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Tolerance enhancement of *Dendrobium officinale* by salicylic acid family-related metabolic pathways under unfavorable temperature

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Abstract

Background Unfavorable temperatures significantly constrain the quality formation of *Dendrobium officinale*, severely limiting its food demand. Salicylic acid (SA) enhances the resistance of *D. officinale* to stress and possesses various analogs. The impact and mechanism of the SA family on improving the quality of *D. officinale* under adverse temperature conditions remains unclear.

Results Combined with molecular docking analysis, chlorophyll fluorescence and metabolic analysis after treatments with SA analogues or extreme temperatures are performed in this study. The results demonstrate that both heat and cold treatments impede several main parameters of chlorophyll fluorescence of *D. officinale*, including the ΦPSII parameter, a sensitive growth indicator. However, this inhibition is mitigated by SA or its chemically similar compounds. Comprehensive branch imaging of ΦPSII values revealed position-dependent improvement of tolerance. Molecular docking analysis using a crystal structure model of NPR4 protein reveals that the therapeutic effects of SA analogs are determined by their binding energy and the contact of certain residues. Metabolome analysis identifies 17 compounds are considered participating in the temperature-related SA signaling pathway. Moreover, several natural SA analogs such as 2-hydroxycinnamic acid, benzamide, 2-(formylamino) benzoic acid and 3-o-methylgallic acid, are further found to have high binding ability to NPR4 protein and probably enhance the tolerance of *D. officinale* against unfavorable temperatures through flavone and guanosine monophosphate degradation pathways.

Conclusions These results reveal that the SA family with a high binding capability of NPR4 could improve the tolerance of *D. officinale* upon extreme temperature challenges. This study also highlights the collaborative role of SA-related natural compounds present in *D. officinale* in the mechanism of temperature resistance and offers a potential way to develop protective agents for the cultivation of *D. officinale*.

Keywords Dendrobium officinale, Temperature tolerance, Salicylic acid family, Chlorophyll fluorescence imaging, Molecular docking, NPR4 protein, Protective agents

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Background

The perennial epiphytic *Dendrobium*, belonging to the *Dendrobium* genus, has been utilized for both culinary and medicinal purposes in Asian, European, and Australian countries for thousands of years [1]. Wild *D. officinale* is prevalent in most tropical regions, and its artificial cultivation has been extensively adopted in subtropical areas, contributing to a production output value of 1.4 billion dollars in China in 2017 [2, 3]. Nevertheless, *D. officinale* is sensitive to environmental factors, particularly temperature, which regulates transcription factors and imposes limitations on the geographical distribution and food yield or quality of *D. officinale* [4, 5]. *D. officinale* frequently experiences significant declines in both productivity and quality due to its hypersensitivity to extreme temperatures in the planting area. Nevertheless, to adapt to seasonal unfavorable temperatures, many temperate herbaceous plants including *D. officinale* also have evolved acclimation mechanisms to derive environmental resistance. These mechanisms not only enhance temperature tolerance but also ensure the maintenance of nutritional contents [6]. Consequently, understanding these adaptive mechanisms is crucial for achieving stable, high-quality production of *D. officinale* and developing innovative protective agents for its cultivation.

Plants respond to adverse environmental factors by actively regulating photosynthetic performance and metabolite changes [7]. Accumulating evidence has drawn a strong correlation between enhanced crop stress tolerance and improved photosynthetic efficiency [8]. Chlorophyll fluorescence parameter detection is used to estimate the light energy absorption and conversion state of photosystem II (PSII). This method also gauges the photoinhibition effect, providing insights into the photosynthetic activity of the plant [9]. However, studies on temperature-related chlorophyll fluorescence parameters in *D. officinale* were limited, particularly regarding the overall distribution within branches [10]. In addition, the metabolism induced by environmental factors affects not only crop development but also the nutritional value and resistance to stress [11]. To comprehensively identify all metabolites in plant samples and elucidate plant metabolic processes, metabolomics is employed [12]. For thousands of years, humans have taken the plant secondary metabolites as food, raw materials, and medicinal purposes [13]. However, the role of metabolites in the response of *D. officinale* to environmental signals remains largely unclear.

Salicylic acid (SA) serves as an endogenous controller of plant physiological processes [14]. SA not only governs the growth and development of plants but also activates stress resistance mechanisms [15]. SA accumulates in response to both biotic and abiotic stimuli in plants, leading to the expression of related genes and the promotion of systemic acquired resistance [16]. Researches indicate that the addition of a small amount of SA can enhance *D. officinale*'s immune systems [1]. The alleviation effect of SA on physiological characteristics caused by low and high-temperature stresses in *D. officinale* has also been previously studied [17, 18]. These results showed that SA significantly increased the antioxidant capacity of *D. officinale*. However, the mechanisms through which SA regulates the metabolite changes in *D. officinale* to resist cold and hot environments are still unknown.

Many analogues of SA have contributed to stress resistance in plants through SA-regulated signaling [19]. Limited research has explored the impact of SA analogues on the resistance of *D. officinale* to high and cold temperature stresses. Investigating whether other SA analogues among these metabolites play a role in the mechanisms employed by *D. officinale* to withstand adverse environmental temperatures is also a worthwhile avenue for study. In addition, conducting molecular docking analysis in conjunction with investigating the impact of SA analogues on enhancing the tolerance of *D. officinale* under adverse temperatures can aid in the development of more effective protective agents for cultivating *D. officinale* under challenging temperature conditions. In this study, metabolomics data mainly analyzed metabolites related to SA signaling pathways in *D. officinale*. In addition, we analyzed which metabolites changed after exposure to cold and hot treatments, which were consistent with those unchanged after SA treatment. These consistent metabolites, as well as the promoted photosynthesis performance may play a role in the mechanism by which SA or its natural analogues regulate the resistance to mitigate the effects of adverse temperature on *D. officinale*.

Results

Changes of chlorophyll fluorescence parameters in response to temperature or SA treatment

Chlorophyll fluorescence measurements were conducted using leaves from both annual and biennial *D. officinale* subjected to adverse temperature treatment or SA treatment alone. In general, adverse temperatures had a more severe impact on biennial *D. officinale* leaves compared to annual ones. The analysis of chlorophyll fluorescence parameters of *D. officinale* revealed that exposure to heat significantly decreased the maximum photosynthetic efficiency (Fv/Fm of PSII) in both annual and biennial leaves. Conversely, cold treatment did not alter Fv/Fm (Fig. 1A and B).

Using the Fv/Fm parameter as a basis, we performed measurements of the actual quantum yield (ΦPSII), non-photochemical quenching (NPQ), and electron transfer rate (ETR) under gradually increasing actinic light. The results indicated that heat substantially impaired ΦPSII across the entire branch under low-light conditions.

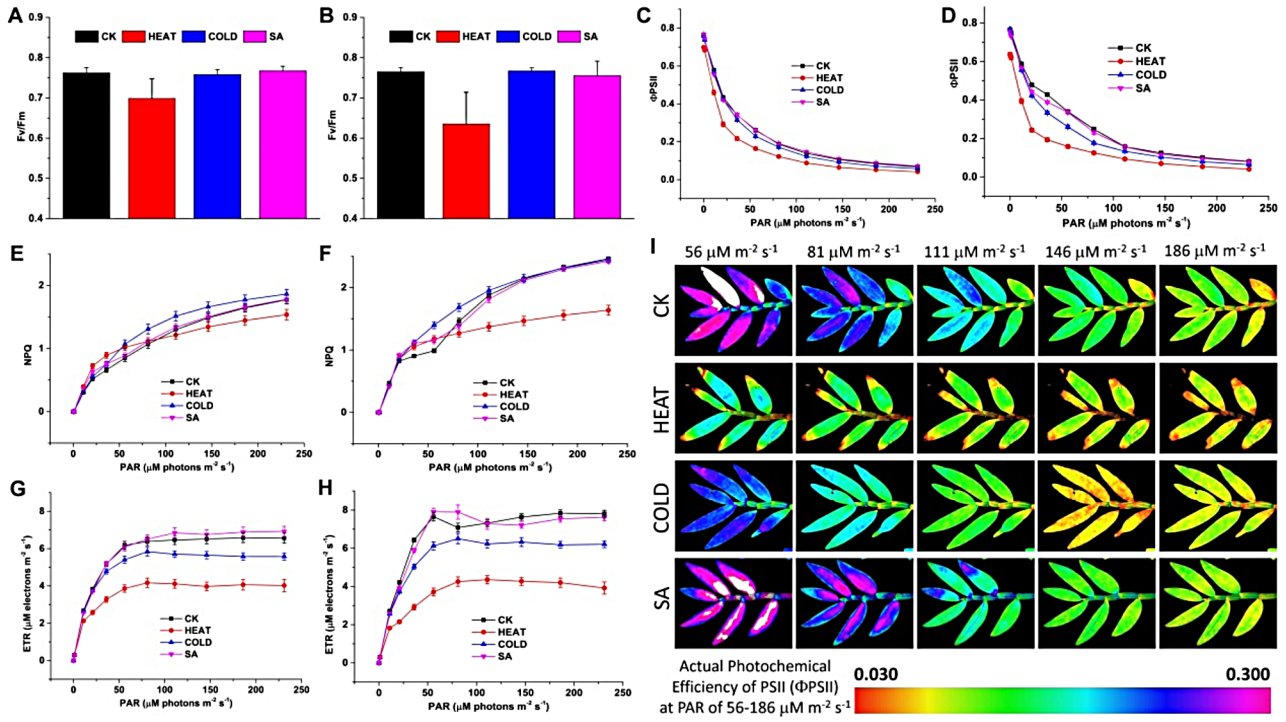

Fig. 1 Changes in chlorophyll fluorescence parameters in *D. officinale* under temperature and SA treatments. **(A and B)** The maximum quantum yield of PSII, Fv/Fm ratio of annual (**A**) and biennial (**B**) *D. officinale*. **(C and D)** Light response curves of quantum yield of PSII, ΦPSII of annual (**C**) and biennial (**D**) *D. officinale*. **(E and F)** Light response curves of non-photochemical quenching, NPQ of annual (**E**) and biennial (**F**) *D. officinale*. **(G and H)** Light response curves of electron transport rate, ETR of annual (**G**) and biennial (**H**) *D. officinale*. *D. officinale* were treated with 1.5mM SA at room temperature (25 °C) for 3 days or transferred to cold (1 °C) or heat (40 °C) stress environment in an incubator for 3 days. Data are the means ± SE ($n \geq 7$). (**I**) Image of the ΦPSII value indicated in (D) under increasing PAR light level in the branch of *D. officinale* after heat, cold, and SA treatments. The scale bar represents the false color of the ΦPSII value from lowest 0.03 to highest 0.3. ΦPSII value under 0.03 or above 0.3 is shown in black or white color, respectively

However, cold treatment only affected ΦPSII in biennial *D. officinale* (Fig. 1C, D, and I). Interestingly, contrary to the decrease in NPQ induced by heat, cold treatment had minimal impact on NPQ under high light but slightly promoted NPQ when leaves were illuminated by low light (Fig. 1E and F). Besides, the hot or cold treatment could significantly inhibit the value of ETR, indicating a disruption in the photosynthetic performance of *D. officinale* due to unfavorable environmental temperature (Fig. 1G and H). Moreover, it's important to note that SA treatment did not affect these chlorophyll fluorescence parameters.

Effects of SA and its analogues on heat and cold resistance of *D. officinale*

The ΦPSII parameter of chlorophyll fluorescence is an important index to study the effect of various environmental stresses on plant temporary behavior [20] and serves as a sensitive indicator for plant growth [8]. In this study, we observed that ΦPSII, measured at PAR of 81 μM photons m^{-2} s^{-1} could quickly and effectively reflect the health status of the *D. officinale* branch after short-term hot and cold treatment. Therefore, this parameter was employed as an indicator for conducting further research. The ΦPSII value from 0.03 to 0.30 across the entire branch, visualized through false-color images (ranging from red to purple), indicates the transition from a stressed state to the normal state of *D. officinale*. The results showed that the ΦPSII value of the *D. officinale*, maintained at room temperature, was approximately 0.24 (Fig. 2A). Following exposure to high temperatures, this value decreased to 0.126, indicating a disruption in the growth of *D. officinale* due to heat stress. Pretreatment using SA or its analogues such as 5-methylsalicylic acid, 3-methylsalicylic acid, 6-methylsalicylic acid, or 3-chlorosalicylic acid could significantly increase the value of ΦPSII by over 40% after the heat treatment. This finding underscores the capacity of SA and some of its analogues to effectively reverse the heat-induced stress experienced by *D. officinale*. False-colour images depicting the distribution of ΦPSII levels on the branch revealed that the impact of heat was pervasive throughout the branch, a condition that could be comprehensively restored by SA or some of its analogues. The response to cold treatment differed from that of heat treatment in some aspects (Fig. 2B). What they share in common is the recovery

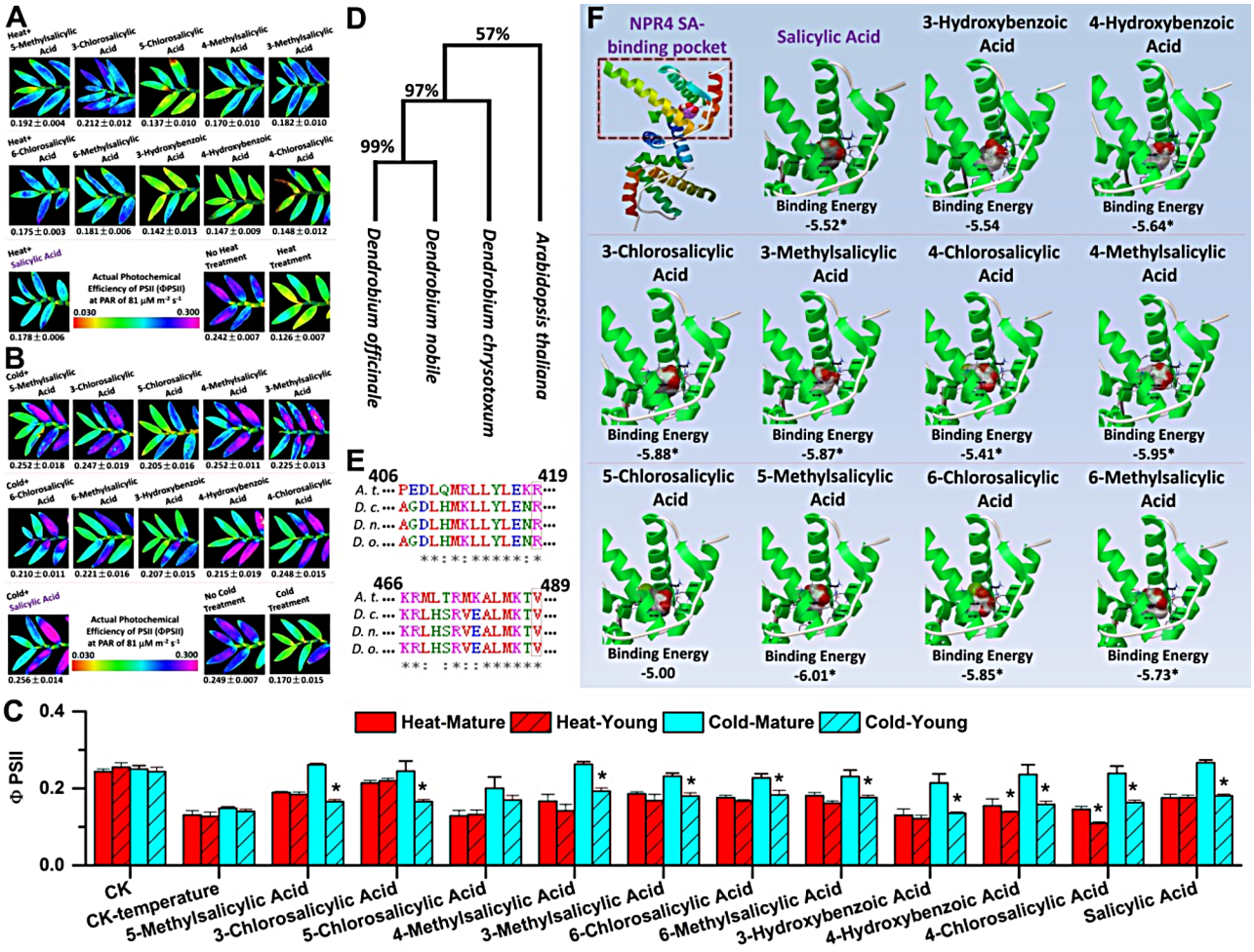

Fig. 2 The binding capacity of the SA analogue is crucial for the temperature resistance of *D. officinale*. **(A and B)** Image of the ΦPSII value under PAR of 81 μM photons m^{-2} s^{-1} light level in the branch of *D. officinale*, which were pretreated by 1.5 mM SA or its analogues and were then exposed to heat (A) or cold (B) stress environment in an incubator for 3 days. Data represent an average of the ΦPSII value from at least 7 different leaves (mean ± SE). (**C**) ΦPSII value of mature or young leaves subjected to heat or cold treatment. The asterisk indicates statistically a significant difference between mature and young leaves under heat or cold treatment ($p < 0.05$). **(D)** Homology tree compiled from the multiple amino acid alignment of the NPR4 genes of *Arabidopsis* and three *Dendrobium* species. The percentage homology is indicated. **(E)** Multiple alignments of the two amino acid sequences of the NPR4 genes of *Arabidopsis* and three *Dendrobium* species. Identical amino acids are indicated with an asterisk and similar amino acids are indicated with double dots. Two important residues R419 and V489 are highlighted by red boxes. (**F**) Molecular docking results of *Arabidopsis* NPR4 SA-binding pocket with SA or its analogues. Data represent the binding energy of SA or each SA analogue. An asterisk indicates the existence of contact of both R419 and V489 with the SA analogue

of cold damage by SA or its analogues, such as 5-methylsalicylic acid and 3-chlorosalicylic acid. In contrast to the heat treatment, the cold treatment resulted in a lower degree of leaf damage, evident in the higher ΦPSII value at 0.170. Furthermore, analysis of ΦPSII level distribution revealed that pretreatment with SA or its analogues could specifically restore the ΦPSII value of mature leaves on the cold-treated branch. Conversely, the alleviation effect could rarely be achieved in the new leaves on top of the *D. officinale* branch (Fig. 2C).

To reveal the molecular mechanism for the diversification of ΦPSII recovery among SA and its analogues, we conducted functional evaluations using molecular docking. This analysis was built upon the established understanding of the interaction between SA and its receptor NPR4 in Arabidopsis. The NPR1-NPR4 complex is known to play a role in heat or cold signaling through the SA-dependent pathway, demonstrating high SA-binding affinity [21–24]. The homology tree compiled from multiple alignments of the NPR4 amino acid sequences in the NCBI database from *Arabidopsis* and three *Dendrobium* species, including *D. officinale* clearly showed that the NPR4 sequences of the *Arabidopsis* have 57% identity with *Dendrobium* species (Fig. 2D). Notably, two important residues, R419 and V489, known for maintaining intact SA-binding activity within the NPR4

SA-binding pocket [25], were highly conserved among the four species (Fig. 2E). This conservation emphasizes their significance in evaluating the interaction abilities of SA and its analogues in *D. officinale*. Generally, the result obtained from molecular docking indicated a positive correlation between the degree of ΦPSII recovery and the binding energies of SA analogues. This correlation was dependent on the interaction with both R419 and V489 residues (Fig. 2F), underscoring the importance of these residues in modulating the efficacy of SA and its analogues in *D. officinale*. Among the 11 SA analogues, 5-methylsalicylic acid, which interacts with R419 and V489, exhibited the lowest binding energy of -6.01 kcal mol^{-1}, leading to a higher ΦPSII recovery ability. In contrast, 3-hydroxybenzoic acid and 5-chlorosalicylic acid demonstrated lower ΦPSII recovery abilities due to their disconnection with V489 residue and the lower binding capacity. These results supported the idea that the position of hydroxyl groups and functional groups on the benzene ring of SA could affect their ability to interact with target proteins of *D. officinale*. This contributes to the diverse protection of the SA family against adverse ambient temperatures.

Widely targeted metabolome analyses among different treatments of *D. officinale*

To reveal the mechanism of SA-induced tolerance promotion of *D. officinale* against adverse environmental temperature, we studied the associated metabolite profile of *D. officinale* that was subjected to hot, cold, or SA treatment. After the treatments, we extracted metabolites from the leaves and performed metabolic profiling using UPLC-MS/MS. Subsequently, we evaluated the quality of the extracts. Then, a total of 883 metabolites were identified (Table S1), categorized into 11 classes, including 143 phenolic acids, 140 flavonoids, 124 lipids, 85 amino acids and derivatives, 81 alkaloids, 58 organic acids, 49 nucleotides and derivatives, 34 quinones, 31 terpenoids, 24 lignans and coumarins and 114 others (Fig. 3A). Furthermore, we conducted a heat map analysis to visually represent the accumulation pattern of metabolites among all samples (Fig. 3B). This analysis revealed that certain metabolites in *D. officinale* were up-regulated in response

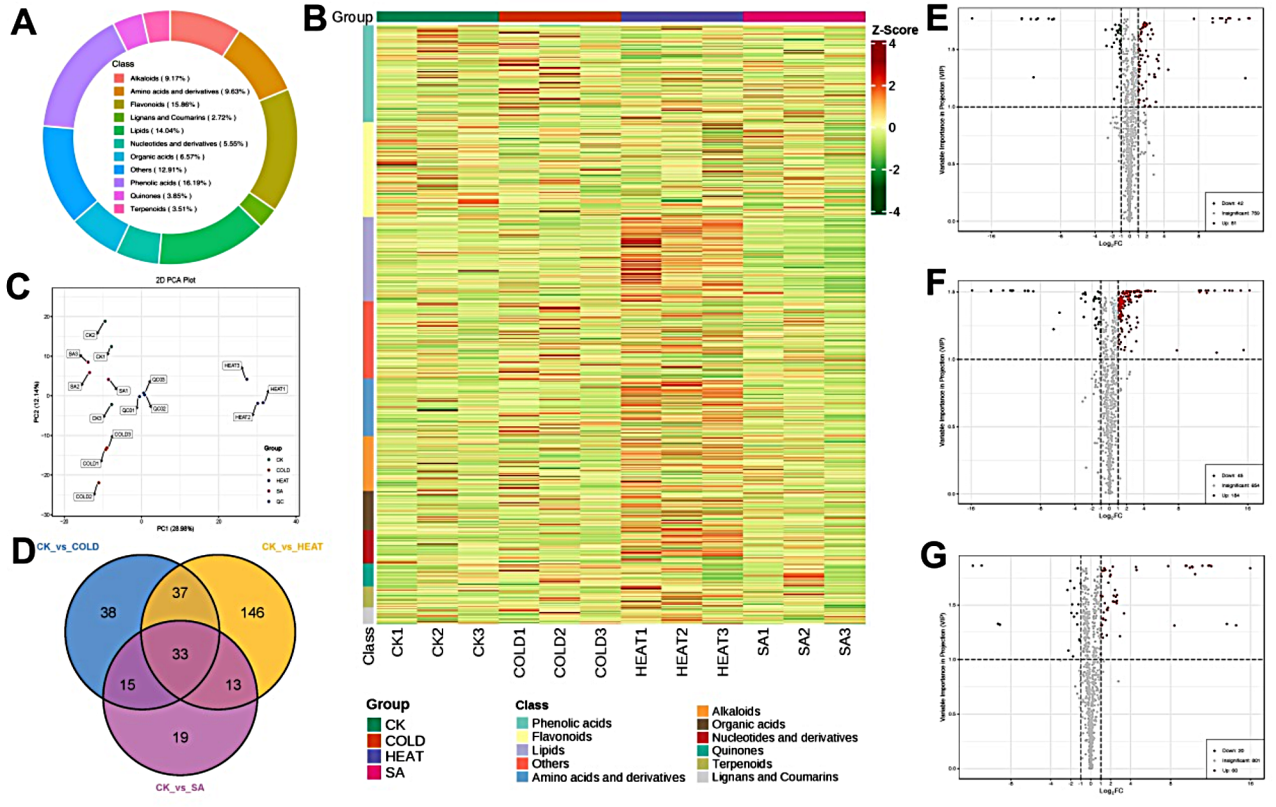

Fig. 3 Overall sample evaluation based on all the metabolites and DAMs. **(A)** Component analysis of all the identified metabolites. The type and proportion of all identified metabolites are presented in the graph. **(B)** Accumulation patterns of all identified metabolites in four different treatments. They are divided into 11 classes, and each coloured rectangle represents the specific compound content. **(C)** PCA score plot. The sampling groups are color-coded as follows: blue, HEAT; orange, COLD; green, CK; red, SA; purple, QC sample. **(D)** Venn diagrams presenting unique and common metabolites of all DAMs in various comparison groups. **(E to G)** The volcano plot shows the differential metabolite expression levels among the CK_vs_COLD (**E**), CK_vs_HEAT (**F**), and CK_vs_SA (**G**). Red, green, and gray dots indicate upregulated, downregulated, and insignificant differentially expressed metabolites, respectively

to heat or cold treatment but down-regulated under SA treatment or in control (CK). This observation suggests that *D. officinale*, when exposed to unfavorable temperatures, undergoes different metabolic processes compared with SA treatment or CK under normal temperatures.

Principal component analysis (PCA) was utilized to visualize the similarity among different treatments of *D. officinale* (Fig. 3C). The analysis revealed distinct separations of samples among different temperature treatments and close clusters of SA and CK treatments. The first principal components of PCA explained 28.98%, accompanied by the second principal components influencing 12.14% of the variance. These results suggest significant biochemical differences with sufficient reproducibility between temperature treatments and SA or CK, while SA had almost no impact on the metabolite profile of *D. officinale*.

Metabolite changes among different treatments of *D. officinale*

We next characterized differentially accumulated metabolites (DAMs) among treatments of *D. officinale* screening for metabolites with a fold change of ≥2 or ≤0.5 and a variable influence on projection (VIP)≥1. As indicated in Venn diagrams (Fig. 3D) and volcano plots that depicted the shared differently expressed metabolites among CK_vs_COLD (Fig. 3E), CK_vs_HEAT (Fig. 3F) and CK_vs_SA (Fig. 3G). In the CK_vs_COLD group, 123 DAMs (81 up-regulated and 42 down-regulated) were detected, while the CK_vs_HEAT group exhibited 229 DAMs (184 up-regulated and 45 down-regulated). The CK_vs_SA group had 80 DAMs (60 up-regulated and 20 down-regulated). In total, there were 301 DAMs identified among three comparison groups (Table S2), with 33 compounds identified as common DAMs.

The sum of all DAMs was then classified into 11 categories that varied among the above three groups. These categories were subjected to KEGG enrichment analysis to obtain the top 20 metabolic and functional insights. Notably, as for DAMs in groups of CK_vs_HEAT (Fig. 4A) and CK_vs_COLD (Fig. 4B), most DAMs were amino acid derivatives and lipids. Furthermore, upon heat treatment, there was a decrease in the contents of flavonoids and quinones, whereas the accumulation of

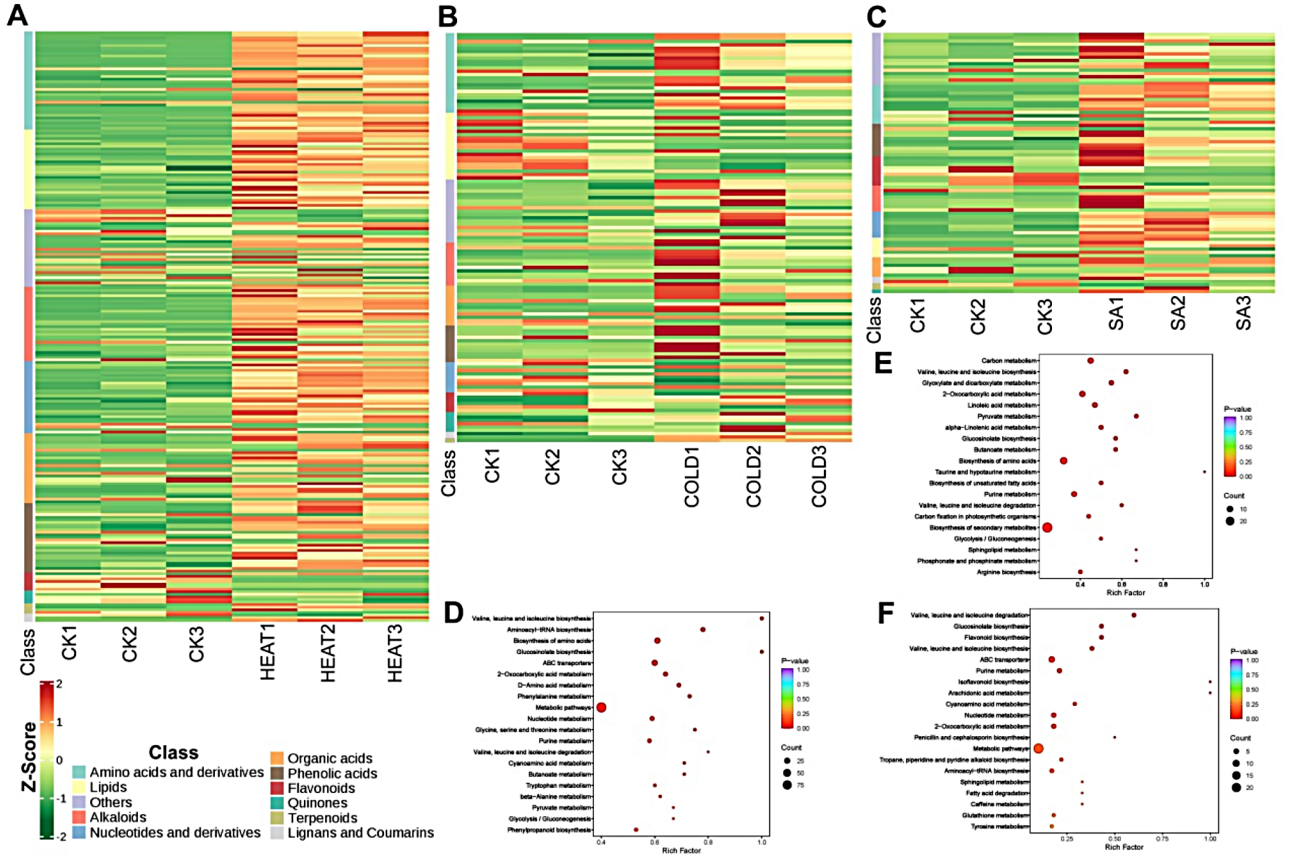

Fig. 4 Analysis of all DAMs for three comparison groups. **(A to C)** Accumulation patterns of class-specific DAMs in CK_vs_HEAT (**A**), CK_vs_COLD (**B**), and CK_vs_SA (**C**). **(D to F)** Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment of DAMs among the groups CK_vs_HEAT (**D**), CK_vs_COLD (**E**) and CK_vs_SA (**F**)

alkaloids, amino acid derivatives, lignans and coumarins, lipids, nucleotide derivatives, organic acids, phenolic acids, and terpenoids increased in response to the heat stress. Specifically, cold treatment led to a reduction in the content of lipids, flavonoids, and nucleotide derivatives, but it promoted the accumulation of compounds in other classes. Compared with the temperature treatments, the CK_vs_SA group exhibited higher levels in the distribution of amino acid derivatives, while the variation in lipids was marginal (Fig. 4C). In addition, only flavonoids had 2/3 of substances that were less in SA than in CK, while the majority of DAMs accumulated more under the SA treatment. KEGG analysis showed that the significant DAMs were mainly involved in metabolic pathways, biosynthesis of secondary metabolites, ABC transporters, biosynthesis of amino acids, 2−oxocarboxylic acid metabolism, carbon metabolism, purine metabolism and nucleotide metabolism (Fig. 4D, E and F). These results suggest that pretreatment of SA might enhance the tolerance of *D. officinale* to unfavorable temperatures, potentially by proactively altering various metabolic pathways, a response that might be delayed to occur when *D. officinale* suffers unfavorable temperatures.

Crucial DAMs related to SA-induced tolerance of unfavorable temperature

To identify key DAMs associated with the mechanism underlying the improvement of *D. officinale* tolerance to heat and cold through SA, we conducted a screening based on the top 20 up-regulated and down-regulated compounds, along with the 33 common DAMs across the three groups. A further comparison between CK_ and_HEAT obtained 20 highest levels of DAMs including amino acid derivatives (count: 4) and organic acids, followed by nucleotide derivatives and flavonoids (Fig. 5A). Moreover, the top 20 DAMs identified in CK_vs_COLD were also amino acid derivatives (count: 4), organic acids (4), and nucleotide derivatives (3) (Fig. 5B). Additionally, the top 20 DAMs altered by SA treatment included 3 organic acids and flavonoids (Fig. 5C). Consequently, 8 compounds that showed the top increase extent were found in all of the 20 DAMs among three groups (Fig. 5D), namely 3 organic acids (ethylmalonic acid, glutaric acid and oxalic acid), 2 nucleotide derivatives (1-methyladenine and xanthine), 2 amino acid derivatives (L-glycyl-L-isoleucine and N-glycyl-L-leucine) and 1 terpenoids (vomifoliol).

In this analysis, peak areas of the 33 mutual DAMs of Fig. 5, which included the 8 top common DAMs, were further studied to reveal their accumulation patterns. As

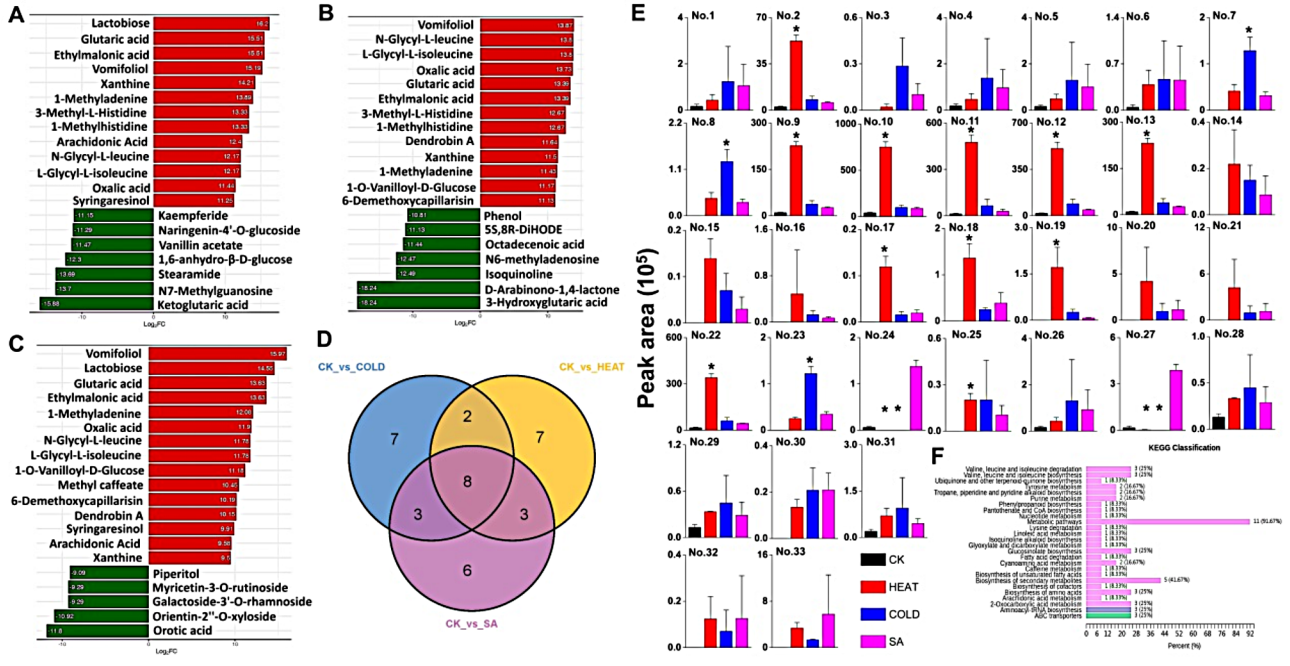

Fig. 5 Crucial natural compounds involved in SA signaling in response to temperature stress in *D. officinale*. **(A to C)** Top 20 up-regulated and down-regulated compounds in CK_vs_HEAT (A), CK_vs_COLD (B), and CK_vs_SA (C), respectively. **(D)** The Venn diagram shows the overlapping of the top 20 DAMs in the three groups. **(E)** Peak areas of 33 common DAMs in three comparison groups. The compound number corresponds to the same in Table S3. The significant level of the difference between SA and heat or cold treatment is indicated by an asterisk * $P < 0.05$ (values are expressed as mean ± SD). **(F)** KEGG classification map of 33 common DAMs. The name of the KEGG metabolic pathway is shown on ordinate, and the abscissa is the number of metabolites annotated to one pathway and their proportion to the total number of metabolites annotated

shown in Fig. 5E, the 33 mutual DAMs consist of 7 amino acid derivatives (No.7–13), 6 phenolic acids (No.27–32) and alkaloids (No.1–6), 4 organic acids (No.20–23), 3 lipids (No.15–17) and 2 nucleotide derivatives (No.18–19) as well as another 5 special compounds (Table S3). These DAMs annotated by KEGG were distributed into 26 pathways (Fig. 5F), with 92% participating in the metabolic pathway and 42% in the biosynthesis of secondary metabolism, respectively. In particular, a total of 17 DAMs were filtered from the mutual DAMs that showed similar contents among heat, cold, and SA treatments to study the downstream events of SA signaling that respond to the temperature stresses. These DAMs included the analogues of feruloyltyramine, dendrobin A, ferulic acid, coumaroyltyramine, syringaresinol, LysoPC, arachidonic acid, ethylmalonic acid, glutaric acid, tyramine, propionic acid, dimethoxybenzaldehyde, vanilloylglucose, coumaric acid, methyl caffeate, and vomifoliol. These all up-regulated DAMs were hypothesized to play crucial roles in SA-induced tolerance improvement in *D. officinale* under unfavorable temperature conditions.

Given that many phenolic acids are natural analogues of SA and might perform similar roles in stimulating *D. officinale* responses to heat or cold stress, the DAMs induced by temperature belonging to phenolic acids with similarities to SA were screened to study the effective SA analogues in nature. The results showed that 25 up-regulated DAMs related to phenolic acids were found by comparison of both CK_vs_COLD and CK_vs_HEAT groups. After subtracting up-regulated DAMs from the CK_vs_SA group to exclude the impact of exogenous SA, 13 DAMs with high SA similarity were revealed (Fig. 6A). Conversely, most of the other DAMs were only induced by the heat treatment, suggesting a greater sensitivity to hot than to cold in *D. officinale* to facilitate SA-like signaling. Of these DAMs, 6 special metabolites (No. 2–3; 6; 8; 10; 12) were evaluated by molecular docking to have potential biological activities against either heat or cold stimulation. This assessment was based on the prediction

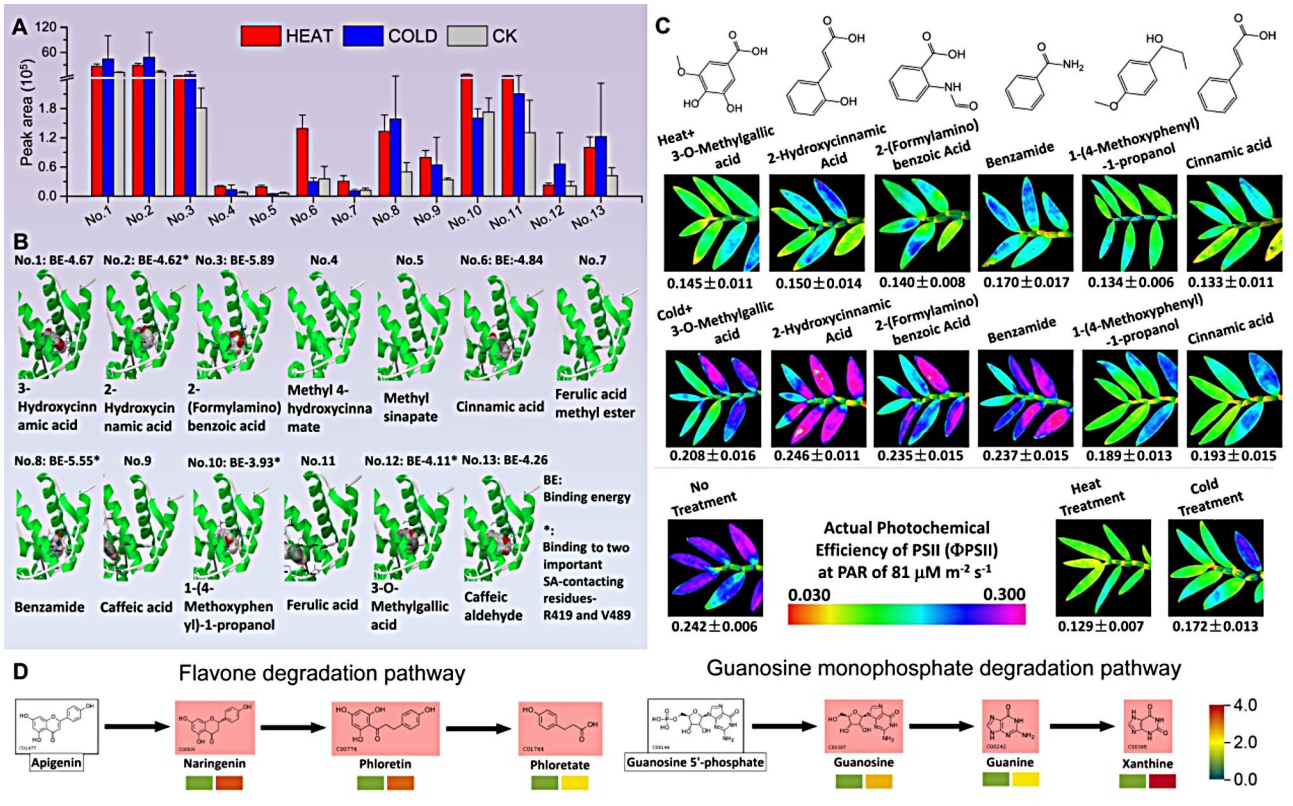

Fig. 6 Functional analysis of SA-similar DAMs in temperature-resistance of *D. officinale*. **(A)** Peak areas of 13 DAMs in CK_vs_HEAT and CK_vs_COLD groups with SA structure similarity. These DAMs were neither up-regulated nor down-regulated in the CK_vs_SA group. **(B)** Molecular docking results of *Arabidopsis* NPR4 SA-binding pocket with the 13 DAMs. The compound number corresponds to the same in (**A**). Data represent the binding energy of each DAM. The asterisk indicates the existence of contact of both R419 and V489 with the 13 DAMs. **(C)** Image of the ΦPSII value under PAR of 81 μM photons m^{-2} s^{-1} light level in the branch of *D. officinale*, which were pretreated by 1.5 mM DAMs of (B) that were predicted to have lower binding energy or both R419 and V489 contact and were then exposed to heat or cold stress environment in an incubator for 3 days. Data represent an average of the ΦPSII value from at least 7 different leaves (mean ± SE). (**D**) Mapping of SA-related DAMs involving flavone and guanine ribonucleotide degradation pathways. The compounds with identifiers were constructed based on the KEGG pathway online. The two rectangles with the color of the scale bar sequentially represent the relative abundance of metabolites with no or SA treatment

of binding energies or the interaction with both R419 and V489 in the NPR4 SA-binding pocket (Fig. 6B).

To test the functional similarity of SA to stimulate hot or cold resistance, the chemical reagents corresponding to these 6 special metabolites were applied to *D. officinale*, which were subsequently exposed to either heat or cold environment. Consistent with the molecular docking data, pretreatment of part of the 6 special metabolites arising from the metabolism of *D. officinale* exhibited higher ΦPSII levels compared to heat or cold treatment alone (Fig. 6C). The high efficiency appeared in 2-hydroxycinnamic acid and benzamide, followed by 2-(formylamino) benzoic acid and 3-o-methylgallic acid. Similarly to SA, the distribution of ΦPSII of the whole branch showed that these SA analogues were difficult to reverse the damage caused by adverse temperatures in young leaves and only showed efficacy in mature leaves. The further screen of SA-related DAMs using KEGG pathways aligned 6 up-regulated compounds, including naringenin, phloretin, phloretate, guanosine, guanine, and xanthine, in most parts of flavone and guanosine monophosphate (GMP) degradation pathways (Fig. 6D). Therefore, activation of both pathways was probably the mechanism of temperature tolerance of SA and its analogues. These results together indicated that the generation of natural metabolites with SA-structural similarity and the activation of flavone and guanine ribonucleotide degradation pathways were the potential mechanisms for *D. officinale* to fight against adverse environmental temperatures.

Discussion

SA is a naturally occurring phenolic acid that could effectively against abiotic stresses, including adverse temperature, water deficiency, and salinity for plants [26]. The application of SA has been shown to influence the physiological behavior of crops, leading to increased quality parameters and alleviation of stress-induced injuries in horticultural commodities [27, 28]. However, these physiological states cannot be observed non-destructively in real-time to evaluate the stress state of a temperature-sensitive plant like *D. officinale*, especially for short-term effects of heat and cold. *D. officinale* in the first or second year does not have as much biomass accumulation and bioactive substances as ones in the third or fourth year [29]. It is prone to death after experiencing adverse temperatures, and cannot propagate at the tillering site like *D. officinale* in the third or fourth year. In addition, even if *D. officinale* dies due to unfavorable temperatures, people can harvest the stems of *D. officinale* to minimize losses. However, it is difficult for annual and biennial *D. officinale* to propagate due to insufficient accumulation of nutrients, and the quality of harvested *D. officinale* cannot meet commercial needs, causing huge losses to the *D. officinale* industry. Therefore, our research focuses on *D. officinale* in the first or second year.

Our study showed that heat stress significantly decreased all the chlorophyll fluorescence parameters in *D. officinale* leaves of different ages (Fig. 1). In contrast to heat treatment, cold treatment had little effect on these parameters, except for ΦPSII and ETR in biennial *D. officinale*. Additionally, SA treatment alone did not influence these parameters. Therefore, false-colour images of ΦPSII enabled us to non-destructively evaluate the short-term thermal and cold effects on *D. officinale* growth condition when pretreated by SA and its analogues in the present study. By using this parameter, we observed the alleviation effect of SA under unfavorable temperatures and subsequently screened out other effective SA analogues (Fig. 2). The decrease in ΦPSII under hot or cold stress could be attributed to the suppression of the linear electron transport ability of the photosynthetic apparatus [30, 31]. Therefore, SA and its analogues play a crucial role in maintaining the biological function of the photosynthetic apparatus in *D. officinale* under heat or cold stress. However, analysis of the branch false-color image distribution revealed that the recovery of ΦPSII value by SA or its analogues was relatively weaker in the younger part on top of the *D. officinale* branch, especially under cold stress. This suggests irreversible damage and hypersensitive of young *D. officinale* during the temperature change. The therapeutic efficacy of SA and its analogues can only work on the mature part of *D. officinale*. This might be due to the maturity-related resistance of plant tissues developmentally regulated independently through the NPR1/4 signaling pathway but depends on prolonged SA accumulation and SA-related transcription factor by environment stimulation [32, 33].

To date, although accumulating SA analogues has been explored to show similar biological effects as SA itself [19, 34], improving crop or fruit tolerance against stresses by SA analogues is still unclear. To study the functional analogues of SA, we first tested the impact of the changed position of the original hydroxyl group or introducing a newly added functional group of the benzene ring of SA, along with examining natural SA analogues. This investigation aimed to understand their influence on mitigating the decline of ΦPSII. The results led to the conclusion that certain SA analogues can stimulate *D. officinale* to withstand cold and hot environments similar to SA (Figs. 2 and 6). Subsequently, we introduced molecular docking analysis to predict the binding capacity and contact of crucial residues in a specific target protein. This approach was utilized to explore the influence of structural changes in SA on ΦPSII recovery, resulting in the identification of more than ten effective SA analogues. Some structural changes might form a more stable catalytic site of the target protein by better recognizing

certain crucial residues via a combination of higher electrostatic interactions, van der Waals forces, and hydrogen bonds [35]. This potential mechanism might enable *D. officinale* to distinguish diverse defense pathways by producing specific SA analogues based on fluctuating environmental temperatures.

Furthermore, the ability to predict molecules with higher biological activity through molecular docking offers advantages in screening or artificially designing potential stress-protective agents or agrochemicals for crops or fruits in the future. Selecting the appropriate model for molecular docking analysis is crucial for accurately predicting and developing biologically active compounds. A previous report has indicated that the NPR1/2/3/4 complex can interact with SA with varying affinities, with NPR4 exhibiting the lowest Kd value [36]. This suggests a strong binding affinity between NPR4 and SA, which could subsequently influence the temperature-regulated signaling of the NPR-SA system [37]. Furthermore, *Dendrobium* species harbor an NPR4 protein similar to the *Arabidopsis* NPR4 protein, which is currently the only experimental model available. This NPR4 protein possesses two conserved residues, R419 and V489, crucial for SA binding (Fig. 2), making it suitable for docking predictions involving functional SA and its analogues implicated in plant temperature response. The identification of naturally occurring plant molecules that can effectively enhance plant heat tolerance holds significant importance for sustainable agriculture and ecosystem adaptation to global warming. Interestingly, some SA analogues, such as 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-chlorosalicylic acid, and 5-chlorosalicylic acid, had impaired binding affinity with NPR4 and failed to induce resistance to adverse temperature. These results demonstrated that the functional SA analogues had the structural characteristics of adjacent hydroxyl and carboxyl groups without electron withdrawing groups in the 4th and 5th positions of the benzene ring.

In the present study, several natural SA analogues were, for the first time, identified as activating photosynthesis recovery akin to SA under heat or cold stress in *D. officinale*. Benzamide derivatives exhibit diverse bioactivities that are still underdeveloped. Some established functions of benzamide include improving plant growth and development, producing higher peroxide and superoxide ions, acting as an abiotic stress agent, and reverting the agronomic trait of the plants [38]. Benzamide and SA were also reported to form co-crystals, potentially enhancing the stability and bioavailability of SA [39]. As another SA analogue originating from plant shikimate biosynthesis pathways, 2-hydroxycinnamic acid derivatives, could act similarly to SA by promoting antioxidant, anti-ultraviolet, anti-inflammatory, antimicrobial, and anti-tyrosinase activities [40]. 3-o-methylgallic acid, a derivative of gallic acid, serves as an anthocyanin metabolite with potent antioxidant capacity, effectively preventing the deterioration and rancidity of fats and oils [41]. Therefore, it is anticipated that 3-o-methylgallic acid can protect lipids from degradation when *D. officinale* is exposed to unfavorable temperatures. In addition, gallic acid could boost the defensive state of plants to fight against cold, ozone, cadmium, and copper stresses [42], while little research has been studied on 3-o-methylgallic acid in plants. Here, we also characterized a new SA analogue 2-(formylamino) benzoic acid that was able to increase cold tolerance for *D. officinale*, although further research is needed to elucidate its regulatory mechanism.

Metabolites undergo complex interactions through parallel pathways, enhancing plant adaptability and stress resistance in response to environmental stresses [43]. This study aims to elucidate the differential metabolites of annual and biennial *D. officinale* subjected to cold or heat stress and SA treatment, providing insights into the mechanisms through which SA and its analogues contribute to the enhancement of *D. officinale* tolerance under heat or cold stress (Figs. 3, 4 and 6). KEGG pathway analysis identified six up-regulated compounds by SA that constitute most parts of flavone and GMP degradation pathways. Both pathways have been reported to participate in plant resistance to adverse temperatures [44, 45]. We speculate that SA and its analogues could mediate the activation of both pathways to combat temperature stresses. In the PCA analysis of temperature-related metabolites, clear separation among the different temperature treatments, suggesting that metabolite accumulation under different treatments was influenced by the temperature, while SA exhibits similarity to the control. This study identified numerous metabolites, with approximately 32% and 9% attributed to temperature- and SA-related DAMs, respectively.

The top temperature-associated DAMs belonged to amino acid derivatives and L-glycyl-L-isoleucine and N-glycyl-L-leucine were the top two common amino acid derivatives, acting downstream of the SA pathway (Fig. 5). Amino acids and their derivatives have a close correlation with SA-related stress adaptation [46]. As two amino acid derivatives, isoleucine and leucine were both found to enhance the antioxidant capacity and resistance to pathogenic microorganisms, contributing to the improvement of crop tolerance and photosynthetic performance [47, 48]. We speculated that these amino acid derivatives of the SA pathway might enable the *D. officinale* to reduce oxidative damage and prevent inhibition of photosynthesis under an uncomfortable environment. Furthermore, three extensively detected organic acids (ethylmalonic acid, glutaric acid, and oxalic acid) demonstrated co-accumulation across the three groups. The biosynthesis of organic acids in roots is often stimulated

by many environmental stresses and can impact the light-independent photosynthetic pathway [49]. In addition to modulating proline metabolism and energy status, oxalic acid is conducive to chilling tolerance by the regulation of membrane integrity and osmotic substance levels in plants [50]. In this study, we also identified vomifoliol, a stomatal aperture regulator [51], which accumulated significantly upon SA treatment and acted downstream of heat and cold metabolic pathways. This finding suggests a potential mechanism for *D. officinale* to cope with adverse temperatures through stomatal regulation. Besides, exposure of *D. officinale* to adverse temperatures resulted in the production of other metabolites that belonged to SA-induced nucleotide derivatives and terpenoids. Among the 17 DAMs identified from the common pool across the three groups, some were classified as the aforementioned DAMs. These metabolites played a role in the collaborative network involving alkaloids, lignans, lipids, organic acids, phenolic acids, and terpenoids in *D. officinale*. This suggests that SA enhances the resistance of *D. officinale* to adverse temperatures through a complex metabolic regulatory network. However, further research is needed to reveal their biological functions in the temperature stress resistance of *D. officinale*.

Conclusion

Understanding the mechanism of SA family-induced temperature stress acclimation is critical for cultivating *D. officinale* of high quality in extreme weather. Our studies disclosed the effects of SA and its analogues on the regulation of metabolic change of *D. officinale* under heat or cold stress, leading to the recovery of photosynthetic performance and tolerance improvement of *D. officinale* (Fig. 7). The function of SA analogues is intricately linked to their binding energies and the interaction with specific residues of the target proteins involved in the temperature tolerance improvement of *D. officinale*. Our metabolite analysis further revealed the metabolites concerning SA-induced resistance against adverse temperature in *D. officinale* by the activation of flavone and GMP degradation pathways, contributing to the demonstration of the regulatory mechanisms of SA and its analogues in response of *D. officinale* to adverse temperature. The present study also discovered four natural SA analogues, such as 2-hydroxycinnamic acid, benzamide, 2-(formylamino) benzoic acid and 3-o-methylgallic acid, that promoted the tolerance of *D. officinale* under temperature stress, indicating the collaborative role among SA chemical family members in reducing heat and cold injury and providing potential protective agents for future *D. officinale* cultivation.

Materials and methods

Plant materials and treatments

Tissue-cultured seedlings of both annual and biennial *D. officinale* were artificially planted and collected on the normal greenhouse seedbeds of the Fujian Institute of Subtropical Botany. The planting substrate consisted of a mixture of sawdust and peat soil (3: 1, v/v). The cultivation temperature was maintained within the range of 15–30 °C throughout the year, and the environmental humidity was maintained at 70–80% under the fluctuating greenhouse sunlight (around 80 to 250 μmol photon m^{-2} s^{-1} of the maximum light intensity at the midday).

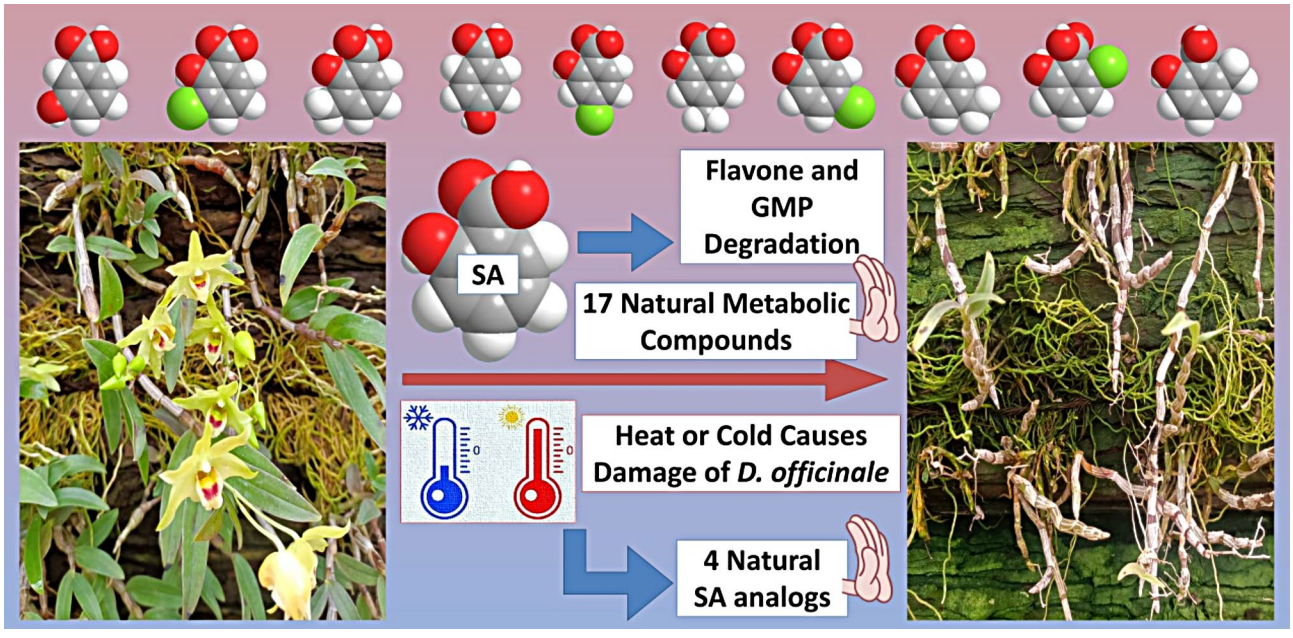

Fig. 7 The mechanism of SA analogs-related tolerance enhancement when *D. officinale* suffers from temperature stress

For the experiments, detached branches with healthy and uniform leaves were carefully chosen. The experiment was performed in April 2023.

For the chlorophyll fluorescence imaging experiment, the annual and biennial *D. officinale* were foliar sprayed with SA or its analogues (1.5 mM) at room temperature for 3 days according to a previous study [17]. Subsequently, they underwent either cold treatment (1 °C), or hot treatment (40 °C), or were maintained at room temperature (25 °C, serving as the control) for an additional 3 days. Fresh leaves of *D. officinale* were then collected for chlorophyll fluorescence imaging. For metabolome analysis, the biennial *D. officinale* were treated by spraying 1.5 mM SA at room temperature for 3 days, or they underwent either cold treatment (1 °C) or hot treatment (40 °C) for 3 days. Then, the fresh detached leaves of *D. officinale* were immediately frozen in liquid nitrogen and stored at -70 °C. Each treatment was replicated three times.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters for leaves treated with SA and its analogues, as well as those subjected to cold and heat treatments, were assessed using a chlorophyll fluorometer (Imaging-PAM-MAXI WALZ, Germany). The entire plant underwent a 30-minute dark adaptation period prior to the experiment in accordance with the methodology described in a recent publication [52]. The branches containing the leaves and stems were harvested and promptly transferred to the mounting stand of the chlorophyll fluorometer for conducting the chlorophyll fluorescence imaging experiment. An additional 5-minute dark adaptation process was implemented and the maximum quantum yield (Fv/Fm) was then measured on the fully expanded leaves. Actual quantum yield (ΦPSII), electron transport rate (ETR), and non-photochemical quenching (NPQ) were then automatically determined by chlorophyll fluorometer after leaves were illuminated for 5 min with gradually increasing actinic light, with more than six measurements conducted per replication, where increasing PAR is the absorbed light from 0 to 231 counted as μmol photon m^{-2} s^{-1} [53].

Conditions for the analysis of UPLC and MS/MS

D. officinale leaves samples, each weighing approximately 3 g, were sent to Wuhan MetWare Biotechnology Co., Ltd and the widely targeted metabolites profiling was conducted by following their self-built database procedures. Briefly, we employed ultra-performance liquid chromatography (UPLC, SHIMADZU Nexera X2) to qualitatively detect mixed samples. Subsequently, tandem mass spectrometry, MS/MS (Applied Biosystems 4500 QTRAP) was utilized for quantification, as detailed in [54]. This approach combines the accuracy of both metabolite identification and quantification. The liquid phase conditions were as follows: UPLC: column, Agilent SB-C18 (1.8 μm, 2.1 mm × 100 mm); mobile phase, solvent A (ultrapure water with 0.1% formic acid): solvent B (acetonitrile with 0.1% formic acid); gradient program (A: B), from 95:5 V/V at 0 min to 5:95 V/V at 9 min, 5:95 V/V at 10 min, then 95:5 V/V at 11.1 min, 95:5 V/V at 14.0 min; flow rate, 0.35 mL/min; column temperature, 40 °C; injection volume 4 μL [55].

ESI (electrospray ionization)-triple quadrupole-linear ion trap-MS was employed for analyzing the ion source of the samples. The ESI source operated with the following settings: ion source - turbine spray; source temperature −550 °C; ion spray voltage −5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I, gas II, and curtain gas were set at 50, 60 and 25.0 psi, respectively, and the collision-activated dissociation was set to high. Use 10 and 100 μmol/L polypropylene glycol solutions for instrument tuning and mass calibration, respectively. A specific set of multiple reaction monitoring transitions was monitored at each period based on the metabolites eluted within that timeframe.

Molecular docking

To explore the resistant activity of potential molecules against adverse temperatures, the screened metabolites of SA analogues were investigated using molecular modeling to evaluate their binding ability to NPR4. AutoDockTools-1.5.6 software [56] was utilized for this purpose. SA analogues were used as the ligands for molecular, referencing the structural basis of the Arabidopsis NPR4 protein (PDB ID: 6WPG) from the RCSB Protein Data Bank (http://www.rcsb.org/pdb) [25]. Hydrogen atoms were added to the SA analogues and NPR4 structures, while water molecules were removed to establish the protonation state throughout the entire process. We set the genetic algorithm runs at 100, with each run undergoing a maximum of 250,000 times of energy evaluation. We used the Lamarckian genetic algorithm to perform calculations for all SA analogues, ranking them based on their lowest binding energy. The binding energy of SA analogues with the NPR4-SA binding pocket and the contacting residues were evaluated using Show Interactions.

Data analysis

The data collected in this study underwent a t-test to ascertain significant differences ($P<0.05$) between different treatments. The graphs and analysis of variance were performed using OriginPro 9.1 (OriginLab Corporation). We assessed the quality and reproducibility of detected metabolites and conducted an analysis of differentially accumulated metabolites, along with their corresponding

heatmaps, following the methodology outlined in a prior study [57].

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions
W.W. and E.H. conceived the research. W.W., M.Z., Z.S., T.L., L.H., Z.L. and T.Y. performed experiments. W.W., Z.S. and T.L. analyzed the data. L.C., F.L. and Y.G. collected the plant materials, helped in the experiment and made suggestions. W.W. and E.H. wrote the manuscript. All authors have read and approved the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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