# Comparative Analysis of the Transcriptomes of Three Varieties Provides Insights Into the Diversity of the Heat Response Mechanisms in Clematis Species

**Hao Zhang**
Shanghai Normal University

**Changhua Jiang**
Shanghai Botanical Garden

**Long Zhang**
Shanghai Normal University

**RuoNan Gai**
Shanghai Normal University

**Siyuan Peng**
Shanghai Normal University

**Yi Zhang**
Shanghai Normal University

**Chanjuan Mao**
Shanghai Normal University

**Yuxia Lou**
Shanghai Normal University

**Shucheng Feng**
Shanghai Botanical Garden

**Feng Ming** ( ✉ fming@fudan.edu.cn )
The Biotechnology Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, 201106, China

---

# Abstract

**Background:** *Clematis* species are commonly grown in western and Japanese gardens. Heat stress can inhibit many physiological processes mediating plant growth and development. The mechanism regulating responses to heat has been well characterized in *Arabidopsis thaliana* and some crops, but not in horticultural plants, including *Clematis* species.

**Results:** In this study, we identified a heat-sensitive *Clematis* variety (*Clematis alpina* 'Stolwijk Gold') and two heat-tolerant *Clematis* varieties (*Clematis vitalba* and *Clematis viticella* 'Polish Spirit') based on heat-related physiological indices. The leaf transcriptomes under normal and heat stress conditions were analyzed by RNA sequencing. Additionally, heat tolerance-related genes (HTGs) were identified and their expression levels were analyzed. Following heat treatments, 41.67% of the differentially expressed HTGs in Stolwijk Gold had down-regulated expression levels, whereas only 9.80% and 21.36% of the differentially expressed HTGs in *C. vitalba* and Polish Spirit, respectively, had the same trend. The HTGs' co-expression and protein–protein interaction networks revealed that the hub genes regulating *Clematis* resistance to heat stress encode heat shock transcription factors (HSFs) and heat shock proteins (HSPs). Moreover, the sensitivity of Stolwijk Gold to heat is mainly due to the heat-induced down-regulated expression of these genes. On the basis of phylogenetic and expression analyses, the differentially expressed HSF and HSP genes in the three examined varieties were divided into three and four clades, respectively, with similar expression profiles common among orthologous family members. Furthermore, we identified two HSF classes in *C. vitalba* that may have diverse functions influencing heat resistance.

**Conclusions:** Our study provides insights into the diversity of the heat response mechanisms among *Clematis* species and may be useful for breeding new heat-resistant ornamental *Clematis* varieties.

# Background

*Clematis* is a genus that includes approximately 300 species belonging to the buttercup family (Ranunculaceae). Hybrids developed from *Clematis* species are popular among gardeners. For example, *Clematis* species play an important role in western and Japanese gardens, and are commonly grown in botanical gardens, parks, and family gardens. However, the potential range of some *Clematis* varieties for cultivation is restricted because of their sensitivity to heat and increasing global warming. To date, research related to *Clematis* species has focused on taxonomy and ecology, and rarely on gene expression.

Plants depend on various genetic regulatory networks to adapt to environmental changes and ensure their survival following exposures to abiotic stresses. High temperatures can significantly affect plant growth and development, leading to phenotypic changes, including morphological changes to the hypocotyl, petiole elongation, early flowering, and reduced stomata formation [1]. However, it remains unclear how plant cells perceive thermal signals [2], although previous research has identified thermosensors such as H2A.Z-containing nucleosomes and phytochromes [3].

Heat stress can adversely affect plant protein structure, conformation, and activity, resulting in denatured and aggregated proteins as well as plant cell death due to biochemical damages and cytotoxicity [1, 2]. Heat shock proteins (HSPs) are molecular chaperones that can stabilize and degrade unfolded proteins [4]. Heat stress significantly increases the production of HSP90, which directly interacts with TRANSPORT INHIBITOR RESPONSE 1 (TIR1) to prevent protein degradation and regulate auxin-mediated plant growth[5]. In addition to HSPs, heat shock transcription factors (HSFs) have conserved and central roles in plants. Decreasing the expression of genes encoding HSFA2, HSF-A1a, -A1b, -A1d, and -A1e, HSFA3, HSFA6b, or HSFB1 and B2b via T-DNA insertions or RNA interference significantly alters the thermotolerance of *Arabidopsis thaliana* [6−11]. The *A. thaliana* genome encodes 21 HSFs, which have been divided into three classes: HSFA, HSFB, and HSFC [12]. Other transcription factors are also responsive to heat stress. For example, *OsSNAC3* expression is induced within a few hours of an exposure to heat and salt stresses, and the encoded protein regulates reactive oxygen species (ROS) homeostasis by directly activating many genes related to ROS clearance [11]. A previous study revealed that OsANN1 is a calcium-binding membrane-bound protein that regulates $H_2O_2$ production by promoting the activities of superoxide dismutase and catalase, thereby making rice resistant to high temperatures [13]. In addition to its effects on transcription, thermal stress also influences other nuclear regulatory processes, including chromatin modification, remodeling, and RNA processing [2, 14−16]. Despite these findings, the

mechanism underlying heat resistance in horticultural plant species, including those belonging to the genus *Clematis*, has not been thoroughly characterized.

In this study, by comparing the primary heat-related physiological indices before and after a high-temperature treatment, we identified a heat-sensitive *Clematis* variety (*Clematis alpina* 'Stolwijk Gold') and two heat-tolerant *Clematis* varieties (*Clematis vitalba* and *Clematis viticella* 'Polish Spirit'). We also analyzed the transcriptomes of these varieties under normal and heat stress conditions. Moreover, we compared the varieties regarding their responses to heat to clarify the differences in their heat resistance. Furthermore, to characterize the considerable heat resistance of *C. vitalba*, we identified two HSF classes with various functions related to heat resistance. The results of this study provide insights into the diversity of the heat response mechanisms among *Clematis* species and may be useful for breeding new heat-resistant ornamental *Clematis* varieties.

## Results

### Heat shock phenotype of different Clematis varieties

*Clematis vitalba* (Cv) is an original *Clematis* specie that produces branched and grooved stems as well as deciduous leaves and green-white flowers with fluffy underlying sepals (Fig. 1A). Because of its disseminatory reproductive system, vitality, and climbing behavior, *C. vitalba* is an invasive plant species in several regions, including New Zealand (http://www.iucngisd.org/gisd/species.php?sc=157). 'Polish Spirit' (PS) and 'Stolwijk Gold' (SG) are ornamental *Clematis* cultivars (Fig. 1A) recognized by the Royal Horticultural Society of England (http://apps.rhs.org.uk/advicesearch/profile.aspx?pid=97).

To compare the heat resistance of three *Clematis* varieties, specific leaf physiological indices were analyzed before and after a high-temperature (HT) treatment (Supplementary Table 1). The resulting data indicated that the relative conductivity and malondialdehyde content were significantly higher in Stolwijk Gold than in *C. vitalba* and Polish Spirit, whereas the opposite trend was observed for the relative water content, soluble protein content, and superoxide dismutase activity (SOD) (Fig. 1B). These observations indicated that the heat-induced membrane damage and peroxidation were greater in Stolwijk Gold than in *C. vitalba* and Polish Spirit. Accordingly, *C. vitalba* and Polish Spirit appeared to be more heat resistant than Stolwijk Gold. Moreover, nitroblue tetrazolium and diaminobenzidine staining revealed the substantial accumulation of ROS in Stolwijk Gold leaves (Fig. 1C) following the heat treatment, which was in contrast to the relatively unchanged ROS contents in *C. vitalba* and Polish Spirit. Thus, the antioxidant systems of Polish Spirit and *C. vitalba* remained active under heat stress conditions (Fig. 1D). These results reflected the heat resistance of *C. vitalba* and Polish Spirit as well as the sensitivity of Stolwijk Gold to heat stress.

## Transcriptome profiles and annotations, differentially expressed genes, and GO and KEGG enrichment analyses

To reveal the molecular basis of the differences in the heat resistance of the three examined *Clematis* varieties, the leaf transcriptomes under normal (control) and heat stress conditions were analyzed by RNA-sEq. Eighteen libraries corresponding to three biological replicates for the control and heat treatments of each variety were constructed and sequenced (Cv_NT_leaf1, Cv_NT_leaf2, Cv_NT_leaf3, Cv_HT_leaf1, Cv_HT_leaf2, Cv_HT_leaf3, PS_NT_leaf1, PS_NT_leaf2, PS_NT_leaf3, PS_HT_leaf1, PS_HT_leaf2, PS_HT_leaf3, SG_NT_leaf1, SG_NT_leaf2, SG_NT_leaf3, SG_HT_leaf1, SG_HT_leaf2, and SG_HT_leaf3). A total of approximately 895 million paired-end reads (raw reads) were generated, filtered, and trimmed, with 40−60 million reads per library (Supplementary Table 2). The raw data have been deposited in the NCBI Sequence Read Archive (PRJNA664279). For each *Clematis* variety, all clean reads were used for a *de novo* sequence assembly using Trinity (https://github.com/trinityrnaseq/trinityrnaseq) (Supplementary Table 3). The obtained unigenes for the three *Clematis* varieties were annotated based on the following six databases: NR (ftp://ftp.ncbi.nlm.nih.gov/blast/db/), Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html), Pfam (http://pfam.xfam.org/), COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG/), GO (Gene Ontology, http://www.geneontology.org), and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) (Supplementary Table 4). For each variety, approximately

half of the unigenes matched *Aquilegia coerulea* sequences (Supplementary Fig. 1), reflecting the close genetic relationship between *Clematis* species and *A. coerulea*. The clean reads were then mapped to the assembled sequence (Supplementary Table 5). The gene expression levels (i.e., transcripts per million reads) were analyzed using RSEM (http://deweylab.github.io/RSEM/). The hierarchical cluster analysis of gene expression among the different samples for each variety indicated the data for the biological replicates were reliable and the error was within the allowable range (Fig. 2A). The differentially expressed genes (DEGs) among the three *Clematis* varieties were analyzed (Supplementary Fig. 2). The number of DEGs and the ratio of the number of DEGs to the total number of genes were highest for Stolwijk Gold, and lowest for *C. vitalba* (Fig. 2C), implying that more biological processes were affected by heat stress in Stolwijk Gold than in *C. vitalba* and Polish Spirit. The GO functional annotation of the DEGs revealed that the heat treatment mainly altered membrane components, with some DEGs in *C. vitalba* and Polish Spirit related to heat responses (Supplementary Fig. 3A). The KEGG pathway enrichment analysis indicated that the pathways affected by heat were mainly associated with secondary metabolism, with fewer pathways affected in *C. vitalba* than in the other examined varieties (Supplementary Fig. 3B). Additionally, some signaling pathways in Stolwijk Gold were modulated by heat stress, including plant hormone signal transduction and the MAPK signaling pathway (Supplementary Fig. 3B). These results suggested *C. vitalba* and Polish Spirit are more heat resistant than Stolwijk Gold.

# Identification of heat tolerance-related genes and an analysis of their differential expression

Regulatory processes in plants are affected by heat stress. On the basis of previous research [1, 2], we divided the regulatory activities mediating plant responses to high temperatures into the following five categories: heat signal transduction, transcriptional regulation, protein homeostasis, ROS homeostasis and RNA homeostasis. To elucidate the molecular mechanism underlying the responses of the three analyzed *Clematis* varieties to heat, we identified the heat tolerance-related genes (HTGs) associated with the five categories (Supplementary Table 7). Specifically, the HTGs were identified via a local blastp search using previously reported HTGs in other species as queries (Supplementary Table 6) and GO term annotations. Additionally, their expression levels in the three examined *Clematis* varieties were compared (Table 1, Fig. 3, Supplementary Table 7). Some of the differentially expressed HTGs in each species had down-regulated expression levels. More specifically, 41.67% of the differentially expressed HTGs in Stolwijk Gold were significantly down-regulated under heat stress conditions, whereas only 9.80% and 21.36% of the differentially expressed HTGs in *C. vitalba* and Polish Spirit, respectively, exhibited the same trend (Fig. 3A). Polish Spirit had the most up-regulated HTGs. *Clematis vitalba* had the fewest down-regulated HTGs (Fig. 3A). These results may help to explain the heat resistance of *C. vitalba* and Polish Spirit.

Table 1

**Heat tolerance-related genes among different cellular processes and their differential expression under heat stress in three** Clematis **varieties.** Identified: all identified HTGs, differ: differentially expressed HTGs, up: up-regulated HTGs, down: down-regulated HTGs.

| Species | Cv | | | | PS | | | | SG | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | identified | differ | up | down | identified | differ | up | down | identified | differ | up | down |
| Heat Signal Transduction | 25 | 0 | 0 | 0 | 24 | 4 | 1 | 3 | 27 | 7 | 1 | 6 |
| Transcriptional Regulation | 42 | 11 | 9 | 2 | 43 | 18 | 13 | 5 | 49 | 13 | 6 | 7 |
| Protein Homeostasis | 227 | 40 | 37 | 3 | 220 | 77 | 66 | 11 | 275 | 46 | 33 | 13 |
| ROS Homeostasis | 15 | 0 | 0 | 0 | 28 | 3 | 0 | 3 | 24 | 6 | 2 | 4 |
| RNA Homeostasis | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 |
| Total | 310 | 51 | 46 | 5 | 315 | 103 | 81 | 22 | 376 | 72 | 42 | 30 |

There were considerable differences in the expression of HTGs in the above-mentioned regulatory categories among the three *Clematis* species. Transcriptional regulation is critical for responses to high temperatures. The HSF family members as well as the ERF/AP2 family transcription factor DREB2A and the NAC transcription factor NAC019 positively affect the heat-activated transcriptional regulatory network [17, 18]. Genes encoding these transcription factors were identified in the *Clematis* transcriptomes (Fig. 3). In response to heat stress, the expression levels of all HSF genes identified in *C. vitalba* were significantly up-regulated, whereas only half of the HSF genes identified in Stolwijk Gold had up-regulated expression levels (the rest had down-regulated expression levels). Moreover, many of the DREB2A and NAC019 transcription factor genes were expressed at lower levels in Stolwijk Gold than in the other varieties. We speculated that the sensitivity of Stolwijk Gold to heat is primarily due to a weak heat-activated transcriptional regulatory network. Additionally, maintaining homeostasis, especially related to protein and ROS contents, is extremely important for stabilizing the biological activities of plants exposed to heat stress [2, 14]. Heat shock proteins, which are molecular chaperones, are important for the stabilization, renaturation, and degradation of unfolded proteins. Following the heat treatment, an analysis of the differentially expressed HSP genes indicated that Polish Spirit had the most up-regulated HSP genes, whereas *C. vitalba* had the highest proportion of up-regulated HSP genes (Fig. 3B). These findings may be related to the differences in the heat resistance mechanisms of the evaluated *Clematis* varieties. In plants, ROS accumulation is a major cellular response to heat stress. Reactive oxygen species contribute to the early plant response to heat; however, high ROS contents lead to the oxidative damage of many cellular components [19, 20]. The HTGs related to ROS homeostasis were not differentially expressed in *C. vitalba*, but had down-regulated expression levels in Polish Spirit, following the high-temperature treatment (Table 1, Fig. 3B). This may have been because the heat resistance mechanism prevented the excessive accumulation of ROS in *C. vitalba* and Polish Spirit. Although the expression of two HTGs related to ROS homeostasis was up-regulated in Stolwijk Gold, four other HTGs related to ROS homeostasis had down-regulated expression levels, resulting in ROS accumulation (Fig. 1C and D, Table 1, Fig. 3B). Additionally, the expression of some HTGs involved in heat signal transduction, such as *CaM1* and *CDPK2*, was down-regulated in Stolwijk Gold, which may adversely affect downstream regulatory processes.

## Genetic regulatory networks in Clematis varieties

To determine the potential interactions or regulatory relationships among the differentially expressed HTGs in the three *Clematis* varieties and to identify hub genes regulating heat resistance, we constructed gene co-expression and protein–protein interaction (PPI) networks (Fig. 4). These networks revealed that HSFs and HSPs, such as HSF30, HSF24, HSP70, and HSP90, have major roles associated with the heat tolerance of the three *Clematis* varieties. We speculated that the down-regulated expression of many genes encoding HSFs and HSPs critical for plant heat tolerance (e.g., HSF70 and HSF90) may be the main cause of the sensitivity of Stolwijk Gold to heat stress. Although *C. vitalba* and Polish Spirit were both resistant to heat stress, their heat-related genetic regulatory networks varied. Specifically, *C. vitalba* had a relatively small regulatory network, with almost no down-regulated HTGs, whereas Polish Spirit had a relatively large regulatory network that included down-regulated HTGs. Accordingly, there are at least two distinct heat resistance mechanisms in *Clematis* species. Furthermore, the gene co-expression network was used to reveal potential targets of heat-responsive transcription factors, including HSFs, DREB2A, and NAC019. The identified gene targets may be useful for future investigations of the heat resistance mechanism in *Clematis* species.

## Phylogenetic relationships and expression-level differences among the genes encoding heat shock transcription factors and heat shock proteins in three Clematis varieties

Considering the importance of HSFs and HSPs for plant heat resistance, we analyzed the phylogenetic relationships of HSF and HSP genes and compared their expression levels to further characterize the differentially expressed HSF and HSP genes among three *Clematis* varieties (Fig. 5). The differentially expressed HSF genes were divided into three clades. The heat treatment down-regulated the expression of Clade 1 genes and *PSHSFA1a* and *SGHSFA5* of Clade 2, suggesting these genes do not induce heat tolerance. All of the *CvHSF* genes belonged to Clades 2 and 3 and had heat-induced up-regulated expression levels, which may be related to the considerable heat tolerance of *C. vitalba*. Among the HSF gene family members, those in Clade 3 were generally more highly expressed than those in Clade 2. Thus, heat stress differentially affected the expression of HSF genes in different phylogenetic clades. We divided the differentially expressed HSP genes into four clades. The 3, 8, and 13 down-

regulated HSP genes in *C. vitalba*, Polish Spirit, and Stolwijk Gold, respectively, were mainly clustered in Clades 1, 2, and 3. The expression levels of most of the HSP gene family members in Clade 4 were up-regulated, indicating this clade is important for the heat tolerance of *Clematis* varieties. The down-regulated expression of many SGHSP genes following the heat treatment may be related to the sensitivity of Stolwijk Gold to high temperatures. Although the expression of a substantial proportion of the PSHSP genes was down-regulated by an exposure to heat, the HSP genes were generally more highly expressed in Polish Spirit than in the other two varieties. Furthermore, our analysis revealed a clear expansion of the HSP gene family members in Clade 4, possibly because of an adaptive evolution to heat.

### Classification and characterization of heat shock transcription factors in Clematis vitalba

Considering *C. vitalba* is an original *Clematis* species with a small and efficient heat resistance genetic regulatory network, we predicted it may be useful for breeding. To further classify and characterize the HSFs in *C. vitalba*, we analyzed the phylogenetic relationships, predicted motifs, and expression of *CvHSF* genes. A phylogenetic analysis revealed that Classes A and B each contained three *CvHSF* genes, which were closely related to the orthologous *AtHSF* genes (Fig. 6A). On the basis of the PPI network, gene co-expression network, and expression profiles, *CvHSF30-1* and *CvHSF30-2* were identified as hub genes critical for the heat tolerance of *C. vitalba*. Thus, the expression of both genes was analyzed in a qRT-PCR assay. Additionally, to clarify the differences between the HSFs in Classes A and B and to functionally characterize the Class B HSFs, the expression of *CvHSFB2a*, which belongs to Class B, was also analyzed. An examination of the predicted motifs revealed that CvHSF30-2 and CvHSFB2a have similar N-terminals, but diverse C-terminals, indicative of functional differences between these two HSFs. As representative HSFs of Classes A and B, CvHSF30-2 has two activator peptide motifs (AHA motifs) and a nuclear export signal at the C-terminal, whereas CvHSFB2a has a repressor domain (Fig. 6B). The qRT-PCR data revealed the increasing *CvHSF30-1* and *CvHSF30-2* expression levels in the first 2 h after a high-temperature treatment (42 °C). Moreover, both genes were more highly expressed than *CvHSFB2a*, suggesting the HSF genes in Class A are important for the heat tolerance of *Clematis* species (Fig. 6C).

# Discussion

Heat stress is a major abiotic factor that plants must adequately respond to [2, 14]. The mechanism regulating the heat tolerance of *Clematis* species remains relatively uncharacterized, which is in contrast to the available information regarding the corresponding mechanisms in traditional model plant species, including *A. thaliana* and *Oryza sativa*. In this study, we first elucidated the molecular basis for the differences in the heat tolerance of three *Clematis* varieties based on a transcriptomic analysis of plants under normal and heat stress conditions. We identified HTGs and compared their expression levels during various regulatory activities (heat signal transduction, transcription regulation, protein homeostasis, ROS homeostasis and RNA homeostasis) (Table 1, Fig. 3B). Compared with Polish Spirit, there were fewer differentially expressed HTGs, but more down-regulated HTGs, in Stolwijk Gold, which may help to explain the sensitivity of Stolwijk Gold to high temperatures. Although *C. vitalba* and Polish Spirit were both confirmed as heat-tolerant varieties, the underlying mechanisms differed. More specifically, although *C. vitalba* had fewer differentially expressed HTGs than Polish Spirit, nearly all of the differentially expressed *CvHTG* genes were up-regulated. Although Polish Spirit had more differentially expressed HTGs, it also had a greater proportion of down-regulated differentially expressed HTGs compared with *Clematis vitalba* (Fig. 3A). Additionally, the differentially expressed HTGs in *C. vitalba* were associated with transcriptional regulation and protein homeostasis, but not heat signal transduction and ROS homeostasis (Fig. 3B). This suggests that *C. vitalba* may quickly respond to heat stress by modulating the activities of intracellular proteins. Moreover, heat stress does not substantially affect the ROS content of *C. vitalba* (Fig. 1C and D), likely because of changes to transcriptional regulation and protein homeostasis. The *C. vitalba* characteristics related to heat resistance may be relevant for breeding new varieties of *Clematis* species.

Gene co-expression and PPI networks revealed the core HSFs and HSPs contributing to the heat stress resistance of *Clematis* species (Fig. 4). Considering the importance of HSFs and HSPs for heat resistance, we analyzed the phylogenetic relationships of the differentially expressed HSF and HSP genes in three *Clematis* varieties. The diversity in the phylogenetic relationships among the differentially expressed HSF and HSP genes (Fig. 5) may be associated with the observed differences in heat resistance among the *Clematis* varieties. Orthologous family members often had similar expression profiles (Fig. 5). Notably, we

detected a clear expansion of the Clade 4 HSP gene cluster in Polish Spirit, which may have influenced the heat resistance of this variety. The related genes should be further analyzed in future studies. It was unclear why the expression levels of some HSF and HSP genes were down-regulated in Polish Spirit and even more so in Stolwijk Gold following the heat treatment. According to previous researches, calcium ($Ca^{2+}$) signaling, ROS signaling, NO signaling and their considerable crosstalk with each other make a difference in heat signal transduction of plant[20–32]. For example, CaM is one of the most important intracellular $Ca^{2+}$ receptors. Knocking out the expression of *AtCaM3* made the resulting mutant more susceptible to HS stress, whereas the overexpression of *AtCaM3* resulted in enhanced plant thermotolerance [33]. We speculated that the down-regulated expression of HTGs related to heat signal transduction (e.g., *CAM1*, *CAM2*, *CPK1*, *CYPA*, and *CDPK2*) may be an important reason. A more thorough functional characterization of these genes may further clarify the mechanism regulating the responses of Polish Spirit and Stolwijk Gold plants to heat stress.

Because of the obvious heat resistance of *C. vitalba*, the differentially expressed *CvHSF* genes were further classified and characterized. Plant HSF family members can be divided into three classes: HSFA, HSFB, and HSFC [12]. The differentially expressed *CvHSF* genes identified in this study are HSFA and HSFB genes. We examined *CvHSF30-1*, *CvHSF30-2*, and *CvHSFB2a* to predict the encoded motifs. Accordingly, we determined that CvHSF30-1 and CvHSF30-2 are A2-type HSFs, whereas CvHSFB2a is a B2-type HSF. Of the HSFA1s-targeted transcription factors/co-activators, HSFA2 is a key regulator of plant thermotolerance [34, 35]. A qRT-PCR experiment revealed the considerable increase in the *CvHSF30-1* and *CvHSF30-2* expression levels in the first 2 h following a high-temperature treatment, which is consistent with the results of previous research on other plants. Therefore, these two HSF genes are likely important for the heat resistance of *C. vitalba*. Additionally, a phylogenetic analysis indicated *CvHSFB2a* is an ortholog of *AtHSFB2b*. Earlier research proved that AtHSFB2b represses the expression of heat-inducible HSF genes, but positively regulates thermotolerance [7]. Hence, CvHSFB2a may be relevant for the molecular breeding of heat-resistant *Clematis* varieties.

## Conclusions

Taken together, in this research, a heat-sensitive *Clematis* variety (*Clematis alpina* 'Stolwijk Gold') and two heat-tolerant *Clematis* varieties (*Clematis vitalba* and *Clematis viticella* 'Polish Spirit') were identified according to primary heat-related physiological indices before and after a high-temperature treatment. Gene expression profiles of three *Clematis* varieties under normal and high temperature based on transcriptome data were firstly reported, which provided valuable resources for the research of *Clematis* species. Moreover, we compared the varieties regarding their responses to heat to clarify the differences in their heat resistance based on HTGs that we identified. Furthermore, to characterize the considerable heat resistance of *C. vitalba*, we identified two HSF classes with various functions related to heat resistance. Our study provided insights into the diversity of heat response mechanisms of *Clematis* species, with implications for the breeding of heat-resistant and ornamental *Clematis* varieties.

## Methods

## Plant material growth conditions and high temperature treatment conditions

Triennial potted plants of three *Clematis* varieties (*Clematis vitalba*, Polish Spirit and Stolwijk Gold) were used in this study. The seeds were derived from the botanical garden in Germany, which were sown into autoclaved nutrient soil and grown for 3 years in 12-inch pots in greenhouse of Shanghai Botanical Garden. For each variety, six plants in great and similar growth condition were used and placed in two constant temperature incubators (one is at 42°C, the other is at 22°C) for 2 hours respectively (three plants per constant temperature incubator). Treated potted plants were used subsequent physiological indexes analysis and RNA sequencing.

## Measurement of physiological indexes before and after high temperature treatment

Relative conductivity: 0.2 g leaves was placed in a 20 mL tube, vacuumized, and stood for 30 min at normal temperature (shaken gently every 5 min during the period). The conductivity was measured with a DDS-11A type conductivity meter. After boiling water bath for 10 min and cooling, the conductivity value was again measured and the relative conductivity was calculated. The relative conductivity per gram of fresh weight was used to represent relative conductivity. Relative water content: Weigh 0.2 g of chopped leaves and place them in a weighing bottle, put them in an oven for 30 min at 105°C for dehumidification, then set them to 80°C for drying to constant weight and calculate the relative water content lastly. Coomassie brilliant blue G 250 method was used to soluble protein quantification. SOD activity was measured by Nitrogen Blue Tetrazolium Photoreduction Method. Malondialdehyde (MDA) content was measured by thiobarbituric acid colorimetric method.

# Nitro blue tetrazole (NBT) and Diaminobenzidine (DAB) staining

Leaves of three Clematis varieties with normal and high temperature treatment were soaked in DAB staining solution at 25 °C for 24 hours or NBT staining solution at 25 °C for 12 h both in the dark, and then soaked in 95% ethanol to remove chlorophyll and took the photo.

# RNA isolation and sequencing

Eighteen samples (Cv_NT_leaf1, Cv_NT_leaf2, Cv_NT_leaf3, Cv_HT_leaf1, Cv_HT_leaf2, Cv_HT_leaf3, PS_NT_leaf1, PS_NT_leaf2, PS_NT_leaf3, PS_HT_leaf1, PS_HT_leaf2, PS_HT_leaf3, SG_NT_leaf1, SG_NT_leaf2, SG_NT_leaf3, SG_HT_leaf1, SG_HT_leaf2 and SG_HT_leaf3) from three Clematis varieties were used for RNA sequencing. RNA was isolated from the leaves of three *Clematis* varieties (*Clematis vitalba, Clematis* "Polish Spirit", *Clematis* "Stolwijk Gold") treated for 2 hours at 42°C and 22°C using TRIzol reagent, respectively. The extracted RNA was quantified using Nanodrop2000, and the RNA was electrophoresed on an agarose gel to check its integrity. Around 0.4 μg of total RNA was used for library construction and sequencing on an Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) at Shanghai Majorbio Bio-pharm Technology Co.,Ltd (Shanghai, China). Prior to library construction, an Agilent 2100 Bioanalyzer (Agilent, CA, USA) was used to confirm the quality and quantity of RNA such that the rRNA ratio (28 s/18 s) was > 1.5 and the RNA integrity number > 7. In brief, 0.1 ~ 0.4 μg total mRNA was purified and fragmented using PCR plates with a magnetic plate stand. Fragmented mRNA was reverse-transcribed to cDNA using random primers and Superscript II (Invitrogen, Carlsbad, CA). Blunt-ended cDNA was generated by end repair and then ligated to yield 30 adenine base overhangs. Oligonucleotide adapters with thymine overhangs were ligated to the cDNA and added to the adapter index for each library. The library fragments were enriched by PCR amplification and ~ 895 million raw pair-end reads were generated on an Illumina Novaseq 6000.

### Trimming, de novo assembly and mapping of reads

Illumina sequence data were assessed using fastx_toolkit_0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/) and filtered using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle). All clean reads of each *Clematis* variety were used *de novo* assembly using Trinity (https://github.com/trinityrnaseq/trinityrnaseq). Assembly results were optimized using TransRate (http://hibberdlab.com/transrate/) and CD-HIT (http://weizhongli-lab.org/cd-hit/) and then assessed using BUSCO (Benchmarking Universal Single-Copy Orthologs, http://busco.ezlab.org). Clean reads of every samples were respectively mapped to *de novo* assemble sequence.

# Transcriptome annotation, identification and enrichment analysis of differential expressed genes (DEGs)

Transcriptome assembly sequences were annotated by NR (ftp://ftp.ncbi.nlm.nih.gov/blast/db/),Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html), Pfam (http://pfam.xfam.org/), COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG/), GO (Gene Ontology, http://www.geneontology.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) six databases, respectively. Gene expression levels were calculated as transcripts per million reads (TPM) using RSEM (http://deweylab.github.io/RSEM/). Differential expressed genes (DEGs) in three Clematis varieties were identified using DEseq2 ($|log2$ FC(HT_leaf/NT_leaf)$|>2$, p-value < 0.05). The software Goatools was used for GO enrichment analysis (FDR < 0.05) of DGEs with Fisher's exact test. All of the DEGs were also subjected to KOBAS 2.0 analysis (http://kobas.cbi.pku.edu.cn/home.do) and significant pathways were selected at a corrected p value < 0.05.

# Hierarchical cluster analysis

The hierarchical clustering and other statistical analyses were carried out using R software (http://www.r-project.org). Pearson correlation was used to calculate distance of different samples.

# Identification of heat tolerance-related genes

On the one hand, we obtained protein sequences based on transcriptome assembly sequence using Transdecoder (https://github.com/TransDecoder) and construct local protein databases of three *Clematis* varieties. Then proteins homologous with previously reported heat tolerance-related genes (HTGs) (Supplementary Table 6) were identified using the local blastp program of BLAST + 2.9.0 (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/) (E-value < 1e-5, identity > 60%) in three *Clematis* varieties. On the other hand, genes of GO term related to heat-resistance in GO enrichment analysis were also regarded as heat-tolerant genes (HTGs). We combined both data to define heat tolerance-related genes (HTGs) (Supplementary Table 7) in three *Clematis* varieties, respectively.

# Differentially expressed analysis of heat tolerance-related genes

TPM data of the overlap between DGEs and HTGs, that is, expression profiles of differentially expressed HTGs were visualized in R software (https://www.r-project.org/). Blue and red represented "down regulated" and "up regulated", respectively.

# Construction of co-expression network and protein-protein interaction network

We used differentially expressed HTGs for further network construction. Co-expression correlation coefficients of differentially expressed HTGs were obtained through Spearman algorithm (corrected p value < 0.05) based on gene expression data and visualized in Cytoscape v3.5.1[36]. PPI networks of differentially expressed HTGs were constructed based on PPIs of *Aquilegia coerulea* in STRING database (https://string-db.org) for three *Clematis* varieties. Networks were also visualized using Cytoscape v3.5.1. Number of edges directly connected with nodes were computed using Network Analyzer in Cytoscape v3.5.1[37].

# Phylogeny and expression analysis of HSFs and HSPs

Phylogenetic trees of HSFs and HSPs from three *Clematis* species were constructed in IQ-TREE v2.0.6[38] with JTT + I + G4 and VT + R3 model respectively. Support for each node was assessed by performing a bootstrap analysis with 1000 replicates. The phylogenetic analysis of HSFs from *Clematis vitalba* and *Arabidopsis thaliana* was inferred by using the Maximum Likelihood method based on the JTT matrix-based model in MEGA-X[39, 40]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed[41]. Fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). Expression profiles (TPM data) of differential expressed HSFs and HSPs were visualized in iTOL (https://itol.embl.de/). Green and red represented "down regulated" and "up regulated" respectively.

# Motif prediction and visualization of HSFs

Motif prediction and visualization of CvHSF30-1, CvHSF30-2 and CvHSFB2a were performed in HEATSTER (https://applbio.biologie.uni-frankfurt.de/hsf/heatster/home.php).

# Quantitative real-time PCR assay

Total RNA was extracted with TRIzol Reagent from leaves of *Clematis vitalba* grown under the normal condition and high temperature treatments with 0.5 h, 1 h, 1.5 h and 2 h. The cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Kyoto, Japan). PCR amplifications were performed using the TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) on the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Gene-specific primers used in the experiments are listed in Supplementary Table 8. Real-time PCRs were done on a Chromo 4™ continuous fluorescence detector with the SYBR RT-PCR Kit (Takara), in a 20 µl reaction volume, which contained 10 µl of SYBR Green I PCR mix, 0.5 µM of each forward and reverse primer, 1 µl of cDNA template, and appropriate amounts of sterile ddH$_2$O. Amplification

conditions were: 2 min at 95℃; 40 cycles of 15 s at 95℃, 30 s at 58℃, and 30 s at 72℃. Fold changes of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method[42] with *CvUBC2D* as an internal control. The entire experiments were repeated three times.

## List Of Abbreviations

HTGs: heat tolerance-related genes; PPI: protein–protein interaction; HSFs: heat shock transcription factors; HSPs: heat shock proteins; TIR1: TRANSPORT INHIBITOR RESPONSE 1; ROS: reactive oxygen species; Cv: *Clematis vitalba*; PS: Polish Spirit SG: Stolwijk Gold; HT: high-temperature; SOD: superoxide dismutase activity; DEGs: differentially expressed genes; AHA motifs: activator peptide motifs; MDA: Malondialdehyde; NBT: Nitro blue tetrazole; DAB: Diaminobenzidine; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its supplementary information files. The RNA-seq data are available from NCBI of sequence Read Archive (SRA project: PRJNA664279).

### Competing interests

The authors declare that they have no competing interests in this paper.

### Author Contributions

F. M. conceived the project and designed the study; H.Z conducted transcriptome analysis based on RNA-seq. C.J provided the RNA-seq data. H. Z, L. Z, R. G, S. P and Y. Z performed physiological experiments and qRT-PCR; H Z wrote the manuscript; F. M and S. F revised the manuscript. All of the authors discussed the results and commented on the manuscript.

## References

1. Li B, Gao K, Ren H, Tang W: **Molecular mechanisms governing plant responses to high temperatures**. *J Integr Plant Biol* 2018, **60**(9):757-779.
2. Zhang J, Li XM, Lin HX, Chong K: **Crop Improvement Through Temperature Resilience**. *Annu Rev Plant Biol* 2019, **70**:753-780.

3. Legris M, Klose C, Burgie ES, Rojas CC, Neme M, Hiltbrunner A, Wigge PA, Schafer E, Vierstra RD, Casal JJ: **Phytochrome B integrates light and temperature signals in Arabidopsis**. *Science* 2016, **354**(6314):897-900.

4. Hendrick JP, Hartl FU: **Molecular chaperone functions of heat-shock proteins**. *Annual review of biochemistry* 1993, **62**:349-384.

5. Wang RH, Zhang Y, Kieffer M, Yu H, Kepinski S, Estelle M: **HSP90 regulates temperature dependent seedling growth in Arabidopsis by stabilizing the auxin co-receptor F-box protein TI R1 (vol 7, pg 1026, 2016)**. *Nat Commun* 2016, **7**:1.

6. Schramm F, Larkindale J, Kiehlmann E, Ganguli A, Englich G, Vierling E, von Koskull-Doring P: **A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis**. *Plant J* 2008, **53**(2):264-274.

7. Ikeda M, Mitsuda N, Ohme-Takagi M: **Arabidopsis HsfB1 and HsfB2b Act as Repressors of the Expression of Heat-Inducible Hsfs But Positively Regulate the Acquired Thermotolerance**. *Plant Physiology* 2011, **157**(3):1243-1254.

8. Liu HC, Liao HT, Charng YY: **The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in Arabidopsis**. *Plant Cell Environ* 2011, **34**(5):738-751.

9. Liu HC, Charng YY: **Common and Distinct Functions of Arabidopsis Class A1 and A2 Heat Shock Factors in Diverse Abiotic Stress Responses and Development**. *Plant Physiology* 2013, **163**(1):276-290.

10. Huang YC, Niu CY, Yang CR, Jinn TL: **The Heat Stress Factor HSFA6b Connects ABA Signaling and ABA-Mediated Heat Responses**. *Plant Physiology* 2016, **172**(2):1182-1199.

11. Fang YJ, Liao KF, Du H, Xu Y, Song HZ, Li XH, Xiong LZ: **A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice**. *J Exp Bot* 2015, **66**(21):6803-6817.

12. Scharf KD, Berberich T, Ebersberger I, Nover L: **The plant heat stress transcription factor (Hsf) family: Structure, function and evolution (vol 1819, pg 104, Year 2017)**. *Biochim Biophys Acta-Gene Regul Mech* 2018, **1861**(1):60-60.

13. Qiao B, Zhang Q, Liu DL, Wang HQ, Yin JY, Wang R, He ML, Cui M, Shang ZL, Wang DK *et al*: **A calcium-binding protein, rice annexin OsANN1, enhances heat stress tolerance by modulating the production of H2O2**. *J Exp Bot* 2015, **66**(19):5853-5866.

14. Li XR, Deb J, Kumar SV, Ostergaard L: **Temperature Modulates Tissue-Specification Program to Control Fruit Dehiscence in Brassicaceae**. *Molecular Plant* 2018, **11**(4):598-606.

15. Kumar SV, Lucyshyn D, Jaeger KE, Alos E, Alvey E, Harberd NP, Wigge PA: **Transcription factor PIF4 controls the thermosensory activation of flowering**. *Nature* 2012, **484**(7393):242-U127.

16. Kumar SV, Wigge PA: **H2A.Z-Containing Nucleosomes Mediate the Thermosensory Response in Arabidopsis**. *Cell* 2010, **140**(1):136-147.

17. Sato H, Mizoi J, Tanaka H, Maruyama K, Qin F, Osakabe Y, Morimoto K, Ohori T, Kusakabe K, Nagata M *et al*: **Arabidopsis DPB3-1, a DREB2A Interactor, Specifically Enhances Heat Stress-Induced Gene Expression by Forming a Heat Stress-Specific Transcriptional Complex with NF-Y Subunits**. *Plant Cell* 2014, **26**(12):4954-4973.

18. Guan QM, Yue XL, Zeng HT, Zhu JH: **The Protein Phosphatase RCF2 and Its Interacting Partner NAC019 Are Critical for Heat Stress-Responsive Gene Regulation and Thermotolerance in Arabidopsis**. *Plant Cell* 2014, **26**(1):438-453.

19. Vacca RA, Valenti D, Bobba A, Merafina RS, Passarella S, Marra E: **Cytochrome c is released in a reactive oxygen species-dependent manner and is degraded via caspase-like proteases in tobacco bright-yellow 2 cells en route to heat shock-induced cell death**. *Plant Physiology* 2006, **141**(1):208-219.

20. Petrov V, Hille J, Mueller-Roeber B, Gechev TS: **ROS-mediated abiotic stress-induced programmed cell death in plants**. *Front Plant Sci* 2015, **6**:16.

21. Liu H-T, Li B, Shang Z-L, Li X-Z, Mu R-L, Sun D-y, Zhou R-g: **Calmodulin is involved in heat shock signal transduction in wheat**. *Plant Physiology (Rockville)* 2003, **132**(3):1186-1195.

22. Finka A, Cuendet AFH, Maathuis FJM, Saidi Y, Goloubinoff P: **Plasma Membrane Cyclic Nucleotide Gated Calcium Channels Control Land Plant Thermal Sensing and Acquired Thermotolerance**. *Plant Cell* 2012, **24**(8):3333-3348.

23. Wang X, Ma XL, Wang H, Li BJ, Clark G, Guo Y, Roux S, Sun DY, Tang WQ: **Proteomic Study of Microsomal Proteins Reveals a Key Role for Arabidopsis Annexin 1 in Mediating Heat Stress-Induced Increase in Intracellular Calcium Levels**. *Mol Cell Proteomics* 2015, **14**(3):686-694.

24. Gao F, Han XW, Wu JH, Zheng SZ, Shang ZL, Sun DY, Zhou RG, Li B: **A heat-activated calcium-permeable channel - Arabidopsis cyclic nucleotide-gated ion channel 6 - is involved in heat shock responses**. *Plant J* 2012, **70**(6):1056-1069.

25. Choudhury FK, Rivero RM, Blumwald E, Mittler R: **Reactive oxygen species, abiotic stress and stress combination**. *Plant J* 2017, **90**(5):856-867.

26. Yao Y, He RJ, Xie QL, Zhao XH, Deng XM, He JB, Song L, He J, Marchant A, Chen XY *et al*: **ETHYLENE RESPONSE FACTOR 74 (ERF74) plays an essential role in controlling a respiratory burst oxidase homolog D (RbohD)-dependent mechanism in response to different stresses in Arabidopsis**. *New Phytol* 2017, **213**(4):1667-1681.

27. Xuan Y, Zhou S, Wang L, Cheng YD, Zhao LQ: **Nitric Oxide Functions as a Signal and Acts Upstream of AtCaM3 in Thermotolerance in Arabidopsis Seedlings**. *Plant Physiology* 2010, **153**(4):1895-1906.

28. Karpets Y, Kolupaev Y, Vayner A: **Functional interaction between nitric oxide and hydrogen peroxide during formation of wheat seedling induced heat resistance**. *Russ J Plant Physiol* 2015, **62**(1):65-70.

29. Niu LJ, Liao WB: **Hydrogen Peroxide Signaling in Plant Development and Abiotic Responses: Crosstalk with Nitric Oxide and Calcium**. *Front Plant Sci* 2016, **7**:14.

30. Shi CY, Qi C, Ren HY, Huang AX, Hei SM, She XP: **Ethylene mediates brassinosteroid-induced stomatal closure via G alpha protein-activated hydrogen peroxide and nitric oxide production in Arabidopsis**. *Plant J* 2015, **82**(2):280-301.

31. Wu D, Chu HY, Jia LX, Chen KM, Zhao LQ: **A feedback inhibition between nitric oxide and hydrogen peroxide in the heat shock pathway in Arabidopsis seedlings**. *Plant Growth Regul* 2015, **75**(2):503-509.

32. Hussain A, Mun BG, Imran QM, Lee SU, Adamu TA, Shahid M, Kim KM, Yun BW: **Nitric Oxide Mediated Transcriptome Profiling Reveals Activation of Multiple Regulatory Pathways in Arabidopsis thaliana**. *Front Plant Sci* 2016, **7**:18.

33. Zhang W, Zhou RG, Gao YJ, Zheng SZ, Xu P, Zhang SQ, Sun DY: **Molecular and Genetic Evidence for the Key Role of AtCaM3 in Heat-Shock Signal Transduction in Arabidopsis**. *Plant Physiology* 2009, **149**(4):1773-1784.

34. Ogawa D, Yamaguchi K, Nishiuchi T: **High-level overexpression of the Arabidopsis HsfA2 gene confers not only increased themotolerance but also salt/osmotic stress tolerance and enhanced callus growth**. *J Exp Bot* 2007, **58**(12):3373-3383.

35. Charng YY, Liu HC, Liu NY, Chi WT, Wang CN, Chang SH, Wang TT: **A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis**. *Plant Physiology* 2007, **143**(1):251-262.

36. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T: **Cytoscape: A software environment for integrated models of biomolecular interaction networks**. *Genome Research* 2003, **13**(11):2498-2504.

37. Assenov Y, Ramirez F, Schelhorn SE, Lengauer T, Albrecht M: **Computing topological parameters of biological networks**. *Bioinformatics* 2008, **24**(2):282-284.

38. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R: **IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era**. *Molecular Biology and Evolution* 2020, **37**(5):1530-1534.

39. Kumar S, Stecher G, Li M, Knyaz C, Tamura K: **MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms**. *Molecular Biology and Evolution* 2018, **35**(6):1547-1549.

40. Jones DT, Taylor WR, Thornton JM: **The rapid generation of mutation data matrices from protein sequences**. *Computer applications in the biosciences : CABIOS* 1992, **8**(3):275-282.

41. Felsenstein J: **CONFIDENCE LIMITS ON PHYLOGENIES: AN APPROACH USING THE BOOTSTRAP**. *Evolution; international journal of organic evolution* 1985, **39**(4):783-791.

42. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods (San Diego, Calif)* 2001, **25**(4):402-408.
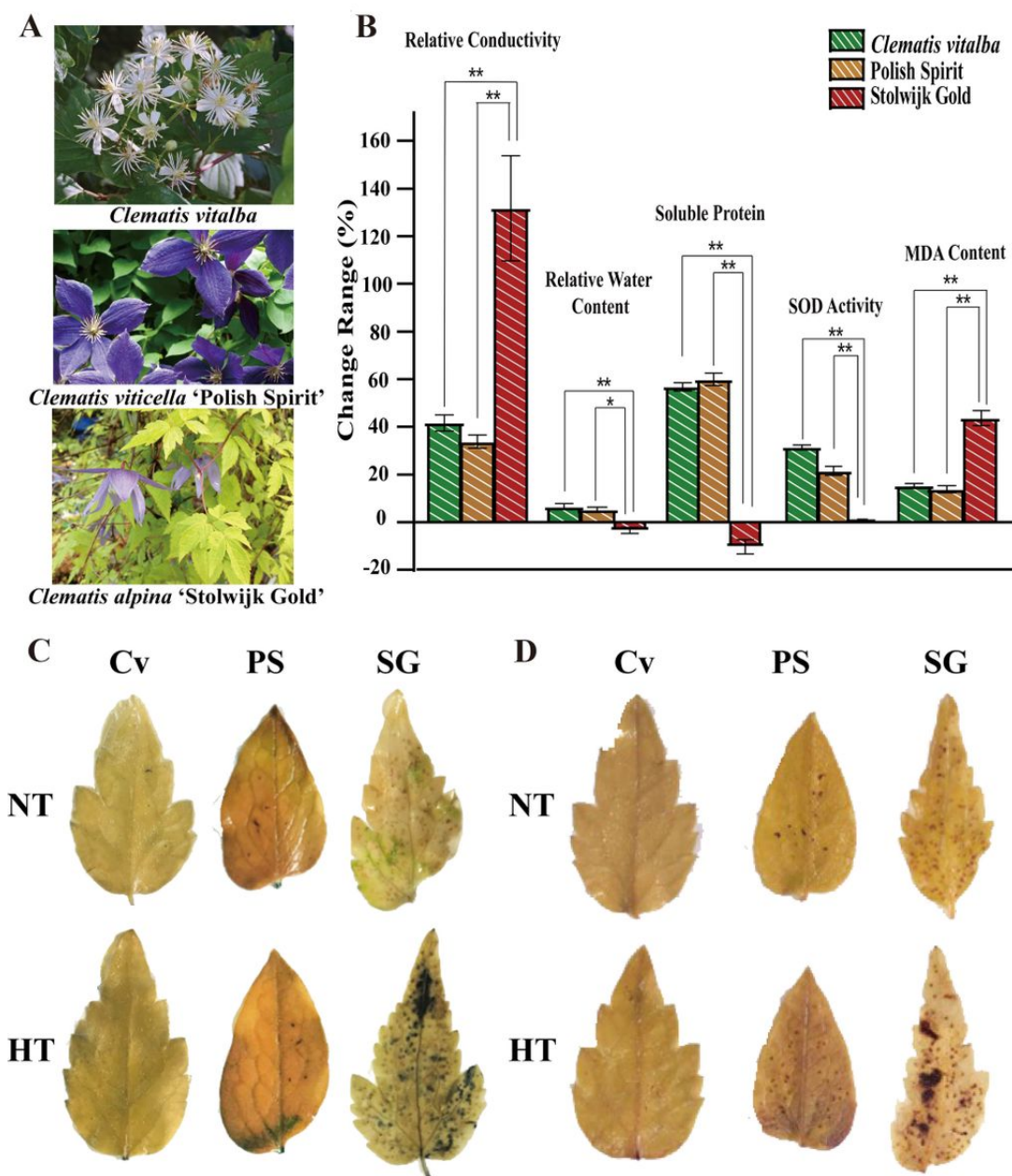
# Figures

**Figure 1**

Three Clematis varieties and physiological change after high temperature treatment. (A) Three Clematis varieties (Clematis vitalba, Clematis viticella 'Polish Spirit' and Clematis alpina 'Stolwijk Gold'). (B) Change of relative conductivity, relative water content, soluble protein, SOD activity and MDA content after high temperature treatment (42°C for 2 hours) among three Clematis varieties. Data were mean ± SE from three biological replicates. * indicate statistically significant differences by student t-test: P<0.05. ** P<0.01. (C) NBT staining of three Clematis varieties leaves after normal (22°C) and high (42°C) temperature treatment for 2 hours. (D) DAB staining of three Clematis varieties leaves after normal (22°C) and high (42°C) temperature treatment for 2 hours.
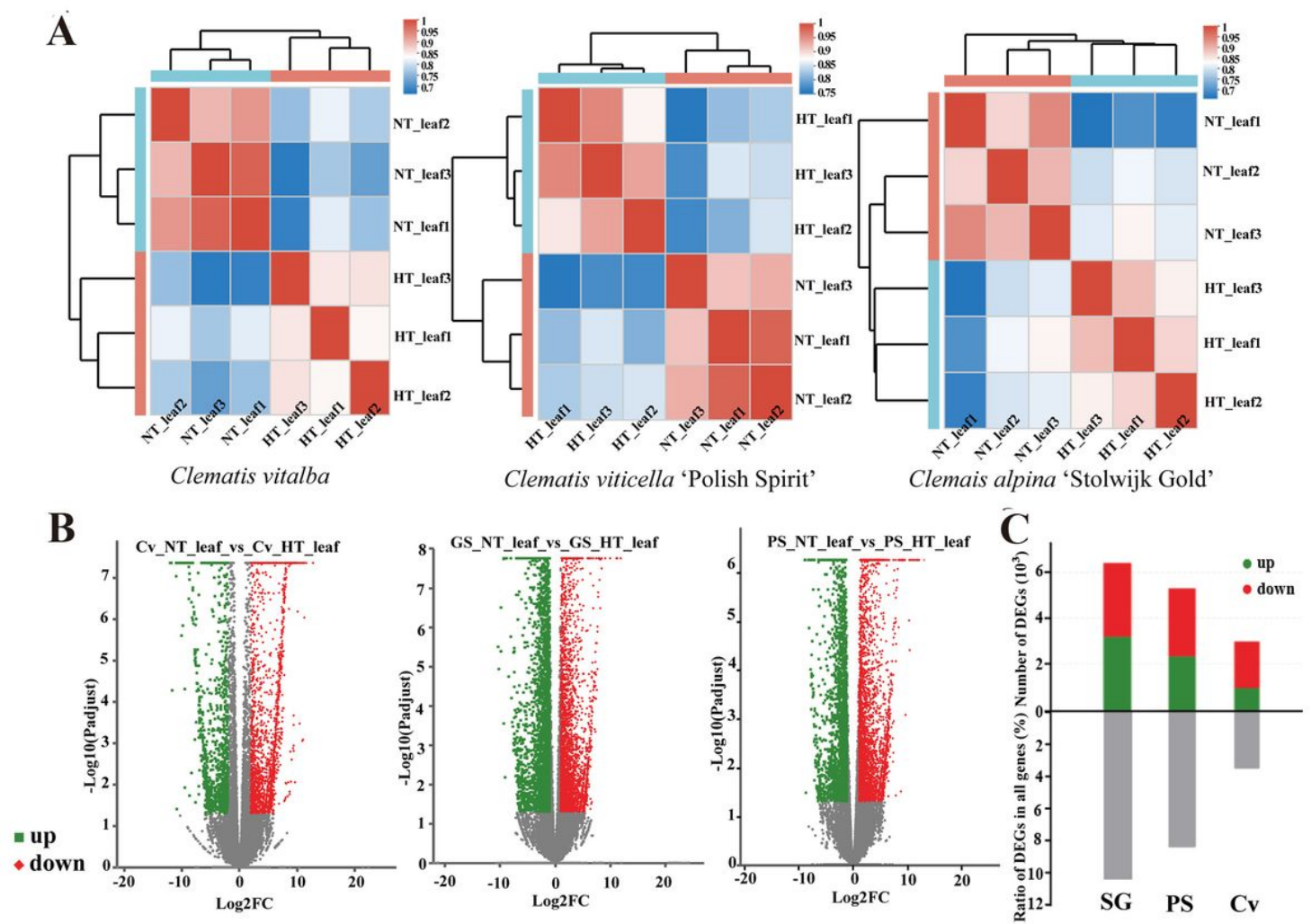
**Figure 2**

Correlations among transcriptomes, visualization of differential expression and statistics of differentially expressed genes. (A) Correlation matrix and cluster dendrogram of the whole dataset of the mapped reads. For every Clematis variety, the analysis was performed by comparing the values of the entire transcriptome in all six samples with three biological replicates. Correlation analysis and hierarchical cluster analysis were performed using R software. Red color indicates a stronger correlation and blue weaker. (B) Volcano plots of differential expression of three Clematis varieties under normal and high temperature. Red and green represent "up regulated" and "down regulated", respectively. (C) Differentially expressed genes' number and proportion in all genes.
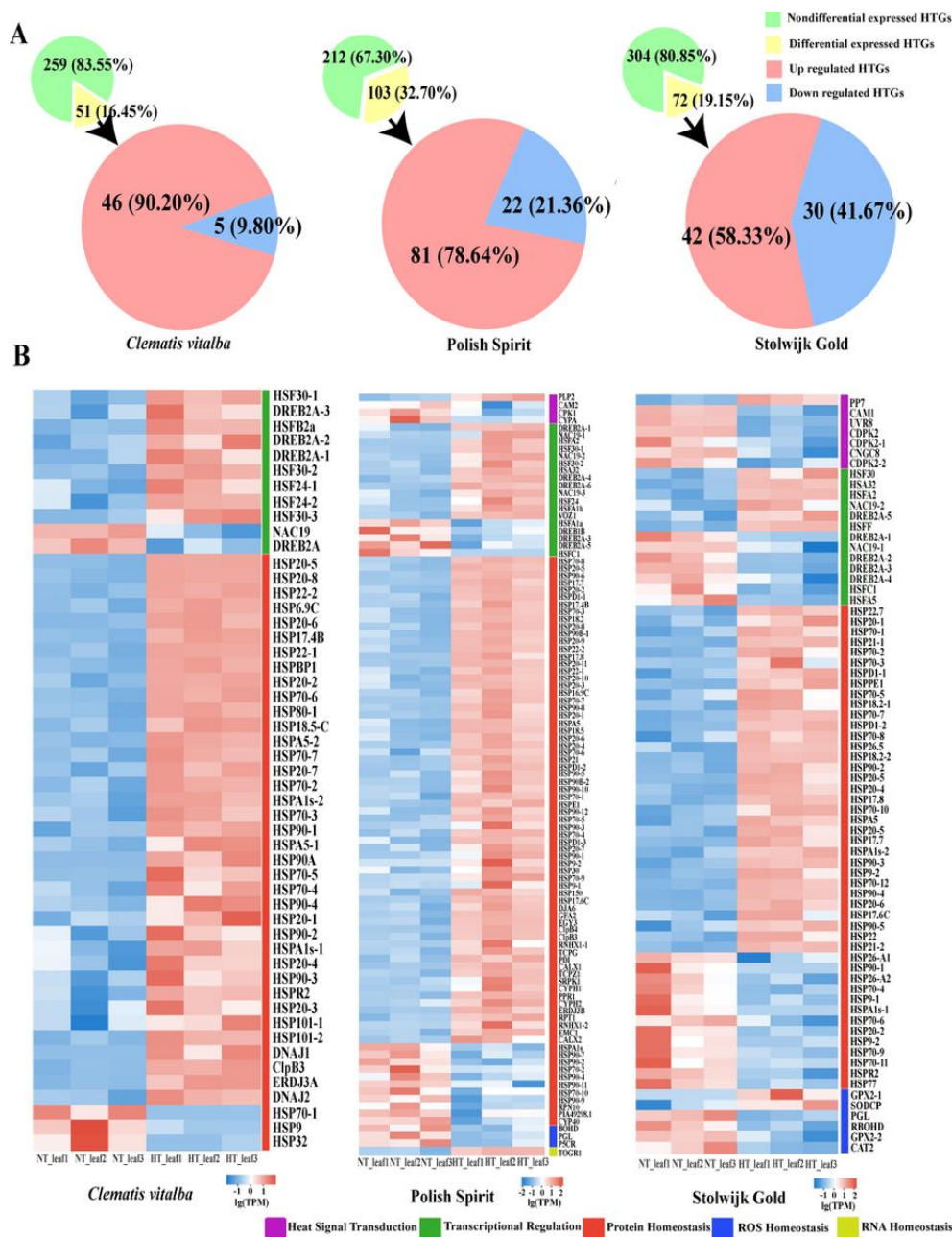
**Figure 3**

The proportion of differential expressed HTGs in all HTGs and expression profiles of differentially expressed HTGs in three Clematis varieties. (A) Pie charts of HTGs (nondifferential expressed HTGs, differential expressed HTGs, up regulated HTGs and down regulated HTGs) (B) Heatmap of differentially expressed HTGs belong to different regulation levels (heat signal transduction, transcriptional regulation, protein homeostasis and RNA homeostasis)
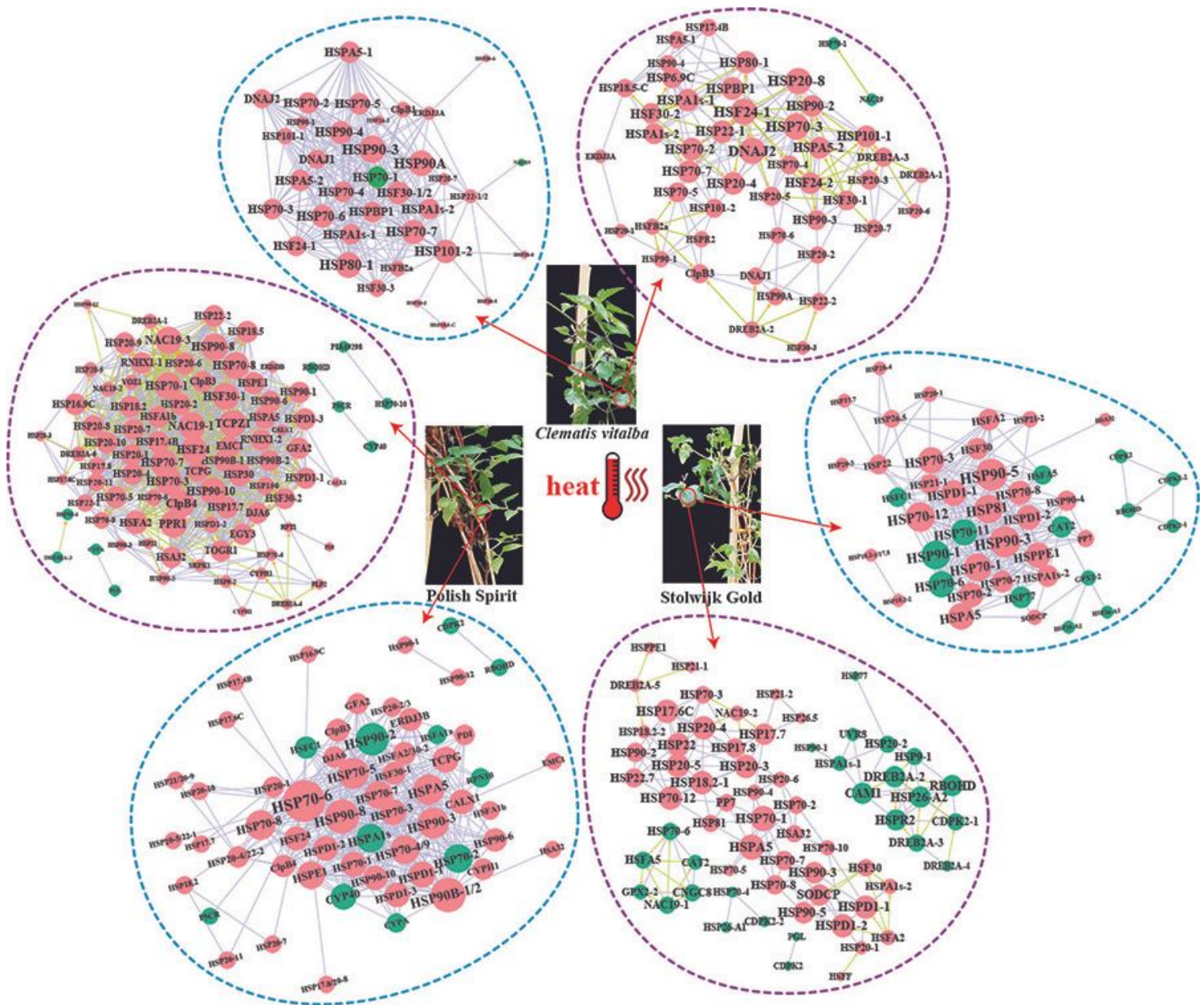
**Figure 4**

Heat-related genetic regulatory network of three Clematis varieties. The networks inside dotted purple line and dotted blue line showed gene co-expression and protein-protein interaction (PPI) relationships, respectively. Red nodes and green nodes represented up regulated and down regulated genes respectively. The size of nodes represented number of edges directly connected with nodes. Yellow arrows pointed at potential targets of heat-responsive transcription factors.
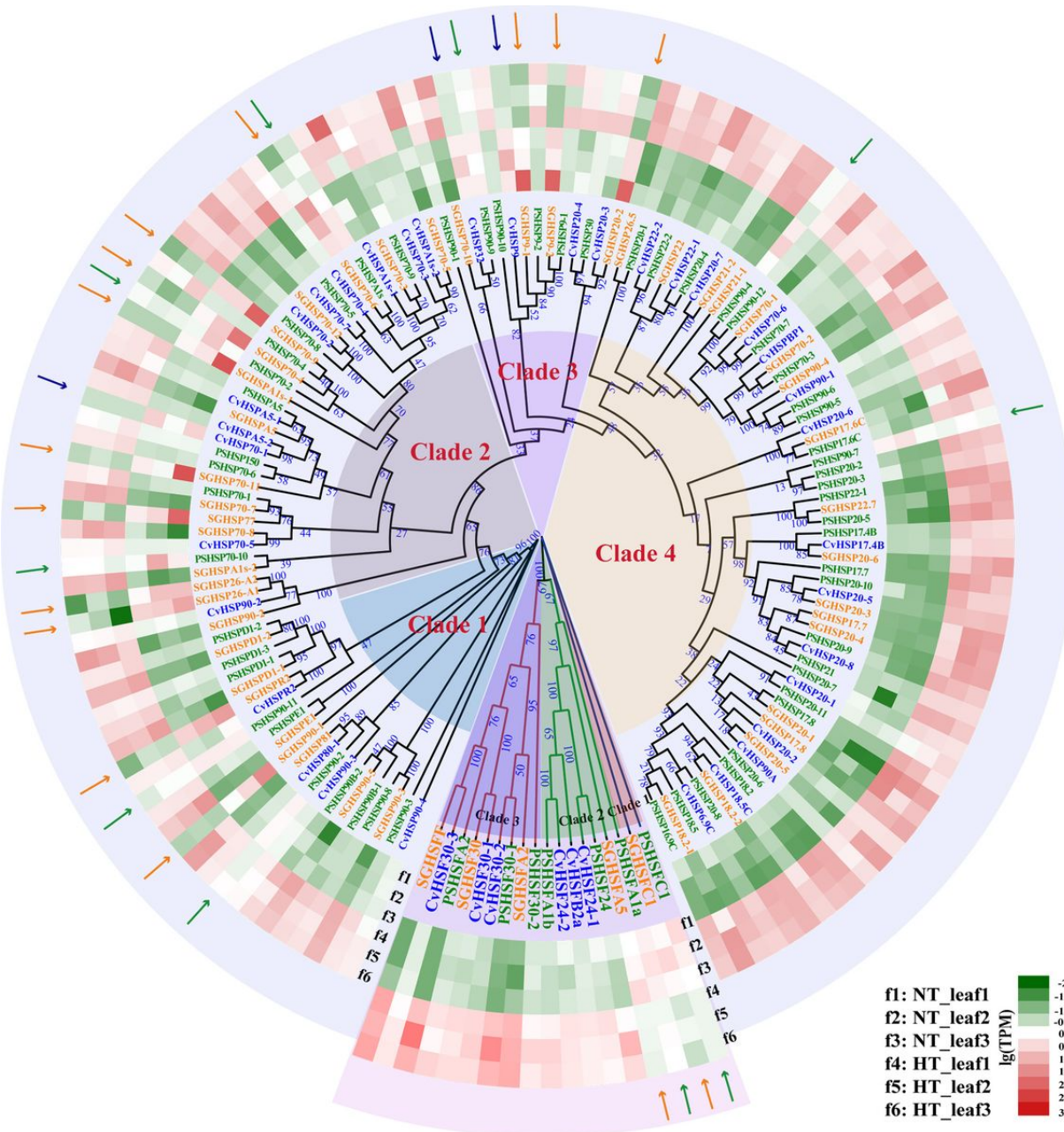
**Figure 5**

Phylogenetic analysis and differential expression of HSF genes and HSP genes in three Clematis varieties. The trees of differential expressed HSFs and HSPs were both constructed in IQ-TREE v2.0.6 with JTT+I+G4 and VT+R3 model and could be divided into 3 and 4 clades, respectively. Support rates were labeled at corresponding branches. The heatmap was generated from the TPM data of transcriptomes. Green and red represented "down regulated" and "up regulated" respectively. Blue, green and yellow arrows pointed at down regulated HSFs or HSPs of Clematis vitalba, Polish Spirit and Stolwijk Gold, respectively.
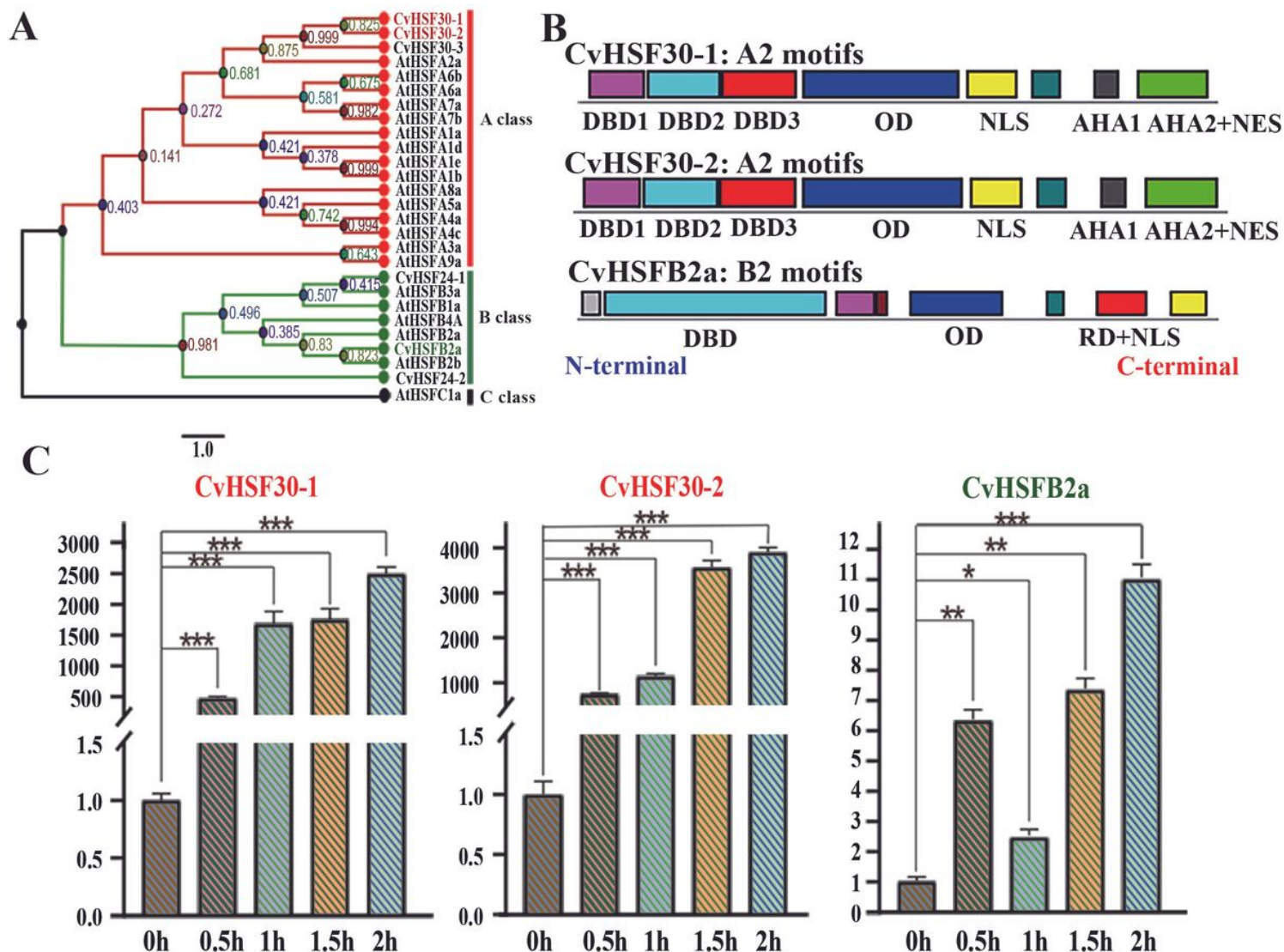
**Figure 6**

Classification, motifs and qRT-PCR analysis of CvHSFs. (A) Phylogenetic analysis of differential expressed CvHSFs and HSF family members of Arabidopsis thaliana. The tree was constructed in MEGA X using the maximum likelihood method and divided into A, B and C three classes. Support rates were labeled at corresponding branches. (B) Motifs of CvHSF30-1, CvHSF30-2 and CvHSFB2a. Motif prediction and visualization were performed in HEATSTER (https://applbio.biologie.uni-frankfurt.de/hsf/heatster/home.php). (C) CvHSF30-1, CvHSF30-2 and CvHSFB2a qRT-PCR analysis from leaves of Clematis vitalba after different high temperature treatment times. The expression of CvUBC2D was used as an internal control. Data were mean ± SE from three biological replicates. * indicate statistically significant differences by student t-test: P<0.05. ** P<0.01.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1Supplementaryfigure1.jpg
- Additionalfile10SupplementaryTable7.xlsx
- Additionalfile11SupplementaryTable8.xlsx
- Additionalfile2Supplementaryfigure2.jpg
- Additionalfile3Supplementaryfigure3.jpg

- Additionalfile4SupplementaryTable1.xlsx
- Additionalfile5SupplementaryTable2.docx
- Additionalfile6SupplementaryTable3.docx
- Additionalfile7SupplementaryTable4.xlsx
- Additionalfile8SupplementaryTable5.docx
- Additionalfile9SupplementaryTable6.xlsx
- SupplementaryInformation.docx