edulis* Sims) is a popular fruit favored by consumers due to its unique taste and health properties around the world. Moreover, it serves as an important source of bioactive compounds, making it highly valuable for industrial exploitation. However, its growth and development are highly susceptible to cold stress, which can result in significant economic losses. Protection against abiotic stress is a significant function of the plant microbiota. In this work, we systematically evaluated the potential of the plant growth-promoting bacterium (PGPB) *Bacillus subtilis* GUCC4, an endophyte isolated from healthy passion fruit leaves, to enhance the cold stress tolerance in passion fruit. The results demonstrated that inoculation with *B. subtilis* GUCC4 significantly enhances the cold tolerance of passion fruit. The strain was shown to rapidly colonize and migrate into both roots and leaves within 48 h of inoculation. Compared to the control, *B. subtilis* GUCC4 inoculation markedly enhanced total chlorophyll content, photosynthetic parameters, antioxidant enzyme activities, osmoregulatory substances, and phytohormone levels in passion fruit seedlings. Notably, *B. subtilis* GUCC4 upregulated genes associated with plant growth and transcription factors responsive to low-temperature stress at 5°C. The mode of action is multifaceted and includes efficient colonization, growth promotion, hormone balancing, and stress protection. Overall, this study demonstrates that *B. subtilis* GUCC4 effectively mitigates the adverse effects of low-temperature stress on passion fruit seedlings, offering a novel and promising strategy for enhancing plant resilience under adverse climate conditions.

1 | Introduction

Passion fruit (*Passiflora edulis* Sims) is a perennial evergreen vine of the family Passifloraceae. It is indigenous to South America and is widely cultivated throughout tropical and subtropical areas (Su et al. 2025). Renowned for significant economic, nutritional, and industrial values, passion fruit is rich in essential amino acids and trace elements, making it popular both in fresh forms and as processed products (He et al. 2020). In addition, passion fruit exhibits potent antioxidant and free radical-scavenging activities, primarily attributed to its high content of vitamin C and polyphenols, as well as its valuable dietary fiber content (Martínez et al. 2012). Furthermore, passion fruits are characterized by their distinctive flavor profile, which blends floral and estery aromas with an exotic tropical sulfur nuance, shaped by a complex mixture of volatile organic compounds (Li et al. 2020). However, the rapidly expanding passion fruit cultivation areas face challenges from a range of

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abiotic stresses that can negatively impact its growth and quality. Among various stressors, low temperature poses a major threat to passion fruit seedlings, significantly hindering their further development (Lin et al. 2025).

Perennial plants are required to withstand various environmental stresses passively throughout their developmental phases, and their responses to cold stress differ according to the species and even variety. However, the leaf is the most sensitive organ of the plant (Sluis and Hake 2015). Low-temperature stress (LTS) disrupts chloroplast structure and reduces chlorophyll content, resulting in leaf chlorosis. Additionally, LTS decreases key physiological indicators such as net photosynthetic rate and stomatal conductance, thereby significantly impairing both photosynthetic parameters and the overall efficiency of the photosynthetic system (Gan et al. 2019). Usually, LTS is accompanied by water stress, which together induce oxidative stress, resulting in increased accumulation of hydrogen peroxide and superoxide anions in plants (Zang et al. 2023). These alterations result in the accumulation of large amounts of reactive oxygen species (ROS). The continuous accumulation of ROS exacerbates lipid peroxidation of cell membranes, resulting in cell damage (Guo et al. 2022). Malondialdehyde is the final product of lipid peroxidation in cell membranes. Its concentration is directly proportional to the extent of cell damage. Increased lipid peroxidation of cellular membranes is associated with higher plasma membrane permeability and increased relative electrical conductivity (Uzal 2022). In response to LTS, plants accumulate osmoregulatory substances such as proline, soluble sugars, and soluble proteins, which help maintain cellular water balance (Guo et al. 2022). Antioxidant enzymes, including peroxidase, superoxide dismutase, and catalase, are also upregulated to scavenge ROS and alleviate cold-induced damage (Uzal 2022). Moreover, indole-3-acetic acid, abscisic acid, gibberellic acid, and jasmonic acid, which are endogenous hormones, also serve crucial functions in modulating plant responses to LTS (Anwar et al. 2018). All plants are holobionts that have co-evolved with their microbiota visible in functional interplay (Cernava and Berg 2022). The microbiota plays a pivotal role in defending plants and mitigating abiotic stress, but the mode of interaction and corresponding effects are not yet fully understood. While it is difficult to change genetic traits, the development of microbiome-based solutions is promising.

Various plant growth promoting bacteria (PGPB) have demonstrated the capacity to improve plant resistance to LTS. For example, inoculation with *Burkholderia phytofirmans* PsJN significantly improved LTS in grapes (Theocharis et al. 2012). Similarly, LTS tolerance was enhanced in tomatoes inoculated with *Streptomyces* sp. TOR3209 (Ma et al. 2023) or *Pseudomonas vancouverensis* OB155-gfp (Subramanian et al. 2015). Additionally, the genus *Bacillus* spp., well-known for producing various secondary metabolites, has demonstrated considerable potential. Li et al. (2023) showed that *Bacillus methylotrophicus* can enhance tomato tolerance to LTS. Similarly, Chen et al. (2022) reported that *Bacillus amyloliquefaciens* GL18 promotes the growth of *Avena sativa* and enhances host plant resistance to LTS. Inoculation with *Bacillus cereus* AR156 and *Bacillus subtilis* SM21 significantly improved tomato survival under LTS (Wang et al. 2016). However, the effects of *Bacillus* spp. as plant growth-promoting rhizobacteria and cold hardiness enhancers in passion fruit remain largely unexplored. To study a promising bioresource for enhancing the resistance of passion fruit seedlings to LTS, this study focuses on evaluating *B. subtilis* GUCC4, which was endophytically isolated from healthy passion fruit leaves and has previously been shown to be effective in controlling leaf blight and promoting plantlet development (Wang et al. 2023). By applying *B. subtilis* GUCC4 to seedlings, the study aimed at investigating its effects on morphological traits, physiological responses, and transcriptomic changes of passion fruit under varying levels of LTS. Furthermore, this study aims to provide insights into the mechanism of action of *B. subtilis* GUCC4, thereby offering an effective approach to enhance the cold tolerance of passion fruit seedlings.

2 | Materials and Methods

2.1 | Plant Variety and Microbial Resources

Seedlings of passion fruit (variety: Qinmi No. 9, one of the most widely cultivated varieties in Guangxi and Guizhou provinces, China) were used as the plant material. The seedlings were supplied by Guizhou Minjie Ecological Agriculture Investment and Development Co. Ltd., located in Rongjiang, Guizhou, China. The strain *B. subtilis* GUCC4, which was previously isolated from healthy passion fruit leaves as an endophyte by this research group (Wang et al. 2023), was obtained from the fungal resource inventory of the International Joint Institute of Plant Microbial Ecology and Resource Management at Guizhou University, Ministry of Agriculture, China Association of Agricultural Science Societies, Guiyang, China (Accession number in NCBI database: ONS82308).

2.2 | Methods of Testing

2.2.1 | Bacterial Preparation and Plant Inoculation

B. subtilis GUCC4 was activated as described by Wang et al. (2023), and the bacterial suspension was adjusted to 1×10^6 CFU mL^{-1} using a hemocytometer (Dao et al. 2018). For plant inoculation, passion fruit seedlings at the same growth stage, approximately 8–10 cm in height, were transplanted into 10×10 cm pots filled with a peat-based soil matrix substrate (Nanning Putianrun Bio-Organic Fertilizer Co. Ltd., Nanning, China). The experimental treatments included: plants inoculated with *B. subtilis* GUCC4 at room temperature (25°C) (GUCC4 room temperature treatment, GRT) and its control group without *B. subtilis* GUCC4 (GRT-CK); plants inoculated with *B. subtilis* GUCC4 at medium temperature (15°C) (GUCC4 medium temperature treatment, GMT) and its control group without *B. subtilis* GUCC4 (GMT-CK); plants inoculated with *B. subtilis* GUCC4 at low temperature (5°C) (GUCC4 low-temperature treatment, GLT) and its control group without *B. subtilis* GUCC4 (GLT-CK).

Each treatment consisted of 30 plants for a total of 180 plants. Ten days after transplanting, seedlings from the GRT, GMT, and GLT groups were inoculated with 50 mL of the *B. subtilis* GUCC4 suspension (1×10^6 CFU mL^{-1}). In parallel, the control groups (GRT-CK, GMT-CK, and GLT-CK) were irrigated with tap water. Subsequently, the seedlings were placed in a growth chamber under the previously mentioned temperature conditions, with a 16 h/8 h photoperiod and 70%–75% relative humidity. At 1, 3-, 5-, 7-, and 9-days post-inoculation, six plants from

each group were randomly selected for phenotypic observation and physiological and biochemical analysis.

2.2.2 | Classification of Plant Chilling Injury

Plant chilling injury was categorized based on the growth responses of passion fruit seedlings subjected to low-temperature treatment according to the criteria established by Cao et al. (2015). Briefly, chilling injury was classified into five levels: Level 0 (no damage); Level 1 (slight edge wilting); Level 2 (less than half of the leaves wilted); Level 3 (more than half of the leaves wilted); Level 4 (most of the leaves and main stem died); and Level 5 (complete plant death). The chilling injury index was calculated as follows:

$$\text{Chilling injury index} = \left[\sum(\text{number of plants at each injury level} \times \text{injury level value})\right] / (\text{total number of plants} \times \text{highest injury level value}) \times 100\%$$

2.2.3 | Root Endophytic Colonization of *B. subtilis* GUCC4

To assess the root endophytic colonization by *B. subtilis* GUCC4, root samples from the GRT and GRT-CK groups were washed with saline solution and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) at 4°C for 24 h. The specimens were then desiccated through a sequential ethanol gradient and lyophilized. The dried samples were mounted on conductive adhesive, gold-coated using a JEOL Smart Coater, and examined under a scanning electron microscope (SEM) (model: JCM 6000).

Total RNA was isolated from the leaves by employing a plant RNA isolation kit (TianGen Biotech (Beijing) Co. Ltd., Beijing, China). The RNA concentration and purity were evaluated with a SMA4000 microspectrophotometer (Merintion Instrument Inc). The isolated RNA was obtained and underwent reverse transcription with FastKing gDNA Dispelling RT Super Mix (TianGen Biotech (Beijing) Co. Ltd., Beijing, China) as per the manufacturer's guidelines for the synthesis of complementary DNA (cDNA). The qRT-PCR for expression analysis of the *B. subtilis gyrB* gene (*BsgyrB*) was conducted on 96 well plates employing the FQD-96A manufactured by BIOER, and the colonization of *B. subtilis* GUCC4 was determined via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The sequences of the primers are listed in Table S1.

2.2.4 | Measurement of Physiological Indicators

Total chlorophyll (Chl) was measured using the method described by Tournaire et al. (2023). The net photosynthetic rate (Pn) and stomatal conductance (Gs) were measured using a portable photosynthesis system (LI-6400, LI-COR, USA). The hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) were determined using kits from Sangon Biotech, Shanghai, China; malondialdehyde (MDA) was determined via the thiobarbituric acid assay (Wang, Gao, et al. 2019); and relative conductivity (REC) was estimated as described by Dhindsa and Matowe (1981). Proline (Pro) was determined by the acidic ninhydrin method according to Ghorbanpour et al. (2018); soluble sugar (SS) and soluble protein (SP) were measured as described by Jhanji et al. (2023) and Ghorbanpour et al. (2018), respectively. Peroxidase (POD) activity was determined via the guaiacol assay (Bestwick et al. 1998); superoxide dismutase (SOD) activity was determined using the NBT reduction assay (Sun et al. 2019); and catalase (CAT) activity was evaluated according to the procedure of Chance and Maehly (1955). Indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellins (GA), and jasmonic acid (JA) were quantified by ELISA (Wu et al. 2011), following the extraction and purification procedures described by Yang et al. (2001).

2.2.5 | Sample RNA Extraction and Transcriptome Sequencing

RNA extraction was carried out as described in section 2.2.3. The RNA samples were subsequently sent for transcriptome sequencing on the Illumina NovaSeq 6000 platform. Clean reads were obtained by quality control of the raw reads and subsequently aligned to a passion fruit reference genome (https://ngdc.cncb.ac.cn/gwh/Assembly/17982/show) using HISAT2. The quantitative metric was TPM (transcripts per million clean tags). Genes exhibiting significant differences in expression (differentially expressed genes, DEGs) were detected through statistical analysis under combined criteria (p-adjust <0.05 and $\log_2$ FC ≥ 1). Gene functions were characterized according to the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using BLAST for homology searches. The raw RNAseq reads from GLT and GLT-CK leaf samples have been deposited in the NCBI SRA database with the accession number PRJNA1281499.

2.2.6 | Transcriptome Sequencing Quality Assessment

After quality control of the raw reads, each sample yielded more than 5 billion clean reads. The average sequencing base error rate for the quality-controlled data of each sample was below 0.1%, and the GC content was approximately 44%, indicating excellent data quality (Table S2). Then, gene expression was quantified for each sample, followed by correlation analysis and principal component analysis (PCA) on the three biological replicates of each group (GLT and GLT-CK). A high correlation was observed among replicates, and significant differences were detected between the two groups (Figure S1), indicating that the sequencing results were reliable.

2.2.7 | Differential Gene qRT-PCR Verification

After cDNA synthesis from GLT and GLT-CK leaf samples, qRT-PCR was performed on the FQD-96A system (BIOER, China) according to the instructions of the SYBR Premix Ex Taq (Takara Bio Inc., Japan), using the *Actin7* gene (*PeACT7*) as a internal control gene. Primers employed in qPCR are shown in Table S1. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

2.3 | Statistics and Data Analysis

The data obtained from the test were processed and analyzed using Excel 2022 and SPSS 22.0. The differences between

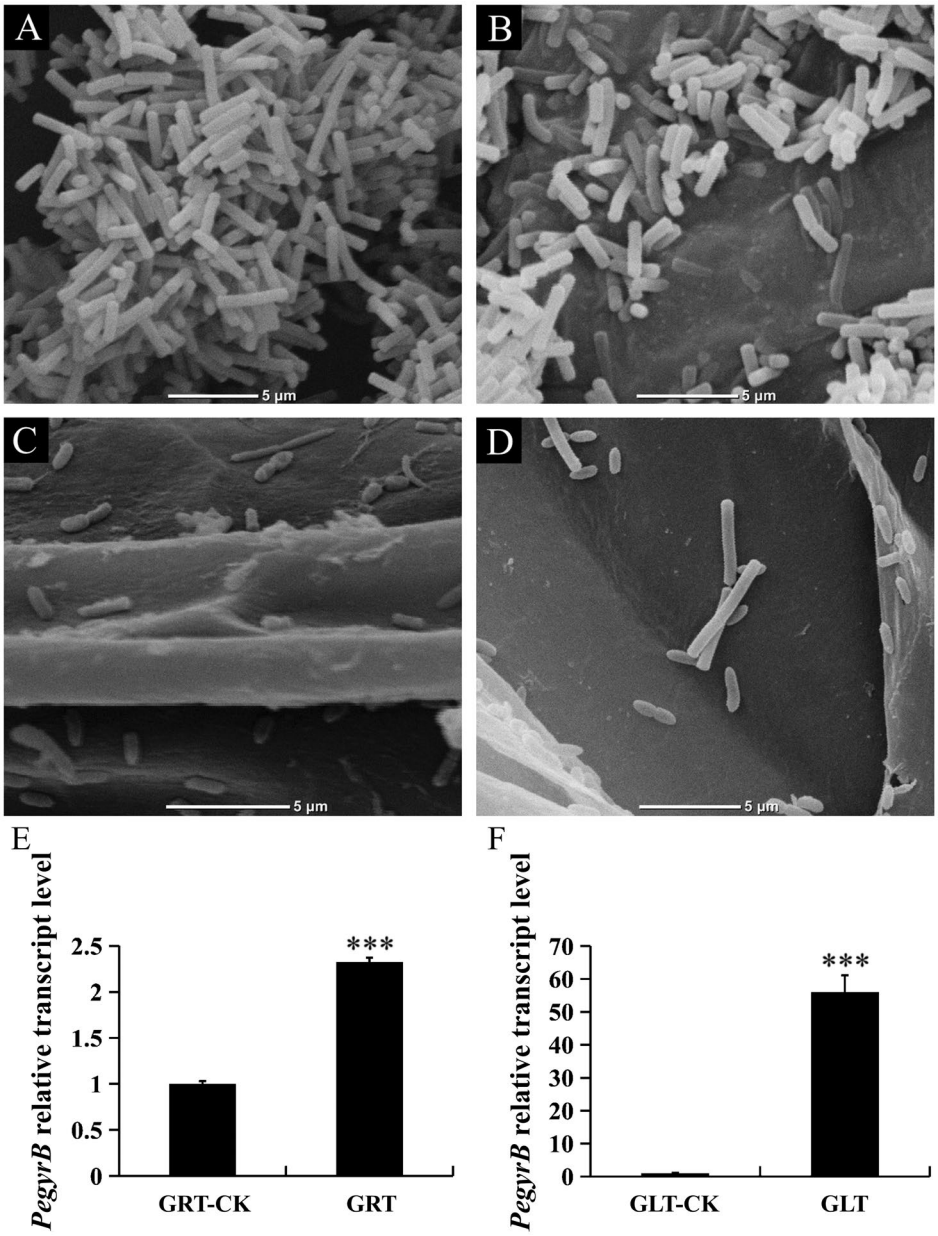


FIGURE 1 | Colonization of *B. subtilis* GUCC4 in passion fruit roots. (A and B: *B. subtilis* GUCC4 strain in pure culture, C and D: Root colonization of the GUCC4 strain, Scale bar: 5 μm) and expression analysis of *gyrB* gene (E and F: Relative expression levels of *gyrB* gene in GRT-CK, GRT, GLT-CK, and GLT). *** indicates a significant difference level of $p < 0.001$.

treatments were evaluated for significance using one-way ANOVA and Duncan's test, with a threshold of $p < 0.05$ deemed significant. The data were then plotted using Origin 2023 and Fig draw.

3 | Results

3.1 | Root Endophytic Colonization of *B. subtilis* GUCC4 in Passion Fruit

SEM images provided clear visual evidence of *B. subtilis* GUCC4 colonization of inner root tissues of passion fruit. Figure 1A–D shows the presence of bacterial cells, indicating a successful colonization. Furthermore, the expression of *gyrB*, a gene specific to *B. subtilis*, was significantly higher in passion fruit leaves treated with *B. subtilis* GUCC4 compared to the control (GRT-CK), as shown in Figure 1E. This increase in gene expression was even more pronounced under LTS, with the GLT group exhibiting an approximately 50-fold higher expression level than GLT-CK after 3 days of stress (Figure 1F). These results are statistically significant and demonstrate that *B. subtilis* GUCC4 can effectively colonize passion fruit leaves.

3.2 | Effects of *B. subtilis* GUCC4 on Morphology and Chilling Injury Index of Passion Fruit Seedlings Under LTS

The application of *B. subtilis* GUCC4 significantly promoted the growth of passion fruit seedlings at room temperature (25°C). Under low temperature stress, both at 15°C and 5°C, passion

fruit leaves gradually wilted and withered with prolonged exposure. Notably, treatment with the *B. subtilis* GUCC4 bacterial suspension significantly delayed the wilting of passion fruit leaves under these conditions (Figure 2). As shown in Table 1, the chilling injury index of the GMT-CK, GMT, GLT-CK, and GLT treatments gradually increased with prolonged stress duration. At 15°C, short-term exposure (0–3 days) did not cause chilling injury to passion fruit, as indicated by a chilling injury index of 0. Chilling injury was first observed at Day 5, and the severity of injury increased with prolonged exposure. After 9 days, the chilling injury index in the *B. subtilis* GUCC4-treated group was markedly reduced compared to the control ($p < 0.05$). Under severe chilling stress (5°C), *B. subtilis* GUCC4 exhibited a pronounced alleviating effect on chilling injury in passion fruit seedlings, which was observed from the first day.

3.3 | Effects of *B. subtilis* GUCC4 on Physiological Characteristics of Passion Fruit Under LTS

3.3.1 | Effects on Chlorophyll Content and Photosynthetic Characteristics

At room temperature, the addition of *B. subtilis* GUCC4 significantly enhanced the photosynthetic parameters of passion fruit seedlings, including total chlorophyll (Chl) content, net photosynthetic rate (Pn), and stomatal conductance (Gs) during the whole experimental period (Figure S2). Under mild LTS (15°C), only Pn showed a consistent increasing trend, whereas Chl and Gs were significantly increased in the *B. subtilis* GUCC4 treatment only during the initial stage (0–3 days) and at the end of the chilling stress period (9 days). Under severe LTS (5°C), *B. subtilis* GUCC4 extensively increased the Chl content and Pn of passion fruit seedlings throughout the entire experimental period. However, Gs appeared to be largely unaffected by *B. subtilis* GUCC4 addition, with only a slight increase observed on Day 3.

3.3.2 | Effects on H_2O_2, O_2^-, Malondialdehyde, and Relative Conductivity Contents

There were no significant differences in the levels of H_2O_2, O_2^-, Malondialdehyde (MDA), and relative conductivity (REC) (Figure S3) at room temperature. Under LTS (15°C and 5°C), H_2O_2 and O_2^- levels initially rose and then declined, while MDA and REC levels displayed a steady elevation throughout the stress treatment. Notably, the application of *B. subtilis* GUCC4 considerably reduced H_2O_2, MDA, and REC levels under LTS (15°C and 5°C) ($p < 0.05$). Regarding O_2^- levels, *B. subtilis* GUCC4 markedly lowered O_2^- levels only during the early phase of stress (0–3 days). Notably, under severe cold stress (5°C), *B. subtilis* GUCC4 significantly reduced O_2^- levels in passion fruit seedlings during both the early (0–3 days) and late (7–9 days) stages of exposure ($p < 0.05$).

3.3.3 | Effects on Osmoregulatory Substances and Antioxidant Enzyme Activities

During the entire period of LTS, the proline (Pro) content in passion fruit seedlings steadily increased with prolonged exposure. In contrast, soluble sugar (SS) and soluble protein (SP) levels, along with the activities of peroxidase (POD) and superoxide dismutase (SOD), exhibited an initial increase followed by a subsequent decline (Figure 3A–E). Catalase (CAT) activity significantly increased under mild LTS (15°C) over the experimental period, while it decreased under severe LTS (5°C) (Figure 3F).



FIGURE 2 | Effect of *B. subtilis* GUCC4 on the phenotype of passion fruit seedlings under low temperature stress. CK, MT, and LT represent the growth conditions of passion fruit seedlings at 25°C, 15°C, and 5°C, respectively. GT, GMT, and GLT represent the growth conditions of seedlings inoculated with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C, respectively, with 24°C as the control. Scale bar represents 10 cm.

TABLE 1 | Chilling injury index assessment for passion fruit seedlings under LTS.

	Chilling injury index (%)				
Treatment	**1 day**	**3 days**	**5 days**	**7 days**	**9 days**
GMT-CK	0	0	8.89 ± 0.77c	13.94 ± 1.80c	19.56 ± 0.77c
GMT	0	0	6.78 ± 0.51c	11.25 ± 1.18c	15.30 ± 1.58d
GLT-CK	15.05 ± 1.65a	19.43 ± 0.99a	30.86 ± 5.94a	46.29 ± 0.99a	57.14 ± 1.15a
GLT	12.00 ± 0.57b	15.46 ± 0.93b	20.19 ± 0.87b	41.33 ± 2.01b	54.10 ± 0.88b

Note: The level of significance difference is $p < 0.05$, and different letters indicate the difference in significance of the same index in each treatment.

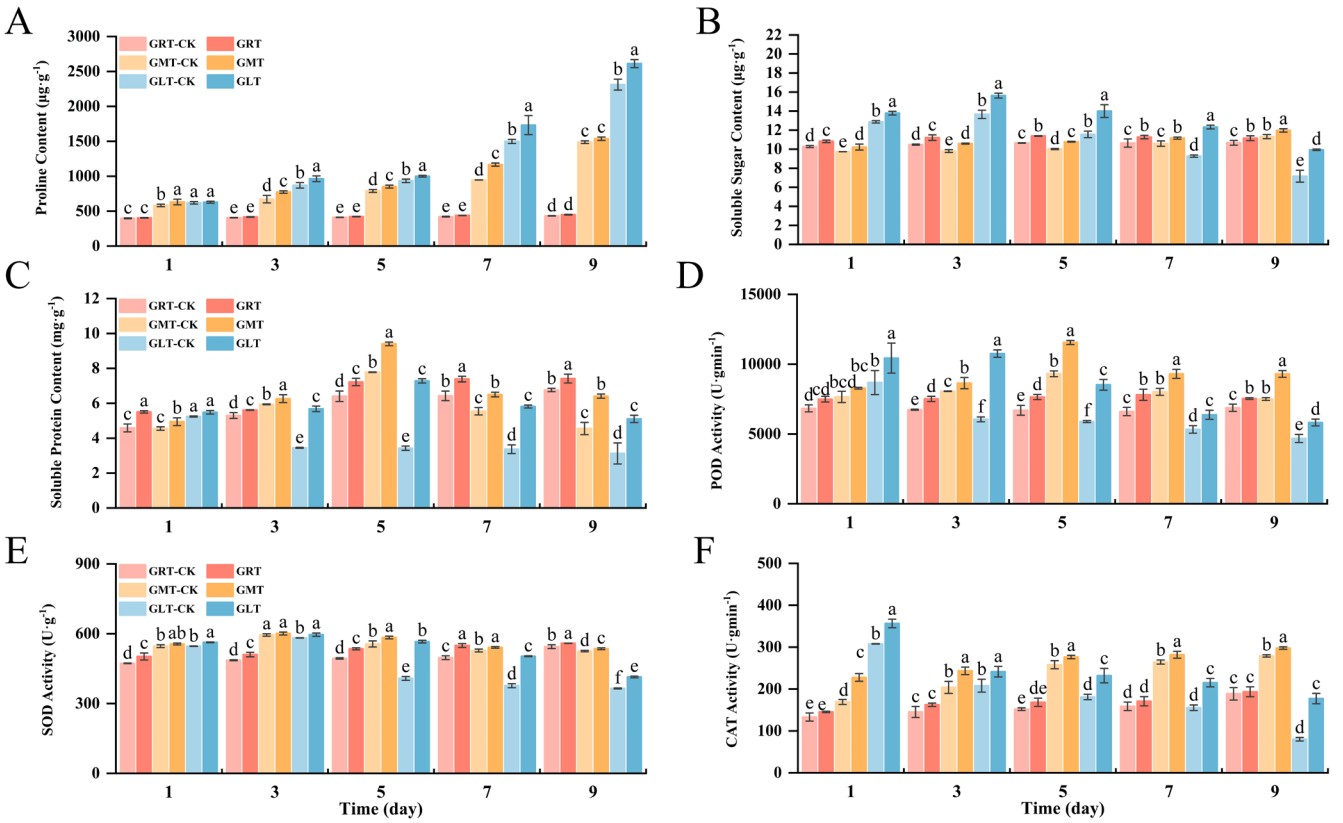


FIGURE 3 | Effects of *B. subtilis* GUCC4 on osmoregulatory substances and antioxidant enzyme activities of passion fruit under LTS. (A) Proline content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (B) Soluble sugar content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (C) Soluble protein content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (D) Peroxidase activity of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (E) Superoxide dismutase activity of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (F) Catalase activity of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. The level of significance difference is $p < 0.05$, and different letters indicate the difference in significance of the same index in each treatment.

Under room temperature, treatment with *B. subtilis* GUCC4 did not significantly alter Pro content or CAT activity. However, it significantly increased SS and SP levels, as well as POD and SOD activities during the experimental period ($p < 0.05$). Treatment with *B. subtilis* GUCC4 under mild LTS (15°C) resulted in sustained increases in SS and SP contents, POD, and CAT activities. Pro content was markedly elevated during the early (0–3 days) and middle (5–7 days) phases of the stress response. In contrast, significant enhancement in SOD activity was observed from the middle (5 days) to the late (≥ 7 days) stages ($p < 0.05$). During severe LTS (5°C), *B. subtilis* GUCC4 treatment resulted in significant SS accumulation and enhanced activities of POD, SOD, and CAT across the experimental period. Conversely, Pro and SP levels did not change on Day 1; however, they were significantly elevated from Day 2 ($p < 0.05$).

3.3.4 | Effects on Endogenous Hormones

Throughout the LTS period, Indole-3-acetic acid (IAA) content in passion fruit seedlings progressively declined with increasing stress duration, while abscisic acid (ABA) content exhibited a contrasting upward trend. Gibberellins (GA) and jasmonic acid (JA) levels initially increased and subsequently decreased over time (Figure S4). Under room temperature conditions, the application of *B. subtilis* GUCC4 had no significant effect on ABA content but significantly increased IAA and JA levels throughout the experimental period. In contrast, GA levels were significantly elevated only on Days 3, 7, and 9 ($p < 0.05$). Under mild LTS (15°C), treatment with *B. subtilis* GUCC4 led to a sustained increase in IAA and ABA contents, while GA levels remained unchanged on Day 1 but rose thereafter. JA content was considerably higher only on Days 3 and 9 ($p < 0.05$). Under severe LTS (5°C), *B. subtilis* GUCC4 significantly enhanced IAA, ABA, and GA contents throughout the experimental period. Although JA levels showed no significant change on Day 5, they were consistently elevated at all other time points relative to the control group ($p < 0.05$).

3.4 | Effects on Gene Expression Under LTS

3.4.1 | Analysis of Differentially Expressed Genes

In this study, a total of 1594 DEGs were identified between GLT and GLT-CK, of which 829 were upregulated, and 765 were down-regulated (Figure 4A). GO enrichment analysis revealed several biological pathways significantly affected by the treatment. As shown in Figure 4B, the top 20 enriched GO

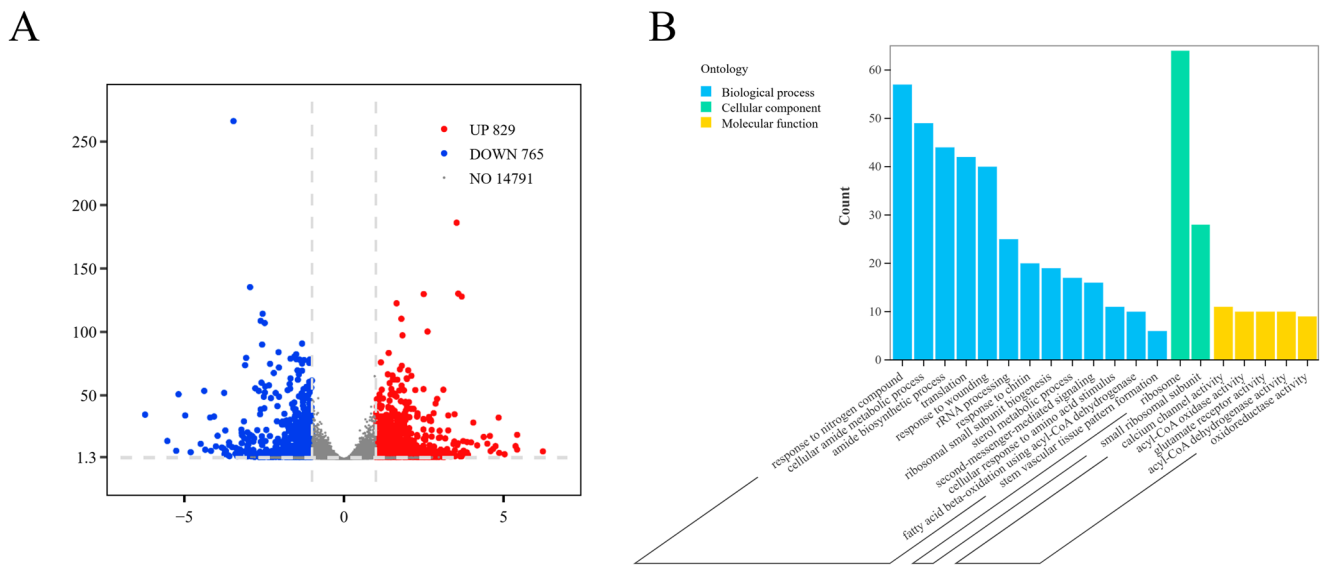


FIGURE 4 | Analysis of differentially expressed genes in passion fruit seedlings between GLT and GLT-CK through RNA-seq. (A) Volcano plot of DEGs between GLT and GLT-CK, with 829 genes up-regulated and 765 genes down-regulated. (B) GO functional enrichment analysis of differentially expressed genes. The *x*-axis represents the GO Term, and the y-axis represents the enrichment significance level of the GO Term. Different colors represent biological process (BP), molecular function (MF), and cellular component (CC), respectively.

terms include molecular functions such as auxin efflux and transmembrane transporter activity, both of which play vital roles in hormone regulation and stress response. Significantly enriched biological processes involve responses to injury, synthetic chitin, moisture and hormone transport, indicating activation of diverse stress response mechanisms. Additionally, cellular components such as ribosomes, which play a crucial role in the synthesis of stress-responsive and defense-related proteins, were prominently enriched. These results indicate that treatment with *B. subtilis* GUCC4 under LTS conditions induces significant changes in gene expression, especially in pathways associated with hormone transport and stress responses.

In addition, KEGG-based enrichment analysis of the DEGs identified several enriched pathways, including plant hormone signal transduction, plant MAPK signaling, sesquiterpenoid and triterpenoid biosynthesis, α-linolenic acid metabolism, and the biosynthesis of various plant secondary metabolites (Figure 5), involving 45, 54, 74, 8, and 7 DEGs, respectively. The top two KEGG pathways with the most significantly enriched DEGs were also assessed. In the plant hormone signaling pathway (Figure S5), genes such as *PeERF2*, *PeMYC2*, *PeJAZ1*, *PeAUX1*, *PeARF*, and *PeIAA27* were significantly upregulated, indicating enhanced hormone signaling and response mechanisms. Additionally, genes related to cell division, including *PeAHP* and *PeB-ARR*, also exhibited significant upregulation. In the MAPK signaling pathway (Figure S6), transcription factors such as *PeMYC2*, which are upstream of jasmonic acid, were induced, along with the upregulation of *PeEBF1/2* and *PeChiB* genes. Notably, the *MKK2* gene in passion fruit, which plays a crucial role in regulating plant responses to salt and cold stress, was also markedly upregulated. Moreover, genes associated with plant-pathogen interactions, such as *PeBAK1*, *PeMKK2*, *PeSUMM2*, and *PePR1*, showed increased expression. These findings highlight the complex regulatory network activated by *B. subtilis* GUCC4 treatment in passion fruit seedlings under LTS.

3.4.2 | Verification of Differences in Gene Expression via Quantitative PCR

To validate the transcriptome sequencing results, nine genes associated with key pathways were analyzed by qRT-PCR (Figure 6). The selected genes, including *PeCML*, *PeALDH*, *PeJAZ1*, *PePYL*, *PePR1*, *PeARR1*, *PeMAPK7*, *PeChiB*, and *PeCoA*, are involved within the phytohormone signaling pathway, MAPK pathway, and peroxisome signaling pathway. The qRT-PCR results indicated that the expression profiles of these genes were consistent with those derived from the transcriptome sequencing data, confirming the reliability of the transcriptomic analysis.

4 | Discussion

4.1 | Endophytic Colonization of *B. subtilis* GUCC4 in Passion Fruit

A key factor in the effectiveness of each PGPB including *B. subtilis* is their ability to successfully colonize plant roots (Lugtenberg and Kamilova 2009). Our results confirmed that *B. subtilis* GUCC4 could stably colonize the roots of passion fruit and significantly increase the abundance of *B. subtilis* in leaves under LTS, possibly through systemic effects after root internalization. This establishes a foundation for the strain to be used to enhance cold resistance of passion fruit. Colonization is essential for bacteria to exert growth-promoting and biocontrol effects on plants, and their success varies largely depending on their ability to persist in the rhizosphere and interact effectively with plants (Padró et al. 2021). Numerous studies have shown that *B. subtilis* and close relatives are prevalent PGPB, capable of stably colonizing plants, producing IAA, fixing nitrogen, and solubilizing phosphorus. It also possesses other desirable characteristics such as ease of formulation and application, non-pathogenicity, and high

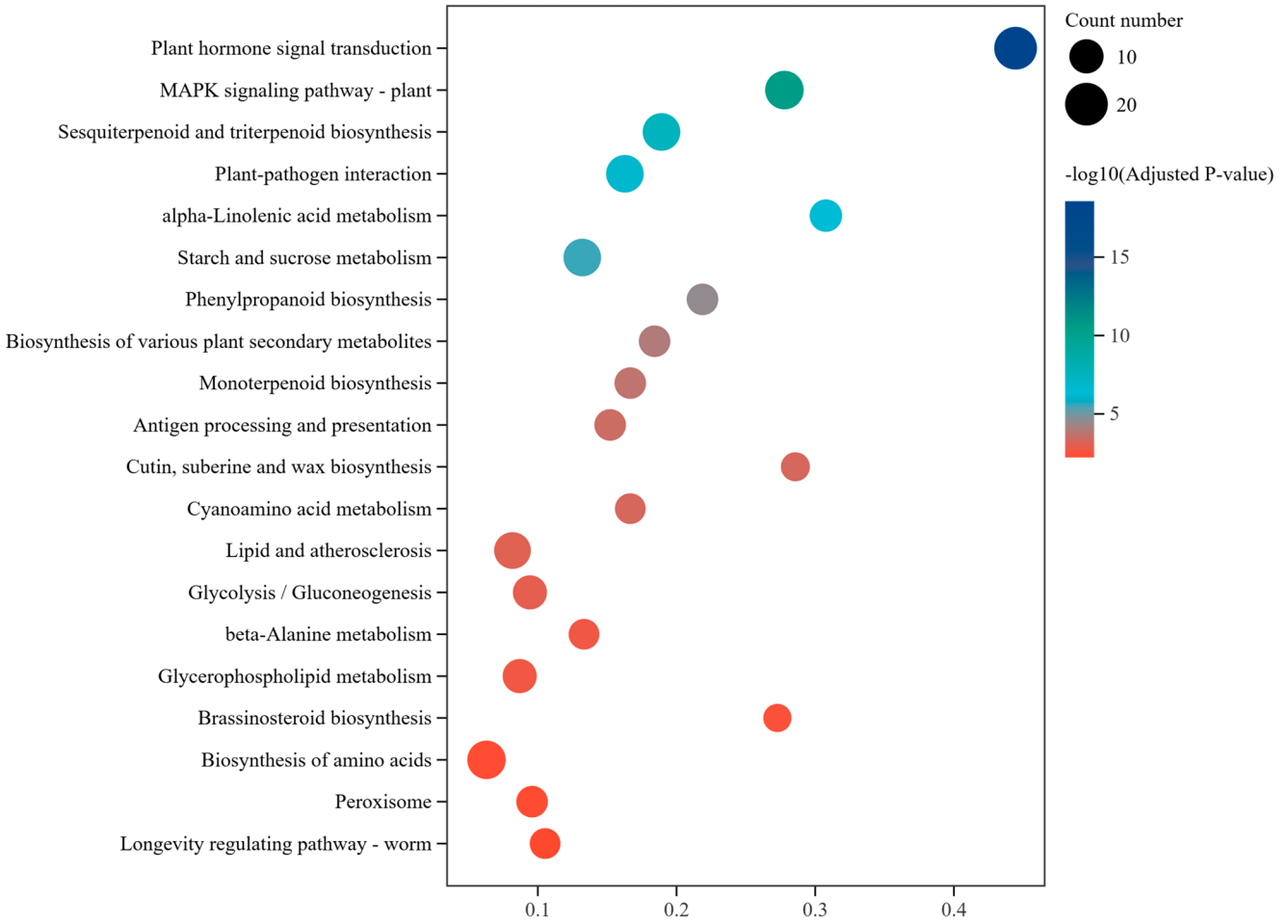


FIGURE 5 | KEGG enrichment of DEGs between GLT and GLT-CK in passion fruit seedlings. The *x*-axis represents the ratio of the number of differentially expressed genes annotated to a KEGG pathway to the total number of differentially expressed genes, and the *y*-axis represents the KEGG pathway.

environmental compatibility (Zhao et al. 2011; Planchon et al. 2021). Additionally, it serves an essential function in promoting growth, disease resistance, and pest resistance during plant development (Lee et al. 2014; Wang et al. 2020). However, the activity of *Bacillus* strains also shows a high variability at strain level (Mülner et al. 2020). Considering this, *B. subtilis* GUCC4 can be considered as highly efficient colonizer and is therefore a promising PGPB.

4.2 | Impact of *B. subtilis* GUCC4 on the Phenotype of Passion Fruit

Low temperature represents a key abiotic stress factor that restricts plant growth and development (Lin et al. 2025). In our study, passion fruit leaves gradually wilted and withered with prolonged exposure both at 15°C and 5°C. Notably, endophytic *B. subtilis* GUCC4 could significantly delay the wilting of passion fruit leaves under these stress conditions. Previous studies have demonstrated that low temperature stress (LTS) significantly impairs the photosynthetic efficiency of plants such as rice (Shimono et al. 2004) and mango (Hmmam et al. 2022), leading to symptoms like leaf yellowing, chlorosis, wilting, and dehydration. This damage likely arises from low-temperature-induced disruption of chloroplasts, which reduces chlorophyll content and triggers negative leaf phenotype changes. Consequently, the photosynthetic system is further compromised, ultimately inhibiting plant growth and development. Our findings corroborate those of Li et al. (2023), who showed that inoculating tomato with *B. methyltrophicus* VL-10 mitigated chilling-induced injuries, thereby enhancing cold tolerance. These evidences have direct implications for agriculture, particularly in the context of climate change-induced abiotic stresses, highlighting the potential of beneficial microbes to improve plant adaptive capacity and sustain productivity (Singh et al. 2018; Hamid et al. 2021).

4.3 | Effects of *B. subtilis* GUCC4 on Physiological Indices of Passion Fruit Under LTS

Under LTS, *B. subtilis* GUCC4 applications significantly enhanced chlorophyll content (Chl), photosynthetic rate (Pn), and soluble sugar (SS) levels, as well as the activities of the antioxidant enzymes peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) in passion fruit seedlings. Furthermore, the strain elevated the concentrations of phytohormones such as indole-3-acetic acid (IAA), abscisic acid (ABA), and gibberellic

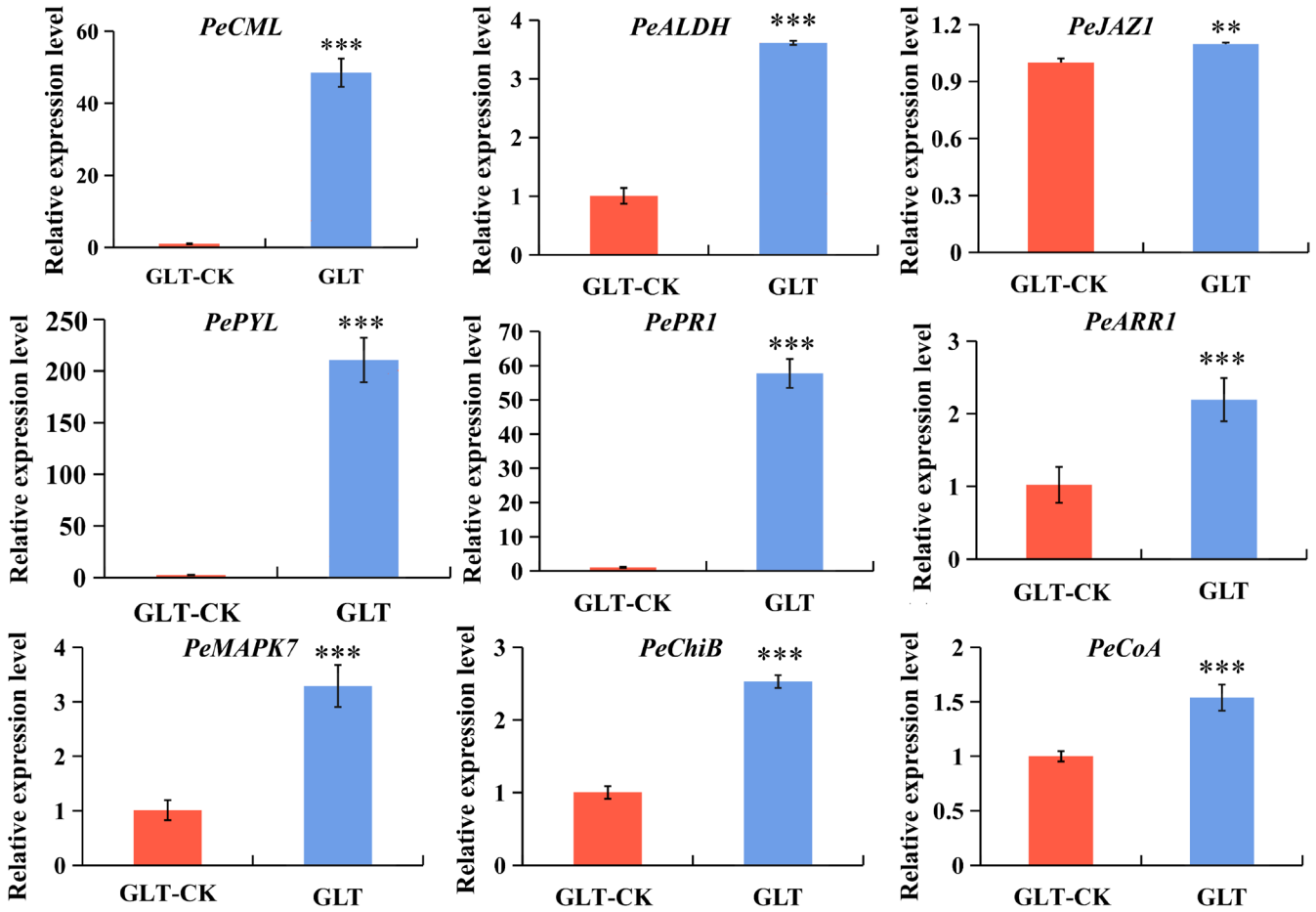


FIGURE 6 | Quantification of DEGs between GLT and GLT-CK in passion fruit seedlings. Real-time qRT-PCR validation of transcription levels of 9 candidate DEGs. ** indicates a significant difference level of $p < 0.01$, and *** indicates a significant difference level of $p < 0.001$.

acid (GA). Conversely, it markedly reduced the accumulation of key biomarkers associated with biotic stress, including hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), along with the relative electrical conductivity (REC).

It is well known that LTS induces accumulation of reactive oxygen species (H_2O_2, O_2^-) and MDA in leaves, subsequently elevating REC (Georgieva et al. 2021; Guo et al. 2022). To counteract these adverse effects, plants have evolved coordinated physiological and biochemical adaptations. These include osmoregulation via Pro, SS, and SP to lower intracellular osmotic potential, thereby suppressing ROS generation (Chen et al. 2022). Concurrently, enzymatic antioxidant systems, including POD, SOD, and CAT, are upregulated to detoxify free radicals and ROS, thereby mitigating MDA accumulation and lipid peroxidation (Jose et al. 2025). Meanwhile, endogenous plant hormones, including IAA, ABA, GA, and JA, are critical regulators of growth and development, morphogenesis, and stress resistance (Yan et al. 2019). Under environmental stress, plants could modulate hormone levels to counteract stress-induced damage (Anwar et al. 2018). Thus, inoculation with *B. subtilis* GUCC4 may enhance osmoregulatory capacity and antioxidant defenses, as well as hormone modulation in passion fruit by inducing systemic resistance, secondary metabolites production and phytohormone homeostasis restorage under LTS. This will further contribute to enhancing the health and resilience of passion fruit under biotic stresses.

In this study, the constrained rhizosphere interactions that are inherent to pot-based experiments may not fully capture the complexity of natural field environments. It remains unclear whether *B. subtilis* GUCC4 can recruit additional beneficial soil microbes under field conditions to facilitate colonization and functional engagement with passion fruit root systems. Therefore, future studies combining field experiments with rhizosphere microbiome analyses are essential for uncovering the ecological processes and application potential of *B. subtilis* GUCC4 in sustainable agroecosystems.

4.4 | Effects of *B. subtilis* GUCC4 on Gene Expression of Passion Fruit Under LTS

By employing GO and KEGG analyses significant enrichments in biological pathways related to hormone regulation, stress/injury responses, dehydration adaptation, and photosynthetic processes, as well as photosynthesis-associated cellular compartments such as plant thylakoids were found. These results indicate that *B. subtilis* GUCC4 application under LTS improves cold tolerance in passion fruit by modulating the expression of key genes involved in hormone signaling and photosynthetic efficiency. Specifically, we observed significant upregulation of cold-responsive genes (*PeMYC2*, *PeAUX1*, and *PeIAA27*) involved in jasmonate and auxin pathways. These findings align with established mechanisms wherein *MYC2* transcription factors

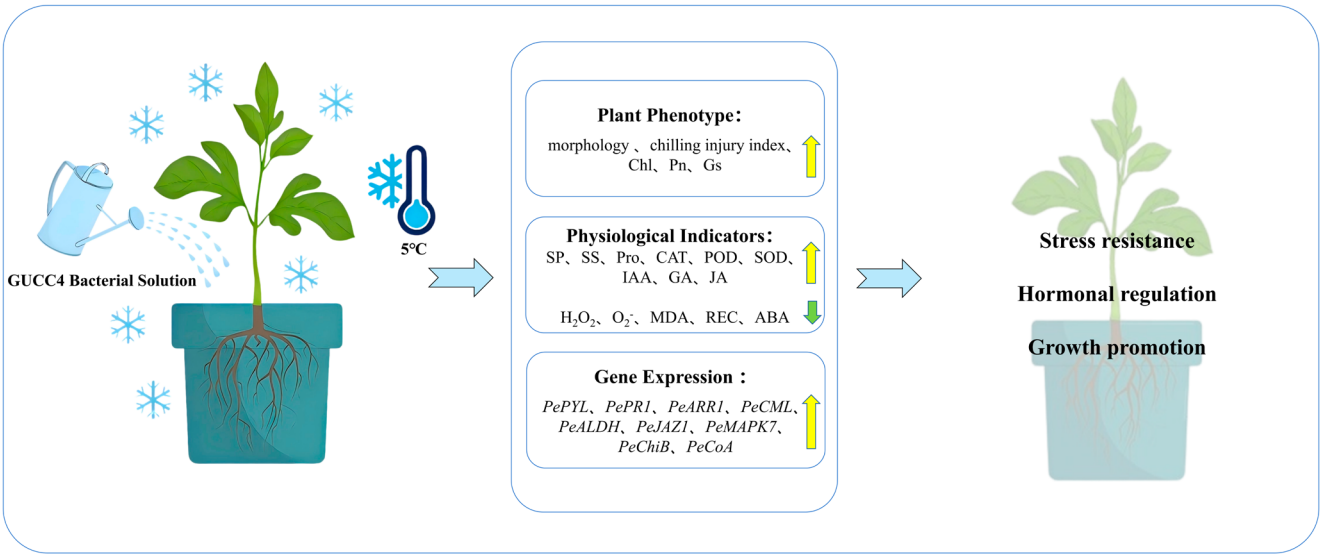


FIGURE 7 | Proposed model of molecular mechanisms employed by *B. subtilis* GUCC4 for mitigating LTS in passion fruit.

regulate stress adaptation through hormonal crosstalk (Wang, Xu, et al. 2019), suggesting a conserved role for microbial-primed phytohormone regulation in cold stress mitigation. Other studies have shown that overexpression of *AtMYC2* and *AtMYB2* transcription factors improves osmotic stress tolerance in transgenic plants (Abe et al. 2003; Yin et al. 2025). Wang, Xu, et al. (2019) also found that exogenous application of methyl jasmonate increased the cold hardiness of apples through the *JAZ-MYC2* pathway under LTS. The results of this experiment showed that inoculation with *B. subtilis* GUCC4 could induce the upregulated expression of *the PeMYC2* gene related to the JA pathway, and the content of JA was increased. Meanwhile, the upregulated expression of plant growth-related genes such as *PeAUX1*, *PeIAA27*, *PeAHP*, and *PeB-ARR* was also induced. This is consistent with previous findings of gene upregulation in plant hormone signaling and MAPK pathways. Overall, *B. subtilis* GUCC4 plays a critical role in enhancing plant resistance to adverse conditions, including LTS, by regulating various biological pathways and gene expression. Additionally, we focused on nine genes that are known to be involved in key biological pathways such as plant hormone signaling and the MAPK pathway (Figure 7), which are critical in plant stress responses (Qi et al. 2019; Sharma et al. 2020). Nonetheless, several limitations remain. Specifically, the functional roles of the candidate genes have yet to be validated through genetic transformation or other functional genomics approaches. Moreover, translating these cold-responsive genes into practical *breeding* applications will require more precise and efficient molecular breeding strategies. Alternatively, the identification of specific *Microbiome* genes (*M* genes) that shape the whole microbiome towards higher cold resistance might be a tangible breeding strategy (Cernava 2024). Employing such strategies could add to the theoretical framework for the genetic improvement of cold resistance in tropical fruit crops.

5 | Conclusion

LTS significantly impairs the growth and physiological functions of passion fruit seedlings, particularly by reducing chlorophyll content, impairing photosynthesis, and increasing harmful ROS and MDA levels, which disrupt cell membrane integrity. Our study demonstrates that inoculation with *B. subtilis* GUCC4 effectively mitigates these adverse effects: it enhances levels of photosynthetic pigments, increases osmoregulatory substances and antioxidant enzyme activities, and positively modulates the hormonal balance by increasing IAA and by decreasing ABA levels, while promoting JA and GA accumulation under LTS. These beneficial effects are likely mediated by the upregulation of key growth-related and stress-response genes such as *PeAUX1*, *PeIAA27*, *PeAHP*, *PeB-ARR*, and *PeMYC2*. Overall, this study demonstrates the promise of *B. subtilis* GUCC4 as a valuable tool to enhance the resilience of passion fruit seedlings to LTS, with promising applications for agriculture in colder regions.

Author Contributions

Shilin Bai, **Xi Wei**, **Hao Ouyang**, **Zeyang Ding:** data curation, software, methodology, writing – original draft. **Guilan Duan:** methodology, writing – review and editing. **Zengliang Liu:** project management, writing – review and editing. **Gabriele Berg:** writing – review and editing. **Tomislav Cernava:** writing – review and editing. **Ivan Baccelli:** writing – review and editing. **Xiaojing Hu** and **Xiaoyulong Chen:** conceptualization, writing – review and editing, funding, supervision.

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[2023] 070, CXTD [2025] 041. The Program for Introducing Talents to Chinese Universities, 111 Program; D20023.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Primer sequences. **Table S2:** Dataset statistics for RNA-seq data. **Figure S1:** Correlation analysis (A) and principal component analysis (PCA) (B). Note: GLT plants inoculated with *B. subtilis* GUCC4 at low temperature (5°C); GLT-CK plants not inoculated (control) at low temperature (5°C). **Figure S2:** Effects of *B. subtilis* GUCC4 on chlorophyll content and photosynthetic characteristics of passion fruit under LTS. (A) Chlorophyll content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (B) Net photosynthetic rate of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (C) Stomatal conductance of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. The level of significance difference is $p < 0.05$, and different letters indicate the difference in significance of the same index in each treatment. **Figure S3:**. Effects of *B. subtilis* GUCC4 on H_2O_2, O_2^-, and malondialdehyde levels as well as relative conductivity of passion fruit under LTS. (A) H_2O_2 content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (B)

O_2^- content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (C) Malondialdehyde content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (D) Relative conductivity content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. The level of significance difference is $p < 0.05$, and different letters indicate the difference in significance of the same index in each treatment. **Figure S4:** Effects of *B. subtilis* GUCC4 on endogenous hormones of passion fruit under LTS. (A) IAA content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (B) ABA content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (C) GA content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. (D) JA content of passion fruit seedlings inoculated or not with *B. subtilis* GUCC4 at 25°C, 15°C, and 5°C. The level of significance difference is $p < 0.05$, and different letters indicate the difference in significance of the same index in each treatment. **Figure S5:** GLT versus GLT-CK plant hormone signal transduction map. Note: (GLT) plants inoculated with *B. subtilis* GUCC4 at low temperature (5°C); (GLT-CK) plants not inoculated (control) at low temperature (5°C). **Figure S6:** GLT versus GLT-CK MAPK signal path map. Note: (GLT) plants inoculated with *B. subtilis* GUCC4 at low temperature (5°C); (GLT-CK) plants not inoculated (control) at low temperature (5°C).