RESEARCH ARTICLE

Enhanced ganoderic acids production by using thermotolerant *Ganoderma tsugae* at high-temperature liquid cultivation

Quan Ma[☯], **Yang-Meng-Jie Jing**[☯], **Li-Yuan Luo**, **Xiao-Long He**, **Yue-Qin Liu***

School of Life Science, Yan'an University, Yan'an, Shanxi, China

☯ These authors contributed equally to this work
* liuyue811223@126.com

Abstract

Ganoderic acids (GAs) are bioactive triterpenoids produced by *Ganoderma* species with demonstrated anticancer properties. While the yield of GAs in *Ganoderma tsugae* is typically low, heat stress has been shown to enhance its production. This study employed atmospheric and room temperature plasma (ARTP) mutagenesis to develop thermotolerant *G. tsugae* mutants for high-temperature cultivation at 35°C. From 59 mutants generated, strain *Ganoderma tsugae* 9 (GT9) demonstrated superior thermotolerance, showing 51.48% increased mycelial growth rate and 76.03% higher biomass compared to wild-type (WT) at 35°C (one-way ANOVA with Dunnett's test, $p < 0.05$). Physiological characterization revealed GT9 possessed enhanced membrane fluidity, elevated intracellular levels of lanosterol (92.49% increase), squalene (1.36-fold increase), trehalose, and ergosterol (66.98% increase) (two-way ANOVA with Tukey's test, $p < 0.05$). Transcriptional analysis revealed significant upregulation of key GAs biosynthetic genes (*hmgr*, *sqs*, *se*, *ls*) and heat shock protein genes (*hsp17.4*, *hsp22*, *hsp70*, *hsp90*). After 10-day cultivation at 35°C, GT9 produced 1.01-fold more GAs than the WT at 35°C and 22.64% more than the WT at 25°C (two-way ANOVA with Tukey's test, $p < 0.05$). However, the difference in GAs production between the WT strain cultured at 25°C and the GT9 strain cultured at 35°C was not significant. ARTP-generated thermotolerant *G. tsugae* mutants enable efficient high-temperature fermentation for enhanced GAs production. This strategy provides significant advantages for industrial-scale application while elucidating the physiological and molecular mechanisms underlying improved GAs biosynthesis under heat stress.

OPEN ACCESS

Citation: Ma Q, Jing Y-M-J, Luo L-Y, He X-L, Liu Y-Q (2026) Enhanced ganoderic acids production by using thermotolerant *Ganoderma tsugae* at high-temperature liquid cultivation. PLoS One 21(3): e0345065. https://doi.org/10.1371/journal.pone.0345065

Editor: Olaf Kniemeyer, Leibniz-Institut fur Naturstoff-Forschung und Infektionsbiologie eV Hans-Knoll-Institut, GERMANY

Received: August 2, 2025

Accepted: February 27, 2026

Published: March 17, 2026

Data availability statement: All relevant data are within the manuscript.

Funding: This work was financially supported by the National Natural Science Foundation of China (No.22268045), the Special Scientific Research Project for Serving Local Areas under

Introduction

Ganoderma is a renowned medicinal fungus in traditional Chinese medicine, valued for over 2000 years for its pharmacological properties in treating various diseases [1].

the Shaanxi Provincial Department of Education (24JC088). The funders of this study had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Ganoderma tsugae, a conifer-associated species predominantly distributed throughout Northeast China [2], produces ganoderic acids (GAs)-oxygenated triterpenoids recognized as critical bioactive metabolites with substantial therapeutic potential. The mevalonate pathway mediates GAs biosynthesis, with squalene and lanosterol serving as key intermediates. The enzymes hydroxymethylglutaryl-CoA reductase, squalene synthase, squalene epoxidase, and lanosterol synthase (encoded by the genes *hmgr*, *sqs*, *se,* and *ls*, respectively) govern the metabolic flux directed toward GAs production [3]. Lanosterol undergoes extensive enzymatic modification including oxidation, reduction, and acetylation to generate either membrane-integral ergosterol or various GAs derivatives [4].

The yield of GAs is commonly used as a key indicator for assessing the overall quality of *Ganoderma* [5]. Despite their important pharmacological value-such as anti-aging, anti-cancer, anti-inflammatory, and metabolic regulation activities [6–9]-the low native production of GAs remains a major limitation for commercial application. Compared to the protracted cycle of traditional fruiting body cultivation, submerged fermentation presents distinct technical advantages for the industrial production of fungal metabolites, primarily through a significantly shortened cultivation period and the ability to achieve consistent, high-yield production in a controlled bioreactor environment [10]. Current research and industrial efforts to enhance GAs yield are primarily focused on several key strategies: culture optimization [11,12], elicitor application [13–15], genetic engineering [16–17], and synthetic biology [18,19]. Transcriptional regulation through transcription factors and signaling pathways presents additional optimization opportunities [20–22].

Environmental conditions are a critical determinant of fungal growth, development, and secondary metabolism (SM) production. Among these conditions, temperature stands out as particularly pivotal [23]. Temperature stress particularly affects GAs biosynthesis in *G. lucidum*, including metabolic restructuring and membrane fluidity adaptation [24,25]. Heat shock proteins (HSPs) and compatible solutes like trehalose provide critical stress protection under heat stress (HS) [26,27].

However, the current research mainly focuses on transient high-temperature stimulation [15,28], sustained high-temperature fermentation remains unexplored. Currently, developing microbial strains with inherent thermotolerance is a major focus in industrial fermentation, as it can reduce cooling costs, minimize contamination risks, and enhance reaction kinetics [7]. *G. tsugae* mycelium exhibits optimal growth at 22–25°C, while temperatures above 25°C (notably 28–30°C) cause clear growth inhibition [1]. This pronounced sensitivity to moderate HS underscores the species' limited thermotolerance. ARTP mutagenesis is a well-established and highly effective approach for microbial breeding, and it has been successfully implemented in diverse fungi such as *Hericium erinaceus*, *Grifola frondosa* and *Aspergillus* [29–31]. Consequently, we employed ARTP mutagenesis to breed mutant *G. tsugae* strains with improved tolerance to 35°C (35°C is defined as a high-temperature stress condition because it represents a severe deviation (over 10°C) from the optimal range, imposing substantial physiological challenges that are expected to profoundly impact cellular processes and secondary metabolism), effectively broadening its optimal

growth range by approximately 10°C and enabling sustained high-temperature liquid fermentation. Then, the extracellular enzyme activity, secondary metabolites and specific gene expression were measured and analyzed. Consequently, our findings demonstrate that high-temperature fermentation using a thermotolerant *G. tsugae* strain is a promising strategy for enhancing GAs yield. This approach not only offers a viable industrial alternative but also provides a valuable platform for further elucidating the biosynthesis of GAs and other secondary metabolites.

Materials and methods

Strains and culture conditions

G. tsugae strain G-1 was originally isolated from a wild fruiting body collected in Mohe City, Greater Khingan Mountains, Heilongjiang Province, China. The field sampling did not require specific permits as the collection location is not privately owned or protected, and the study involved only common fungal species not listed as endangered or protected under Chinese regulations. Strain identification was confirmed by ITS sequencing (GenBank accession unmber: JQ781853). The optimal culture conditions for mycelial growth were determined as: glucose as carbon source, 60 g/L Tsuga sawdust extract, incubation temperature of 25°C, and initial pH 5.0. The wild-type (WT) strain was maintained on potato dextrose agar (PDA) medium (200 g/L potato infusion, 20 g/L glucose, 20 g/L agar) at 4°C. The WT and mutant *G. tsugae* strains were cultivated on solid PDA or in liquid fermentation medium at 25°C in darkness. The liquid fermentation medium was prepared using a Tsuga sawdust extract solution (60 g/L, pH 5.0) and contained the following components per liter: potato infusion (from 200 g peeled and diced potato); glucose, 30 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; and vitamin B, 0.02 g.

Protoplast preparation

Protoplast preparation was performed according to Feng et al.with modifications [10]. Briefly, *G. tsugae* strains were cultured on PDA plates at 25°C for 7 days. Then, four mycelial blocks (1 cm diameter) were inoculated into 250 mL flasks containing 100 mL liquid fermentation medium and incubated at 180 rpm, 25°C for 7 days. The cultures were homogenized for 15 seconds and subsequently returned to shaking for 24 hours. Mycelia were collected by filtration through nonwoven fabric and washed three times with 0.5 M mannitol. After centrifugation at 8,000 × g for 15 minutes, the pellet was treated with 2% lysing enzyme solution (1 mL per 300 mg mycelia) and incubated at 180 rpm, 30°C for 3 hours. The enzymatic hydrolysate was filtered, mixed with 0.5 M mannitol (1:1 ratio), and centrifuged at 4,000 × g for 5 minutes. This washing process was repeated three times. Protoplast concentration was adjusted to 10^8 cells/ mL with 0.5 M mannitol.

ARTP mutagenesis

ARTP mutagenesis was performed according to previously described methods [10,29,30] with minor modifications. Aliquots (90 μL) of the protoplast suspension were mixed with 10 μL sterile glycerol, and 20 μL of this mixture was subjected to ARTP treatment using the ARTP-IIS system(Wuxi Yuanqing Tianmu Biotechnology Co., Ltd., Jiangsu, China). The mutagenesis parameters were: temperature 25°C, treatment time 50 seconds, helium flow rate 10 L/min, irradiation distance 2 mm. After treatment, the protoplasts were transferred to 650 μL of 0.6 M mannitol solution, and 200 μL aliquots was spread onto regeneration medium (109.3 g/L mannitol, 10 g/L glucose, 5 g/L maltose, 5 g/L yeast extract, 20 g/L agar). Plates were incubated in darkness at 25°C for 5 days. Larger colonies were selected and transferred to fresh PDA plates for further cultivation at 25°C for 10 days in darkness. Regenerated colonies were numbered by GT numbers.

Screening of thermotolerant mutant

Mutant identification was performed using an established antagonism assay [10]. Putative mutant and WT strains were inoculated on opposite sides of PDA plates (2 cm apart) and cultured at 25°C in darkness. The presence of an inhibition zone confirmed genetic differentiation. For thermotolerance screening, mycelial blocks (1 cm diameter) from

fifth-generation subcultures were inoculated onto PDA plates and cultured at 35°C for 8 days. Strains showing superior growth rates were selected for secondary screening in liquid culture. Four mycelial blocks of each candidate strain were inoculated into 250 mL flasks containing 100 mL fermentation medium and incubated at 180 rpm, 35°C for 8 days. Mycelial biomass were collected, washed with deionized water three time, and dried at 50°C to a constant weight. According to the dry weight, the strain exhibiting the highest biomass production at 35°C was selected for further study.

High-temperature culture

Four mycelial blocks of thermotolerant mutants and WT strains were inoculated into 250 mL flasks containing 100 mL fermentation medium. Cultures were initially maintained statically at 35°C for 2 days, followed by shaken incubation at 180 rpm for 8 days. Parallel WT strain was cultured under optimal condition (25°C) using the same protocol. Mycelia were harvested for subsequent analyses of biomass, membrane fluidity, secondary metabolites, and gene expression.

Extracellular enzyme activity assays

Culture supernatants were collected by centrifugation at 8 000 × g for 20 minutes and used as crude enzyme extract. Amylase, cellulase and laccase activities were determined using commercial assay kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to manufacture's instructions.Absorbance was measured at 660 nm, 540 nm, and 420 nm for amylase, cellulase and laccase, respectively.

Gene expreession analysis by qRT-PCR

Total RNA was extracted from liquid nitrogen-powdered mycelia using TRI zol reagent. c DNA synthesis was performed using PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). qRT-PCR was conducted with the LightCycler SYBR Green I Master Kit (Roche Diagnostics, Basel, Switzerland) on a LightCycler 96 system. The housekeeping gene 18S rRNA served as the internal control, and relative expression levels were calculated using the 2-ΔΔCT method [32]. Gene-specific primers were designed based on the *G. lucidum* genome sequence [26,33].

Membrane fluidity measurement

Membrane fluidity was assessed by fluorescence anisotropy using a HORIBA FluoroMax-4 spectrofluorometer (HORIBA Scientific, Edison, NJ, USA) at 37°C, following established protocols [25]. Decreased fluorescence anisotropy values indicated increased membrane fluidity.

Trehalose quantification

Trehalose content was determined using the anthrone method with a commercial trehalose assay kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). Mycelia samples (100 mg) were extracted and mixed with 1.0 mL working solution, heated at 95°C for 10 minutes, then cooled rapidly. Absorbance was measured at 620 nm.

Metabolite analysis

GAs, ergosterol, squalene and lanosterol were extracted and analyzed using an ultra-performance liquid chromatography (UPLC) system (Waters Acquity, Waters Corporation) according to published methods [13,17]. Separation was achieved on a Waters Acquity UPLC HSS T3 C18 column (150 × 2.1 mm, 1.8 μm) using an isocratic mobile phase of 100% methanol at 1.0 mL/min. Detection wavelengths were 254 nm for GAs, 282 nm for ergosterol, 195 nm for squalene, and 210 nm for lanosterol. Quantification used external standards: ganoderic acid A (Solarbio, ≥ 98%), ergosterol, squalene, and lanosterol (Sigma, ≥ 98%).

Statistical analysis

All experiments were conducted with at least three independent biological replicates. Quantitative data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (version 26.0, IBM Corp., Armonk, NY, USA), and a *p* < 0.05 was considered statistically significant. Specific tests were applied as follows.

Gene expression data were log-transformed to meet the homogeneity of variance assumption for parametric tests. Outliers, defined as data points exceeding ± 3 SD from the mean, were retained in the primary analyses. No missing data required exclusion.

Differences in mycelial growth rates between the WT and the 59 mutant strains were analyzed by one-way analysis of variance (ANOVA). Upon identifying a significant overall effect, post-hoc comparisons against the WT were performed using Dunnett's test. The effects of strain (WT vs. GT9), culture temperature (25°C vs. 35°C), and their interaction on all measured outcomes were assessed using two-way ANOVA, followed by Tukey's test for post-hoc multiple comparisons. To evaluate the relationships between GAs content and key physiological and molecular variables, a multiple correlation analysis was conducted. Pearson correlation coefficients (r) were calculated between GAs content and the following parameters: expression levels of GAs biosynthetic genes (*hmgr, sqs, se, ls*), extracellular enzyme activities (amylase, cellulase, laccase), membrane properties (fatty acids (FAs) unsaturation degree, membrane fluidity), and stress-response indicators (trehalose content, *hsp70* and *hsp90* expression). The strength of the correlations was interpreted based on the absolute value of r as follows: |r| = 0.00–0.19, very weak; 0.20–0.39, weak; 0.40–0.59, moderate; 0.60–0.79, strong; and 0.80–1.00, very strong. The standard *p*-value for each correlation was calculated. Additionally, to robustly validate the strongest positive correlations identified, their significance was re-evaluated using a non-parametric bootstrap resampling method with 10,000 iterations, which generates a reliable confidence interval without assuming data normality. Exact *p*-values are provided in the results section.

Results

Thermotolerant mutant development

ARTP mutagenesis generated 59 mutant strains. These were first distinguished from WT via antagonism assays (Fig 1) and then screened for thermotolerance based on mycelial growth on PDA plates and biomass yield in liquid culture at 35°C. Most mutants exhibited reduced growth, though eight strains (GT1, GT9, GT28, GT35, GT41, GT44, GT47, GT52) outperformed WT. The GT9 strain demonstrated superior performance with mycelial growth rate of 2.56 ± 0.41 mm/d and biomass of 8.52 ± 0.76 g/L, which were 51.48% and 76.03% higher than the WT at 35°C, respectively (growth rate: *p* = 0.013 biomass: *p* = 0.008; Table 1).

Growth kinetics under high-temperature culture

The growth kinetics of WT and GT9 strains were monitored under different temperature (Fig 2). The biomass of GT9 at 35°C was significantly higher than that of the WT at 35°C, though slightly lower than WT at its optimal temperature of 25°C. The maximum dry weight of GT9 reached 9.18 ± 0.54 g/L on day 10, which was 2.23-fold higher than that of the WT at 35°C (*p* = 0.003). Notably, there was no significant difference in the growth kinetics between WT(25°C) and GT9 (35°C) when comparing their maximum biomass accumulation and growth trends, reflecting the stable adaptability of GT9 to high-temperature conditions without compromising growth potential relative to WT under optimal temperature.

Extracellular enzyme activity

The activities of amylase, cellulase, and laccase were assessed on day 10 (Table 2). GT9 at 35°C showed significantly enhanced amylase (46.85%, *p* = 0.028), cellulase (36.65%, *p* = 0.035), and laccase (36.05%, *p* = 0.037) activities compared to the WT at 25°C, while the enzyme activities in the WT at 35°C were also higher than in WT at 25°C, the

Fig 1. Antagonism assay between WT and GT9 mutant strain. Clear zone indicates genetic differentiation between strains.

https://doi.org/10.1371/journal.pone.0345065.g001

differences were not statistically significant. The enhanced enzyme activity in GT9 suggests improved substrate utilization efficiency under HS. The correlation analysis demonstrated weak positive correlations of GAs production with amylase ($r = 0.34$, $*p* = 0.024$), cellulase ($r = 0.28$, $*p* = 0.048$), and laccase ($r = 0.31$, $*p* = 0.035$) activities. These significant relationships indicate that the superior extracellular enzyme activity in GT9 is functionally linked to its enhanced biosynthesis and accumulation of GAs under HS.

GAs biosynthesis and gene expression

The contents of lanosterol and squalene, along with GAs themselves, and the expression of key biosynthetic genes (*hmgr*, *sqs*, *se*, *ls*) were analyzed (Fig 3). The GT9 at 35°C showed significantly increased accumulation of lanosterol, squalene, and GAs from day 6 onward compared to the WT at 35°C (Fig 3A-C). Final contents reached 12.05 ± 0.78 μg/g DW (lanosterol), 1.98 ± 0.82 μg/g DW (squalene), and 9.75 ± 0.75 mg/g DW (GAs) on day 10, which were 92.49% ($*p* = 0.007$), 1.36-fold ($*p* = 0.023$), and 1.01-fold increase ($*p* = 0.041$) over the WT at 35°C, respectively. Notably, the GAs content in GT9 was also comparable to, or slightly higher than, that in the WT at 25°C, but the difference was not statistically significant. The squalene content in GT9 indicated a significant increase compared to that in the WT at 25°C. Crucially, the expression of all four key genes was markedly upregulated in GT9 under sustained HS compared to the WT at both 25°C and 35°C, whereas their expression was significantly suppressed in WT at 35°C compared to the WT at 25°C (Fig 3D). The correlation analysis demonstrated moderate positive correlations between the expression of key genes (*hmgr*, *sqs*, *se*, *ls*) and the final GAs content ($r = 0.46–0.54$, $*p* < 0.0001$). Furthermore, the contents of squalene and lanosterol, were also weak positive correlated with GAs yield ($r = 0.32$ and 0.38, respectively, $*p* < 0.001$). This analysis

Table 1. Mycelial growth rate and biomass of *G.tsugae* strains at 35°C.

Strain number	Mycelial growth rate (mm/d)[1]	Biomass (g/L)[2]	Strain number	Mycelial growth rate (mm/d)	Biomass (g/L)
#WT	1.69 ± 0.23^{bc}	4.84 ± 0.57^{bc}	#GT30	1.36 ± 0.22^{bc}	2.89 ± 0.12^{d}
#GT1	1.95 ± 0.18^{ab}	6.38 ± 0.85^{ab}	#GT31	1.13 ± 0.41^{c}	5.04 ± 0.45^{bc}
#GT2	1.37 ± 0.15^{bc}	4.37 ± 0.43^{bc}	#GT32	1.86 ± 0.17^{ab}	5.23 ± 0.66^{bc}
#GT3	1.53 ± 0.24^{bc}	3.22 ± 0.52 cd	#GT33	1.44 ± 0.26^{bc}	3.08 ± 0.28 cd
#GT4	1.39 ± 0.31^{bc}	5.51 ± 0.34^{bc}	#GT34	1.67 ± 0.34^{bc}	4.54 ± 0.61^{bc}
#GT5	1.25 ± 0.17^{c}	2.04 ± 0.25^{d}	#GT35	2.06 ± 0.33^{ab}	6.26 ± 0.54^{ab}
#GT6	1.54 ± 0.16^{bc}	3.18 ± 0.29 cd	#GT36	1.71 ± 0.22^{b}	4.12 ± 0.36^{c}
#GT7	1.27 ± 0.33^{c}	2.87 ± 0.47^{d}	#GT37	1.65 ± 0.16^{bc}	3.08 ± 0.43 cd
#GT8	1.82 ± 0.28^{ab}	5.23 ± 0.62^{bc}	#GT38	1.39 ± 0.15^{bc}	3.39 ± 0.32 cd
#GT9	2.56 ± 0.41^{a}	8.52 ± 0.76^{a}	#GT39	1.79 ± 0.18^{b}	4.85 ± 0.57^{bc}
#GT10	1.87 ± 0.34^{ab}	4.03 ± 0.43 cd	#GT40	1.66 ± 0.31^{bc}	3.76 ± 0.64 cd
#GT11	1.33 ± 0.38^{bc}	4.59 ± 0.69^{bc}	#GT41	2.03 ± 0.25^{ab}	6.31 ± 0.75^{ab}
#GT12	1.38 ± 0.12^{bc}	3.56 ± 0.36 cd	#GT42	1.55 ± 0.09^{bc}	4.17 ± 0.48^{c}
#GT13	1.34 ± 0.11^{bc}	3.47 ± 0.28 cd	#GT43	1.45 ± 0.11^{bc}	4.08 ± 0.22^{bc}
#GT14	1.28 ± 0.12^{c}	2.81 ± 0.22^{d}	#GT44	2.14 ± 0.23^{ab}	6.07 ± 0.54^{ab}
#GT15	1.85 ± 0.16^{ab}	6.02 ± 0.54^{ab}	#GT45	1.74 ± 0.14^{b}	4.31 ± 0.16^{c}
#GT16	1.82 ± 0.24^{ab}	4.93 ± 0.28 cd	#GT46	1.48 ± 0.12^{bc}	3.18 ± 0.24 cd
#GT17	1.24 ± 0.25^{c}	2.65 ± 0.23^{d}	#GT47	1.97 ± 0.13^{ab}	5.75 ± 0.33^{bc}
#GT18	1.62 ± 0.13^{bc}	3.27 ± 0.18 cd	#GT48	1.27 ± 0.37^{c}	3.16 ± 0.11 cd
#GT19	1.67 ± 0.18^{bc}	3.41 ± 0.21 cd	#GT49	1.76 ± 0.23^{b}	4.25 ± 0.35^{c}
#GT20	1.22 ± 0.09^{c}	2.05 ± 0.14^{d}	#GT50	1.74 ± 0.19^{b}	4.06 ± 0.27^{c}
#GT21	1.35 ± 0.15^{bc}	3.55 ± 0.26 cd	#GT51	1.83 ± 0.25^{ab}	4.42 ± 0.32^{bc}
#GT22	1.80 ± 0.21^{b}	4.92 ± 0.48^{bc}	#GT52	2.04 ± 0.17^{ab}	6.22 ± 0.56^{ab}
#GT23	1.77 ± 0.14^{b}	4.67 ± 0.23^{bc}	#GT53	1.18 ± 0.12^{c}	2.25 ± 0.25^{d}
#GT24	1.38 ± 0.08^{bc}	3.36 ± 0.15 cd	#GT54	1.55 ± 0.16^{bc}	5.06 ± 0.39^{bc}
#GT25	1.26 ± 0.08^{c}	5.34 ± 0.21^{bc}	#GT55	1.64 ± 0.21^{bc}	3.15 ± 0.23 cd
#GT26	1.42 ± 0.32^{bc}	3.83 ± 0.55 cd	#GT56	1.73 ± 0.15^{b}	5.39 ± 0.55^{bc}
#GT27	1.84 ± 0.27^{ab}	4.37 ± 0.65^{bc}	#GT57	1.12 ± 0.38^{c}	2.45 ± 0.42^{d}
#GT28	1.96 ± 0.43^{ab}	5.63 ± 0.73^{bc}	#GT58	1.27 ± 0.37^{c}	3.16 ± 0.11 cd
#GT29	1.85 ± 0.38^{ab}	5.92 ± 0.56^{b}	#GT59	1.56 ± 0.19^{bc}	5.13 ± 0.49^{bc}

Footnotes: [1] mm/d, millimeters per day. [2] g/L, grams per liter. #WT, wild-type strain. #GT1–GT59, mutant *G. tsugae* strains 1–59. Note: Data are presented as mean ± SD (n = 6). Different lowercase letters (e.g., a, b, c,...) within the same column indicate significant differences (*p* < 0.05, one-way ANOVA with Dunnett's post-hoc test), while sharing the same letter indicates no significant difference. Letters are for within-column comparisons only.

https://doi.org/10.1371/journal.pone.0345065.t001

confirms that the enhanced GAs content in GT9 is directly associated with the sustained upregulation of its biosynthetic pathway under HS.

Cell membrane adaptation to HS

The intracellular FAs composition was analyzed to investigate membrane adaptation under HS (Fig 4). The major fatty acids in *G. tsugae* were indentified as C14:0, C15:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C22:1 (Fig 4A). The GT9 exhibited substantial membrane remodeling with decreased saturated FA (C16:0: −32.72%; C18:0: −40.92%) and increased unsaturation FAs (C18:1: + 29.29%; C18:2: + 46.15%) compared to the WT at 25°C (Fig 4A). Additionally, C18:0 content in GT9 was 36.56% lower than in the WT at 35°C (*p* = 0.008). The FAs unsaturation degree was significantly

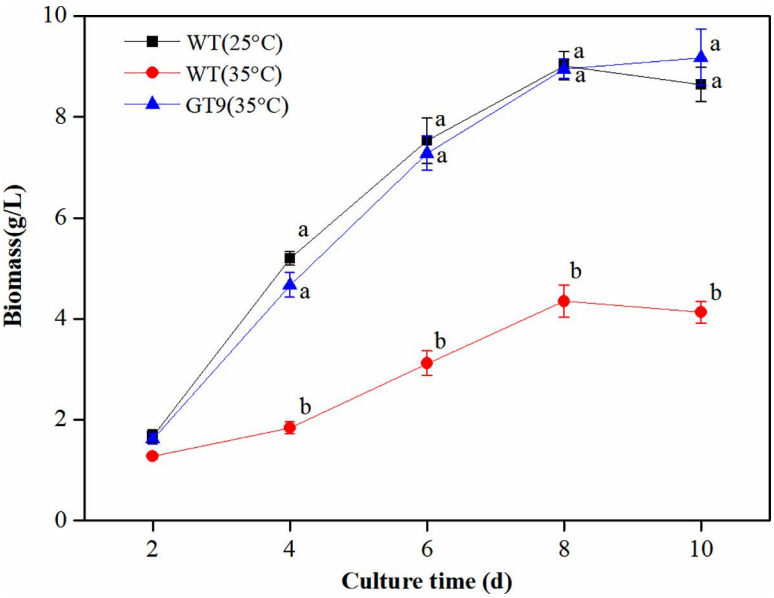

Fig 2. Time profile of biomass production under high-temperature culture conditions. WT(25°C): wild-type strain at 25°C; WT(35°C): wild-type strain at 35°C; GT9 (35°C): GT9 mutant strain at 35°C; Error bars represent SD (n = 6). Note: Data are presented as mean ± SD (n = 6). Different lowercase letters indicate significant differences (two-way ANOVA, Tukey's test, *p* < 0.05), while sharing the same letter indicates no significant difference.

https://doi.org/10.1371/journal.pone.0345065.g002

Table 2. Extracellular enzyme activities on day 10.

Strains and culture temperature (°C)	Amylase activity (U. mL^{-1})	Cellulase activity (U. mL^{-1})	Laccase activity (U. mL^{-1})
WT (25°C)	10.83 ± 0.87^{a}	42.62 ± 3.18^{a}	8.96 ± 0.42^{a}
WT (35°C)	12.32 ± 0.94^{ab}	50.73 ± 3.23^{ab}	10.78 ± 0.65^{ab}
GT9 (35°C)	15.89 ± 1.03^{b}	58.24 ± 3.27^{b}	12.19 ± 0.86^{b}

Table comparing enzyme activities across strains and temperatures. Footnotes: U mL^{-1}, Enzyme unit per milliliter. Note: Data are presented as mean ± SD (n = 6). Different lowercase letters within the same column indicate significant differences (two-way ANOVA, Tukey's test,*p* < 0.05).

https://doi.org/10.1371/journal.pone.0345065.t002

higher in GT9-approximately 2.67-fold (*p* < 0.001) and 2.14-fold (*p* = 0.003) greater than in the WT at 25°C and 35°C, respectively (Fig 4B). We further assessed membrane fluidity, which was notably elevated in GT9, as indicated by a significantly lower fluorescence anisotropy value relative to the WT (Fig 4C). These results demonstrate that the GT9 exhibits increased FAs unsaturation and enhanced membrane fluidity. Ergosterol is an important component of fungal membranes and is synthesized from lanosterol in *G. tsugae*, which plays an important role in ensuring the cell membrane integrity, membrane fluidity, cell viability and cell material transport. Therefore, in this study, the ergosterol content of strains was determined. The ergosterol content in GT9 was highest, reaching 6.93 ± 0.48 mg/g DW-a 66.98% increase over the WT at 25°C (*p* = 0.005) and significantly higher than the WT at 35°C throughout the cultivation period (Fig 4D). Furthermore, relative to the WT at 35°C, the GT9 showed significant increases of approximately 70.03% on day 6 (*p* = 0.012), 1.86-fold on day 8 (*p* = 0.003), and 2.86-fold (*p* < 0.001) on day 10. In contrast, ergosterol content in WT at 35°C was significantly lower than at 25°C throughout the cultivation period (*p* < 0.001). The correlation analysis revealed that the FAs unsaturation degree was positively correlated with GAs yield (r = 0.57, *p* < 0.0001). A moderate positive correlation was also observed between membrane fluidity and GAs content (r = 0.45, *p* < 0.0001). These findings confirm that the

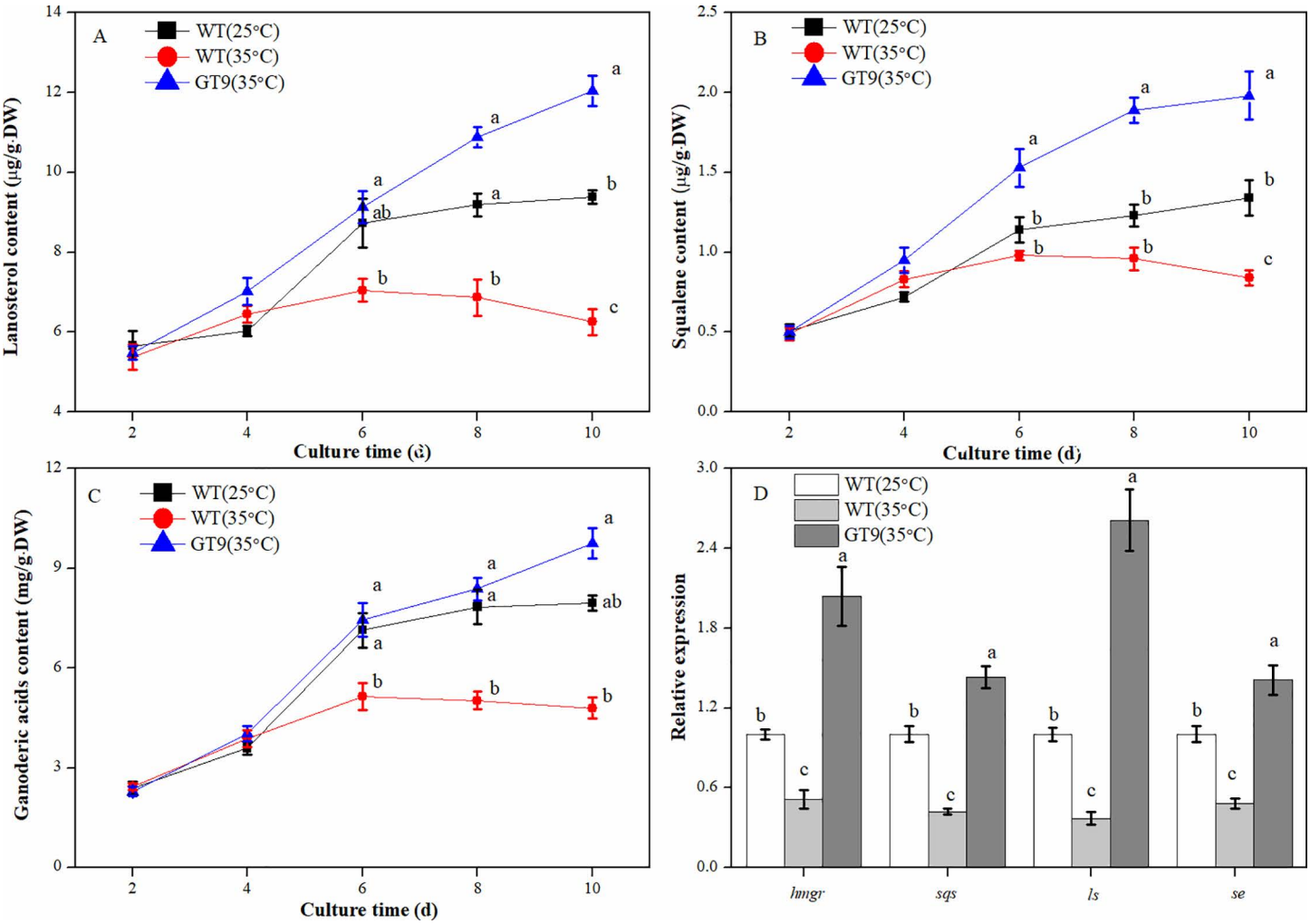

Fig 3. Time profiles of lanosterol (A), squalene (B), GAs content (C) and gene expression (D). Note: Error bars represent SD. Data are presented as mean ± SD (n = 6). Different lowercase letters indicate significant differences(two-way ANOVA, Tukey's test,*p* < 0.05).

https://doi.org/10.1371/journal.pone.0345065.g003

membrane adaptations in GT9-specifically, increased unsaturation and fluidity-are closely associated with its enhanced capacity for GAs biosynthesis

Thermal protection responses

To assess whether GT9 exhibits increased trehalose accumulation relative to the WT strain, Trehalose content was measured at 35°C. The WT cultured at 35°C displayed a higher trehalose content than when cultured at 25°C, indicating that HS promotes trehalose accumulation. In comparison to the WT strain at both 25°C and 35°C, trehalose accumulation in GT9 was significantly higher, reaching 67.39 ± 3.5 mg/L on day 8 (vs. WT at 25°C: *p* = 0.007; vs. WT at 35°C: *p* < 0.001) and 73.24 ± 4.3 mg/L on day 10 (vs. WT at 25°C: *p* = 0.002; vs. WT at 35°C: *p* < 0.001)(Fig 5A). These results suggest that the elevated trehalose content in GT9 reflects altered glycolytic flux, which may endow it with better thermotolerance. Previous studies have reported that HS upregulates HSP gene expression in *G. tsugae*. Therefore, here, we analyzed the expression of *hsp17.4*, *hsp22*, *hsp70,* and *hsp90* in both strains. Under HS condition (35°C), the WT strain showed

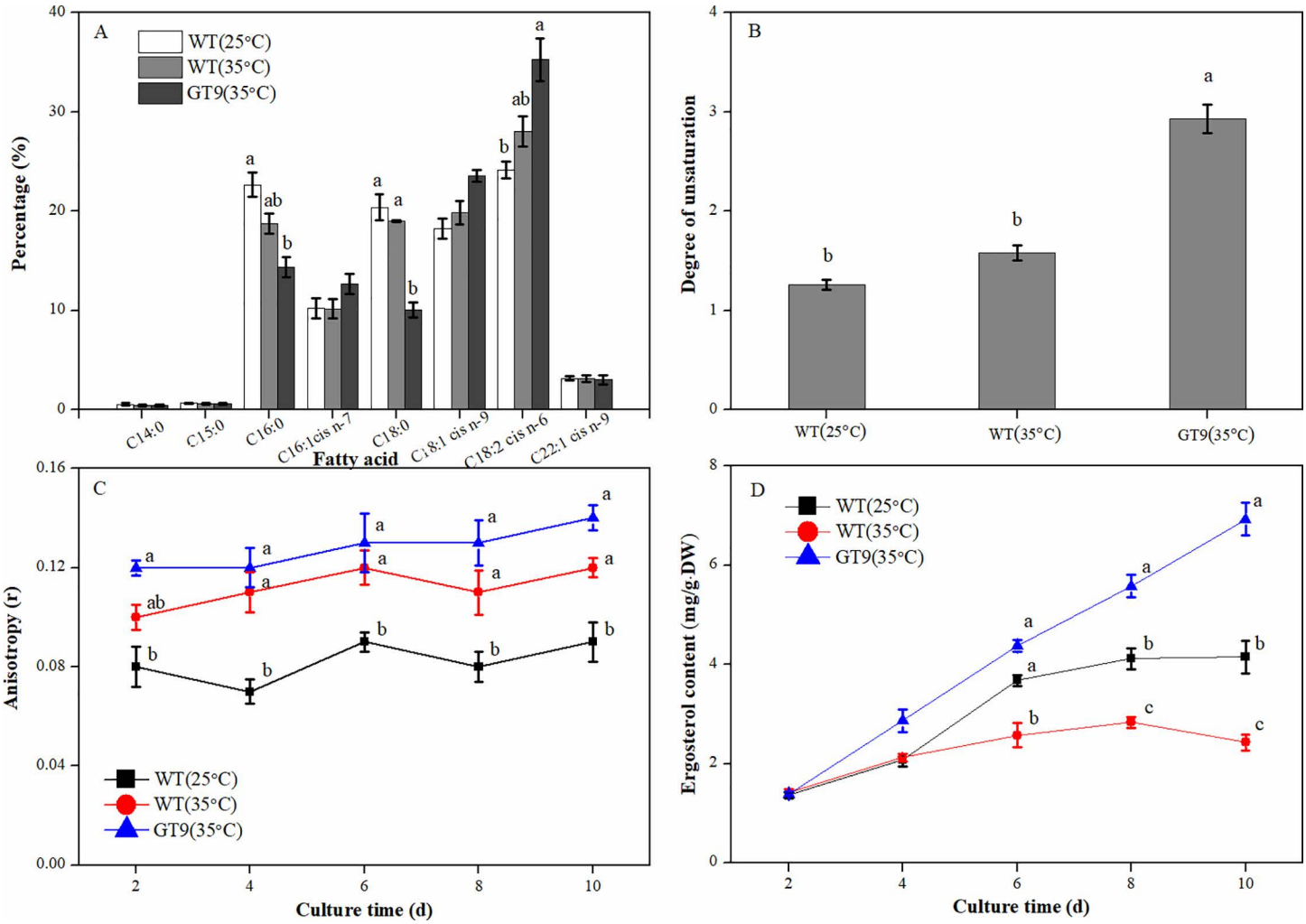

Fig 4. Analyses of FAs composition (A), unsaturation degree(B), membrane fluidity (C) and ergosterol content (D). Note: Error bars represent SD. Data are presented as mean ± SD (n = 6). Different lowercase letters indicate significant differences (two-way ANOVA, Tukey's test,*p* < 0.05).

https://doi.org/10.1371/journal.pone.0345065.g004

significant upregulation of HSP genes relative to the WT at 25°C: *hsp17.4* (4.01-fold, *p* < 0.001), *hsp22* (2.21-fold, *p* = 0.003), *hsp70* (2.33-fold, *p* = 0.001), and *hsp90* (2.22-fold, *p* = 0.002), confirming that HS induces substantial HSP expression. Notably, it can also be seen that the GT9 exhibited significantly elevated expression of *hsp17.4*, *hsp22*, *hsp70,* and *hsp90* compared to the WT at 25°C (Fig 5B). Furthermore, The GT9 also showed increased of *hsp17.4* and *hsp70*, though no signifiant difference was observed for *hsp22* when compared to the WT at 35°C. The correlation analysis revealed a significant positive correlation between trehalose and GAs content (r = 0.72, *p* < 0.0001). Furthermore, the expression of *hsp70* and *hsp90* genes were also positively correlated with GAs content (r = 0.65 and r = 0.63, respectively, *p* < 0.0001). These relationships indicate that the enhanced accumulation of trehalose and the upregulation of specific HSP genes in GT9 are functionally associated with its improved capacity for GAs biosynthesis.

Discussion

HS significantly influences fungal growth, development and secondary metabolism [13,23–26,34]. Therefore, it is very important to improve the thermotolerance of *Ganoderma* species to meet the demands of large-scale industrial

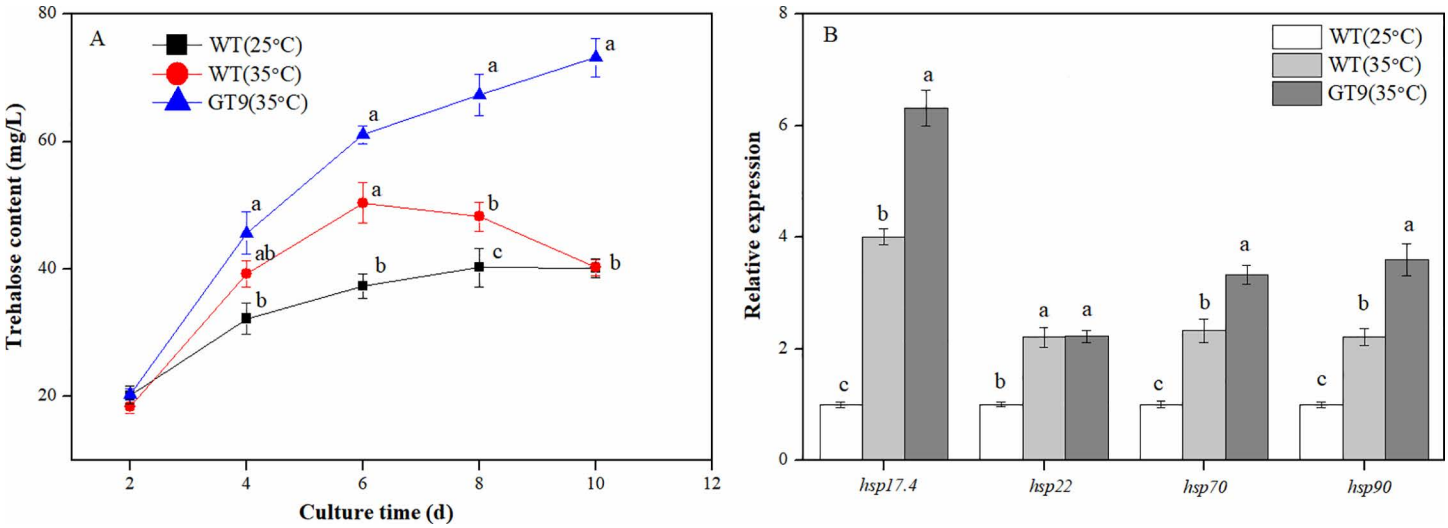

Fig 5. Analyses of trehalose (A) and gene transcription levels (B). Note: Error bars represent SD. Data are presented as mean ± SD (n = 6). Different lowercase letters indicate significant differences (two-way ANOVA, Tukey's test,*p* < 0.05).

https://doi.org/10.1371/journal.pone.0345065.g005

fermentation. In this study, a thermotolerant mutant strain of *G. tsugae*, designated GT9, was obtained through ARTP mutagenesis. The GT9 strain exhibited a superior mycelial growth rate and increased biomass production under high-temperature condition, indicating successful acquisition of thermotolerance through mutagenesis.

Temperature is a critical factor regulating enzyme activity, and the optimal temperature for microbial growth often differs from that for metabolite accumulation. A moderately elevated fermentation temperature can enhance enzyme activity and promote the yield of target products [35]. In this work, the GT9 strain showed increased enzyme activities of amylase, cellulase, and laccase compared to the WT under liquid high-temperature culture. This suggests that improved thermotolerance contributes to higher catalytic efficiency and metabolic flux, supporting more efficient cellular biosynthesis [36].

GAs are biosynthesized via the mevalonate pathway, in which squalene and lanosterol are two key intermediates. The genes *hmgr*, *sqs*, *se,* and *ls* encode enzymes that catalyze critical steps in this pathway, and their upregulation is often associated with enhanced GAs yield [4]. Although transient HS has been reported to induce GAs biosynthesis [26,37], the GT9 maintained elevated transcription levels of these key genes, along with increased accumulation of GAs, squalene, and lanosterol under sustained liquid high-temperature culture. To systematically evaluate the interrelationships among physiological and molecular factors contributing to enhanced GAs content, we performed multiple correlation analysis. The results revealed that GAs content showed moderate positive correlations with the expression of GAs biosynthetic genes *hmgr*, *sqs*, *se*, and *ls* (r = 0.46–0.53, *p* < 0.0001), and weak positive correlations with extracellular enzyme activities (r = 0.28–0.34, *p* < 0.05). These findings demonstrate that the improved GAs content in GT9 is linked to the sustained upregulation of these key pathway genes and enhanced metabolic activity—a correlation also observed in *Antrodia cinnamomea* and *Centella asiatica* [38,39]. Notably, our results reiterate findings from numerous studies that highlight the complexity of GAs production regulation. Even with the successful acquisition of thermotolerance and coordinated upregulation of key biosynthetic genes, the magnitude of increase in GAs production was relatively modest, the difference in GAs production between WT (25°C) and GT9 (35°C) was not statistically significant, which further supports that GAs biosynthesis is a multifactorial process influenced by intricate interactions between strain characteristics, environmental conditions, and metabolic networks rather than a single regulatory factor.

The cell membrane plays important roles in sensing environmental change [34], especially for temperature upshifts in fungi. The fluidity and stability of membranes are largely governed by the composition of FAs, particularly the degree of

unsaturation [25]. In this study, GT9 displayed altered FAs composition, increased FAs unsaturation, enhanced membrane fluidity, and elevated ergosterol content compared to the WT. Notably, correlation analysis indicated that FAs unsaturation ($r = 0.57$, $*p* < 0.0001$) and membrane fluidity ($r = 0.45$, $*p* < 0.0001$) were moderate positive correlated with GAs accumulation, suggesting that membrane adaptability under HS is crucial for maintaining metabolic homeostasis and supporting secondary metabolite biosynthesis.

Reportedly, producing a large amount of HSPs is a widely adapted mechanism [26,35]. HSPs are a family of proteins that protect proteins from being irrevocably denatured, misfolded or aggregated and help organisms to modulate stress responses and protect organisms from cellular damage, including HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs based on molecular mass, which are considered to play an important role in conferring thermotolerance [40,41]. Additionally, compatible solutes such as trehalose act as effective thermoprotectants in fungi [27]. In this study, our results showed that GT9 accumulates more trehalose and exhibits higher expression of HSP genes than the WT. Correlation analyses further confirmed significant positive correlations between the GAs content and trehalose accumulation ($r = 0.72$, $*p* < 0.0001$), as well as the expression of *hsp70* and *hsp90* genes ($r = 0.68$ and 0.63, respectively; $*p* < 0.0001$), supporting the conclusion that these molecular adaptations collectively bolster thermotolerance and create favorable conditions for GAs biosynthesis. In summary, the thermotolerant GT9 strain enhances its high-temperature adaptability through multiple coordinated mechanisms: modulating membrane composition and fluidity, accumulating protetive molecules, and sustaining the expression of biosynthetic and stress-responsive genes. The correlation patterns observed provide statistical evidence that GAs overproduction under HS is an integrated outcome of robust growth, efficient substrate utilization, enhanced biosynthetic capacity, and comprehensive stress protection. These adaptations facilitate robust growth and efficient GAs production under HS. The strategy developed in this study-combing ARTP mutagenesis with high-temperature cultivation-provides an effective approach for improving GAs production in *Ganoderma* species.

Conclusions

We developed a thermotolerant *G. tsugae* mutant (GT9) via ARTP mutagenesis and demonstrate that its enhanced GAs production under HS results from multi-level adaptations. Correlation analyses confirmed that GAs overproduction is linked to sustained upregulation of biosynthetic genes, heightened extracellular enzyme activities, an adaptable cell membrane with increased FAs unsaturation, and the efficient accumulation of trehalose and HSPs. In particular, the increased trehalose content played the most critical role in enhancing GAs content. However, the difference in GAs production between the WT strain at 25°C and the GT9 strain at 35°C was not statistically significant, indicating that the improvement from high-temperature fermentation mainly comes from the stability and adaptability of the thermotolerant strain rather than a dramatic increase in individual titers. Our results support the view that GAs biosynthesis is a complex and tightly regulated process. This study validates that combining ARTP mutagenesis with high-temperature cultivation is a viable industrial breeding strategy to enhance secondary metabolite production.

Author contributions

Conceptualization: Xiao-Long He, Yueqin Liu.

Data curation: Li-Yuan Luo.

Formal analysis: Quan Ma, Yueqin Liu.

Funding acquisition: Yueqin Liu.

Investigation: Yueqin Liu.

Methodology: Quan Ma, Yang-Meng-Jie Jing.

Software: Li-Yuan Luo, Xiao-Long He.

Supervision: Xiao-Long He, Yueqin Liu.

Validation: Li-Yuan Luo, Xiao-Long He.

Visualization: Quan Ma, Yang-Meng-Jie Jing.

Writing – original draft: Quan Ma, Yang-Meng-Jie Jing.

Writing – review & editing: Yueqin Liu.

References

1. Xu A, Yang X, Li Y, Jacob MS, Zhang B, Li X. Fresh-eating Lingzhi becomes possible: Comparative evaluation of nutritional and taste profile of Ganoderma tsugae at different fruiting body morphogenesis stages. LWT. 2024;201:116234. https://doi.org/10.1016/j.lwt.2024.116234
2. Xia L, Tan X, Wang P, Yang D, Zhang Y, Cui Y, et al. Evaluation of two different treatments for larch logs as substrates to cultivate ganoderma tsugae in the forest. Life (Basel). 2024;15(1):39. https://doi.org/10.3390/life15010039 PMID: 39859979
3. Xu X, Li C, Wu F, Zhao S, Chen T, You H, et al. Integrated transcriptomic and targeted metabolomic analysis reveals the key genes involved in triterpenoid biosynthesis of ganoderma lucidum. J Fungi (Basel). 2025;11(1):57. https://doi.org/10.3390/jof11010057 PMID: 39852476
4. Wang Q, Xu M, Zhao L, Wang F, Li Y, Shi G, et al. Transcriptome dynamics and metabolite analysis revealed the candidate genes and regulatory mechanism of ganoderic acid biosynthesis during liquid superficial-static culture of Ganoderma lucidum. Microb Biotechnol. 2021;14(2):600–13. https://doi.org/10.1111/1751-7915.13670 PMID: 32975886
5. Yang Y, Jian Y, Liu B. Rapid determination of diverse ganoderic acids in ganoderma using UPLC-MS/MS. Curr Anal Chem. 2024;20(3):191–200. https://doi.org/10.2174/0115734110289769240125115919
6. Wang H, Tai M, Li W, Li Y, Zhang Z, Zhang D, et al. Ganoderma lucidum extract reduces skin aging by reducing mitochondrial stress and controlling mitochondrial numbers. Fitoterapia. 2025;184:106627. https://doi.org/10.1016/j.fitote.2025.106627 PMID: 40381851
7. Lei B, Zhang M, Shi X, Feng N, Yin J, Dong R, et al. Ganoderic acid T, a novel activator of pyruvate carboxylase, exhibits potent anti-liver cancer activity. Metabolism. 2025;170:156321. https://doi.org/10.1016/j.metabol.2025.156321 PMID: 40480453
8. Shi D, Xu X, Wang J, Bu T, Sun P, Yang K, et al. Synergistic anti-inflammatory effects of Ganoderma lucidum polysaccharide and ganoderic acid A on LPS-induced RAW264.7 cells by inhibition of TLR4/NF-κB activation. Int J Biol Macromol. 2025;309(Pt 2):143074. https://doi.org/10.1016/j.ijbiomac.2025.143074 PMID: 40220822
9. Lu F, Ju S, Ying Y, Liu J, Yang C, Li M, et al. Ganoderic Acid A Alleviates High-Fat Diet-Induced Lipid Metabolism Disorders in Mice by Inhibiting Intestinal Farnesoid X Receptor. Food Frontiers. 2025;6(6):3108–21. https://doi.org/10.1002/fft2.70120
10. Feng J, Tang C-M, Liu Y-F, Tang C-H, Zhang J-S. Enhancing high-efficiency breeding and microbial microdroplet cultivation techniques for Ganoderma lucidum. World J Microbiol Biotechnol. 2024;40(7):225. https://doi.org/10.1007/s11274-024-04011-0 PMID: 38822208
11. Kumar V, Ahluwalia V, Saran S, Kumar J, Patel AK, Singhania RR. Recent developments on solid-state fermentation for production of microbial secondary metabolites: challenges and solutions. Bioresour Technol. 2021;323:124566. https://doi.org/10.1016/j.biortech.2020.124566 PMID: 33390315
12. Tajik A, Samadlouie HR, Salek Farrokhi A, Ghasemi A. Optimization of chemical conditions for metabolites production by Ganoderma lucidum using response surface methodology and investigation of antimicrobial as well as anticancer activities. Front Microbiol. 2024;14:1280405. https://doi.org/10.3389/fmicb.2023.1280405 PMID: 38318131
13. Liu R, Zhu T, Chen X, Wang Z, Yang Z, Ren A, et al. GSNOR regulates ganoderic acid content in Ganoderma lucidum under heat stress through S-nitrosylation of catalase. Commun Biol. 2022;5(1):32. https://doi.org/10.1038/s42003-021-02988-0 PMID: 35017648
14. Chen J, Xu W, Wang Z, Tian L, Liu W, Ren A, et al. Mitochondrial pyruvate carrier influences ganoderic acid biosynthesis in Ganoderma lucidum. Appl Microbiol Biotechnol. 2023;107(4):1361–71. https://doi.org/10.1007/s00253-022-12357-4 PMID: 36635397
15. Liu Y-N, Chen Y-L, Zhang Z-J, Wu F-Y, Wang H-J, Wang X-L, et al. Phosphatidic acid directly activates mTOR and then regulates SREBP to promote ganoderic acid biosynthesis under heat stress in Ganoderma lingzhi. Commun Biol. 2024;7(1):1503. https://doi.org/10.1038/s42003-024-07225-y PMID: 39537975
16. Xu J-W, Yue T-H, Yu X, Zhao P, Li T, Li N. Enhanced production of individual ganoderic acids by integrating Vitreoscilla haemoglobin expression and calcium ion induction in liquid static cultures of Ganoderma lingzhi. Microb Biotechnol. 2019;12(6):1180–7. https://doi.org/10.1111/1751-7915.13381 PMID: 30821132
17. Wang Q, Liu H-J, Xu Y, Wang Z-X, Sun B, Xu J-W. Development of a 2A peptide-based multigene expression system and its application for enhanced production of ganoderic acids in Ganoderma lucidum. J Biotechnol. 2024;393:109–16. https://doi.org/10.1016/j.jbiotec.2024.08.001 PMID: 39103018
18. Yuan W, Jiang C, Wang Q, Fang Y, Wang J, Wang M, et al. Biosynthesis of mushroom-derived type II ganoderic acids by engineered yeast. Nat Commun. 2022;13(1):7740. https://doi.org/10.1038/s41467-022-35500-1 PMID: 36517496
19. Liu X, Wang W. Biosynthesis of a novel ganoderic acid in Saccharomyces cerevisiae and research of its antitumor activity. Appl Biochem Micro. 2023;59(2):184–9. https://doi.org/10.1134/S0003683823020072

20. Ren A, Shi L, Zhu J, Liu R, Zhao MW. Biosynthetic pathway and signal transduction regulation of ganoderic acid biosynthesis in Lingzhi. The Lingzhi Mushroom Genome. Cham: Springer International Publishing. 2021. p. 147–64. https://doi.org/10.1007/978-3-030-75710-6_8
21. Liu Y-N, Wu F-Y, Tian R-Y, Shi Y-X, Xu Z-Q, Liu J-Y, et al. The bHLH-zip transcription factor SREBP regulates triterpenoid and lipid metabolisms in the medicinal fungus Ganoderma lingzhi. Commun Biol. 2023;6(1):1. https://doi.org/10.1038/s42003-022-04154-6 PMID: 36596887
22. Han J, Wang L, Tang X, Liu R, Shi L, Zhu J, et al. Glsirt1-mediated deacetylation of GlCAT regulates intracellular ROS levels, affecting ganoderic acid biosynthesis in Ganoderma lucidum. Free Radic Biol Med. 2024;216:1–11. https://doi.org/10.1016/j.freeradbiomed.2024.02.029 PMID: 38458391
23. Xie H, Wan L, Han J, Huang C, Li J, Yao Q. TMT-based proteomic and transcriptomic analysis reveal new insights into heat stress responsive mechanism in edible mushroom Grifola frondosa. Sci Hortic. 2024;323:112542. https://doi.org/10.1016/j.scienta.2023.112542
24. Tao Y, Han X, Ren A, Li J, Song H, Xie B, et al. Heat stress promotes the conversion of putrescine to spermidine and plays an important role in regulating ganoderic acid biosynthesis in Ganoderma lucidum. Appl Microbiol Biotechnol. 2021;105(12):5039–51. https://doi.org/10.1007/s00253-021-11373-0 PMID: 34142206
25. Liu Y-N, Zhang T-J, Lu X-X, Ma B-L, Ren A, Shi L, et al. Membrane fluidity is involved in the regulation of heat stress induced secondary metabolism in Ganoderma lucidum. Environ Microbiol. 2017;19(4):1653–68. https://doi.org/10.1111/1462-2920.13693 PMID: 28198137
26. Zhang X, Ren A, Li M-J, Cao P-F, Chen T-X, Zhang G, et al. Heat stress modulates mycelium growth, heat shock protein expression, ganoderic acid biosynthesis, and hyphal branching of ganoderma lucidum via Cytosolic Ca2. Appl Environ Microbiol. 2016;82(14):4112–25. https://doi.org/10.1128/AEM.01036-16 PMID: 27129961
27. Shi L, Lian L, Wang L, Zhang S, Han Q, Shi L, et al. General control nonderepressible 4 activates the transcription of trehalose phosphorylase to improve trehalose production and abiotic stress tolerance in Ganoderma lucidum. Int J Biol Macromol. 2025;311(Pt 3):143840. https://doi.org/10.1016/j.ijbiomac.2025.143840 PMID: 40318725
28. Liu R, Zhu T, Yang T, Yang Z, Ren A, Shi L, et al. Nitric oxide regulates ganoderic acid biosynthesis by the S-nitrosylation of aconitase under heat stress in Ganoderma lucidum. Environ Microbiol. 2021;23(2):682–95. https://doi.org/10.1111/1462-2920.15109 PMID: 32483888
29. Zhu L, Wu D, Zhang H, Li Q, Zhang Z, Liu Y, et al. Effects of Atmospheric and Room Temperature Plasma (ARTP) Mutagenesis on Physicochemical Characteristics and Immune Activity In Vitro of Hericium erinaceus Polysaccharides. Molecules. 2019;24(2):262. https://doi.org/10.3390/molecules24020262 PMID: 30641994
30. Liu W, Yang W, Wu J, Cheng Y, Wei Z, Wang T, et al. ARTP Mutagenesis to Improve Mycelial Polysaccharide Production of Grifola frondosa Using a Mixture of Wheat Bran and Rice Bran as Substrate. Journal of Food Quality. 2021;2021:1–11. https://doi.org/10.1155/2021/6110743
31. Asitok A, Ekpenyong M, Akwagiobe E, Asuquo M, Rao A, Ubi D, et al. Interspecific protoplast fusion of atmospheric and room-temperature plasma mutants of Aspergillus generates an L-asparaginase hyper-producing hybrid with techno-economic benefits. Prep Biochem Biotechnol. 2023;53(7):827–40. https://doi.org/10.1080/10826068.2022.2150643 PMID: 36449415
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609
33. Ren A, Liu R, Miao Z-G, Zhang X, Cao P-F, Chen T-X, et al. Hydrogen-rich water regulates effects of ROS balance on morphology, growth and secondary metabolism via glutathione peroxidase in Ganoderma lucidum. Environ Microbiol. 2017;19(2):566–83. https://doi.org/10.1111/1462-2920.13498 PMID: 27554678
34. Lara-Martínez D, Tristán-Flores FE, Cervantes-Montelongo JA, Silva-Martínez GA. Fungal Stress Responses and the Importance of GPCRs. J Fungi (Basel). 2025;11(3):213. https://doi.org/10.3390/jof11030213 PMID: 40137251
35. Liu R, Zhang X, Ren A, Shi D-K, Shi L, Zhu J, et al. Heat stress-induced reactive oxygen species participate in the regulation of HSP expression, hyphal branching and ganoderic acid biosynthesis in Ganoderma lucidum. Microbiol Res. 2018;209:43–54. https://doi.org/10.1016/j.micres.2018.02.006 PMID: 29580621
36. de Carvalho Silvello MA, Teixeira GS, Bueno D, Goldbeck R. CRISPR/Cas9-engineered thermotolerant Saccharomyces cerevisiae JAYET for enhanced 3G-ethanol production from Chlorella vulgaris biomass. Algal Research. 2025;90:104275. https://doi.org/10.1016/j.algal.2025.104275
37. Liu W, Sun Y, Yue S, Kong Y, Cong Q, Lan Y, et al. ATP deficiency triggers ganoderic acids accumulation via fatty acid β-oxidation pathway in Ganoderma lucidum. Microb Cell Fact. 2025;24(1):62. https://doi.org/10.1186/s12934-025-02668-2 PMID: 40069729
38. Chen Y-T, Shen Y-C, Chang M-C, Lu M-K. Precursor-feeding strategy on the triterpenoid production and anti-inflammatory activity of Antrodia cinnamomea. Process Biochemistry. 2016;51(8):941–9. https://doi.org/10.1016/j.procbio.2016.05.001
39. Baek S, Ho T-T, Lee H, Jung G, Kim YE, Jeong C-S, et al. Enhanced biosynthesis of triterpenoids in Centella asiatica hairy root culture by precursor feeding and elicitation. Plant Biotechnol Rep. 2019;14(1):45–53. https://doi.org/10.1007/s11816-019-00573-w
40. Hou L, Wang J, Li T, Zhang B, Yan K, Zhang Z, et al. Transcriptome Analysis Revealed That Cell Wall Regulatory Pathways Are Involved in the Tolerance of Pleurotus ostreatus Mycelia to Different Heat Stresses. J Fungi (Basel). 2025;11(4):266. https://doi.org/10.3390/jof11040266 PMID: 40278087
41. Nie C, Wei S, Wu S, Qi L, Feng J, Wang X. Transcriptomic, and metabolic profiling reveals adaptive mechanisms of Auricularia heimuer to temperature stress. PeerJ. 2025;13:e19713. https://doi.org/10.7717/peerj.19713 PMID: 40708823