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## Research article

# High temperature and UV-C treatments affect stilbenoid accumulation and related gene expression levels in *Gnetum parvifolium*



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## ABSTRACT

**Background:** *Gnetum parvifolium* stems and roots have been used for a long time in traditional Chinese medicines. Stilbenes are bioactive compounds present in *G. parvifolium* plants, and they possess antioxidative and anticancer properties. However, little is known about the responses of *G. parvifolium* stilbene biosynthetic pathways to stress conditions. Therefore, we investigated stilbene biosynthesis, including the expression of relevant genes, in *G. parvifolium* exposed to high-temperature and ultraviolet-C treatments.

**Results:** High temperatures did not influence the accumulation of total stilbenes in stems but decreased stilbene concentrations in roots at 3 h, with a subsequent restoration to control levels. In contrast, ultraviolet irradiation induced the accumulation of total stilbenes in stems but not in roots. We also observed that high temperatures inhibited the production of resveratrol and piceatannol in *G. parvifolium* stems and roots, whereas ultraviolet treatments initially inhibited their accumulation (up to 6 h) but induced their production at later time points. Analyses of specific genes (i.e., *PAL*, *C4H*, *4CL*, *STS*, and *CYP*) revealed that their expression levels generally increased in stress-treated stems and roots, although there was some variability in the expression profiles during treatments.

**Conclusions:** Our results indicated that high temperatures and ultraviolet irradiation differentially affect the biosynthesis of specific stilbenes in *G. parvifolium* stems and roots. Therefore, cultivating *G. parvifolium* seedlings under optimal stress conditions may increase the biosynthesis of specific stilbene compounds.

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## 1. Introduction

The genus *Gnetum* consists of 35–40 species and is one of three unique groups of the phylum Gnetophyta [1]. This genus forms a morphologically and ecologically diverse monophyletic group with special cytological features [2,3,4]. There are some controversies regarding its phylogenetic position among seed plants, and resolving this issue may provide important insights into the evolution and origin of flowers [5]. Several *Gnetum* spp. are edible or used as paper pulp, which makes them economically valuable in many regions of Africa and Asia [6]. Moreover, these *Gnetum* spp. are important sources of raw materials for traditional medicines in many countries [1]. Stem and root extracts have been used to cure ocular complications,

relieve swelling, treat acute respiratory infections, and cure chronic bronchitis [7]. In addition, numerous studies have indicated that these extracts contain a wide range of natural bioactive compounds, including stilbenoids [8,9,10]. Together with plants from the families Pinaceae, Cyperaceae, Vitaceae, Dipterocarpaceae, and Fabaceae, *Gnetum* spp. are considered one of the best sources of stilbenoids among plants [11].

Stilbenoids are a family of polyphenols known for their diverse biological activities [11,12]. Some of these compounds exhibit hypotensive, antioxidant, anticancer, and antibacterial effects [13]. About 100 different types of stilbenoids have been identified in at least 15 *Gnetum* spp., representing almost the full spectrum of natural stilbenoids [11,14]. Some stilbenoids, such as resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), have attracted considerable attention. Resveratrol is believed to be involved in the health benefits associated with the moderate consumption of red wine [15] and is currently one of the most extensively studied natural products [16]. Numerous studies have revealed that it can inhibit the progression of diverse illnesses, including cancer, HIV disease, and cardiovascular

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disease. Resveratrol can also prolong the lifespan of various organisms by activating sirtuin deacetylases [17,18,19]. Furthermore, its hydroxylated analog, piceatannol, exhibits considerable antitumorigenic and antileukemic activities [20,21].

Stilbenes are synthesized by stilbene synthases (STSs) together with chalcone synthases that share a common upstream pathway [22,23]. Key enzymes [i.e., phenylalanine ammonia-lyase (PAL) and cinnamic acid 4-hydroxylase (C4H)] catalyze the deamination of L-phenylalanine to *trans*-cinnamic acid during the first step of phenylpropanoid biosynthesis. The resulting *p*-coumaroyl-CoA through a reaction catalyzed by 4-coumarate: CoA ligase (4CL). Three acetate extender units (derived from malonyl-CoA) are added to produce a linear tetraketide intermediate. The subsequent folding and cyclization of the generated intermediate lead to the production of a chalcone or stilbene ring structure depending on the polyketide synthase activity [22]. Resveratrol may be further hydroxylated by some members of the CYP gene family (e.g., cytochrome P450) to produce piceatannol (Fig. 1). We previously analyze *Gnetum parvifolium* transcriptome and determine that these genes are primarily involved in stilbenoid biosynthesis [1].

*G. parvifolium* is mainly distributed in tropical and subtropical regions of southern China. Its stems and roots have been used in traditional Chinese medicines for more than 1500 years [14]. Our previous studies indicate that *Gnetum* spp. are rich in stilbenes [1,24,25]. We also determine that stilbene accumulation differs among *G. parvifolium* tissues, with concentrations mediated by the expression of relevant biosynthetic genes [1]. In addition, several stilbene compounds have been detected in *G. africanum* stems [10,26,27] or *G. parvifolium* stems and roots [1]. Stilbene production is induced by high temperature and ultraviolet (UV) treatments [1], implying that stress factors can stimulate the accumulation of stilbenes in leaves. In

this study, we analyzed young *G. parvifolium* stems and roots treated with high temperature or UV light. We focused on the accumulation of total stilbenes, including resveratrol and piceatannol, and the expression of genes responsible for stilbenoid biosynthesis. A more comprehensive characterization of the responses of *G. parvifolium* stilbene biosynthetic pathways to environmental stresses may contribute to the breeding of stilbene-rich plants.

## 2. Materials and methods

### 2.1. Materials and treatments

All experiments were conducted using 1-year-old *G. parvifolium* (Warb.) C.Y. Cheng ex Chun seedlings cultivated in the greenhouse. The seedlings were acclimated in a climate chamber for several days and then divided into two equal groups. One group was exposed to high temperature (40°C), whereas the other group was treated with UV-C irradiation (20 W; wavelength: 200–275 nm). Both the groups were treated for 3, 6, 12, 24, and 48 h. We did not use the within 1-h UV-C treatment applied in many earlier studies [28,29]. We applied relatively long irradiation times in our experiments because of the thick leathery leaves in this *Gnetum* plant. Our earlier results of photosynthetic experiments suggested that *Gnetum* was not sensitive to strong irradiation and was able to withstand several hours of UV-C treatment without any noticeable loss of viability, except for withering after the extreme 48-h treatment (data not shown). In addition, long UV processing time (9 h) has been applied to *Arabidopsis thaliana* [30]. These observations provided us with an opportunity to check much longer range of UV-C treatments than are commonly applied in plants. The experiments were completed using four biological replicates. All

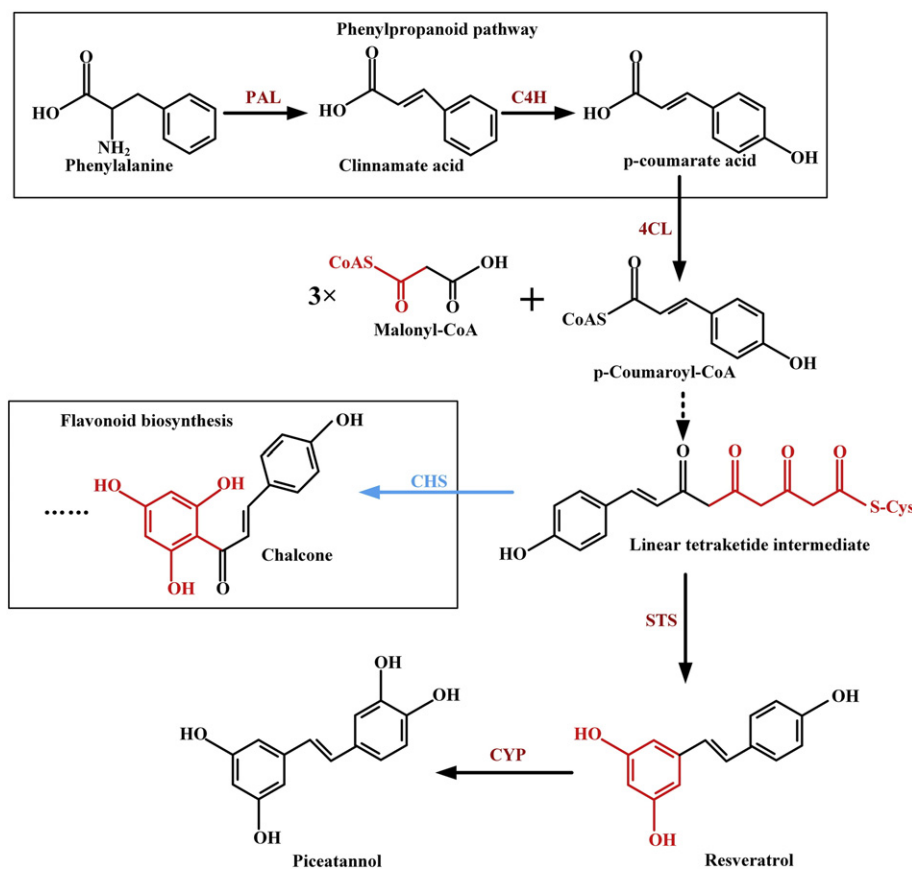


Fig. 1. The biosynthesis pathway of stilbenes and candidate genes involved in stilbenoids biosynthesis in *G. parvifolium*. PAL: phenylalanine ammonia-lyase, C4H: cinnamic acid 4-hydroxylase, 4CL: 4-coumarate: CoA ligase, CYP: cytochrome P450 gene, STS: stilbene synthase.

treated stems and roots were harvested and immediately stored in liquid nitrogen.

## 2.2. Stilbenoid extraction and quantification

### 2.2.1. Extraction

All 96 harvested samples were oven-dried and ground into a powder. Each sample (10 mg) was resuspended in 500  $\mu$ L 80% methanol before undergoing an ultrasonic treatment for 30 min. The samples were then incubated at 4°C overnight. The homogenates were centrifuged at 12,000 rpm for 10 min, and supernatants were stored at 4°C until analyzed.

### 2.2.2. Total stilbenoids

Sample extracts (50  $\mu$ L) were diluted in ddH<sub>2</sub>O to a volume of 500  $\mu$ L. The absorbance of the samples was measured at 333 nm, and total stilbenoid content was determined with 80% methanol as the reference and resveratrol (Tongtian Biotech) as the standard.

### 2.2.3. Stilbene analysis by high-performance liquid chromatography

According to Deng et al.'s method [1], 20  $\mu$ L extracts were analyzed using an HPLC 1260 system (Agilent, CA, USA) with an Eclipse XDB-C18 reverse phase column (4.6  $\times$  150 mm; particle size: 5  $\mu$ m). Solvent A was 0.1% formic acid in water, whereas solvent B consisted of 0.1% formic acid in acetonitrile. The flow rate was 1 mL min<sup>-1</sup>. Compounds were separated with a linear gradient (5–70% solvent B over 30 min). A photodiode array detector (Agilent) was used to detect the UV-visible absorption (190–600 nm).

## 2.3. RNA isolation

Total RNA was isolated from 100 mg samples (stored at –80°C) using TRIzol (Invitrogen, CA, USA). The purity of the RNA was checked using a NanoPhotometer spectrophotometer (Implen, CA, USA), and concentrations were determined with a Qubit RNA Assay kit and Qubit 2.0 Fluorometer (Life Technologies, CA, USA). Degradation and contamination were evaluated in 1% agarose gels, and RNA integrity was assessed using a Nano 6000 Assay Kit and the Bioanalyzer 2100 system (Agilent).

## 2.4. Determination of gene expression levels by quantitative reverse transcription polymerase chain reaction

Equal amounts of extracted total RNA were reverse-transcribed by Superscript III Reverse Transcriptase (Invitrogen). The polymerase chain reaction (PCR) experiments were conducted using the SYBR Premix Ex Taq kit (Takara, Dalian, China) and LightCycler 480 (Roche, USA). Primers specific for selected *G. parvifolium* genes were designed using Primer3 (version 0.4.0; <http://frodo.wi.mit.edu/primer3/>) and are listed in Table 1. The PCR experiments were completed using the Bio-Rad Sequence Detection System and the following program: 95°C for 10 s and 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative expression levels were calculated using the 2<sup>- $\Delta\Delta$ CT</sup> method [31].

**Table 1**

Gene-specific primers used for quantitative reverse transcription polymerase chain reaction analyses.

Gene	Forward primer	Reverse primer
PAL 1	CCTTCAACTCCTCTCGAACACGC	GCITTCGTACATTGACAACC
PAL 2	CCTTCAACTCCTCTCGAACACGC	GCITTCGTACATTGACAACC
4CL	TGCCAATCCCAACCTTCACT	CCACCCTACGGACGACAATC
C4H	GGGAGCAAACACGACCAG	GGAGACGACCTCAATCACAG
STS	CAGCCTGCCAATGTGATAAAC	TTCAGCATCTCTCCGTGAG
CYP	GAAATCCGTATCATCAATAGG	TGCCACCAAGAGGGTCATAT
Actin	TTGTAGGTCGCCCTCGTC	CTCCCTGTAGCCITTTGG

## 2.5. Data analysis and statistics

Data were obtained from four biological replicates, and the results are presented as the mean  $\pm$  standard deviation. All statistical analyses and plottings were completed using R software (version 3.2.4).

## 3. Results

### 3.1. Effects of high temperature and UV treatments on total stilbene accumulation

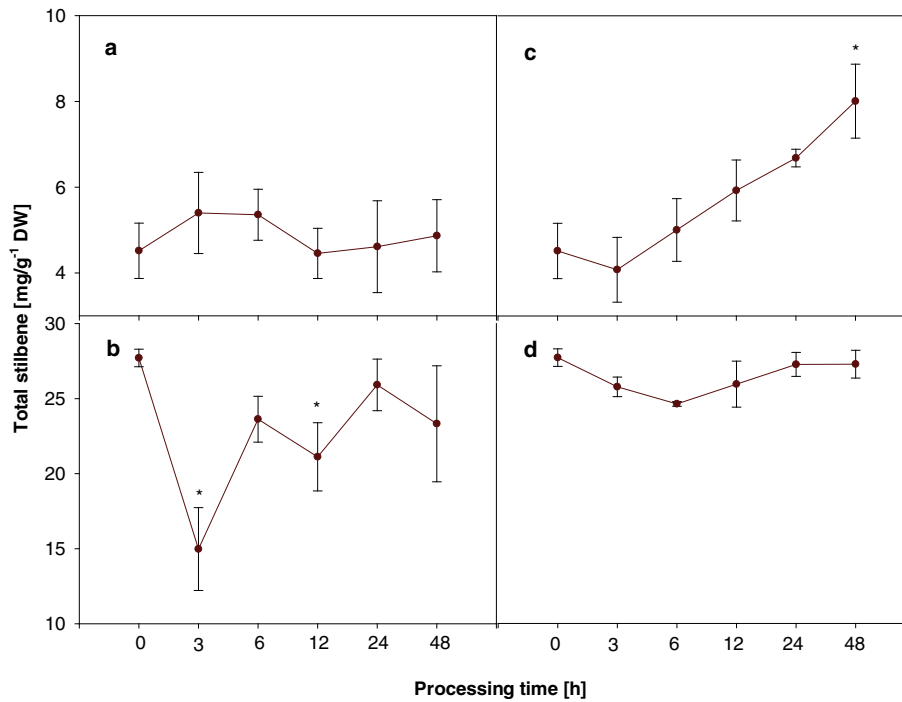
Our previous study reveals that high temperature and UV treatments affect stilbene production in young *G. parvifolium* leaves [1]. In the present study, there were no significant changes to stilbene content in high temperature-treated stems over time (Fig. 2a). However, we observed significant fluctuations in the roots (Fig. 2b). During UV treatments, the stem stilbene content increased in an almost linear fashion and peaked at 48 h [8.0 mg g<sup>-1</sup> dry weight (DW)], which corresponded to an increase of 78% over the control level (Fig. 2c). However, UV treatments did not influence the root stilbene content (Fig. 2d). These results suggested that high temperature and UV stresses affected stilbene accumulation differently.

### 3.2. Effects of high temperature and UV treatments on resveratrol and piceatannol accumulation

The stem resveratrol and piceatannol contents exhibited similar trends following high-temperature treatments (Fig. 3a,b), with the concentrations of both compounds decreasing significantly after 6 h (resveratrol) or 12 h (piceatannol) and then stabilizing (Fig. 3a,b). During UV treatments, the concentrations of both compounds decreased up to 6 h and then increased, peaking at 48 h with values of 202  $\mu$ g g<sup>-1</sup> DW (resveratrol; Fig. 3c) and 964  $\mu$ g g<sup>-1</sup> DW (piceatannol; Fig. 3d). The changes in resveratrol and piceatannol contents in roots were similar to those in stems (Fig. 4). Under high temperature conditions, the concentrations of the compounds gradually decreased over time, reaching the lowest levels at 48 h (Fig. 4a,b). In contrast, during exposure to UV light, the lowest resveratrol and piceatannol levels occurred at 6 h, followed by an increase up to 671  $\mu$ g g<sup>-1</sup> DW (resveratrol; Fig. 4c) and 3728  $\mu$ g g<sup>-1</sup> DW (piceatannol; Fig. 4d) at 48 h. These results indicated that *G. parvifolium* stems and roots contained a relatively large amount of resveratrol and piceatannol. In addition, the accumulation of these compounds was induced by UV irradiation, whereas high temperature treatments had the opposite effect.

### 3.3. Expression patterns of candidate genes affecting the stilbenoid biosynthesis pathway under stress conditions

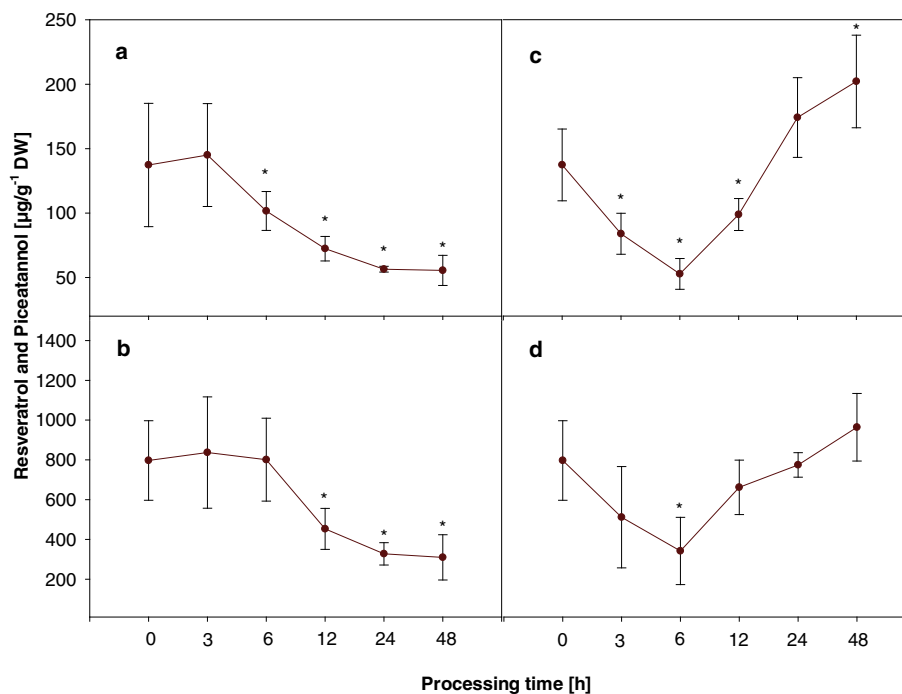
Our previous transcriptomics study identifies candidate unigenes involved in the stilbenoid biosynthesis pathway (i.e., PAL, C4H, 4CL, STS, and CYP) [1]. In stems, high temperature and UV treatments increased the expression level of all of these genes (Fig. 5). For the upstream genes (i.e., PAL, C4H, and 4CL), high-temperature treatments significantly increased gene expression levels by up to 5–60-fold, with peaks at 24 or 48 h. Similarly, UV irradiation increased the expression of these genes by up to 33–3367-fold at 24 or 48 h. For the key biosynthetic genes (i.e., STS and CYP), high-temperature treatments induced the expression of STS at 24 h, with levels over 17-fold higher than the control values. Exposure to UV light resulted in a >200-fold increase in the STS expression level at the same time point. Similarly, the CYP expression level in high temperature-treated samples was >100-fold higher than that of the controls after 24 h, whereas it was >1000-fold higher than that of the controls 24 h after initiating the UV treatments. The specific gene expression patterns in stems depended on the treatment duration. In addition, the effects of the high temperature and UV treatments varied among the tested genes (Fig. 5).



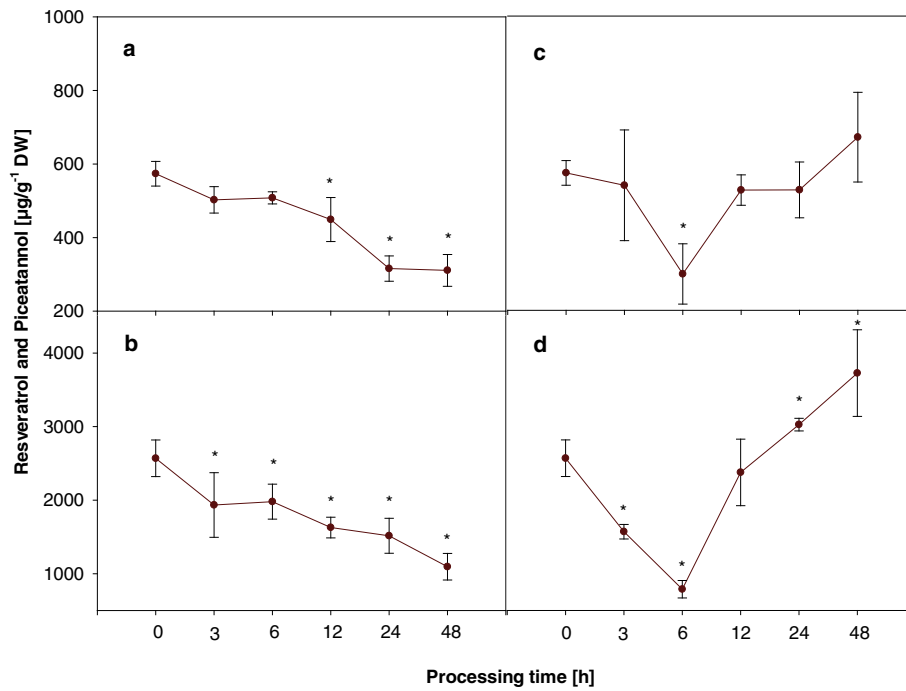
**Fig. 2.** Contents of total stilbenes in the stems and roots of *G. parvifolium* seedlings under stress conditions. X-axis represents the treatment time and Y-axis represents the content of total stilbene [ $\text{mg g}^{-1}$  DW]. Total stilbene in stems (a) and roots (b) under high temperature and in stems (c) and roots (d) under UV treatment. Means  $\pm$  SD,  $n = 4$ . \* and \*\* indicate significant difference at  $P < 0.05$  and  $P < 0.01$ , respectively, between the treated and control plants.

The expression patterns of the analyzed genes in treated roots were generally less uniform than those in the stems (Fig. 6). The high-temperature treatments induced significant increases in the *PAL*, *C4H*, and *4CL* expression levels at 3 h (for *4CL*) or 6 h (for *PAL* and *C4H*). The expression levels of these three genes in the high temperature-treated plants were 2–25-fold higher than the control

values. Similarly, UV light significantly upregulated the expression of these genes after only 3 h, with expression levels 4–41-fold higher than the control values. In addition, exposure to high temperature increased *STS* expression by >6-fold, with the highest values observed at 6 h. In contrast, UV irradiation increased *STS* expression by >159-fold at 24 h. Unlike in stems, root *CYP* expression levels decreased in high



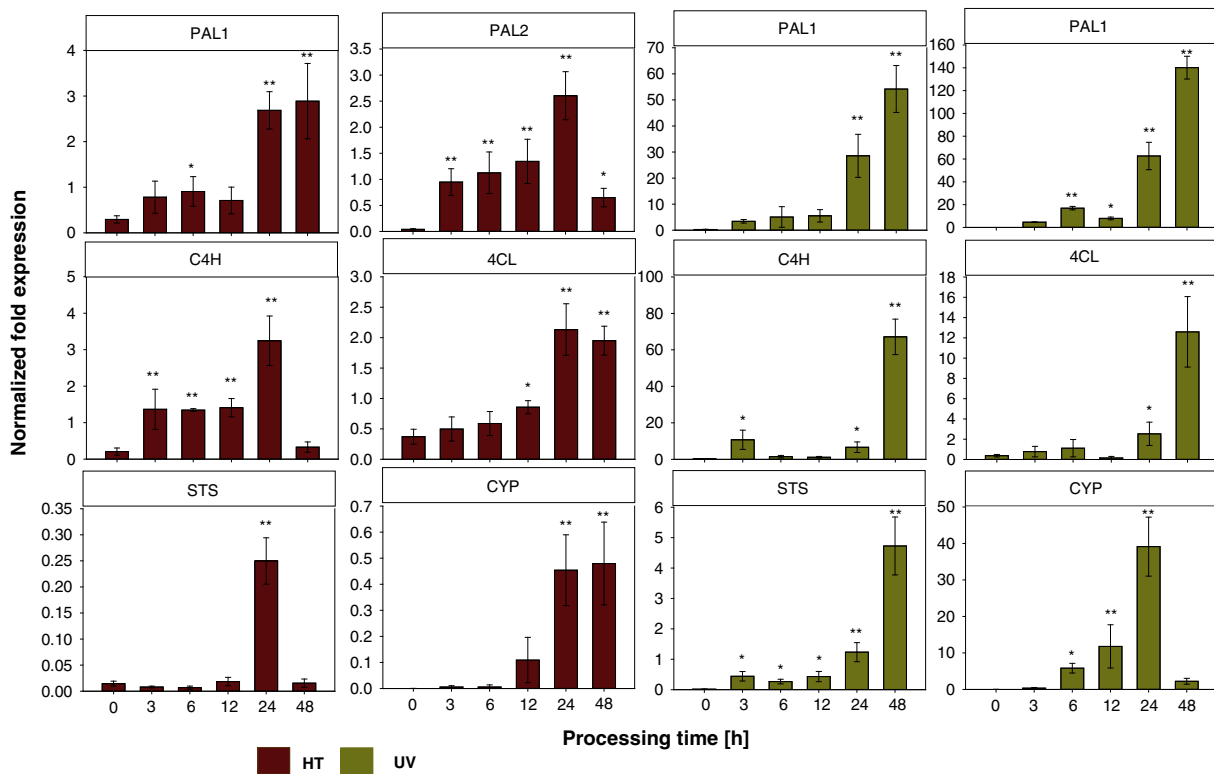
**Fig. 3.** Contents of resveratrol and piceatannol in the stems of *G. parvifolium* seedlings under stress conditions. X-axis represents the treatment time and Y-axis represents the content of resveratrol and piceatannol [ $\mu\text{g g}^{-1}$  DW]. Resveratrol (a) and piceatannol (b) in stems under high temperature and resveratrol (c) and piceatannol (d) in stems under UV treatment. Means  $\pm$  SD,  $n = 4$ . \* and \*\* indicate significant difference at  $P < 0.05$  and  $P < 0.01$ , respectively, between the treated and control plants.



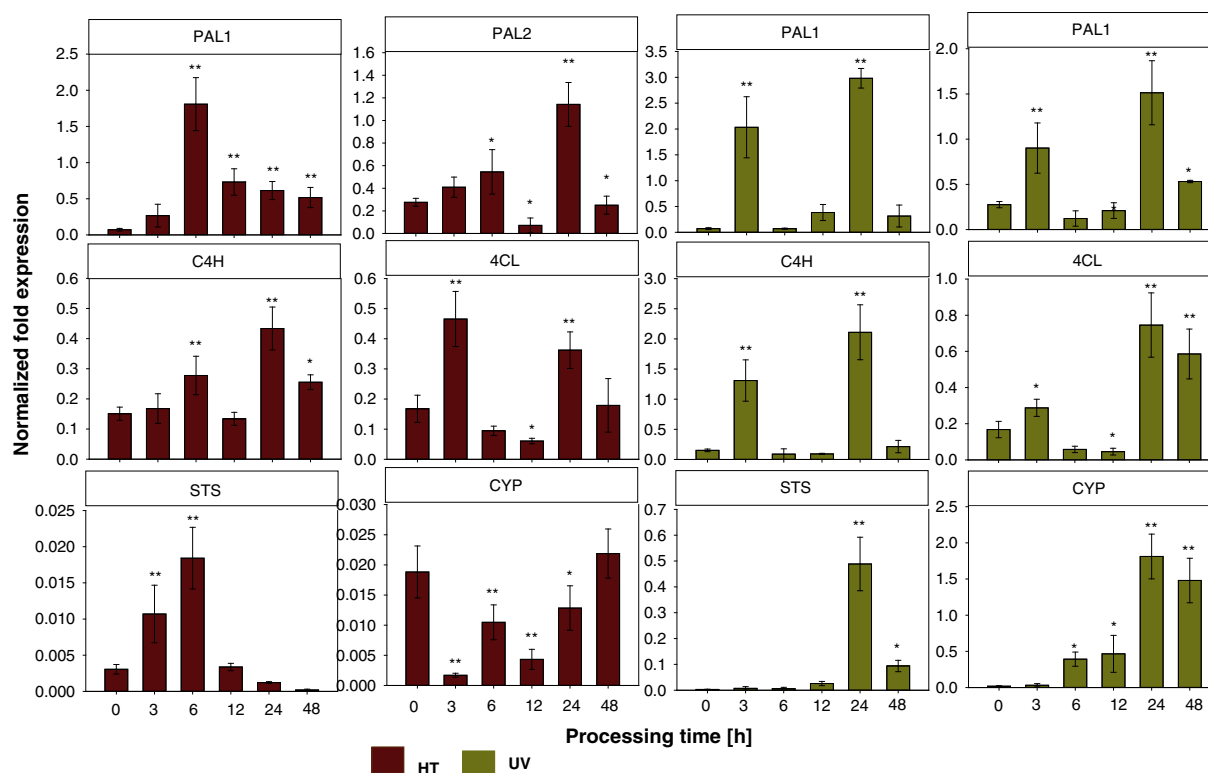
**Fig. 4.** Contents of resveratrol and piceatannol in the roots of *G. parvifolium* seedlings under stress conditions. X-axis represents the treatment time and Y-axis represents the content of resveratrol and piceatannol [ $\mu\text{g g}^{-1}$  DW]. Resveratrol (a) and piceatannol (b) in roots under high temperature and resveratrol (c) and piceatannol (d) in roots under UV treatment. Means  $\pm$  SD,  $n = 4$ . \* and \*\* indicate significant difference at  $P < 0.05$  and  $P < 0.01$ , respectively, between the treated and control plants.

temperature-treated plants after only 3 h. However, the expression levels returned to control levels at 48 h. The stem *CYP* expression level at 24 h of UV irradiation was 96-fold higher than control levels. Root *CYP* expression

exhibited similar patterns. These results indicated that exposure to high-temperature and UV light stresses affected the expression of all the analyzed stilbene biosynthesis genes.



**Fig. 5.** Expression patterns of candidate genes involved in stilbenoids biosynthesis in the stems of *G. parvifolium* seedlings under stress conditions. X-axis represents the treatment time and Y-axis represents the fold change in expression; HT: high temperature, UV: UV-C irradiation. Means  $\pm$  SD,  $n = 4$ . \* and \*\* indicate significant difference at  $P < 0.05$  and  $P < 0.01$ , respectively, compared to control (0 h).



**Fig. 6.** Expression patterns of candidate genes involved in stilbenoids biosynthesis in the roots of *G. parvifolium* seedlings under stress conditions. X-axis represents the treatment time and Y-axis represents the fold change in expression; HT: high temperature, UV: UV-C irradiation. Means  $\pm$  SD,  $n = 4$ . \* and \*\* indicate significant difference at  $P < 0.05$  and  $P < 0.01$ , respectively, compared to control (0 h).

#### 4. Discussion

Approximately 450–475 million years ago, the ancestors of modern vascular plants adapted to growth in terrestrial environments [32]. This colonization led to several physiological changes, including the evolution of new specialized secondary metabolites, including stilbenes and flavonoids [33]. These plant products may be of considerable use as potential drugs, nutraceuticals, and food additives [33,34,35]. Polyphenolics possess a broad range of pharmacological and therapeutic effects, including antioxidative and anticancer activities. Gnetaceae species produce polyphenolics, such as stilbenes and flavonoids, as their major secondary metabolites, and they may be unique in this respect [1]. Therefore, physiological and molecular investigations of *Gnetum* spp. may lead to the discovery of new bioactive and health-related compounds. We previously determined that *G. parvifolium* roots contain  $28 \text{ mg g}^{-1}$  DW stilbene, which is more than 6.1- and 3.4-fold higher than the leaf and stem stilbene contents, respectively [1]. The accumulation of stilbenes in roots has also been observed in *Cayratia trifolia* [33]. Under normal conditions, *G. parvifolium* stems and roots contain  $137$  and  $573.70 \text{ } \mu\text{g g}^{-1}$  DW resveratrol, respectively [1]. These concentrations are much higher than those of mulberry ( $50.61 \text{ } \mu\text{g g}^{-1}$  DW), jamun seed ( $34.87 \text{ } \mu\text{g g}^{-1}$  DW), and grape seed ( $5.89 \text{ } \mu\text{g g}^{-1}$  DW) [36]. These results imply that *G. parvifolium* plant is one of the natural sources that produce specific stilbenes with pharmaceutical properties.

The leaves of *Gnetum* spp. are often consumed as a healthy vegetable. Our earlier experiments revealed that high temperature and UV treatments (0, 3, 6, 12, 24, and 48 h) promote the accumulation of stilbenes, including resveratrol and piceatannol, in the leaves of young *G. parvifolium* seedlings [1]. The stems and roots of *Gnetum* spp. are used in traditional Chinese medicines and have been regarded as potential sources of stilbene compounds [10,26,27]. We used the same *G. parvifolium* plant materials from our earlier study [1] to investigate the responses of stilbene biosynthetic pathways to high temperature

and UV treatments in the stems and roots. Neither treatment resulted in the accumulation of total stilbenes in roots; however, UV irradiation increased total stilbene content in stems (Fig. 2). Resveratrol and piceatannol contents decreased following high-temperature treatments, whereas their concentrations significantly increased in stems and roots after prolonged (24–48 h) UV treatment (Fig. 3, Fig. 4). This study revealed that these two stress factors have contrasting effects on the accumulation of resveratrol and piceatannol in stems and roots. These findings in the stems and roots differed from those in the leaves from our earlier results in *G. parvifolium*, although they were collected in the same treatments [1]. This may be because of variations in the genetic regulation of secondary metabolism in different tissues, leading to diverse (and even contrasting) responses to stresses.

Several plants, including *Polygonum cuspidatum*, *Gnetum* spp., and *Pinus* spp., accumulate large amounts of stilbenes [1,11]. Most studies conducted on the cells and leaves of peanut, grapevine, and pine seedlings concluded that stilbenes are present at only very low levels under normal conditions [37]. However, following exposure to stress conditions, these compounds can accumulate through increases in the expression of relevant biosynthetic genes and coordinated activation of the expression of genes upstream from the general phenylpropanoid pathway (e.g., *PAL*, *4CL*, and *C4H*) [37]. Earlier studies determined that *STS* expression is highly induced in response to biotic and abiotic stresses such as infection, wounding, UV-C exposure, and chemical treatments [37,38]. In many plant species that are rich in stilbenes, including peanut and eastern white pine, *STS* genes exist as a family of closely related genes (i.e., two *STS* genes [39,40]). However, grapevine is the only stilbene-rich plant whose genome has been sequenced. Little is known about the genes affecting stilbene biosynthesis in stilbene-rich *Gnetum* spp. In the present study, gene expression analyses (Fig. 5, Fig. 6) indicated that *PAL*, *4CL*, and *C4H* were transcriptionally activated after high temperature and UV treatments. The inductions of *4CL* and *C4H* expression were closely coordinated with that of *PAL*. In addition, the expression levels of *STS* and *CYP*, which play an important

regulatory role during the biosynthesis of resveratrol and other stilbene derivatives, increased significantly following high temperature and UV treatments. However, the gene expression changes did not correspond with the changes in stilbene production. This inconsistency suggests that stilbene biosynthesis is influenced by post-transcriptional regulation.

This study represents one of the first attempts to clarify the molecular mechanisms underlying *Gnetum* sp. responses to stress factors. We focused on the regulation of secondary metabolites in different plant tissues. A more thorough characterization of these mechanisms may help us understand how *Gnetum* spp. adapted to tropical and subtropical habitats and ultimately will enable to breed stilbene-rich *Gnetum* spp.

### Conflict of interest

The authors declared that they have no conflict of interest.

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