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Effect of Environmental Stresses on Physiophytochemical Responses and Expressional Profiling Analysis of Flavone C-Glycoside-Associated Genes in Three Bamboo Species

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Abstract

Bamboo, valued for its sustainability and versatility, holds potential in various domains, including food and medicine, due to its abundance of antioxidant compounds such as flavone C-glycosides (FCG) found in bamboo leaf extracts. However, the effects of environmental stress on bamboo growth, phytochemical synthesis, and the genetic regulation of FCG biosynthesis remain unclear. This study investigated the response mechanisms of 3 bamboo genotypes (Pai Sang-Mon, Pai-Liang, and Pai Khao-Lam) under different environmental stresses: Acidity (pH 4.0), heavy metal zinc (200 µM $ZnSO_4 \cdot 7H_2O$), salinity (100 mM NaCl), and water deficit stress over 0, 1, 2, 4, and 8 days. The results indicated that water deficit stress had the most significant impact on growth and photosynthesis, with Pai Khao-Lam and Pai Sang-Mon exhibiting greater drought tolerance than Pai-Liang. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis revealed diverse responses in phytochemical content. Pai Sang-Mon showed increased caffeic acid and apigenin under salt stress, while Pai-Liang exhibited decreased phytochemical content under acid, zinc, and water deficit stresses. In contrast, Pai Khao-Lam showed elevated *p*-coumaric acid, chlorogenic acid and vitexin levels under multiple stresses, indicating robust antioxidant capabilities. Gene expression analysis using quantitative real-time PCR indicated differential gene responses among the bamboo genotypes. Pai Sang-Mon showed reduced vitexin levels correlating with decreased expression of the C-glycosyltransferase (CGT1) gene. Pai Khao-Lam exhibited increased expression of the flavanone 2-hydroxylase (F2H) and CGT1 genes under water deficit stress, suggesting a molecular response to maintain cellular homeostasis through phytochemical accumulation. In contrast, Pai-Liang showed increased expression of the sucrose nonfermenting 1-related protein kinase 1 (SnRK1) gene under short-term salt stress, indicating the sensitivity to osmotic stresses. These findings highlight the complex relationship between gene regulation, phytochemical accumulation, and stress tolerance in bamboo, offering valuable insights for breeding bamboo with enhanced stress tolerance and optimizing environmental conditions to maximize phytochemical production in bamboo leaf extracts.

Keywords: Abiotic stress, Bamboo resilience, C-glycosyltransferase, Orientin, Phenolic acid, Phytochemical profiling, Vitexin

Introduction

Bamboo, a monocotyledonous plant belonging to the Poaceae family, subfamily Bambusoideae, encompasses 1,670 species within 125 genera [1]. Widely distributed in tropical and subtropical regions, Thailand hosts approximately 15 - 20 genera and 80 - 100 species of bamboo [2,3]. *Dendrocalamus sericeus* (Pai Sang-Mon), *Thyrsocalamus liang* Sangkaew & Goh (Pai-Liang), and *Schizostachyum pergracile* (Munro) Majumdar (Pai Khao-Lam) are species that are extensively cultivated in Thailand [2]. Renowned for its rapid growth, bamboo is utilized for various purposes including food, medicine, housing, ornamentals, and

rituals, making significant economic and cultural contributions [3-5]. Apart from its applications in construction, furniture, packaging, and cuisine, bamboo leaves are recognized for their abundance of phytochemicals with antioxidant properties. These phytochemicals exert anti-inflammatory, neuroprotective, hepatoprotective, antidiabetic, antifatigue, antimicrobial, antimelanogenic, antityrosinase, and anticancer activities [1,4,6].

Phytochemicals are compounds primarily produced by plants that are classified into 2 main groups: Primary metabolites, such as carbohydrates, proteins, and fats, which play roles in plant growth and development; and secondary metabolites, including alkaloids, terpenoids, and phenolic compounds. Secondary metabolites, particularly phenolic compounds, play significant roles in plant defense against both biotic and abiotic stresses [7,8]. Phenolic compounds encompass various groups, including flavonoids, phenolic acids, stilbenes, lignans, and tannins, with flavonoids being a large category widely found in plants, possessing a broad spectrum of health-promoting effects, including antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic activities [8,9]. Previous studies have reported significant amounts of phytochemicals and their antioxidants in bamboo-leaf extracts (BLE), making them valuable for medicinal and nutritional purposes [4,6,10,11]. BLE serve as rich sources of essential phenolic acids, such as *p*-coumaric acid, chlorogenic acid, caffeic acid, ferulic acid, syringic acid, protocatechuic acid, and flavonoids, including apigenin, tricin, isoorientin, isovitexin, orientin, and vitexin [1,4,11]. FCG are particularly abundant among the flavonoids and have been used as markers for determining the quality of commercial bamboo-leaf flavonoid products [1,11,12]. The mechanism by which FCG are synthesized starts with the convergence of *p*-coumaroyl-CoA from the phenylalanine pathway and malonyl-CoA from fatty acid biosynthesis, resulting in naringenin, a precursor from which all flavonoid compounds are synthesized. This process relies on the enzyme flavanone 3-hydroxylase (F3H), which converts naringenin into eriodictyol. Subsequently, the initial enzyme involved in FCG synthesis, F2H, converts naringenin and eriodictyol into 2-hydroxylnaringenin and 2-hydroxyleriodictyol, respectively. Following this, the enzyme CGT catalyzes the conversion into C-glucosylated intermediates, which are unstable molecules. Dehydration then occurs, resulting in the loss of a water molecule, yielding 4 types of FCG: Orientin, isoorientin, vitexin, and isovitexin [13]. Not only are the phytochemical compounds in BLE, such as apigenin, luteolin, tricin, and FCG, beneficial for human health, but they are also crucial for plants in mitigating environmental stress-induced oxidative damage and apoptosis [4]. These findings underscore the potential therapeutic applications of FCG in medicine and plant defense responses under abiotic stresses. However, there have been few studies on the effects of environmental stress on the levels of phytochemicals, especially FCG, in bamboo plants.

Environmental stressors, including temperature fluctuations, water scarcity, salinity, heavy metal contamination, and pH variations, induce oxidative stress in plants, resulting in the accumulation of reactive oxygen species (ROS). These ROS can damage cellular components, inhibit plant growth and development, and affect the synthesis of phytochemicals [14-17]. To counteract oxidative stress, plants produce antioxidants, such as phenolics, to mitigate cellular damage. For example, moso bamboo [*Phyllostachys edulis* (Carrière) J. Houz.] responds to water deficiency by accumulating anthocyanins, flavonoids, organic acids, amino acids, sugars, and alcohols [17]. Conversely, salinity induces ionic stress and reduces soil water potential, hindering water uptake by plant roots and triggering mechanisms to minimize water loss through stomatal closure [18]. Pulavarty and Sarangi [15] studied 3 bamboo species, namely *D. strictus*, *D. longispathus*, and *Bambusa bambos*, under salt stress conditions and observed reductions in shoot and root length, decreased chlorophyll content, and increased proline accumulation, indicating physiological and phytochemical changes induced by the stress. Similarly, research by Syed *et al*. [16] demonstrated that bamboo (*B. balcooa*) subjected to drought and salt stress undergoes changes in the expression of antioxidant enzyme genes and increases in the levels of soluble sugars, proline, malondialdehyde (MDA), total amino acids, and H_2O_2. Additionally, exposure to heavy metals in plants induces toxicity via them binding to protein sulfhydryl groups, altering protein structure and enzyme activity, and increasing ROS levels, leading to cellular

damage [19]. In bamboo (*Indocalamus latifolius*), exposure to heavy metals (Cu, Pb, and Zn) was shown to decrease growth indices and photosynthetic rates [20]. Furthermore, studies on moso bamboo found that a lower concentration of Zn (20 - 100 µM) in solution led to increased levels of MDA and proline [14], while growth restriction and abnormalities in root ultrastructure were evident at higher-Zn treatments (200 - 400 µM) [21]. Acidic soil also has adverse effects on agriculture due to its negative impacts on plant growth and nutrient availability. Acidification from fertilizer and chemical use increases Al and Mn solubility, leading to plant toxicity and nutrient leaching due to reductions in plant growth and productivity [22]. Furthermore, different environmental conditions and seasonal variations affect the accumulation of flavonoids and phenolics in bamboo, indicating the dynamicity of plant responses to stress [23,24].

Despite extensive research on the physiological and biochemical responses of bamboo to environmental stress and the overall quantity and antioxidant properties of its phytochemicals, investigations into the regulatory mechanisms controlling the synthesis of specific compounds, particularly FCG such as orientin, isoorientin, vitexin, and isovitexin, remain limited. Against this background, the present study aims to elucidate the effects of environmental stressors such as acidity (pH 4.0), heavy metal exposure (200 µM $ZnSO_4 \cdot 7H_2O$), salinity (100 mM NaCl), and water deficiency on the physiological responses, phytochemical contents, and expression of genes involved in FCG synthesis in 3 bamboo species: *D. sericeus* (Pai Sang-Mon), *S. pergracile* (Pai Khao-Lam), and *T. liang* (Pai-Liang). Specifically, we will investigate the expression of the CGT1 and F2H genes, which are responsible for controlling FCG synthesis [13], as well as the SnRK1 gene, which has been reported to be upregulated when bamboo is placed under stress [25]. This research could provide fundamental data for further intensive studies on the physiology and molecular mechanisms by which bamboo responds to environmental stress. It may also contribute to advancing knowledge in bamboo cultivation and the development of BLE for pharmaceutical, cosmetic, or nutraceutical applications.

Materials and methods

Plant materials, growth conditions, and stress treatments

Branch cuttings from 3 bamboo genotypes, namely, *D. sericeus* (Pai Sang-Mon), *T. liang* (Pai-Liang), and *S. pergracile* (Pai Khao-Lam), aged 6 months (with branch heights of ~120 cm), served as the plant materials. These cuttings were transplanted into soil-filled pots of 13 cm in diameter and 20 cm in height. To prevent waterlogging, trays were placed under these pots. The bamboo plants were subjected to 5 different conditions, each with 4 replicates, as follows: 1) Daily irrigation with 150 mL of water at pH 7.2 - 7.8 (control), 2) Daily irrigation with 150 mL of water at pH 4.0 (acid stress), 3) Daily irrigation with 150 mL of 200 µM $ZnSO_4 \cdot 7H_2O$ (heavy metal stress from zinc), 4) Daily irrigation with 150 mL of 100 mM NaCl (salt stress), and 5) No irrigation (water deficit stress). Observations and sample collection were conducted on days 2, 4, and 8 after stress induction. Photographs were taken to record the characteristics of the leaves of the stressed bamboo under each set of experimental conditions. The leaves evaluated were taken from 6-month-old bamboo plants and included the 1st to 7th leaves from the apex of the branch. The maximum quantum efficiency of photosystem II (Fv/Fm) was measured specifically from the 3rd leaf from the tip of the branch using a Handy PEA chlorophyll fluorimeter (Hansatech Instruments Ltd., UK). Subsequently, samples were prepared for extraction by drying at 60 °C for 3 days, followed by grinding with liquid nitrogen into a fine powder. The powdered samples were stored at −20 °C until further extraction for analysis of secondary metabolites, pigments, and antioxidant assays. Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TChl), and total carotenoids (Caro) were analyzed following the method outlined by Cha-um *et al*. [26]. Analyses of F_v/F_m and photosynthetic pigments were performed on the 4th leaf from the shoot tip of bamboo branches. Additionally, leaf samples for gene expression analysis were collected from positions 2 - 3 from the top of bamboo branches and these samples were immediately submerged in liquid nitrogen and stored at −80 °C for further analysis.

Extraction and determination of phenolics and flavonoids

Powdered leaf samples collected on days 4 and 8 were extracted using a 60 % ethanol solvent, in accordance with a modified version of the protocol reported by Kasemsukphaisan and Maksup [11]. The extraction process involved adding 6 mL of 60 % ethanol to 0.5 g of leaf powder, followed by vortexing for 30 s and incubation in a water bath at 60 °C for 20 min. After centrifugation for 10 min, the supernatant was collected, and the extraction process was repeated twice, with 1.5 mL of solvent added each time. The supernatants were pooled into 1 tube, and the volume was adjusted to 5 mL with 60 % ethanol before storage at 4 °C for subsequent analysis.

The analysis of total phenolic content (TPC) was performed in accordance with a modified version of a method previously reported by Karawak *et al.* [10]. In this procedure, 0.05 mL of sample extract and 0.25 mL of 10 % Folin-Ciocalteu reagent were added to a vial, followed by incubation in the dark for 1 min. Subsequently, 0.75 mL of 75 % sodium carbonate (Na_2CO_3) solution was added, and the total volume was adjusted to 2 mL with distilled water. After being left to stand at room temperature for 30 min, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Optizen 3220UV, Mecasys Co., Ltd, Daejeon, Republic of Korea), with distilled water as the blank. The absorbance readings were compared to a gallic acid standard curve, and the TPC was expressed as mg of gallic acid equivalents per gram of dry weight of bamboo-leaf sample (mg GAE g^{-1} DW).

The analysis of total flavonoid content (TFC) was conducted using the aluminum chloride colorimetric method [10]. In this method, 0.25 mL of sample extract and 0.75 mL of methanol were mixed in a vial, followed by the addition of 0.05 mL of 10 % aluminum chloride and 0.05 mL of 1 M potassium acetate. The total volume was adjusted to 2.5 mL with distilled water. After being left to stand at room temperature for 30 min, the absorbance was measured at 415 nm using a UV-Vis spectrophotometer, with distilled water as a blank. The absorbance readings were compared to a quercetin standard curve, and the TFC was calculated as µg of quercetin equivalents per gram of dry weight of bamboo-leaf sample (µg QE g^{-1} DW).

Analysis of antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was assessed using a modified version of a protocol previously reported by Karawak *et al*. [10]. Briefly, 2 mL of DPPH solution was mixed with 100 µL of Trolox (standard antioxidant compound) or the BLE, and the absorbance was promptly measured (OD_{t0}) at 515 nm using a UV-Vis spectrophotometer. Trolox standards (0 - 0.4 mM) or BLE (0 - 0.8 mg mL^{-1}) were incubated with the DPPH solution for 30 min in the absence of light. Subsequently, the absorbance was remeasured (OD_{t30}) and the rate of DPPH inhibition was calculated using the following formula: % DPPH inhibition = $[(OD_{t0} - OD_{t30})/OD_{t0}]\times100$. A concentration-response curve was constructed to illustrate the relationship between the concentration of sample extracts or standards and the rate of DPPH inhibition. The antioxidant capacity of the sample extracts was determined relative to Trolox standards and is expressed as Trolox equivalents antioxidant capacity (TEAC) in mM TE g^{-1} DW.

Analysis of FCG and phenolic acids

The identification and quantification of FCG and phenolic acids in the sample extracts were performed using RP-HPLC. After evaporation to dryness, the crude extracts were re-dissolved in 60 % ethanol to achieve a concentration of 1 mg mL^{-1}. The filtered samples were injected into an HPLC system composed of the Agilent HPLC 1260 Infinity II bioinert (Agilent Technologies, Germany) coupled with a Waters 2996 diode array detector equipped with a C18 column [Agilent TC-C18(2), 4.6×250 mm, 5 µm]. The HPLC running conditions were similar to those outlined by Kasemsukphaisan and Maksup [11]. Detection was conducted at 335 nm, and compound identification was accomplished by comparison with standard compounds (isoorientin, orientin, isovitexin, vitexin, chlorogenic acid, caffeic acid, *p*-coumaric acid, and apigenin). All samples were analyzed in triplicate.

Study of gene expression levels using quantitative real-time PCR (qRT-PCR)

RNA extraction, cDNA synthesis, and qRT-PCR were performed on leaf samples of all 3 bamboo species from each experimental group following the method

described by Maksup *et al*. [27]. Approximately 100 mg of each sample was used for total RNA extraction with Trizol reagent, in accordance with the manufacturer's guidelines (Invitrogen, CA, USA). The RNA pellet was dissolved in 20 µL of RNase-free water. Subsequently, the obtained RNA samples were quantified and assessed for quality using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. RNA samples (500 ng) were subjected to cDNA synthesis using the ImProm-II™ Reverse Transcription System (Promega Corporation, USA), in accordance with the manufacturer's instructions, and the synthesized cDNA was stored at −20 °C until further use.

Primers for F2H and CGT1 genes of the 3 bamboo species were designed from the conserved sequences of these genes. Additionally, the SnRK1 gene, known to be responsive to stress in moso bamboo (*P. edulis*), and the serine/threonine protein 2 (PP2A) gene, previously reported to have stable expression levels in moso bamboo [25], were used as reference genes **(Table 1)**. The cDNA from leaf samples of all 3 bamboo species in each experimental group was analyzed for the expression levels of CGT, F2H, and SnRK1 genes using qRT-PCR with the Applied Biosystems 7500 Real-Time PCR System.

Table 1 Information of reference genes and other gene-specific primers used in this study.

Gene description	Primer pairs (5' → 3')	Amplicon size (bp)	References
C-glycosyltransferase (CGT1)	F: 5' CTTGTGGGTGGTGAAGAGCA 3'	181	This study
	R: 5' CCAACCACAGTGGCTGATGA 3'		
Flavanone 2-hydroxylase (F2H)	F: 5' GACATCCTCATGGACGTGG 3'	158	This study
	R: 5' TAGGCCGGGTGGTTGATCA 3'		
Sucrose nonfermenting 1- related protein kinase 1 (SnRK1)	F: 5' AGCTCGACGATGAAACCCTT 3'	248	Pan [25]
	R: 5' TTCCATAGAACCGTACTGCCTA 3'		
Serine/threonine protein 2 (PP2A) (Reference gene)	F: 5' ACGCCCCCGACACCAACT 3'	141	Pan [25]
	R: 5' TCTGCTCTCATAATTTCCTCTC 3'		

Note: *Sequences and percent identities of the studied genes against the NCBI database are shown in Supplementary **Table S1**.

The reaction mixture contained 1.2 µL of cDNA, 4.32 µL of nuclease-free water, 0.24 µL of each primer (10 pmol), and 6 µL of KAPA SYBR FAST qPCR Master Mix (2×) in each well. The qRT-PCR was then conducted with the following steps: 1) Holding stage at 95 °C for 3 min, 2) Cycling stage consisting of denaturation at 95 °C for 3 s, and annealing and extension at 60 °C for 30 s, repeated for 40 cycles, and 3) Melting curve analysis. Each set of experimental conditions was applied to 3 biological replicates, and each qRT-PCR analysis was conducted with 2 technical replicates. The melting curve analysis was performed using the PP2A gene as a reference. The relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method [28].

Statistical analysis

The statistical significance of differences in mean values and standard deviations was analyzed using one-way ANOVA followed by post hoc tests using Duncan's multiple range tests (DMRT), implemented in IBM SPSS Statistics software.

Results and discussion

Impact of environmental stress on bamboo growth and physiology

Bamboo species exhibit notable adaptability to various environmental stresses, including water deficit, salinity, heavy metals, and acidity. These stressors can induce significant changes in the growth and physiological responses of bamboo plants

[14,16,20,29]. The complexity of plant responses is influenced by the duration and intensity of stress, plant genotype, the combination of different stresses, and the developmental stage at which plants are exposed to the stress [30]. This study investigated the growth characteristics and physiological responses of Pai Sang-Mon, Pai-Liang, and Pai Khao-Lam under environmental stress conditions, including acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit stress for 4 and 8 days. The results revealed that these environmental stresses each influenced the growth and leaf characteristics of bamboo differently (**Figure 1** and **Table 2**). Here, water deficit stress had more pronounced impacts on leaf color and photosynthetic efficiency than the other stressors in bamboo samples. After 8 days of water deficit stress, Pai Sang-Mon and Pai-Liang leaves exhibited complete desiccation and turned brown, losing all green pigmentation, while Pai Khao-Lam leaves, although wilted, retained some green coloration, highlighting a differential response to water deficit stress among the bamboo varieties. In contrast, the leaves of all 3 bamboo varieties remained turgid under acid, zinc, and salt stresses, displaying only minor yellowing in some leaflets, thereby underscoring the more severe impact of water deficit stress on leaf color and overall health. Typically, water deficit stress leads to stomatal closure, reduced transpiration rates, decreased leaf chlorophyll content, and alterations in photosynthetic efficiency, as evidenced by changes in parameters such as Fv/Fm [31,32]. A study on the effects of 3-month drought treatments on 1-year-old potted seedlings of moso bamboo found that drought stress also decreased root and shoot dry weight and altered root architecture [33]. Furthermore, the reduction in Fv/Fm values and chlorophyll b content observed in Pai-Liang after 8 days of water deprivation is closely associated with the significant browning of its leaves, underscoring its vulnerability to water deficit stress (**Table 2**). In contrast, the increase in Fv/Fm values in Pai Khao-Lam on day 4, following both zinc and water deficit stress, coupled with the retention of some green coloration in its leaves across all environmental stress conditions, suggests a potential resilience linked to enhanced pigment stability under stress. This interplay between photosynthetic efficiency and pigment content underscores the importance of these parameters in evaluating the overall health and stress response of bamboo species.

In this study, the acid, zinc, and salt stress conditions resulted in mild stress, as demonstrated by the maintenance of cell turgidity, and did not adversely affect the Fv/Fm values or photosynthetic pigment contents of Pai Khao-Lam and Pai Sang-Mon; however, negative effects have been observed in other bamboo species. Pulavarty and Sarangi [15] reported that *D. strictus* and *B. bambos*, species that are defined as being salt-tolerant, exhibited reduced growth (shoot length, root length, number of leaves, and fresh weight) and increased chlorophyll degradation after treatment with 100 mM NaCl. These adverse effects were less evident than those in the sensitive species *D. longispathus*. Elsewhere, Ahmad *et al*. [34] evaluated the growth performance of 4 bamboo species (*D. giganteus*, *D. strictus*, *D. hamiltonii*, and *D. longispathus*) under salinity stress. Their results showed that, as NaCl concentration/salinity increased, morphological parameters declined and biomass production decreased, with *D. giganteus* being the least affected, indicating its higher salt tolerance. In another study, Emamverdian *et al*. [20] showed that 2-year-old *I. latifolius* treated with Zn at different concentrations (0, 500, 1000, and 2000 mg kg^{-1}) exhibited reduced photosynthetic-related indices, transpiration, and growth at high Zn concentrations (1000 and 2000 mg kg^{-1}). However, photosynthesis and growth were enhanced at low Zn concentrations (500 mg kg^{-1}), suggesting that Zn at low levels acts as a micronutrient [35,36]. This aligns with our finding that Fv/Fm of Pai Khao-Lam increased after 4 days of exposure to 200 µM $ZnSO_4 \cdot 7H_2O$ (**Table 2**). Soil pH significantly influences nutrient solubility and availability to plants; highly acidic soil (pH < 5.5) can hinder plant growth primarily by reducing the availability of essential nutrients and enhancing the toxicity of heavy metals, along with negatively impacting microbial communities [37-39]. Although there have been few studies on the effect of acidic soil or low pH in bamboo, long-term application of N fertilizer in moso bamboo forests in Zhejiang Province, China, has been shown to increase soil acidification rates, enhance metal ion mobility (Cu, Pb), and ultimately affect bamboo growth [38].

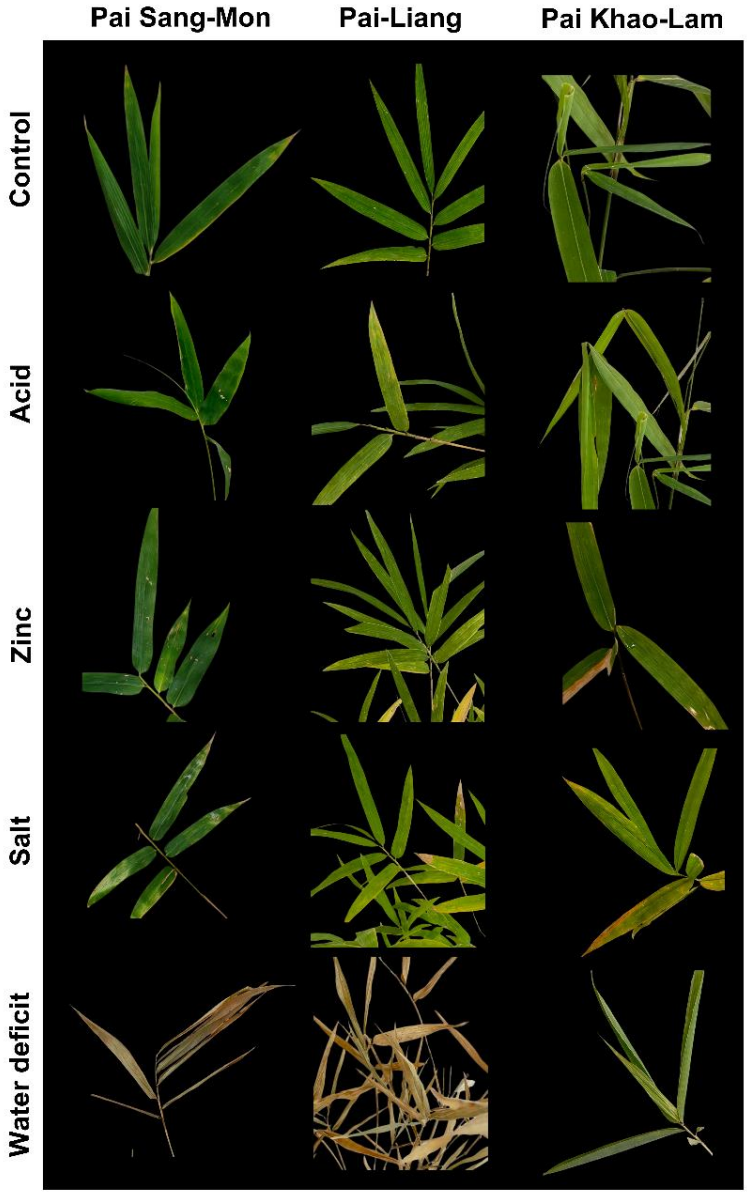

Figure 1 Phenotypes of leaves taken from the apex of the branches of 3 bamboo genotypes after 8 days of exposure to acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit stress.

Table 2 The changes in maximum quantum efficiency of PSII (Fv/Fm), and chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TChl), and carotenoid contents in Pai Sang-Mon, Pai-Liang, and Pai Khao-Lam leaves after exposure to acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit stress for 4 and 8 days.

Time	Species	Stress	Fv/Fm	Chl a	Chl b	TChl	Caro
Day 4	Pai Sang-Mon	Control	0.77 ± 0.03	9.50 ± 0.58	6.92 ± 1.87	16.42 ± 1.80	2.49 ± 0.73
		Acid	0.77 ± 0.01	6.50 ± 3.15	6.18 ± 3.96	12.68 ± 3.17	1.18 ± 2.06
		Zinc	0.78 ± 0.03	7.92 ± 0.90	4.71 ± 1.90	12.63 ± 2.63	2.36 ± 0.34
		Salt	0.75 ± 0.03	9.03 ± 0.84	6.68 ± 0.95	15.71 ± 1.66	2.32 ± 0.37
		Water deficit	0.74 ± 0.05	9.18 ± 0.89	7.27 ± 2.57	16.44 ± 3.41	2.35 ± 0.46^{ns}
		ANOVA	NS	NS	NS	NS	NS
	Pai-Liang	Control	0.73 ± 0.03^{a}	6.63 ± 1.75	3.23 ± 1.24	9.86 ± 2.98	2.17 ± 0.48

Time	Species	Stress	Fv/Fm	Chl a	Chl b	TChl	Caro
		Acid	0.74 ± 0.02[a]	7.30 ± 2.24	3.65 ± 1.60	10.95 ± 3.80	2.51 ± 0.61
		Zinc	0.72 ± 0.02[a]	7.33 ± 1.30	3.62 ± 0.92	10.96 ± 2.21	2.43 ± 0.13
		Salt	0.72 ± 0.04[a]	6.00 ± 1.29	2.57 ± 0.78	8.57 ± 2.03	2.59 ± 0.23
		Water deficit	0.30 ± 0.28[b]	6.30 ± 2.49	3.39 ± 1.87	9.68 ± 4.35	3.00 ± 0.21
		ANOVA	*	NS	NS	NS	NS
	Pai Khao-Lam	Control	0.59 ± 0.12[b]	8.44 ± 0.74	4.65 ± 0.63	13.09 ± 1.21	2.99 ± 0.47
		Acid	0.51 ± 0.06[b]	8.80 ± 1.25	5.52 ± 2.10	14.31 ± 3.18	2.86 ± 0.43
		Zinc	0.72 ± 0.04[a]	8.09 ± 0.65	4.11 ± 0.15	12.19 ± 0.75	3.34 ± 0.15
		Salt	0.62 ± 0.07[ab]	8.29 ± 1.70	4.39 ± 1.48	12.68 ± 3.16	3.08 ± 0.41
		Water deficit	0.73 ± 0.05[a]	7.24 ± 1.80	3.63 ± 1.20	10.87 ± 2.91	3.03 ± 0.64
		ANOVA	*	NS	NS	NS	NS
Day 8	Pai Sang-Mon	Control	0.77 ± 0.05	9.36 ± 0.91	7.70 ±1.76[ab]	17.06 ± 2.28	2.35 ± 0.60
		Acid	0.80 ± 0.02	8.94 ± 1.46	4.73 ±1.49[c]	13.67 ± 2.87	3.19 ± 0.40
		Zinc	0.79 ± 0.03	9.20 ± 0.91	5.32 ± 1.15[bc]	14.52 ± 1.89	3.01 ± 0.42
		Salt	0.81 ± 0.01	9.90 ± 0.73	8.66 ± 2.14[a]	18.56 ± 2.24	2.38 ± 0.73
		Water deficit	0.59 ± 0.40	8.88 ± 1.35	7.12 ± 2.00[abc]	16.00 ± 3.14	2.26 ± 0.15
		ANOVA	NS	NS	*	NS	NS
	Pai-Liang	Control	0.74 ± 0.01[a]	7.61 ± 1.51[a]	4.12 ± 1.54	11.73 ± 3.02	2.63 ± 0.31
		Acid	0.72 ± 0.02[a]	7.62 ± 1.38[a]	3.76 ± 1.29	11.38 ± 2.67	2.72 ± 0.30
		Zinc	0.74 ± 0.03[a]	7.83 ± 1.62[a]	4.33 ± 1.98	12.15 ± 3.53	2.57 ± 0.23
		Salt	0.72 ± 0.03[a]	8.59 ± 1.08[a]	5.21 ± 1.72	13.80 ± 2.63	2.81 ± 0.39
		Water deficit	0.19 ± 0.36[b]	3.55 ± 4.06[b]	2.36 ± 2.36	5.91 ± 6.42	1.43 ± 1.36
		ANOVA	*	*	NS	NS	NS
	Pai Khao-Lam	Control	0.66 ± 0.05	9.22 ± 1.60	8.07 ± 3.08	17.28 ± 4.38	2.41 ± 0.74
		Acid	0.70 ± 0.14	9.60 ± 0.83	7.50 ± 1.76	17.09 ± 2.27	2.72 ± 0.63
		Zinc	0.75 ± 0.04	9.51 ± 0.96	7.53 ± 3.02	17.04 ± 3.67	2.79 ± 0.81
		Salt	0.67 ± 0.05	9.50 ± 1.62	7.52 ± 3.08	17.01 ± 4.66	2.66 ± 0.38
		Water deficit	0.58 ± 0.37	8.62 ± 2.14	6.64 ± 3.30	15.26 ± 5.29	2.86 ± 0.69
		ANOVA	NS	NS	NS	NS	NS

Note: The values presented are expressed as mean ± SD (n = 4). The mean values were compared among different stress conditions of each bamboo genotype based on DMRT. NS and * indicate non-significant and significant difference at $p \leq 0.05$, respectively.

Phytochemical responses and gene expression patterns associated with FCG synthesis

Plants exhibit a wide array of responses to abiotic stresses, encompassing changes in gene expression, physiology, plant architecture, and both primary and secondary metabolism [40]. The biosynthesis of secondary metabolites or phytochemicals often increases as a defense mechanism against oxidative stress, enhancing plant resistance to environmental stresses [17,40-42]. These intricate changes enable plants to tolerate and adapt to adverse conditions [40].

In this study, the changes in TPC, TFC, and antioxidant activity analyzed by spectroscopy-based techniques were not statistically significant, corresponding to the slight changes observed in photosynthetic efficiency and leaf photosynthetic

pigments (**Figure 2**). Similar findings were reported by Karawak *et al*. [10], who found no significant differences in TFC, anthocyanin content, and antioxidant activity in Pai Sang-Mon and Pai-Liang. Kasemsukphaisan and Maksup [11] also noted that TPC, TFC, and antioxidant activity in leaves of 11 different bamboo genotypes collected from Uttaradit Province, Thailand, varied among genotypes but showed no significant differences among Pai Sang-Mon, Pai Khao-Lam, and Pai-Liang. Additionally, they found that, while TPC and antioxidant activity in leaves of Pai Sang-Mon and Pai-Liang harvested in summer and winter did not differ, TFC was significantly higher in winter, suggesting that flavonoids, including FCG, may increase under abiotic stress.

However, further RP-HPLC-based analysis revealed significant changes in certain flavonoid levels under the applied abiotic-stress conditions (**Figures 3** and **4**). It was observed that different bamboo genotypes exhibited distinct phytochemical profiles, indicating genotype-dependent responses to environmental stressors. Under unstressed conditions, the predominant phytochemicals in Pai Sang-Mon were vitexin (6.1 - 6.2 mg g^{-1} DW), isoorientin (1.4 - 1.6 mg g^{-1} DW), and apigenin (0.1 - 0.3 mg g^{-1} DW), whereas Pai-Liang showed an abundance of *p*-coumaric acid (10.0 - 12.2 mg g^{-1} DW), isoorientin (1.6 - 2.1 mg g^{-1} DW), and isovitexin (1.4 - 1.9 mg g^{-1} DW). Caffeic acid (0.12 - 0.17 mg g^{-1} DW) and orientin (0.6 - 2.7 mg g^{-1} DW) were found in comparable quantities in 3 bamboo species. Moreover, chlorogenic acid was absent from the BLE of Pai Sang-Mon and Pai-Liang, and apigenin was not detected in the BLE of Pai Khao-Lam (**Figure 3**). These findings align with previous studies showing that vitexin is a major compound in Pai Sang-Mon (2.33 mg g^{-1} DW) and *p*-coumaric acid is abundant in Pai-Liang (4.12 mg g^{-1} DW) [11].

Similarly, the BLE of *Phyllostachys nigra*, *Lophatherum gracile*, and *Pleioblastus amarus* contain various phytochemicals, with *P. nigra* having 4 times more chlorogenic acid than the other 2 species, and exhibiting an abundance of isoorientin and isovitexin. Orientin was most abundant in *P. amarus*, and cynaroside dominated in *L. gracile* [43]. In *B. multiplex* cv. Fernleaf, isoorientin was consistently the most dominant flavonoid, accounting for approximately 50 % of total flavonoids in different seasons [44]. Furthermore, 9 chemical compounds, including 3 FCG, 2 flavone O-glycosides, 3 flavone C-diglycosides, and 1 flavone di-C,O-glycoside, were identified in the BLE of *P. edulis* using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry, highlighting the complexity and variation of flavonoids in bamboo species [45,46].

The response of different species to environmental stresses is a complex phenomenon, which varies from 1 species to the next. Abiotic factors have been shown to stimulate multi-gene responses, leading to modifications in the accumulation of secondary metabolites [40,47]. Our study revealed that the expression of FCG-associated genes and the phytochemical profiles changed in different ways across the bamboo genotypes under stress conditions (**Figures 3 - 5**). The proposed biosynthetic pathway of 8 phytochemicals and FCG-associated genes identified in this study is presented in **Figure 6**.

For Pai Sang-Mon, total phytochemical contents tended to increase only under salt stress (**Figure 4**). Specifically, caffeic acid and apigenin levels increased in the leaves of this species after exposure to salt stress (**Figures 3(b)** and **3(h)**). Conversely, vitexin levels decreased after exposure to salt and water deficit stresses for 4 days, correlating with lower expression of the CGT1 gene (**Figures 3(f)** and **5(d)**), which regulates the final step in vitexin biosynthesis in bamboo [13]. Since the biosynthesis of caffeic acid occurs upstream and apigenin uses the same substrate (naringenin) as vitexin, the increases in caffeic acid and apigenin might lead to a reduction in vitexin [13,48,49]. Similarly, Hodaei *et al*. [50] showed that water deficiency stimulates the synthesis of luteolin, quercetin, rutin, and apigenin in *Chrysanthemum morifolium*, with these phytochemicals playing crucial roles in the enhancement of antioxidant activity and adaptation to environmental stress [51].

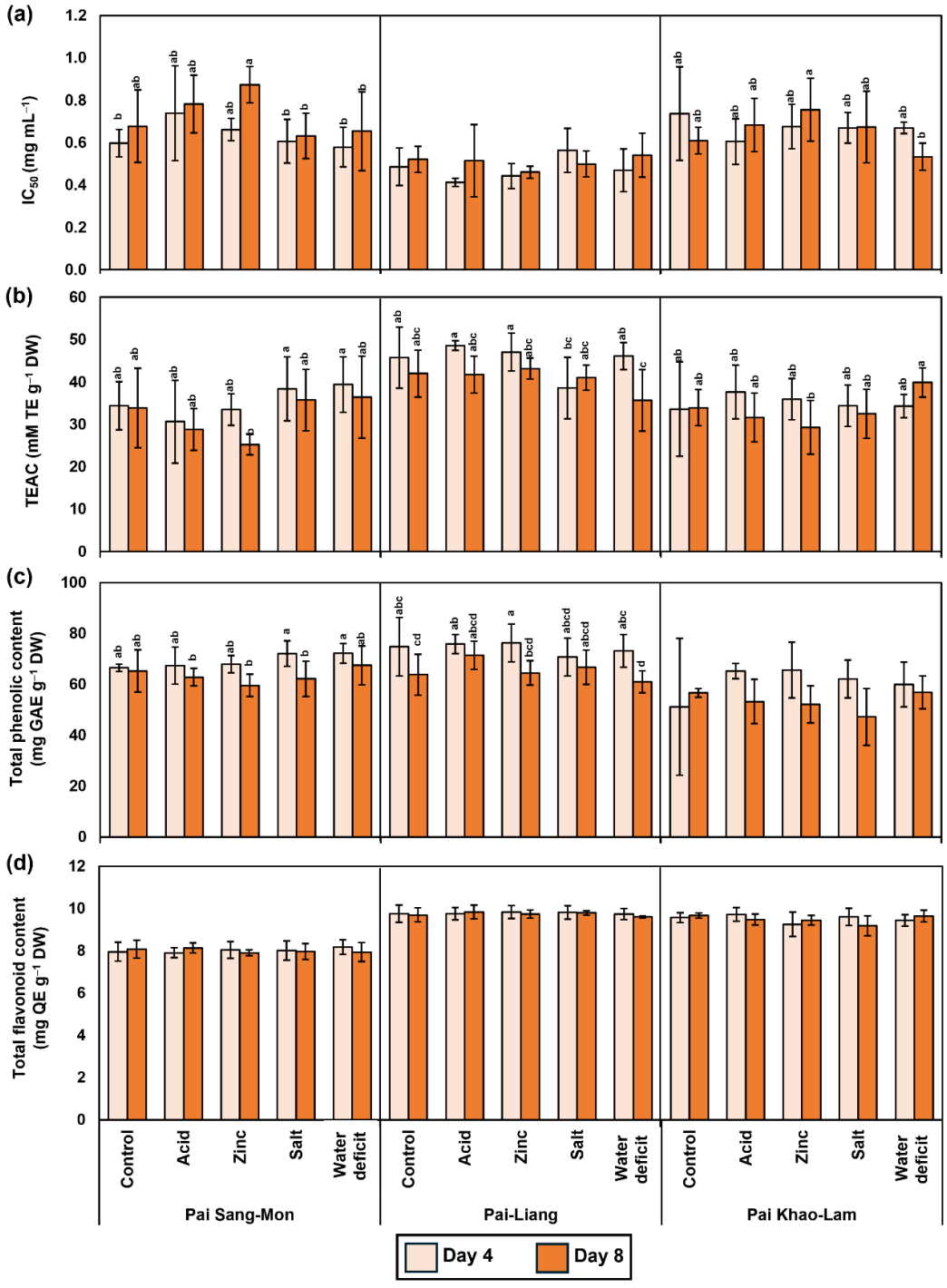

Figure 2 Determination of TEAC **(a)**, IC_{50} **(b)**, TPC **(c)**, and TFC **(d)** in BLE of 3 bamboo genotypes after exposure to acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit) stress for 0, 4, and 8 days. Data represent mean ± SD (n = 4). The mean values were compared among different stress conditions of each bamboo genotype based on DMRT ($p \leq 0.05$).

Our study found that the expression of the SnRK1 gene, essential for carbon metabolism and stress responses in annual herbaceous plants [25], remained unchanged during salt and water deficit stresses (**Figure 5(g)**). It was reported that stress caused by 200 mM NaCl induced SnRK1 expression in *P. edulis* seedlings, with transgenic SnRK1-overexpressing seeds showing significantly higher germination rates and longer roots under high-salt conditions, indicating that SnRK1 plays an important regulatory role in the stress tolerance of

bamboo [25]. These results suggest that Pai Sang-Mon has the potential to grow under various environmental stresses; however, salt stress (100 mM NaCl) may affect the quality of its BLE by reducing vitexin production.

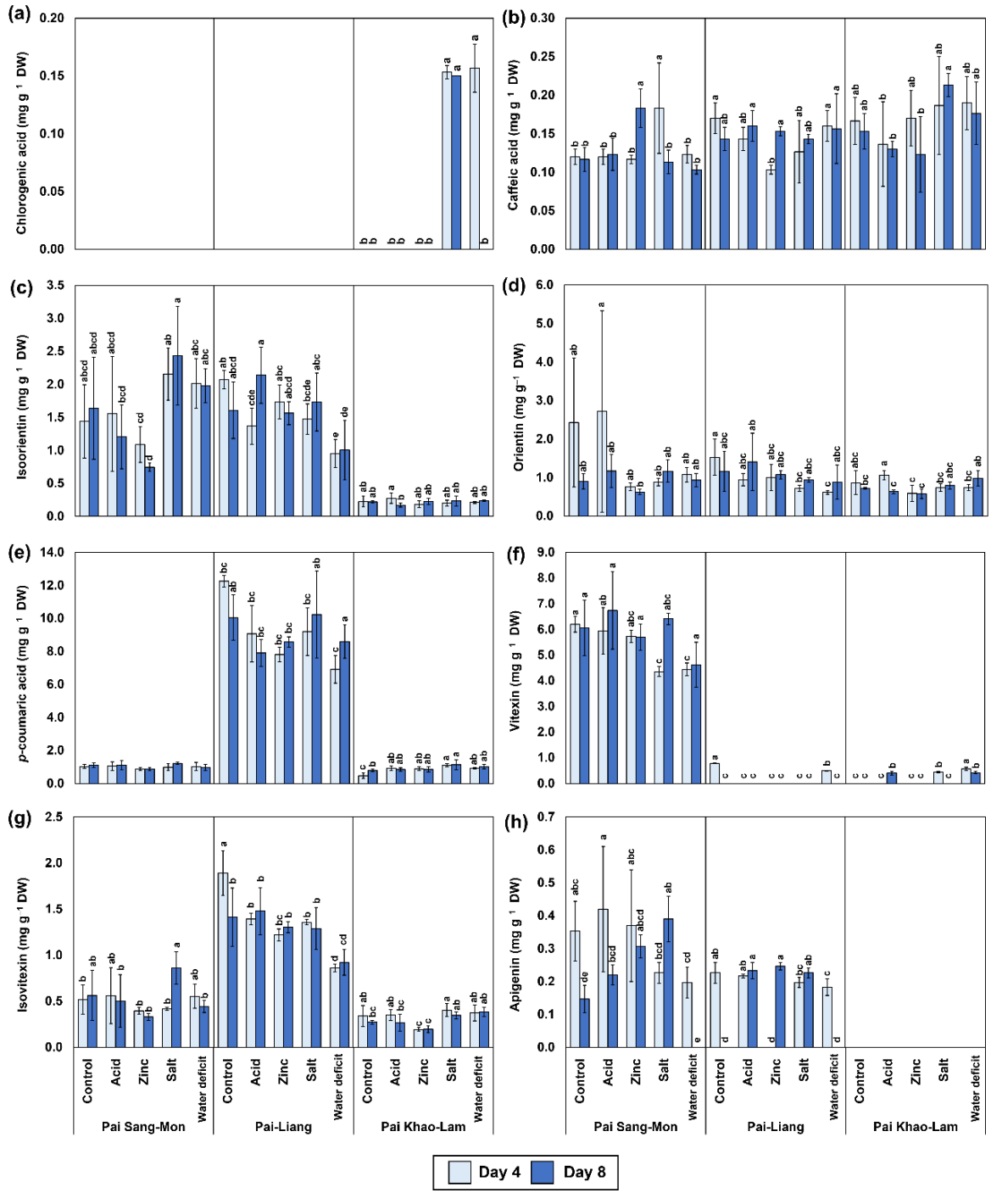

Figure 3 The contents of 8 phytochemicals in BLE of Pai Sang-Mon, Pai-Liang, and Pai Khao-Lam after exposure to acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit stress for 4 and 8 days. Data represent mean ± SE from 3 biological replicates. The mean values were compared among different stress conditions of each bamboo genotype at $p \leq 0.05$ based on DMRT.

For Pai-Liang, an increase in apigenin was observed after 8 days of acid, zinc, and salt stresses, while isoorientin, orientin, vitexin, and isovitexin exhibited decreases in their levels after exposure to various stress conditions, particularly after acid and water deficit stresses for 4 or 8 days (**Figure 3**). Total

phytochemical contents tended to decrease under acid, zinc, or water deficit stresses, corresponding to decreased levels of isoorientin, orientin, vitexin, and isovitexin (**Figures 3** and **4**). These reductions align with the decreased expression of the CGT1 gene after exposure to acid, zinc, or water deficit stresses for 4 days (**Figure 5(e)**). Conversely, total phytochemical contents were observed to increase under salt stress (**Figure 4(b)**), potentially due to elevated apigenin content after 8 days of acid, zinc, and salt stresses (**Figure 3(h)**), suggesting that FCG and apigenin biosynthesis are competitive pathways [49]. Furthermore, increased expression of the SnRK1 gene was observed after 2 days of salt stress (**Figure 5(h)**). This implies that Pai-Liang's mechanism of response to salt stress involves influencing the expression of stress-inducible genes, controlling cell proliferation, balancing energy requirements, and consequently inducing stress tolerance [52-54]. However, significant downregulation of the SnRK1 gene after exposure to acid, zinc, salt, and water deficit stresses for 8 days (**Figure 5(h)**) suggests that Pai-Liang may eventually fail to maintain cellular homeostasis under long-term stress conditions or may rely on alternative methods [53]. Thus, Pai-Liang is more sensitive to environmental stress than Pai Sang-Mon and Pai Khao-Lam, and the quality of its BLE is reduced upon growth under stressed conditions.

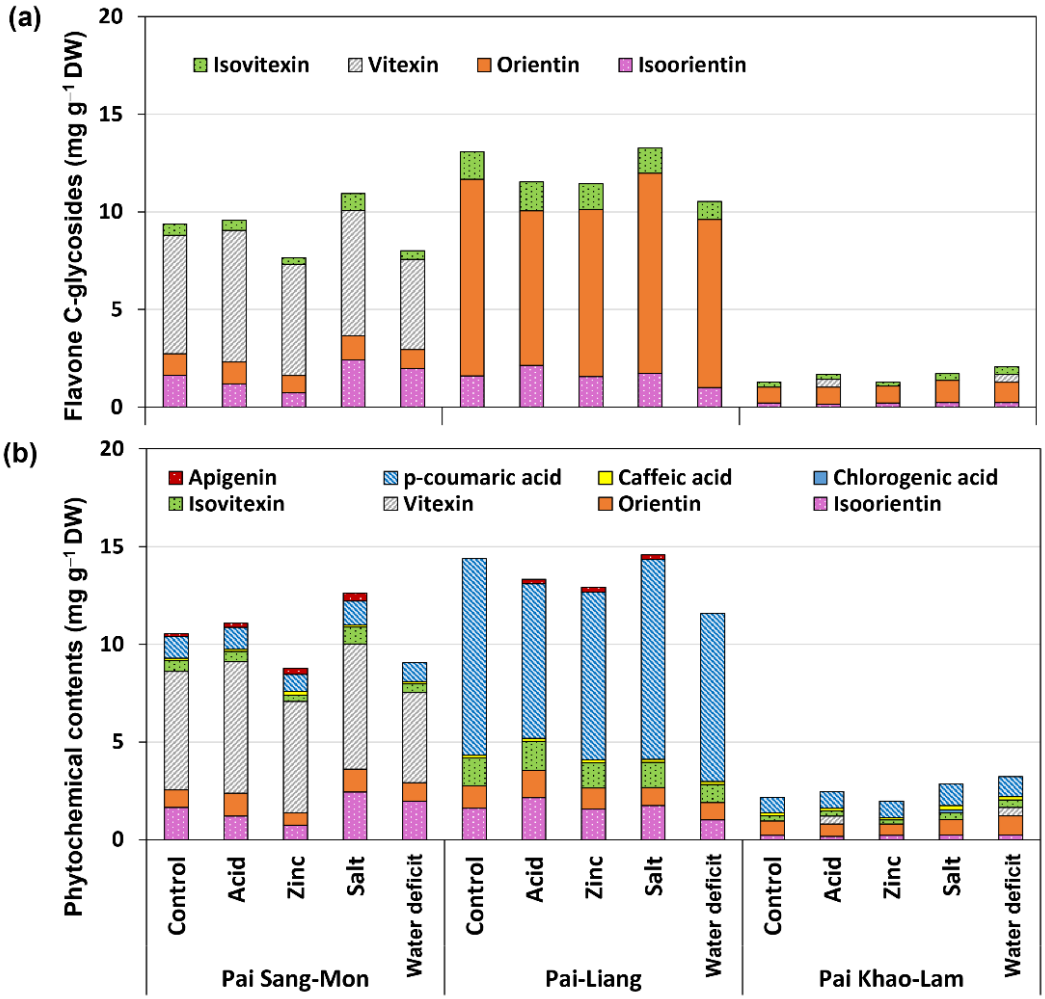

Figure 4 An overview of flavone C-glycoside (a) and 8 phytochemical contents (b) analyzed by RP-HPLC in BLE of 3 bamboo genotypes after exposure to acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit stress for 8 days.

In the case of Pai Khao-Lam, although the overall quantity of phytochemicals was lower than for the other 2 species (**Figure 4(b)**), chlorogenic acid increased after salt and water deficit stresses (**Figure 3(a)**), while *p*-

coumaric acid increased after acid, zinc, salt, and water deficit stresses for 4 days (**Figure 3(e)**). Vitexin increased after acid, salt, and water deficit stresses for 4 or 8 days (**Figure 3(f)**). Moreover, when considering the total content of FCG and 8 phytochemical contents at 8 days, it was found that the quantities in Pai Sang-Mon and Pai-Liang tended to increase after salt stress but decrease after zinc and water deficit stresses. Although the total quantity of phytochemicals was lower in Pai Khao-Lam than in the other 2 species, FCG and phenolic acids tended to increase in it after exposure to environmental stresses (**Figure 4**). This was reflected in the increased levels of chlorogenic acid under salt and water deficit stresses and *p*-coumaric acid under acid, zinc, salt, and water deficit stresses. Chlorogenic acid and *p*-coumaric acid are phenolic compounds that play important roles in defense against various abiotic stresses, including heavy metals, UV radiation, cold, and drought [40]. The increased accumulation of these compounds suggests that Pai Khao-Lam has the ability to scavenge ROS, maintain cellular osmotic potential, and consequently adapt to and resist environmental stress [40,41].

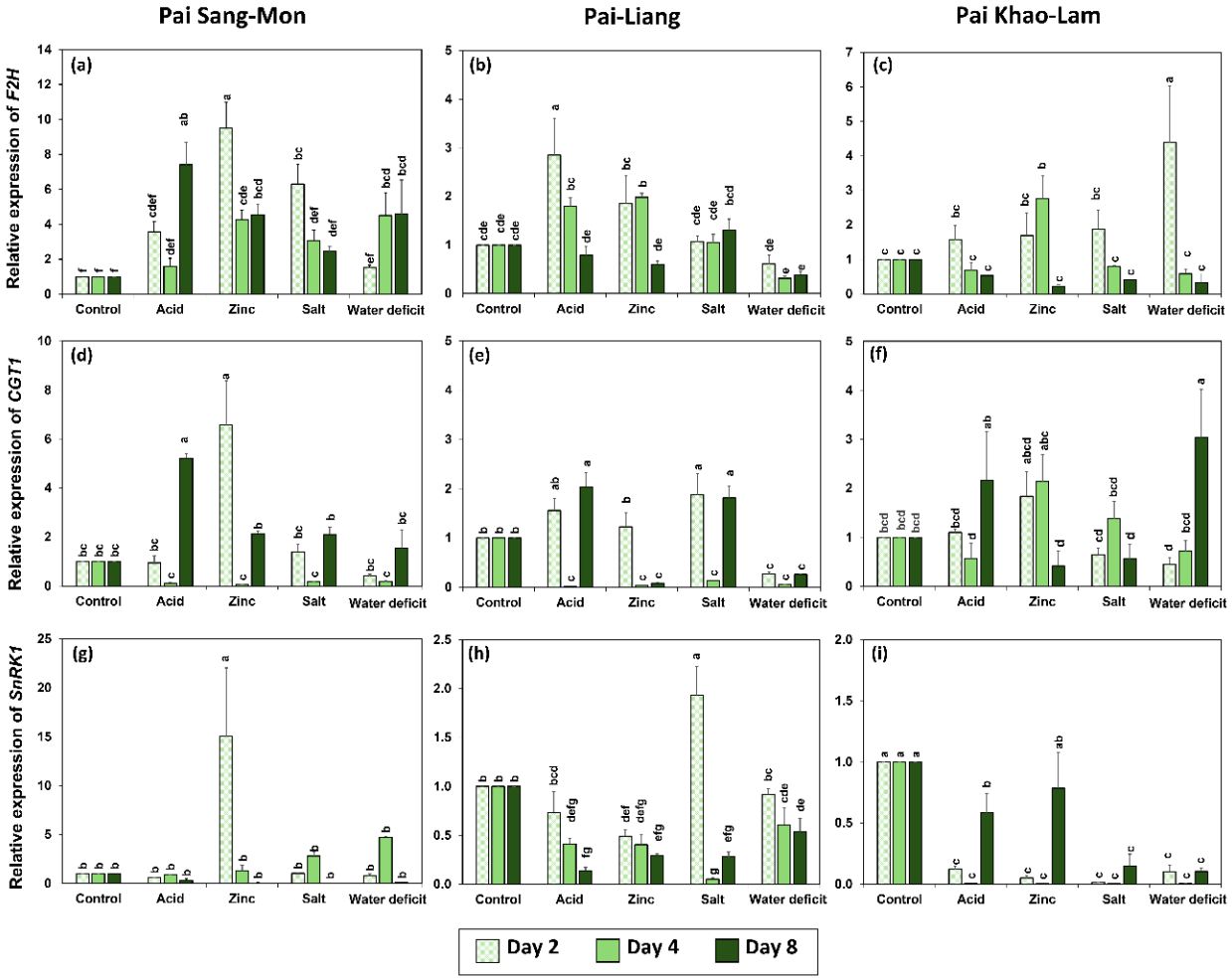

Figure 5 Relative expression of GCT1, F2H, and SnRK1 genes in leaves of 3 bamboo genotypes after exposure to acid stress (pH 4.0), zinc stress (200 µM $ZnSO_4 \cdot 7H_2O$), salt stress (100 mM NaCl), and water deficit stress for 2, 4, and 8 days. Data represent mean ± SD (n = 3). The mean values were compared among different stress conditions of each bamboo genotype based on DMRT ($p \leq 0.05$).

Additionally, vitexin levels increased under acid, salt, and water deficit stresses, while apigenin was not detected, indicating that naringenin was preferentially used for vitexin synthesis rather than apigenin. Similarly, Lim *et al*. [55] reported that vitexin content increased in buckwheat (*Fagopyrum esculentum* M.)

sprouts after exposure to NaCl stress (10, 50, 100, and 200 mM) for 7 days. However, upregulation of the F2H and CGT1 genes was observed only after 2 and 8 days of water deficit stress, respectively (**Figures 5(c)** and **5(f)**). A slight correlation was observed between phytochemical content and gene expression, likely influenced by the presence of multiple gene families with similar functions, which may complicate the relationship between these variables [13,56,57]. Additionally, the downregulation of the SnRK1 gene under all stress conditions (**Figure 5(i)**) suggests that Pai Khao-Lam utilizes alternative mechanisms to resist environmental stress, potentially through different pathways that promote the accumulation of phenolic compounds to maintain cell turgidity and photosynthetic pigments [11]. This alternative strategy may enable the plant to sustain homeostasis and improve its chances of survival under adverse conditions.

This study underscores the intricate relationship between gene regulation and the presentation of phytochemicals, particularly FCG, in response to environmental stressors in bamboo species (**Figure 6**). Evaluation of the expression of FCG-associated genes can shed light on the molecular responses of bamboo species to environmental challenges. Understanding how these genes are regulated under stress conditions can enhance our knowledge of bamboo resilience and inform breeding or conservation efforts. There is a need for further studies to elucidate the complex regulatory network governing bamboo's adaptive responses to environmental stress.

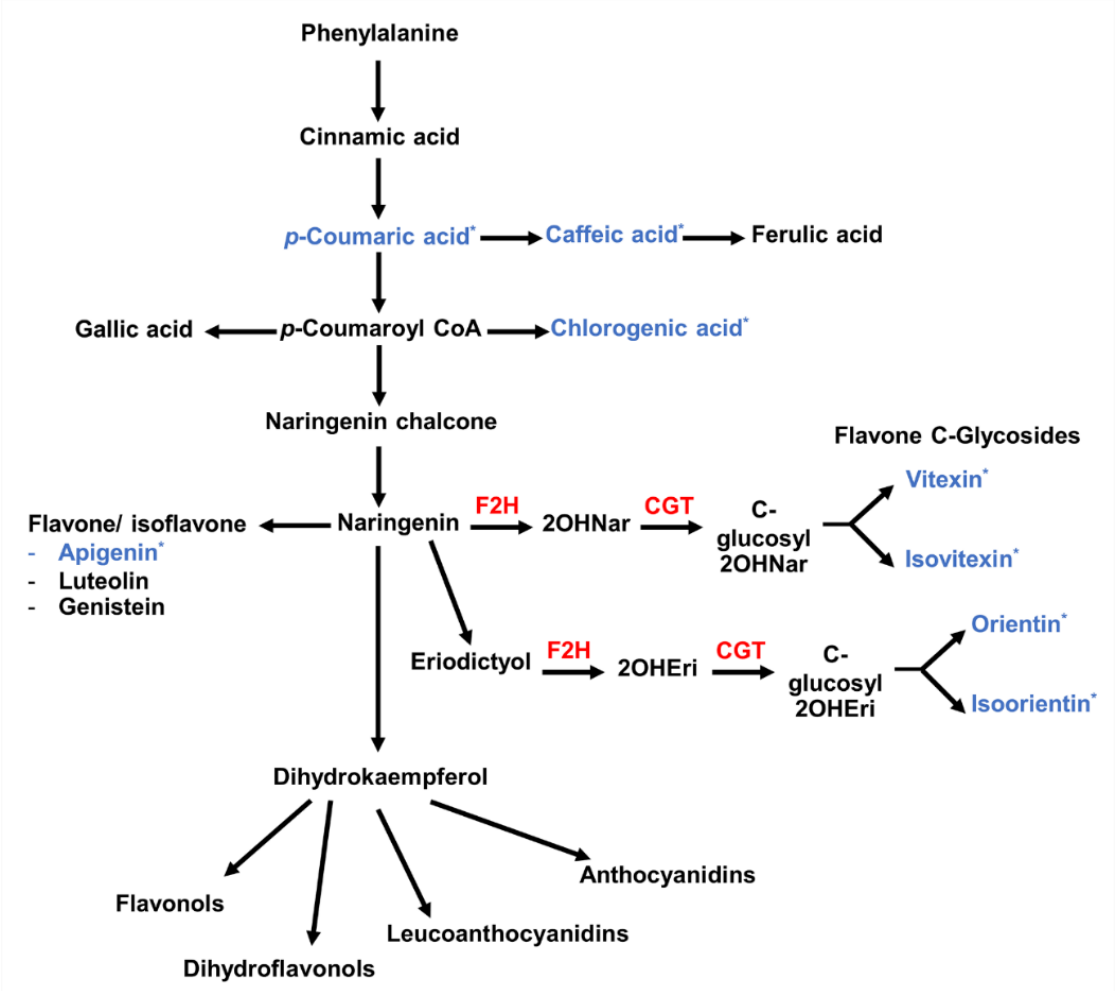

Figure 6 Overview of the proposed biosynthetic pathway of flavonoids in bamboo leaves. Abbreviations: F2H, flavanone 2-hydroxylases; CGT, C-glycosyltransferases; 2OHNar, 2-hydroxylnaringenin; 2OHEri, 2-hydroxyleriodictyol. Asterisks indicate 8 phytochemicals as determined in this study.

Conclusions

This study investigated the growth, physiological responses, phytochemical profiles, and gene expression patterns of 3 bamboo genotypes (Pai Sang-Mon, Pai-Liang, and Pai Khao-Lam) under acid, zinc, salt, and water deficit stresses. Our findings reveal distinct mechanisms these bamboo types employ to adapt to environmental stressors, with water deficit stress having the most significant impact on growth and photosynthesis. Pai Khao-Lam and Pai Sang-Mon demonstrated greater tolerance than Pai-Liang, as indicated by stable or increased Fv/Fm values and minimal leaf wilting. Phytochemical profiling and gene expression analyses showed varied responses. Pai Sang-Mon increased caffeic acid and apigenin levels under salt stress, which was accompanied by a reduction in vitexin, correlating with lower CGT1 expression. In contrast, Pai-Liang generally exhibited decreased phytochemical content under environmental stresses, corresponding with the downregulation of CGT1. Although Pai-Liang's SnRK1 expression increased under short-term salt stress, it decreased with prolonged exposure. Conversely, Pai Khao-Lam exhibited increased levels of *p*-coumaric acid, chlorogenic acid, and vitexin, according to the upregulation of F2H and CGT1 under water deficit stress. This suggests a coordinated genetic response to sustain phytochemical synthesis and enhance antioxidant capabilities. Overall, this study underscores the complex relationship among gene regulation, phytochemical presentation, and stress tolerance in bamboo, emphasizing the need for genotype-specific strategies in breeding and conservation efforts. Future research should explore the regulatory networks governing bamboo's adaptive responses to stress.

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