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TECHNICAL REPORT

Mechanism of Engineered Rhizobia Overexpressing Cytokinin Improve Heat Stress Response in Alfalfa

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ABSTRACT

Heat stress is a major abiotic stress that limits alfalfa (*Medicago sativa* L.) productivity. To test whether cytokinin-overexpressing engineered rhizobia could enhance heat tolerance, we inoculated alfalfa with modified rhizobia and evaluated their physiological and molecular responses. We found that inoculation with engineered rhizobia significantly increased trans-Zeatin content in alfalfa. Compared with control rhizobia-inoculated plants, engineered rhizobia-inoculated plants exhibited increased plant height, fresh/dry weight, relative water content, and photosynthetic efficiency (total chlorophyll and carotenoids), alongside reduced hydrogen peroxide (H_2O_2) and superoxide ($O_2.^-$) levels under heat stress. RNA-seq analysis revealed that engineered rhizobia upregulated heat stress-responsive genes in alfalfa, which was further verified by qRT-PCR. Metabolomics analyses showed significant alterations in phenylpropanoid, flavonoid, phenolic acid, and salicylic acid metabolic pathways in engineered rhizobia-inoculated plants under heat stress. Contrary to conventional approaches, our results demonstrate that cytokinin-overexpressing rhizobia not only enhance alfalfa heat tolerance but also activate multi-pathway stress responses. Collectively, these findings propose a novel strategy for developing heat-tolerant alfalfa through engineered rhizobia-mediated cytokinin biosynthesis, which helps to promote the development of sustainable alfalfa breeding for heat-tolerant varieties.

1 | Introduction

Heat stress, driven by global climate change, is a major abiotic stressor impairing plant growth and development (Janni et al. 2020). Extreme heat events threaten crop and forage productivity (Galushko and Gamtessa 2022). Heat stress disrupts physiological functions by damaging cell membranes, inhibiting photosynthesis (such as breaking the structure of chloroplasts, particularly the thylakoid membranes, causing degradation of photosynthetic pigments and a decline in photosynthetic efficiency), accumulating reactive oxygen species (ROS) and inducing oxidative stress (Mathur et al. 2021). These disruptions impair water/nutrient homeostasis (like increasing

Abbreviations: ACC, aminocyclopropane-1-carboxylate; CKs, cytokinins; DAB, diaminobenzidine; DW, dry weight; FDR, false discovery rate; FW, fresh weight; H_2O_2, hydrogen peroxide; IAA, indole-3-acetic acid; LC-MS, liquid chromatography-mass spectrometry; MDA, malondialdehyde; NBT, nitroblue tetrazolium; $O_2^{\cdot-}$, superoxide anions; PGPR, plant growth-promoting rhizobacteria; POD, peroxidase; ROS, reactive oxygen species; RT-qPCR, quantitative real-time PCR; RWC, relative water content; SOD, superoxide dismutase; tZ, trans-Zeatin.

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plant respiration, accelerating starch breakdown, and raising the levels of soluble sugars), imbalance of plant hormones (suppressing the production of auxin and gibberellins), reducing growth and yield (Grover et al. 2013). Consequently, developing heat-tolerant crop systems is now a priority for sustainable agriculture (Grover et al. 2013).

To counteract the detrimental effects of elevated temperatures, plants have developed an array of heat tolerance mechanisms (Hasanuzzaman et al. 2013). Among these, stomatal modulation plays a crucial role in minimizing water loss and maintaining cellular hydration under thermal stress, a process governed by intricate hormonal and environmental signaling networks (Xu et al. 2025). Concurrently, plants upregulate heat shock proteins (*HSPs*) that serve as molecular chaperones, facilitating the refolding of thermally denatured proteins and safeguarding proteostasis (Selinga et al. 2022). At the metabolic level, plants undergo a comprehensive reprogramming to bolster antioxidant defenses, including enhanced production of key enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which collectively scavenge deleterious ROS and prevent oxidative damage (Xu et al. 2013). Certain species employ additional strategies, such as the selective modulation of flavonoid biosynthesis and other secondary metabolic pathways, to further enhance heat tolerance (Jan et al. 2021). These integrated physiological and molecular adaptations function in concert to maintain cellular homeostasis and ensure plant viability under prolonged heat stress conditions.

As a key plant hormone, cytokinin (CK) significantly enhances plant thermotolerance during high-temperature adaptation by regulating mechanisms such as metabolic reprogramming, antioxidant defense, signal transduction, and hormone interactions (Mas-ud et al. 2025). Under heat stress, the expression of *isopentenyltransferase* (*IPT*) genes is significantly upregulated, promoting the synthesis of isopentenyladenosine precursors, which in turn increases the levels of active CKs (such as zeatin) (Yang et al. 2022). High temperatures also suppress the activity of cytokinin oxidase (CKX), reducing CK degradation. CK signaling, transmitted through the histidine kinase (*AHK2/AHK3*), phosphotransfer protein (*AHP*), and response regulator (*ARR*) pathway, transcriptionally activates downstream target genes (such as heat shock proteins, *HSPs*), thereby enhancing heat resistance (Mas-ud et al. 2025). CK directly upregulates the genes encoding antioxidant enzymes like SOD and CAT, mitigating the membrane lipid peroxidation induced by high temperatures (e.g., reducing MDA content). Under heat stress conditions, CK helps maintain chlorophyll content and the integrity of thylakoid membrane structure, alleviating the decline in Photosystem II photochemical efficiency (F_v/F_m). In a high-temperature environment, CK establishes a dynamic balance with multiple other hormones: it antagonizes the salicylic acid (SA)/jasmonic acid (JA) pathways; it synergizes with brassinosteroids (BRs) to enhance antioxidant capacity and carbon assimilation efficiency (Chen et al. 2021); and it balances auxin levels, regulating root architecture to adapt to temperature stress (Tiwari and Lovelace 2023).

As the preeminent temperate forage legume, *Medicago sativa L.* underpins global livestock agriculture, delivering unparalleled ecosystem services through nitrogen fixation and carbon sequestration (Xia et al. 2023). While its nutritional supremacy (18%–25% leaf protein content) and abiotic stress resilience have sustained cultivation across 32 million hectares worldwide (Hadidi et al. 2023). Additionally, alfalfa demonstrates notable resilience to various environmental stresses, particularly drought and heat (Wang et al. 2016). Publications demonstrated that high temperatures lead to a significant reduction in chlorophyll content in alfalfa leaves, as well as a notable decline in photosynthetic efficiency of Photosystem II (Liu et al. 2014). Additionally, heat stress causes an increase in the relative membrane permeability of leaves and the content of malondialdehyde (MDA), while the activity of the antioxidant enzyme system, including POD, SOD, and CAT, undergoes significant changes. Furthermore, it results in a decrease in leaf area per individual leaf, stunted plant growth, and reduced yield. As climate change continues to intensify, there is an urgent and growing need to explore and implement strategies to further enhance its heat tolerance, safeguarding its crucial role in sustainable grassland agriculture (Wassie et al. 2020). There has been a report on the successful construction of genetically modified alfalfa lines using plant genetic engineering techniques, where the senescence-associated promoter *SAG12* drives the expression of the *IPT* gene, thereby delaying leaf senescence (Calderini et al. 2007). However, it is regrettable that this genetic material was not further analyzed for stress tolerance.

In the domain of research focused on improving plant stress resistance, a variety of scientific methodologies have produced significant findings in wheat, rapeseed, and rice. Specifically, in the case of wheat, quantitative trait loci (QTL) analysis was performed using the *SeriM82×Babax* recombinant inbred line population, leading resulting into the successful identification of QTLs associated with agronomically important traits, including heading date and thousand-grain weight. Furthermore, this analytical approach enabled the screening of stable and trait-specific QTLs, while establishing indirect selection criteria that incorporate heading date as a key indicator (Tahmasebi et al. 2014, Tahmasebi et al. 2017). In rapeseed research, an integrated multi-omics approach—combining genome-wide association studies (GWAS), metabolomic profiling, and transcriptomic sequencing data from 119 diverse accessions—facilitated the identification of relevant single nucleotide polymorphisms (SNPs), the discovery of candidate genes with functional relevance to stress resistance, and the characterization of trait-specific SNPs amenable to marker-assisted selection (MAS) (Salami et al. 2024). For rice, gene expression meta-analysis was employed to systematically identify genes with robust upregulation under multiple stress conditions, while further elucidating the cis-regulatory elements that modulate the transcriptional regulation of stress resistance pathways (Shariatipour and Heidari 2020). Collectively, these investigations have yielded essential candidate genes, reliable molecular markers, and validated selection criteria for enhancing stress tolerance in the three crops, thereby providing valuable insights to accelerate the advancement of stress-resistant breeding programs.

The symbiotic interactions between alfalfa and rhizobia not only induce the formation of nitrogen-fixing nodules to assimilate nitrogen gas into chemical nitrogen nutrition but also enhance the plant tolerance to exogenous stress through various pathways, similar to other endophytic microorganisms (Kumawat et al. 2023; Shaffique et al. 2022), including the tolerance to salinity, cold, alkalinity, and heat (Irshad et al. 2021;

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Song et al. 2021; Wang et al. 2016). By leveraging the multiple interactions between rhizobia and alfalfa, such as rhizosphere colonization, tissue (intercellular spaces) endophytism, infection thread encapsulation, and the formation of nitrogen-fixing bacteroids within nodules, synthetic biology techniques are used to express exogenous metabolic pathway genes in rhizobia to synthesize specific plant hormones like auxin (IAA), cytokinin (trans-zeatin), or flavodoxin, which inoculation of alfalfa not only increases nodulation and nitrogen fixation and growth, but also enhances the stress resistance of the plants (Bianco and Defez 2009; Defez et al. 2017; Redondo et al. 2012; Xu et al. 2012). For example, inoculation of IAA-producing rhizobia improves salt and drought tolerance of the alfalfa plants, while producing-trans-zeatin bacteria enhances drought tolerance of the plants (Bianco and Defez 2009; Defez et al. 2017; Ji Xu et al. 2012). Expressing exogenous genes to synthesize active small molecules that regulate plant growth and development in rhizobia is easier, faster, and more economical than doing so in alfalfa.

In our previous work, we constructed engineered strains of *Sinorhzibium meliloti* with an exogenous *ipt* gene integrated into the genome, generating trans-zeatin (tZ), which significantly enhanced the tolerance of alfalfa to extreme drought after inoculation (Yu et al. 2024). Given the correlation between drought and high temperature, in this work, we thoroughly investigated whether inoculation of engineered strains enhances tolerance to high temperature or heat stress of alfalfa plants, as well as the underlying mechanisms.

2 | Materials and Methods

2.1 | Plant Materials and Stress Treatment

This study employed four rhizobial strains. The control strain *Sinorhizobium meliloti* 1021 (Sm1021) served as the wild-type rhizobium. The transfection control strain pK19 (pK19mob2ΩHMB) carried an empty vector due to the deletion of the isopentenyl transferase (*ipt*) gene. Two genetically engineered strains were additionally constructed: P*lac-ipt* and P*KatG-ipt*. The Plac-ipt strain utilized a constitutive P*lac* promoter, while P*KatG-ipt* carried a ROS-responsive P*katG* promoter. Both promoters drove expression of the *ipt* gene derived from Agrobacterium tumefaciens.

Alfalfa (Xinjiang large-leaf variety) was selected as the experimental plant material. This study employed pot experiments using 10 cm × 10 cm vases. Plants were cultivated in a growth chamber under a 14-h photoperiod (24°C day-time/20°C night-time) and 60% relative humidity.

Twenty-eight days post-germination, the incubator temperature was adjusted to 38°C (day)/35°C (night), with all other conditions remaining unchanged. Experimental parameters measured on treatment Day 0 were established as the control group. Each experiment was measured with three biological replicates with three technical replicates, respectively.

2.2 | Measurement of Endogenous Hormones

Endogenous hormone extraction was initiated by homogenizing 0.1 g of leaf tissue in a 2-mL centrifuge tube, followed by the addition of 1 mL of ice-cold ethyl acetate and vortex-mixing for 5 min. The mixture was then incubated at 4°C for 12 h. After incubation, centrifugation was performed at 12,000 × *g* for 3 min at 4°C. The pellet was resuspended in 1 mL of fresh ethyl acetate, and the extraction procedure was repeated. Combined supernatants from both extractions were stored at −20°C overnight, then lyophilized under N_2 gas flow at 25°C. The dried extract was reconstituted in 100 μL of HPLC-grade methanol, vortexed for 40 s, and filtered through a 0.22-μm PTFE syringe filter into amber vials for LC-MS/MS analysis.

2.3 | Determination of ROS and Antioxidant Enzymes

2.3.1 | *NBT, DAB Staining*

To prepare the NBT staining solution, dissolve 2.38 g of HEPES in 100 mL of ultrapure water and adjust the pH to 7.5, followed by adding 0.49 g of nitroblue tetrazolium (NBT) with thorough mixing until complete dissolution. The solution should be stored in the dark to prevent photodegradation. For staining, incubate leaf samples in the NBT solution for 2 h, then dehydrate in 95% ethanol at 50°C for 2 h before photographic documentation. For DAB staining, prepare the working solution by dissolving 0.1 g of 3,3′-diaminobenzidine (DAB) in 100 mL of ultrapure water with pH adjustment to 3.8, and store protected from light. Immerse leaf samples in the DAB solution and incubate at 37°C for 9 h, followed by decolorization in ethanol at 80°C for 2 h prior to image capture for analysis.

2.3.2 | *Measurement of H_2O_2 and O_2^- Content*

To quantify hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) levels, the samples were collected from the third to fifth leaves (counted acropetally from the apex) at baseline (Day 0) and after 14 days of heat stress treatment. The analyses were performed using commercial assay kits (Solarbio, Beijing, China; H_2O_2: BC4748, O_2^-: BC3595) following the manufacturer's protocols. All physiological parameters were assessed in biological replicates to evaluate treatment effects.

2.3.3 | *Measurement of POD Activity and MDA Contents*

The reaction mixture, containing 1.5 mL PBS, 0.3 mL each of Met, NBT, EDTA-Na_2, and riboflavin (200 μM) solutions, 0.1 mL enzyme extract, and 0.5 mL water, was incubated at 25°C–35°C for 30 min under light. Dark-incubated tubes served as blanks. POD activities were determined by measuring absorbance at 470 nm, respectively, using a spectrophotometer.

The contents of MDA were measured using commercial assay kits (Solarbio, Beijing, China; MDA: BC0025) following the manufacturer's protocols.

2.4 | Measurements of Chlorophyll Fluorescence and Photosynthesis Rate

Chlorophyll fluorescence measurements were performed using a LI-6800 photosynthesis analyzer (LI-COR Biosciences). Leaves were dark-adapted for 30 min prior to measurements. For each treatment group, five leaves were selected with ten

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measurements replicated per leaf. The LI-6800 was configured with a flow rate of 500 μmol s^{-1}, 60% relative humidity, 400 μmol mol^{-1} CO_2 concentration, fan speed at 10,000 rpm (revolutions per minute), 25°C chamber temperature, and initial photosynthetic photon flux density (PPFD) set at 0 μmol m^{-2} s^{-1}. Under steady-state light conditions (PPFD: 800 μmol m^{-2} s^{-1}), photosynthesis-related parameters, including transpiration rate (*E*), net photosynthetic rate (*A*), intercellular CO_2 concentration (Ci), and stomatal conductance to water vapor (gsw) were measured. Five leaves per treatment group were assessed with five replicate measurements per leaf.

For chlorophyll content determination, the second fully expanded leaf from the apex was collected and extracted with 1.2 mL of 96% (v/v) ethanol. Samples were kept in darkness at room temperature for 24 h. Chlorophyll a, Chlorophyll b, carotenoids, and total chlorophyll contents were calculated by reading the absorbance at 665, 649, and 470 nm using a spectrophotometer.

2.5 | Transcriptome Sequencing and qRT-PCR Analyses

For transcriptome profiling, leaves from alfalfa (*Medicago sativa*) plants inoculated with either P*lac-ipt* or *S. meliloti* 1021 (Sm1021) were harvested under both control (25°C) and heat stress (38°C) conditions. cDNA library construction and high-throughput sequencing were performed by Metware Biotechnology Co. Ltd. (Wuhan, China) using the Illumina NovaSeq. 6000 platform. Bioinformatics analyses were conducted using the OmicStudio platform (https://www.omicstudio.cn). Gene expression levels were normalized and quantified as fragments per kilobase of transcript per million mapped reads. Differentially expressed genes (DEGs) were identified using the DESeq. 2 package (version 1.30.1) in R, with significance thresholds set at absolute log2 fold change ≥1 and false discovery rate (FDR) < 0.05.

Total RNA from leaves of alfalfa (*Medicago sativa* L.). RNA isolation was performed using the Eastep Super Total RNA Extraction Kit (Promega Biotech Co. Ltd.), with genomic DNA (gDNA) contamination removed via the kit's built-in DNase I treatment. Isolated total RNA was subsequently reverse-transcribed into complementary DNA (cDNA) using the Hi-Script III RT SuperMix for qRT-PCR (Vazyme Biotech Co. Ltd.), strictly following the manufacturer's recommended protocol. Quantitative real-time PCR (qRT-PCR) was performed on a 96-well real-time PCR system (LightCycler 480 II; Roche Diagnostics) using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co. Ltd.). The qRT-PCR reaction conditions were set as follows: [e.g., initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s], in line with the kit's recommended protocol. Each qRT-PCR reaction was performed in three technical replicates to account for intra-assay variability, and three biological replicates were included for alfalfa leaves to minimize inter-sample variability. To normalize qRT-PCR data and correct for variations in RNA input and reverse transcription efficiency, the internal reference genes selected were *MsActin* (a constitutively expressed gene in *M. sativa* L.). The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ (delta-delta CT) method, as described previously by Livak and Schmittgen (2001), for relative quantification of gene expression. The primers were listed in Supplementary Table S1.

2.6 | Measurement of Secondary Metabolites and Soluble Sugar Content

For secondary metabolite extraction, leaf tissue (0.3 g) was homogenized in 3 mL of methanol-formic acid solution (75:23:2, v/v, methanol: ultrapure water: formic acid). Samples were sonicated for 30 min, followed by incubation at 4°C for 48 h. After drying under a nitrogen stream, residues were re-suspended in 200 μL methanol and filtered through a 0.22-μm organic membrane into amber vials.

For soluble sugar analysis, ground samples (0.1 g) were mixed with 1.4 mL ice-cold 75% methanol and 100 μL ribitol solution (4 mg/mL) in 2-mL centrifuge tubes. Samples were incubated in a metal bath at 70°C with 950 rpm shaking for 30 min. After centrifugation at 12,000 × *g* for 10 min, 800 μL supernatant was combined with 750 μL ice-cold HPLC-grade chloroform and 1.4 mL ultrapure water, then vortexed for 10 s. A 100-μL aliquot was vacuum-concentrated for 4 h, derivatized with 40 μL methoxyamine hydrochloride (5 mg/mL) at 37°C with 950 rpm shaking for 2 h, and stored in amber vials for GC-MS analysis.

2.7 | Statistical Data

Data analysis was performed using IBM SPSS Statistics v19.0 (SPSS IBM). One-way analysis of variance (ANOVA) was employed to compare differences between treatment groups, with significance levels set at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Data are presented as the mean ± standard error (SE) of the mean from independent replicates. GraphPad Prism 9.5.1 (San Diego) software was used for data plotting.

3 | Results

3.1 | Engineered Rhizobia Inoculation Enhanced Alfalfa Growth Under Heat Stress

The engineered rhizobia strains were successfully constructed through triparental conjugation, with *S. meliloti* 1021 serving as the chassis strain and the helper strain *Escherichia coli* MT616/pRK600 (Yu et al. 2024). Specifically, the P*lac-ipt* strain was generated by introducing the suicide plasmid pK19*mob*2ΩHMB-*SMc00275*-P*lac-ipt*, while the P*katG-ipt* strain was created through integrating the plasmid pK19*mob*2ΩHMB-*SMc00275*-P*katG-ipt* into the genome. For experimental controls, we utilized both the wild-type *S. meliloti* 1021 and its derivative carrying the empty vector pK19*mob*2ΩHMB- *SMc00275* (designated as pK19). These engineered strains were used for the inoculation of alfalfa seedlings.

After 4-week co-inoculation, the growth and developmental phenotypes of alfalfa under control and heat stress conditions were evaluated. The results revealed that alfalfa inoculated with engineered rhizobia P*lac-ipt* and P*katG-ipt* strains demonstrated significantly reduced incidence of lodging, leaf dehydration, wilting, tissue damage, and chlorosis compared with the wild-type

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Sm1021 and pK19 strains following heat stress, whereas no significant differences were observed under control conditions (Figure 1A–C). Specifically, alfalfa inoculated with the P*lac-ipt* and P*katG-ipt* strains exhibited significantly increased plant height (12.3% and 17.6%), dry weight (2.15-fold and 2.59-fold that of the pK19 strain-inoculated group, respectively), fresh weight (4.00-fold and 4.33-fold that of the pK19 strain-inoculated group, respectively), and relative water content (77.1% and 81.1%) compared to those inoculated with the pK19 strain (Figure 1D–G), though the stem thickness was not varied. These results indicate that inoculation of engineered rhizobia by integrating an exogenous *ipt* gene enhanced alfalfa tolerance to heat stress.

3.2 | Engineered Rhizobia Inoculation Enhance Photosynthetic Efficiency Under Heat Stress

Initial observations indicated that leaves of alfalfa inoculated with P*lac-ipt* or P*katG-ipt* exhibited enhanced chlorophyll retention and reduced wilting compared to those inoculated control strains (Figure 1C). Quantitative analysis revealed that there were no significant differences in the levels of photosynthetic pigments and photosynthetic parameters between the leaves of alfalfa inoculated with the engineered strain and those inoculated with the control strain before heat stress treatment (Figure 2). However, after high temperature treatment, the levels of chlorophyll a (6.09-fold and 5.67-fold), chlorophyll b (2.35-fold and 2.38-fold), total chlorophyll (4.30-fold and 4.09-fold), and carotenoids (1.03-fold and 1.72-fold) in the leaves of alfalfa inoculated with engineered rhizobia were significantly higher than those in the plants inoculated with the control strains (Figure 2A–D). Additionally, the net photosynthetic rate (4.94-fold and 6.56-fold), transpiration rate (7.96-fold and 10.51-fold), intercellular CO_2 concentration (1.14-fold and 1.23-fold), stomatal conductance (8.80-fold and 10.50-fold) and chlorophyll fluorescence parameters (25.3%) and (25.4%) of the alfalfa inoculated with the engineered strains were significantly increased compared to that of the alfalfa inoculated with the control strain (Figure 2E–I). Therefore, inoculation with the engineered strain can enhance the photosynthetic capacity of alfalfa under heat stress.

3.3 | Engineered Rhizobia Inoculation Enhance ROS Scavenging Capacity under Heat Stress

High temperature stress induces a large amount of ROS in leaves, which accelerates leaf senescence. Using NBT and DAB histochemical staining analysis, we found that before high temperature treatment, there was no significant difference in the levels of superoxide anions and H_2O_2 in the leaves of alfalfa inoculated with the engineered strains and the control strains (Figure 3A,B). However, after high temperature stress, the levels of superoxide anions and H_2O_2 in the leaves of alfalfa inoculated with the engineered strain were lower than those in the plants inoculated with the control strain (Figure 3A,B). Quantitative analysis showed that after high temperature treatment, the levels of superoxide anions and H_2O_2 in the leaves of alfalfa inoculated with the engineered strain P*katG-ipt* decreased by 14.9% and 41.4%, respectively, compared to the alfalfa inoculated with the control strain, while only the level of H_2O_2 in the leaves of alfalfa inoculated with the engineered strain P*lac-ipt* decreased by 50.7% (Figure 3C,D). Additionally, by detecting the activity of oxidoreductases in the leaves, it was found that after high temperature stress, the POD activity in the leaves of alfalfa inoculated with the engineered strains increased by 28.1% and 20% compared to the plants inoculated with the control strains (Figure 3E). We also detected the MDA content in the leaves and found it was significantly lower inoculated with the engineered rhizobia than the control strain-inoculated group after heat stress (Figure 3F). These results indicate that inoculation with the engineered strain can significantly reduce the accumulation of ROS and MDA in alfalfa leaves under heat stress conditions.

3.4 | Engineered Rhizobia Inoculation Modified Phytohormone Levels in Alfalfa Leaves Under Heat Stress

We previously reported that both engineered rhizobial strains produced trans-Zeatin (tZ) in free-living medium (Yu et al. 2024). Since tZ is translocated from roots to leaves, its concentrations in alfalfa leaves were quantified at 4, 8, 12, 24, and 72 h post heat treatment. The levels of tZ in alfalfa leaves showed a marked decline between 4 and 8 h of high-temperature treatment, remained relatively stable from 8 to 24 h, and were at their lowest after 72 h of treatment (Figure 4A). In alfalfa plants inoculated with the P*katG-ipt* engineering strain, the tZ levels in the leaves were consistently higher than those in plants treated with the control strain (Figure 4A). Additionally, in alfalfa plants inoculated with the P*lac-ipt* engineering strain, the tZ levels were significantly higher than those in the control strain-treated plants at 4, 12, and 72 h after high-temperature treatment (Figure 4A). These results indicate that alfalfa plants inoculated with the engineering strains had higher tZ levels in their leaves compared to plants inoculated with the control strain.

Including tZ, the levels of five additional phytohormones, indole-3-acetic acid (IAA), salicylic acid (SA), abscisic acid (ABA), Zeatin (Z), and 1-aminocyclopropane-1-carboxylic acid (ACC) were also quantified via liquid chromatography-mass spectrometry (LC-MS). The levels of zeatin and ACC in the leaves of alfalfa inoculated with the engineered strains were always higher than those in the leaves of plants inoculated with the control strains, both under heat treatment and without treatment (Figure 4B and Supporting Figure S1). Interestingly, the IAA levels in the leaves of alfalfa inoculated with the engineered strain were lower than those in the leaves of plants inoculated with the control strains when not exposed to high temperatures, but showed the opposite trend after heat treatment (Figure 4B). Surprisingly, the levels of SA in the leaves were completely opposite to those of IAA; that is, the levels of SA in the leaves of plants inoculated with the engineered strains were significantly higher than those in the leaves of plants inoculated with the control strains when not exposed to high temperatures, but heat stress treatment reversed this trend (Figure 4B and Supporting Figure S1). In other words, after heat stress treatment, the levels of IAA in the leaves of alfalfa inoculated with the engineered strains increased significantly, while SA levels decreased significantly; the opposite trend was observed in the leaves of plants inoculated with the control strains. After high temperature treatment, the levels of ABA in the leaves of alfalfa inoculated with the P*katG-ipt* engineered

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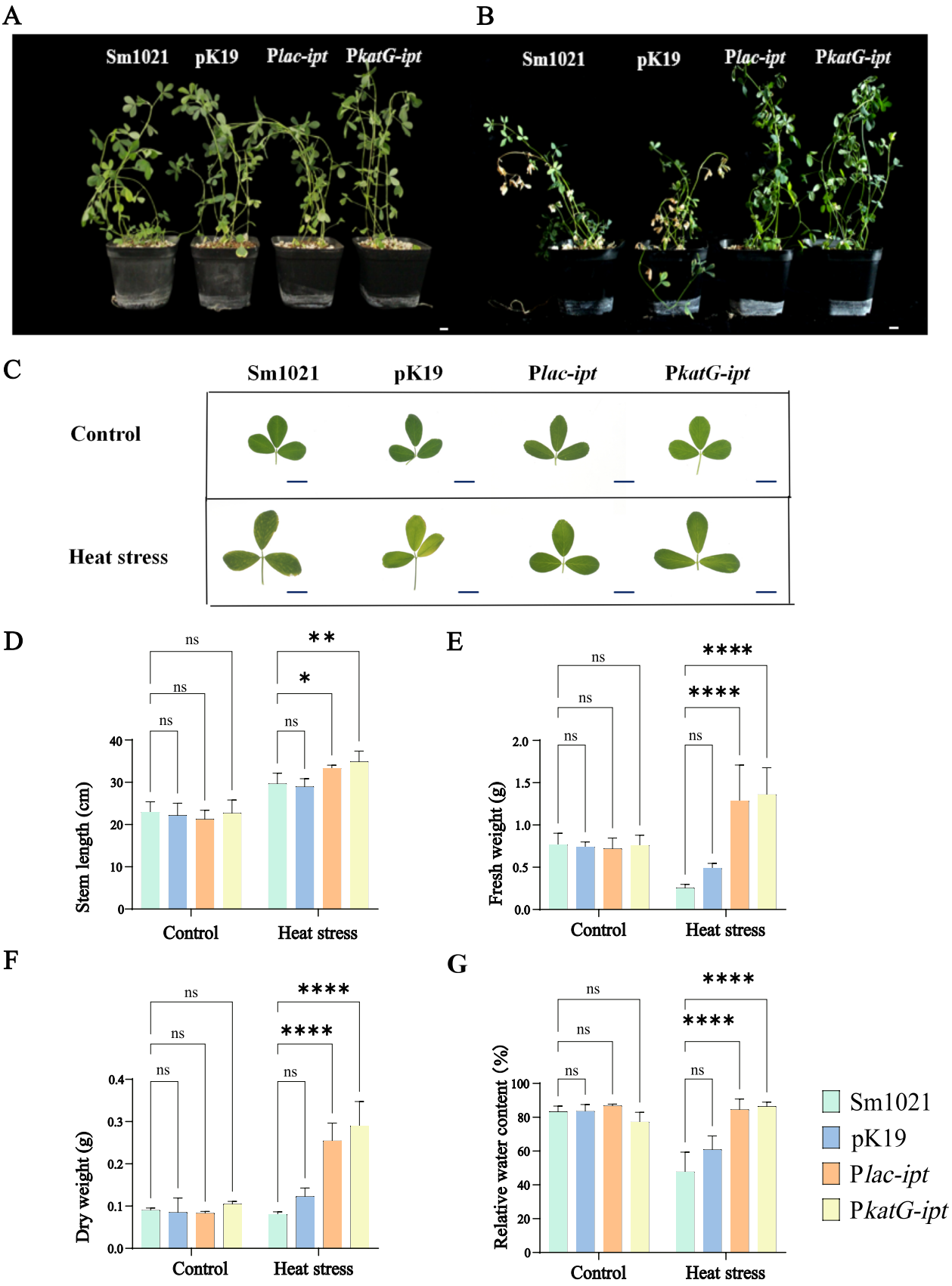


FIGURE 1 | Inoculation of engineered *S. meliloti* expressing the *ipt* gene enhances the tolerance of alfalfa to high temperature stress. Growth phenotypes under control (A). Growth phenotypes after heat stress (B). Leaf morphologies under control and heat treatment conditions (C). Bars = 1 cm. Stem length (D). Fresh weight (E). Dry weight (F). Relative water content (G). One-way ANOVA (Tukey's test) was performed statistically significant differences were indicated by: (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

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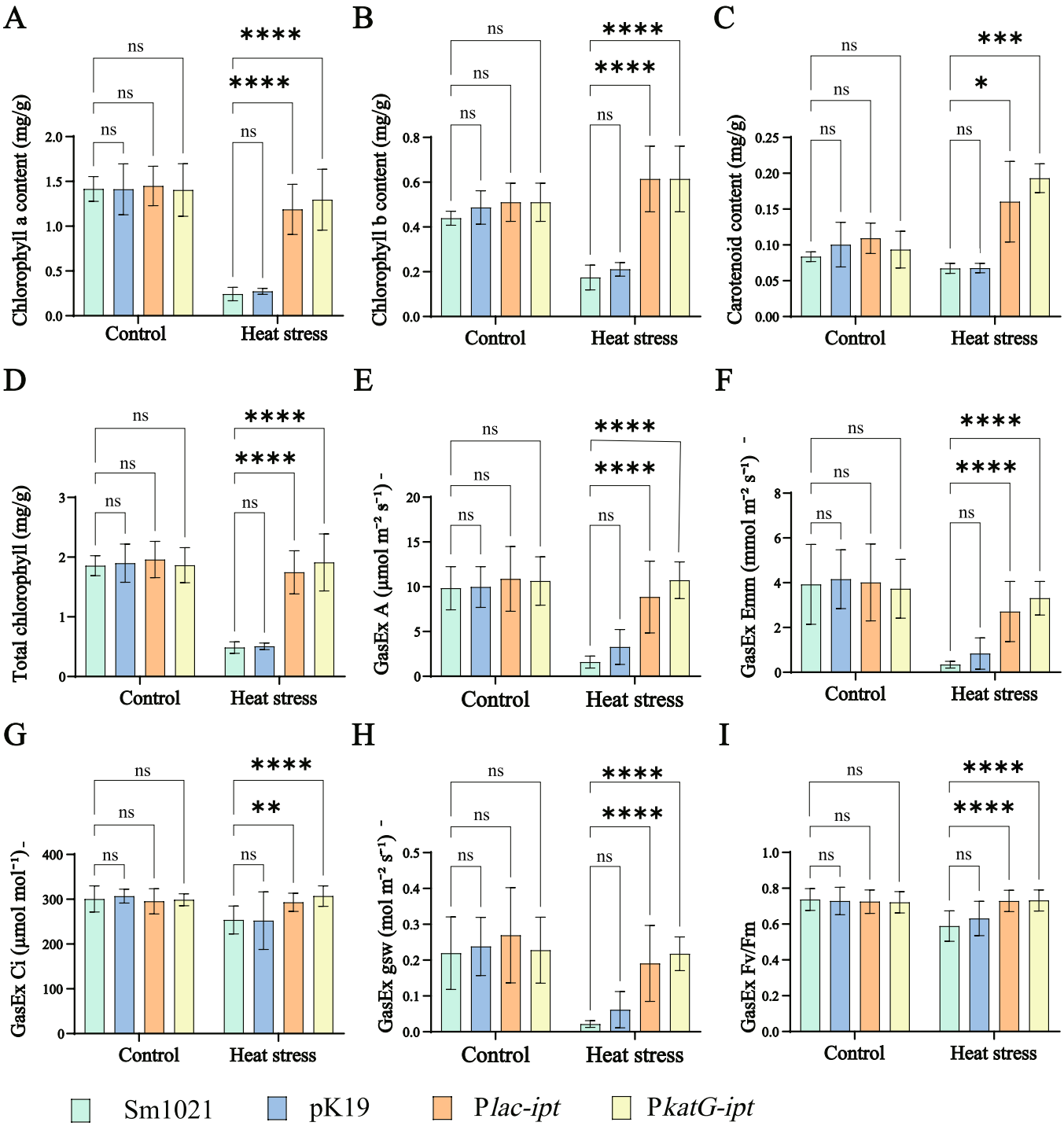


FIGURE 2 | Inoculation of engineered *S. meliloti* strains expressing the ipt gene enhanced the photosynthesis of alfalfa leaves under heat stress conditions. Chlorophyll a content (A), Chlorophyll b content (B), Carotenoids content (C), Total chlorophyll content (D), Net photosynthetic rate (GasExA) (E), Transpiration rate (GasExEmm) (F), Intercellular CO_2 concentration (GasExCi) (G), Stomatal conductance (GasExgsw) (H), and Leaf chamber fluorescence (GasExFv/Fm) (I) in the leaves of alfalfa inoculated with four different Rhizobium strains under control and after heat stress. One-way ANOVA (Tukey's test) was performed statistically significant differences were indicated by: (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

strain tended to decrease compared to the plants that did not undergo heat treatment, while the change in the P*lac-ipt* engineered strain was not significant (Figure 4B and Supporting Figure S1). Thus, inoculation with the engineered strains altered the levels of multiple plant hormones in the leaves of alfalfa under heat stress.

3.5 | Engineered Rhizobia Inoculation Significantly Modulates Leaf Metabolite Profile of Alfalfa Under Heat Stress

To determine the changes in levels of small molecule compounds in the leaves of alfalfa inoculated with the engineered strain, excluding phytohormones, we systematically analyzed

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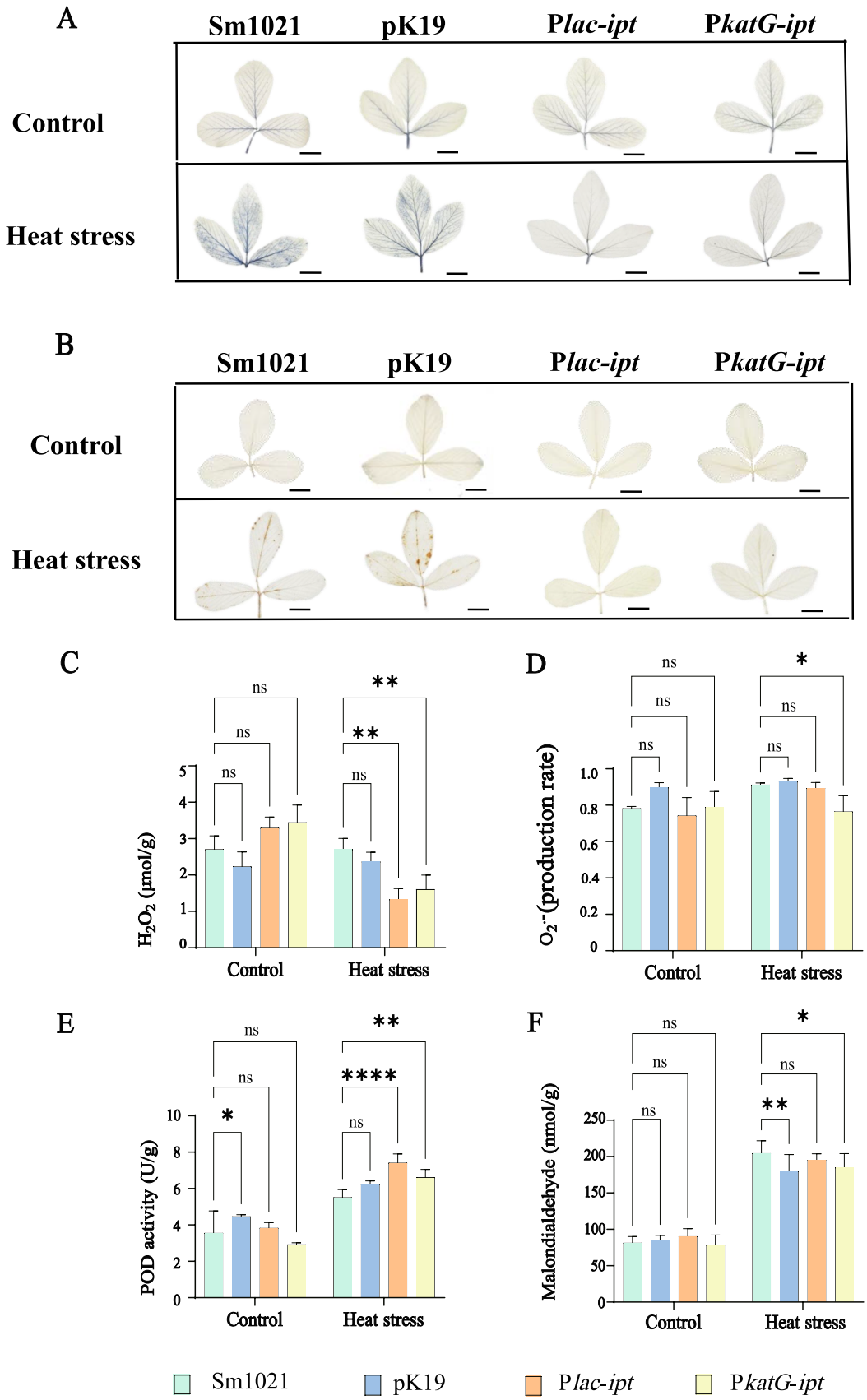


FIGURE 3 | Inoculation of engineered *S. meliloti* strains expressing the *ipt* gene enhanced the oxidative detoxification capacity of alfalfa leaves under heat stress conditions. DAB and NBT staining of alfalfa leaves under control and after heat stress (A, B). Bars = 1 cm. H_2O_2 content (C), $O_2^{\cdot-}$ production rate (D), POD activity (E), and Malondialdehyde content (F) under control and after heat stress. One-way ANOVA (Tukey's test) was performed and statistically significant differences were indicated by: (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

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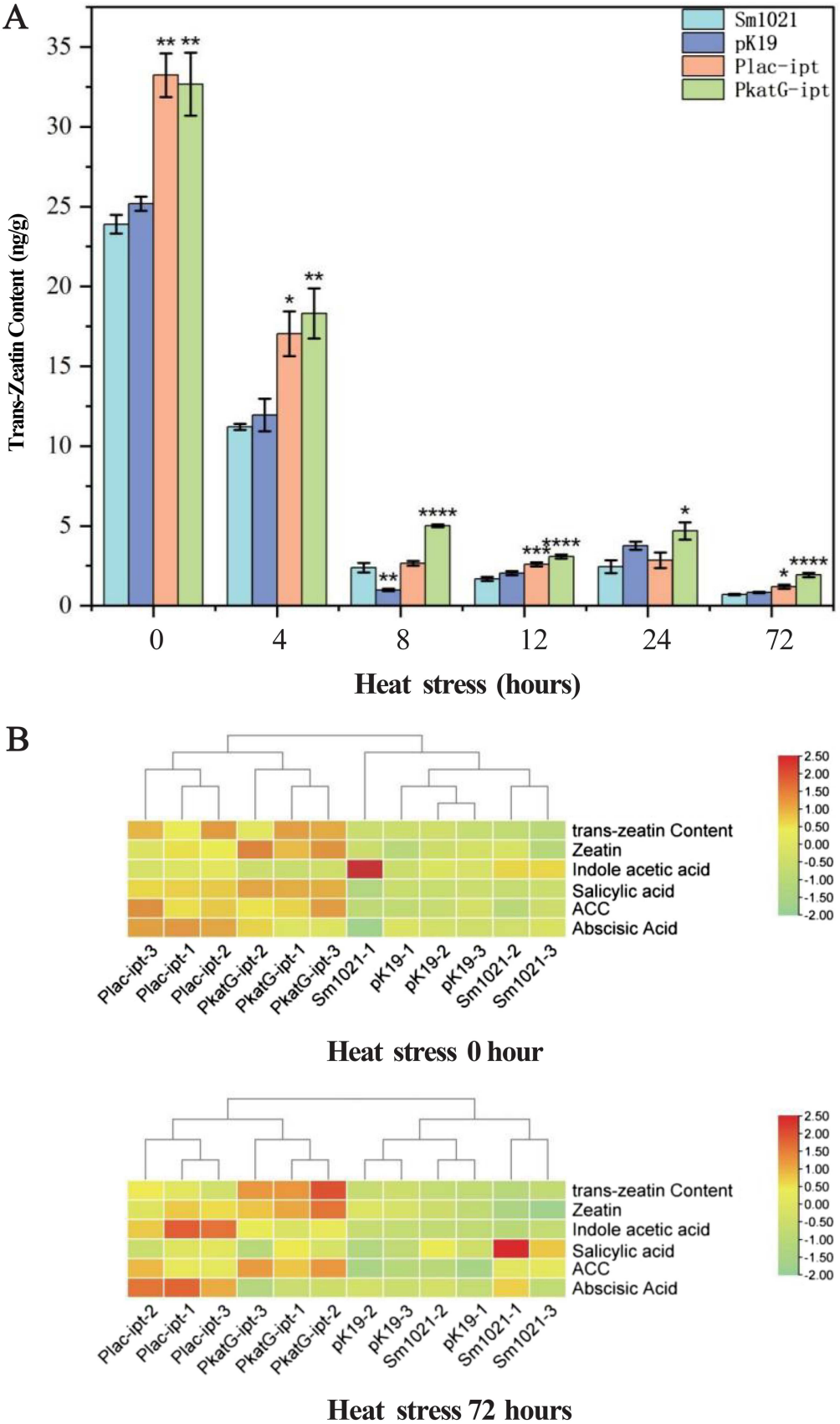


FIGURE 4 | Inoculation of engineered *S. meliloti* strains expressing the *ipt* gene increased the levels of various plant hormones in the leaves of alfalfa. Trans-Zeatin content at 0, 4, 8, 12, 24, and 72 h after heat stress (A). Heat map analysis of hormone content in the leaves of alfalfa inoculated with four different Rhizobium strains under control and heat stress for 72 h (B). One-way ANOVA (Tukey's test) was performed for data and statistically significant differences were indicated by: (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

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the metabolic profiles of alfalfa leaves inoculated with the P*lac-ipt* engineered strain and the Sm1021 control strain using metabolomics techniques. The results showed that, without heat stress treatment, compared to the alfalfa inoculated with the control strain, the leaves of the plants inoculated with the engineered strain had significantly increased levels of protocatechuic acid and including salicylic acid; while the contents p-coumaric acid were significantly reduced (Figure 5A–F). After heat stress treatment, compared to the alfalfa inoculated with the control strain, the leaves of the plants inoculated with the engineered strain had significantly increased levels of gallic acid, ferulic acid, apigenin, proanthocyanidins, and chlorogenic acid, including salicylic acid; while the contents of gentisic acid, isorhamnetin, kaempferol, and p-coumaric acid were significantly reduced (Figure 5G–L). Further analysis revealed that the metabolites increased in the leaves of alfalfa inoculated with both the engineered and control strains after heat stress, included gallic acid, gentisic acid, and fructose-1-phosphate, while the metabolites decreased, included salicylic acid, ferulic acid, proanthocyanidins, p-coumaric acid, and protocatechuic acid. The control strain decreased while the engineered strain increased the levels of apigenin, and the control strain increased while the engineered strain decreased the levels of isorhamnetin and kaempferol (Figure 5A–L). Additionally, the metabolomics data also revealed that before heat stress treatment, there were no significant differences in the levels of organic acids (such as malic acid, quinic acid, citric acid, and succinic acid) and soluble sugars (like fructose, glucose, galactose, and lactose) between the leaves of alfalfa inoculated with the engineered strain and those inoculated with the control strain (Figure 5M,N). However, after heat stress treatment, the levels of these two classes of compounds in the leaves of alfalfa inoculated with the engineered strain showed a significant trend of decrease compared to those inoculated with the control strain (Figure 5O,P). These results indicate that the metabolic profiles in the leaves of alfalfa inoculated with the tZ-overproducing engineered strain undergo significant changes under heat stress.

3.6 | Engineered Rhizobia Inoculation Induced the Differential Gene Expression of Alfalfa Leaves Under Heat Stress

To determine the transcriptional regulatory mechanisms underlying the enhanced heat tolerance in alfalfa inoculated with the engineered strain, we selected leaves from alfalfa inoculated with P*lac-ipt* and Sm1021, extracted mRNA, and performed RNA-seq transcriptomic analysis. Firstly, we selected three representative heat stress genes for PCR quantitative analysis, and samples were collected at 0, 1, 2, 7, and 14 days of heat stress. According to the quantitative expression results, it was found that the expression levels of heat-responsive genes were significantly elevated on day 7 before and after inoculation of engineered rhizobia, as shown in the accompanying figure. Therefore, 7 days and 14 days were selected as the two time points for statistical analysis in the heat stress treatment (Supporting Figure S4). The results showed that after heat treatment, compared to the alfalfa inoculated with the control strain, the alfalfa inoculated with the engineered strain had 3,420 differentially expressed genes ($|\log2FC| > 1$, FDR < 0.05), including 1975 up-regulated genes and 1445 down-regulated genes. In contrast, the alfalfa without heat stress treatment had only 734 differentially expressed genes, among which 343 were up-regulated and 391 were down-regulated (Figure 6A). When comparing the heat-treated and untreated alfalfa inoculated with the engineered strain, there were 5096 differentially expressed genes, including 2606 up-regulated genes and 2490 down-regulated genes, while in the alfalfa inoculated with the control strain, there were 6517 differentially expressed genes, including 3308 up-regulated genes and 3209 down-regulated genes (Figure 6A). The Venn diagram clarified the up-regulated and down-regulated genes after transcriptome sequencing. A total of 1550 up-regulated genes and 1467 down-regulated genes in alfalfa inoculated with the engineered rhizobium (P*lac-ipt*-7-D) compared with control strain (Sm1021-7-D) after 7-day heat treatment; while 288 up-regulated genes and 275 down-regulated genes in alfalfa inoculated with the engineered rhizobium (P*lac-ipt*-0-D) compared with the control strain (Sm1021-0-D) under non-heat treatment (Figure 6B,C). Metmap and KEGG pathway enrichment analysis revealed that the differentially expressed genes in the leaves of alfalfa inoculated with either the engineered or control strain, without heat treatment, were mainly involved in soyasaponin biosynthesis, isoflavones aglycones biosynthesis, and tomatine steroidal glycoalkaloids biosynthesis (Figure 6D and Supporting Figure S2). After heat stress treatment, the differentially expressed genes were primarily enriched in benzoic acid derivatives biosynthesis, soyasaponin biosynthesis, tricin O-glycosides biosynthesis, and tomatine steroidal glycoalkaloids biosynthesis (Figure 6E and Supporting Figure S2). Further analysis indicated that the differentially expressed genes in the leaves of alfalfa inoculated with either of the two rhizobia strains after heat treatment were mainly enriched in heat shock proteins and molecular chaperones, photosynthesis-related proteins, isoflavone synthase, and components of the ROS detoxification system (Figure 6F and Supporting Table S2). This result indicates that the alfalfa inoculated with the engineered strain induced the expression of a large number of genes related to heat stress response and detoxification in the leaves under heat treatment conditions.

Transcriptome analysis results showed that the expression levels of the following genes were significantly different after high-temperature treatment. By reviewing the literature, 12 candidate validation genes in the effective heat stress pathways were identified, including Heat Shock Protein 90 (*MsGHSP90*), Heat Shock Factor A2 (*MsGHsfA2*), Histone H2B (*MsGH2B*), Heat Stress Transcription Factor C1 (*MsGHSFC1*), TGA Transcription Factor 9 (*MsGTGA9*), Sec.1/Sec.4 Homolog 1 (*MsGSEC. 1*), DnaJ Homolog Subfamily B Member 4 (*MsGDNAJB4*), Host Cell Factor 1 (*MsGHCF136*), Flavonoid 3′-Hydroxylase (*MsGF3′H*), Methionine Sulfoxide Reductase B5 (*MsGMSRB5*), and Pyruvate Dehydrogenase Complex II (*MsGPDH2*).

Specifically, each gene plays a crucial and distinct role in plant response to high-temperature stress: *MsGHSP90* functions as a molecular chaperone that assists in the correct folding of other proteins under high-temperature conditions, maintaining intracellular protein homeostasis to enhance plant heat tolerance (Ohama et al. 2017); $MsGHsfA_2$ serves as a pivotal node in the gene expression regulatory network for high-temperature stress responses, activating the transcription of a series of heat

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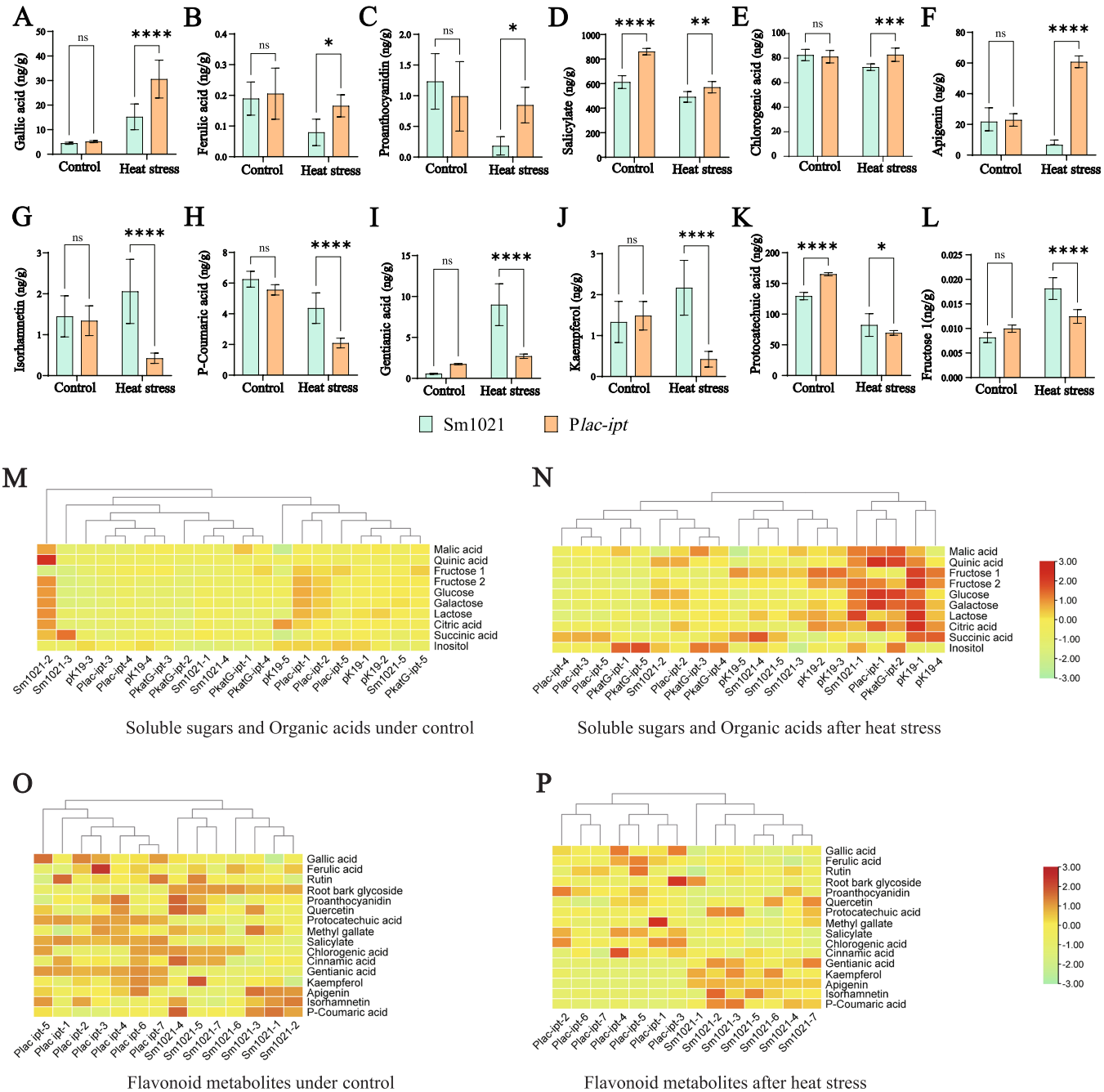


FIGURE 5 | The key metabolites in alfalfa leaves under heat stress after inoculation of engineered rhizobia determined by Flavonoid metabolism. Gallic acid content (A), Ferulic acid content (B), Proanthocyanidin content (C), Salicylic acid content (D), Chlorogenic acid content (E), Apigenin content (F), Isoquercetin content (G), Cinnamic acid content (H), Gentisic acid content (I), Kaempferol content (J), Protocatechuic acid content (K), Furctose 1 content (L). Cluster analysis of sugar and acid metabolites in alfalfa inoculated with four different rhizobial strains (M, N). Cluster analysis of flavonoid metabolites in alfalfa inoculated with four different rhizobial strains (O, P). One-way ANOVA (Tukey's test) was performed statistically significant differences were indicated by: (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

stress-responsive genes to promote plant adaptation to high temperatures (Lee et al. 2022); *MsGH2B* regulates chromatin structure to influence gene accessibility, playing a vital role in modulating gene expression during plant responses to high-temperature stress (Banti et al. 2010); *MsGHSFC1* deeply participates in high-temperature stress signal transduction pathways and is involved in regulating the expression of downstream heat stress-responsive genes (Bobde et al. 2022); *MsGTGA9* interacts with other transcription factors to coordinately regulate physiological responses in plants under high-temperature stress (Zhuang et al. 2018); *MsGSEC. 1* is involved in vesicular transport, which is crucial for maintaining normal intracellular material transport and vesicle fusion under high temperatures, thereby sustaining stable cellular physiological functions (Yoon et al. 2019); *MsGDNAJB4* possesses chaperone activity to assist in protein folding and repair, improving protein stability in high-temperature environments (Karnik et al. 2013); *MsGHCF136* regulates multiple cellular

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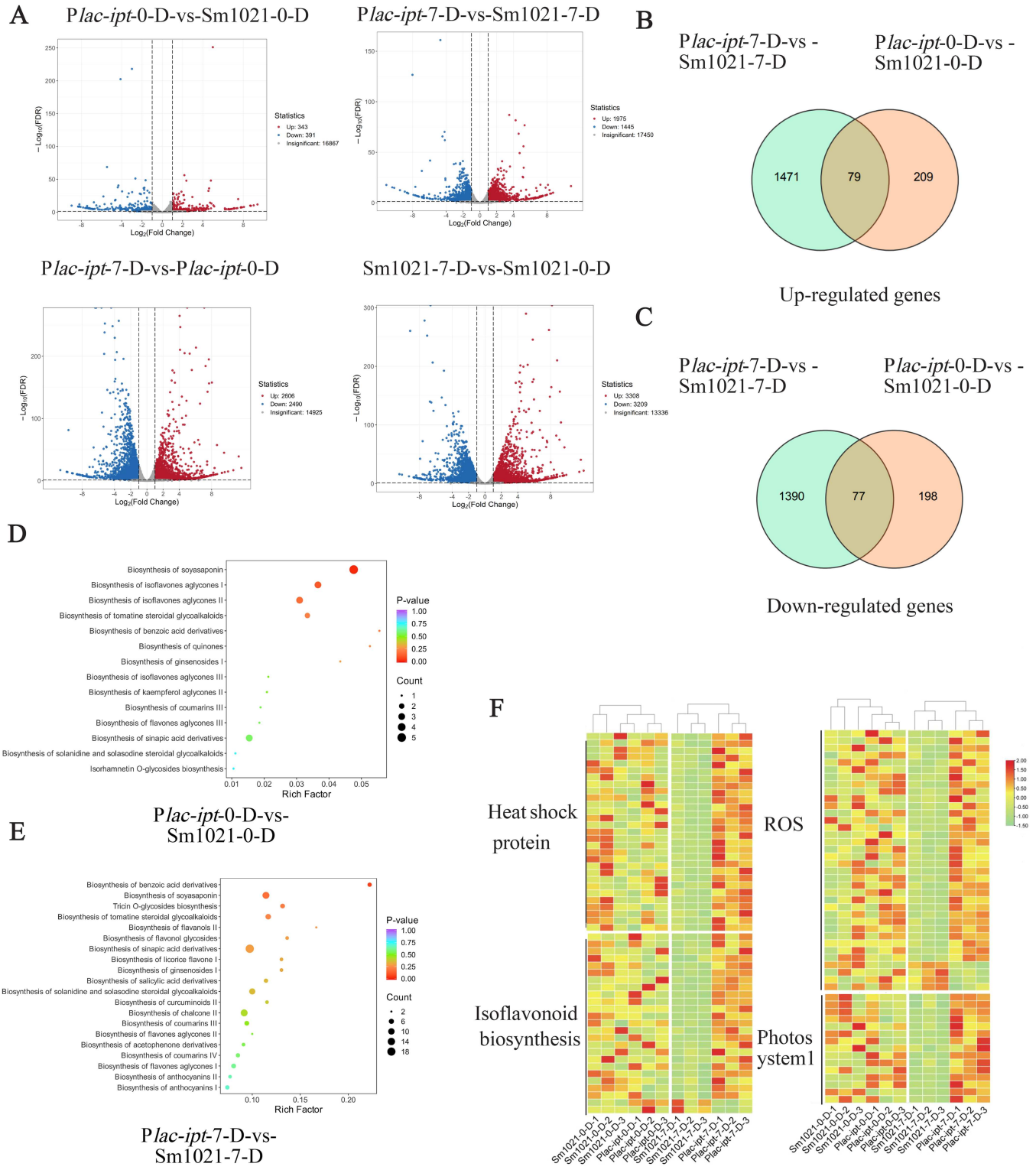


FIGURE 6 | Inoculation with engineered rhizobia altered the expression of antioxidant and photosynthetic genes in alfalfa leaves under heat stress determined by transcriptome analysis. Volcano plots of differentially expressed genes (DEGs) (A). Venn diagram of upregulated DEGs (B). Venn diagram of downregulated DEGs (C). Metabolic pathway enrichment scatter plots: P*lac-ipt*-0-D versus Sm1021-0-D (D), P*lac-ipt*-7-D versus Sm1021-7-D (E). Heat map analysis of DEGs in P*lac-ipt*-7-D vs Sm1021-7-D, including heat shock protein, isoflavonoid biosynthesis, ROS, photosystem 1 (F). [Color figure can be viewed at wileyonlinelibrary.com]

physiological processes, providing positive support for high-temperature stress responses (Fang et al. 2023); *MsGF3'H*, as a key enzyme in the flavonoid metabolic pathway, exhibits up-regulated expression to promote the synthesis of flavonoid compounds, enhancing plant antioxidant capacity to defend against high-temperature stress (Gilg et al. 2016); MsMSRB5 can repair proteins damaged by oxidative stress, serving as a core factor in maintaining cellular protein function and heat

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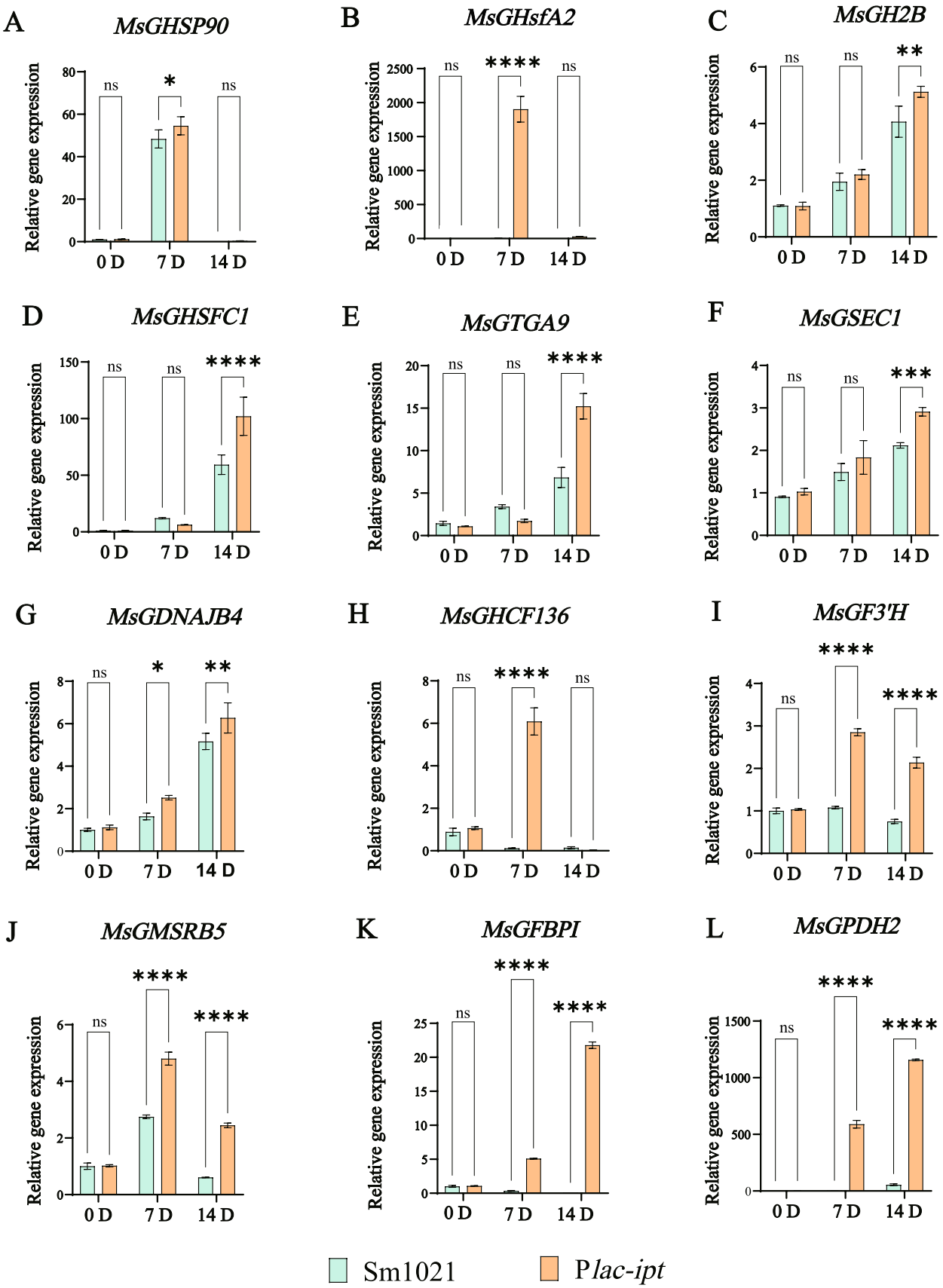


FIGURE 7 | Legend on next page.

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stress tolerance (Yu et al. 2023); *MsPDH*$_2$ participates in carbohydrate metabolism to supply energy for cells under high-temperature conditions, ensuring the normal operation of physiological metabolism (Braga 2014).

These findings indicate that the aforementioned genes exhibit significant positive regulation in response to high-temperature stress treatment in alfalfa inoculated with engineered rhizobia. Through their distinct biological functions, they synergistically enhance alfalfa's tolerance to high-temperature stress across multiple levels, including protein homeostasis maintenance, gene expression regulation, cellular material transport, antioxidant defense, and energy metabolism. This provides crucial experimental evidence and theoretical support for elucidating the molecular mechanisms by which engineered rhizobia enhance alfalfa's stress resistance.

4 | Discussion

As global warming accelerates and abnormal weather becomes more frequent, high temperatures or heat stress pose a significant threat to crop production. Applying the principles of microbe-plant interactions, developing new biotechnologies, such as agricultural synthetic biology, offers novel strategies to address this global issue. PGPR regulate the growth and development of host plants by synthesizing hormones, enhancing their tolerance to stresses like drought, salinity, and high temperatures or heat. Cytokinins play a crucial role in plant growth, development, and stress tolerance. *IPT*, the rate-limiting enzyme in cytokinin synthesis, has been extensively used in the cultivation of transgenic plants. Most of these transgenic plant lines, driven by senescence-related promoters, exhibit exceptional drought tolerance. However, apart from creeping bentgrass (*Agrostis stolonifera* L.) (Xu et al. 2009), there have been no reports on the heat tolerance of these transgenic plants. In alfalfa, *IPT* transgenic lines have been reported to effectively delay leaf senescence, but no evidence of tolerance to abiotic stresses such as drought, salinity, or high temperatures has been documented. Our previous research leveraged the symbiotic interaction between alfalfa and soil rhizobia, creatively overexpressing the *ipt* gene in rhizobia to produce tZ, which significantly enhanced alfalfa's drought tolerance after inoculation (Xu et al. 2012; Yu et al. 2024). However, the heat tolerance of alfalfa inoculated with these genetically engineered rhizobia strains has not been studied. This paper is the first to systematically investigate the heat tolerance of alfalfa inoculated with engineered rhizobia producing tZ, revealing that it significantly enhances the heat tolerance of the host plant. The physiological mechanism involves a noticeable increase in tZ levels in the leaves, altering the normal hormone homeostasis (increased IAA levels and decreased SA levels), reducing the damage of heat stress on chlorophyll, maintaining or even improving photosynthetic efficiency, increasing the expression of antioxidant enzymes and the levels of antioxidant molecules, and changing the metabolism balance, thereby delaying or preventing heat stress-induced leaf senescence and plant death (Figure 8).

Firstly, alfalfa inoculated with the engineered rhizobia producing tZ exhibited similar phenotypes to *IPT* transgenic plants when compared with controls after high-temperature treatment. This included slight wilting of the leaves, which basically did not turn yellow (Figure 1B,C and Xu et al. 2009), and an increase in chlorophyll content (Figure 2A-D and Xu et al. 2009). However, unlike the *IPT* overexpressing creeping bentgrass, alfalfa inoculated with the engineered rhizobia showed a significant increase in yield (fresh and dry weight) after high-temperature treatment (Figure 1E,F and Xu et al. 2009). This demonstrates that using engineered bacteria producing tZ to inoculate host plants can result in heat tolerance phenotypes similar to or even better than *IPT* transgenic plants.

The heat and drought tolerance of alfalfa inoculated with the engineered bacteria was significantly improved (Figure 1 and Yu et al. 2024), with higher chlorophyll levels under drought and high temperature stress compared to the control strains (Figure 2 and Yu et al. 2024). Additionally, the levels of superoxide anion and hydrogen peroxide were lower, while the activity of the reducing enzyme POD was increased in the inoculated plants compared to the controls (Figure 3 and Yu et al. 2024). Notably, under high temperature conditions, the engineered bacteria maintained photosynthetic efficiency better than under drought conditions (Figure 2 and Yu et al. 2024). Under drought stress, alfalfa inoculated with the engineered strain showed significantly reduced MDA content and increased levels of proline, SOD, and APX activity (Yu et al. 2024). Under high-temperature stress, the proline content in P*lac-ipt* decreased by 57.4% compared to Sm1021, and the proline content in P*katG-ipt* decreased by 54.7% compared to Sm1021. The ascorbate peroxidase (APX) content in Plac-ipt decreased by 49.5% compared to Sm1021, and the APX content in P*katG-ipt* decreased by 53.6% compared to Sm1021 (Supporting Figure S3). This suggests that after inoculation with the engineered rhizobia producing tZ, alfalfa exhibits different physiological mechanisms in responding to and adapting to drought and high temperature stress.

The disruption of phytohormone balance in the leaves of alfalfa inoculated with the engineered rhizobia producing tZ after high-temperature treatment is the main reason for its enhanced heat tolerance. Firstly, the levels of IAA reversed before and after high-temperature treatment (Figure 4B), indicating that there may be an antagonistic interaction between cytokinins and auxins before high-temperature treatment, while a positive

FIGURE 7 | Validation of the key differential expression genes in alfalfa leaves under heat stress after inoculation of engineered rhizobia by qRT-PCR. Relative expression of DEGs in alfalfa leaves inoculated with Sm1021- and Plac-ipt- strains under control and after heat stress. Heat Shock Protein 90 (MsGHSP90) (A), Heat Shock Transcription Factor A2 (MsGHSfA2) (B), Histone H2B (MsGH2B) (C), Heat Stress Transcription Factor C1 (MsGHSFC1) (D), TGA Transcription Factor 9 (MsGTGA9) (E), Sec. 1/Sec. 4 homology protein 1 (MsGSEC. 1) (F), DnaJ homolog subfamily B member 4 (MsGDNAJB4) (G), Host Cell Factor 1, 36 kDa (MsGHCF136) (H), Host Cell Factor 1, 36 kDa (MsGF3'H) (I),(I), Methionine Sulfoxide Reductase B5 (MsGMSRB5) (J), Fructose-1-phosphate kinase (MsGFBPI) (K), Pyruvate Dehydrogenase Complex 2 (MsGPDH2) (L). One-way ANOVA (Tukey's test) was performed for data and statistically significant differences were indicated by: (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

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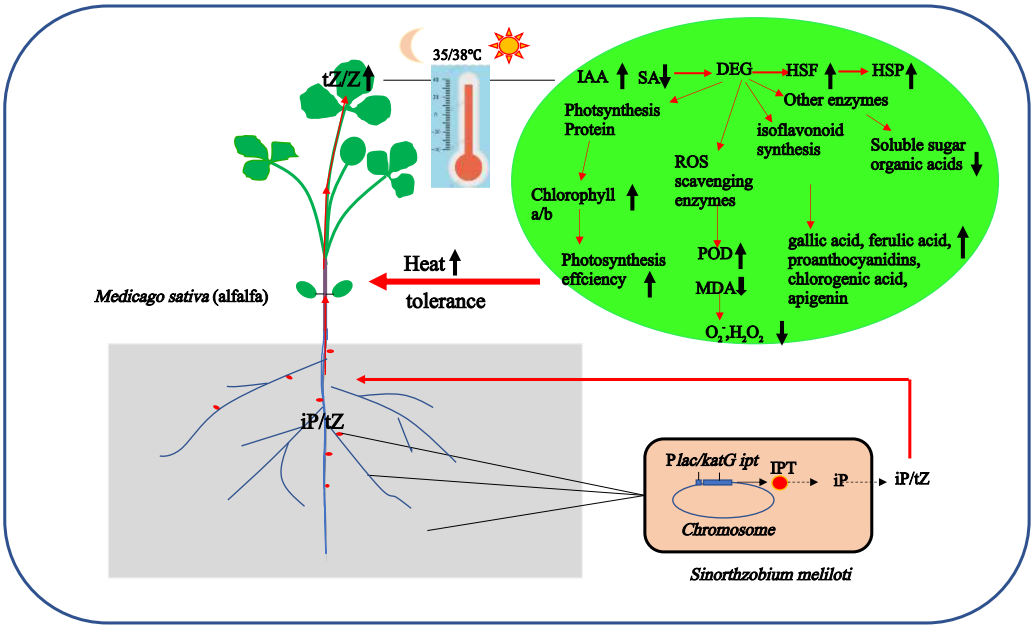


FIGURE 8 | Possible Mechanism of Enhanced Heat Tolerance in Alfalfa (*Medicago sativa*) Inoculated with an Engineered *Sinorhizobium meliloti* Strain Expressing the *ipt* Gene: the engineered rhizobia synthesize trans-zeatin or its precursors in the roots or root nodules, which are then transported to the leaves via stems, leading to elevated levels of trans-zeatin in the leaves. Under high-temperature stress, this process induces an increase in IAA (Indole-3-acetic acid) levels and a decrease in SA (Salicylic acid) levels, resulting in the differential expression of a large number of genes. These differentially expressed genes are involved in metabolic pathways including: the heat shock response (transcription factors and heat shock proteins), the photosynthetic response (photosynthetic proteins, chlorophyll synthesis, and photosynthetic efficiency), ROS degradation (detoxifying enzymes such as POD, and the decomposition of superoxide anions and hydrogen peroxide), flavonoid biosynthesis (enzymes and metabolites), and the synthesis of soluble sugars and organic acids. The differentially expressed genes from these various metabolic pathways, along with the metabolites the encoding enzymes produced, contribute to varying degrees to the heat stress tolerance of alfalfa. [Color figure can be viewed at wileyonlinelibrary.com]

synergistic effect appears after high-temperature treatment. This is an important finding of this paper and corresponds to previous reports that cytokinins balance IAA levels, alter root system architecture, and adapt to cold stress (Tiwari and Lovelace 2023). Secondly, the levels of SA also reversed before and after high-temperature treatment, but the pattern was completely opposite to that of auxins (Figure 4B). This suggests that before high-temperature treatment, cytokinins and SA have a synergistic effect, while after high-temperature treatment, they exhibit an antagonistic effect. This is consistent with previous reports that under high-temperature stress, cytokinins and SA are antagonistic, affecting the expression of oxidoreductases (Chen et al. 2019). Therefore, cytokinins and auxins, along with SA, synergistically regulate the senescence process of alfalfa leaves under heat stress, which provides new insights for further research on the mechanisms of leaf senescence in alfalfa.

Alfalfa inoculated with genetically modified rhizobia exhibited significantly enhanced heat tolerance due to the accumulation of multiple isoflavonoid compounds, including gallic acid, ferulic acid, proanthocyanidins, chlorogenic acid, and apigenin. This accumulation likely represents the key mechanism underpinning the increased heat resistance, with these flavonoid molecules potentially exerting their effects by participating in ROS detoxification processes during thermal stress. Concurrently, the characteristic accumulation of lower levels of soluble sugars and organic acids (Figure 5M,N) suggests that metabolic homeostasis regulated by cytokinins may also participate in the regulation of heat tolerance. This finding resonates with studies on oilseed rape (*Brassica napus* L.) under varying moisture conditions. These investigations demonstrate that genotype, growth stage, and water status collectively influence the plant's growth, physiological and biochemical characteristics, and functional activity (Salami et al.2023). Furthermore, drought-tolerant genotypes can be identified through scientifically validated screening criteria. Specifically, analysis of 12 rapeseed genotypes under field water-holding capacities of 10% (drought) and 30% (control) revealed that while drought reduced growth traits by 23%–47%, it significantly enhanced pigment, phenolic, and flavonoid content alongside antioxidant activity. In genotypes *G01* and *G08*, specific compounds such as epicatechin showed positive correlations with anti-glycation activity, and phenolic content in flowering-stage extracts exceeded that in leaves and seeds. Further evaluation of 119 rapeseed genotypes (combining drought tolerance indices, PCA, and MGIDI) revealed that drought caused reductions in fresh weight, dry weight, and relative moisture content. The MGIDI model demonstrated significant efficacy in screening drought-tolerant genotypes. This study provides a theoretical foundation for drought tolerance screening and high-value utilization of rapeseed (Salami et al. 2025).

The differential gene expression induced by heat stress in alfalfa inoculated with the engineered strain producing trans-zeatin is the main reason for its enhanced heat tolerance. These differentially expressed genes include heat shock regulators such as $HsfA_2$ and *HSFC1* genes, photosynsis proteins, antioxidant enzymes, and biosynthesis enzymes for small molecule

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compounds (Figures 6 and 7), which are basically consistent with the previously observed phenotypes (Figures 1–3) and quantitative results (4–5). Our results are consistent with the proteomic data reported in creeping bentgrass, where several heat shock proteins, antioxidant-related enzymes, and photosystem II proteins were upregulated in shoots overexpressing an *IPT* gene (Xu et al. 2009). This is mainly consistent with the results of high-temperature treatment (Figure 7), whereas SOD and APX were upregulated during drought treatment but downregulated or showed no difference during high-temperature treatment, which is consistent with the enzyme activity results (Figure 3E,F). These data reflect the different effects of the engineered rhizobia on alfalfa under two different stresses.

During the course of our research, we also found that there was no significant difference in the amount of tZ synthesized and the effect on inoculated alfalfa between the strains P*katG-ipt* and P*lac-ipt*. In the future, we will continue to construct engineered strains that express *ipt* induced by hydrogen peroxide or heat stress, aiming to achieve expression only under stress conditions. Additionally, it needs further verification whether the rhizobia synthesize and transport trans-zeatin after forming nodules with plants, or synthesize isopentenyladenine and then transport it to the plants, and tZ is generated in the leaves.

In conclusion, inoculating alfalfa with engineered rhizobia that produce tZ has indeed improved its heat tolerance, with an effect almost comparable to that of *IPT*-overexpressing transgenic plants. The mechanism involves altering the hormone balance in the leaves, regulating the expression of genes related to photosynthesis and detoxification, and delaying leaf senescence (Figure 8). This study not only reveals that the engineered rhizobia that produce tZ can enhance the drought tolerance of alfalfa but also its heat stress tolerance, and potentially its ability to withstand combined drought and heat stress, providing a new technical approach for the production of alfalfa in an era of global warming and abnormal climate.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Figure S1: Determination of phytohormones content in alfalfa under control and heat stress at 0, 4, 8, 12, 24, and 72 hours.

Supplementary Table S1: Primer sequences used for RT-qPCR.

Supplementary Table S2: Differentially expressed genes involved in the heat stress response for heat map analysis in alfalfa.

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