



## Identification of Key Transcription Factors in Cucumber (*Cucumis sativus*): Linking the Circadian Clock with Heat Stress Response

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### Abstract

Climate change-induced heat stress is a major threat to cucumber (*Cucumis sativus*) productivity, impairing growth, photosynthesis, and yield. To identify key transcriptional regulators of the heat stress response, we conducted an RNA-seq analysis on leaves of cucumber seedlings exposed to 42°C at 3- and 6-hour post-treatment (accession PRJNA634519). Using stringent criteria (FDR < 0.00001, |log<sub>2</sub>FC| ≥ 4), we identified 166 significantly differentially expressed genes (DEGs). Among these, eight were identified as transcription factors (TFs), including members of the HSF, MYB, WRKY, NAC, Trihelix, GARP, and B3 families. Notably, several of these TFs, such as REVEILLE 6 (RVE6), REVEILLE 8 (RVE8), and PCL1, are known regulators of the circadian clock, suggesting a close link between circadian rhythms and heat stress response in cucumber. Additionally, up-regulation of NAC, WRKY, and HSF TFs underscores their established roles in abiotic stress defense. The significant downregulation of a B3 domain-containing TF implies it may act as a repressor relieved under stress. Our findings highlight a complex regulatory network where circadian-associated TFs potentially coordinate the timely activation of heat-responsive genes. These key TFs provide valuable targets for future functional validation and breeding strategies to enhance thermotolerance in cucumber.

#### Keywords

Circadian clock, *Cucumis sativus*, Heat stress, Transcription factor, RNA-seq analysis

### Introduction

Climate change and rising global temperatures have profoundly affected the growth and productivity of crop plants. Among abiotic stresses, heat stress is one of the main limiting factors for growth, photosynthesis, fertility, and yield in many plant species including cucumber (*Cucumis sativus*). Cucumber, being temperature-sensitive, suffers from reduced fruit quality and yield under high heat conditions, posing significant challenges for sustainable agriculture and food security. In response to heat stress, plants utilize complex mechanisms involving changes in gene expression, signaling pathway regulation, production of heat shock proteins, and activation of transcription factors. Transcription factors are key regulators of defense responses, binding to cis-elements in promoter regions of target genes to activate or repress their expression. Families such as HSF, NAC, WRKY, MYB, and bZIP play crucial roles in regulating responses to temperature stress (Liang *et al.*, 2024). The circadian clock orchestrates the expression of key transcription factors, enabling plants to synchronize their heat stress responses with the predictable daily rise in temperature. With the advancement of next-generation sequencing technologies, RNA-seq has become a powerful tool for comprehensive gene expression analysis under different conditions. This technology enables the identification of differentially expressed genes (DEGs), activated biological pathways, and regulatory factors involved in stress responses. In this study, RNA-seq was used to analyze gene expression changes in cucumber leaves under heat stress. Genes with significant differential expression were identified, and transcription factors among them were extracted. The aim was to identify key regulators and molecular pathways involved in cucumber's heat stress response, providing a genetic basis for breeding heat-tolerant varieties.

### Materials and methods

#### RNA-Seq Data Collection and Processing

We used RNA-seq data available from the Sequence Read Archive (SRA) under accession PRJNA634519 (Chen *et al.*, 2020). The dataset comprised nine samples in total from cucumber seedling leaves (cultivar '9930') exposed to high temperature (42°C). Samples were collected at three time points: before treatment (0 hours), 3 hours, and 6 hours after heat stress treatment. The goal was to study gene expression changes in response to heat. RNA-seq data were analyzed using the Galaxy platform (<https://usegalaxy.org>). Raw reads quality was assessed with Fast QC, showing good quality not requiring trimming. The cucumber reference genome and annotation files were downloaded from Ensembl Plants (<https://plants.ensembl.org>). Reads were aligned to the reference genome using HISAT2. Gene transcript counts were obtained using Feature Counts based on the annotation. Raw counts were normalized in DESeq2 by estimating size factors, and differential expression testing was based on the DESeq2 output. Genes with adjusted p-value ≤ 0.00001 and |log<sub>2</sub> fold change| ≥ 4 were considered significantly differentially expressed. We used conservative thresholds to reduce false positives and report only robust, reliable expression changes, particularly given the relatively small number of biological replicates (n = 9) and the need for downstream validation.

#### Transcription Factor Identification

Differentially expressed genes were queried in Ensembl Plants (<https://plants.ensembl.org>) and their sequences were compared against the iTAK database (<http://itak.feilab.net>), which contains plant transcription factors and regulators. This allowed identification of transcription factors among the differentially expressed genes.

### Results and discussion

The analysis showed that exposure to 42°C caused significant gene expression changes in cucumber leaves at 3 and 6 hours post-treatment. Using strict criteria (FDR < 0.00001 and |log<sub>2</sub>FC| ≥ 4), 166 DEGs were identified, including upregulated and downregulated genes in response to heat stress. Among these, eight genes were identified as TFs based on the iTAK database matching (Table 1).

Gene Ontology and KEGG pathway enrichment analysis revealed distinct functional categories for the upregulated and downregulated DEGs. Among the upregulated genes, molecular function (MF) terms were significantly enriched for *unfolded protein binding* (Count = 6, FDR = 3.94E-06) and *calcium ion binding* (Count = 7, FDR = 3.04E-05). KEGG pathway analysis indicated enrichment for *Protein processing in endoplasmic reticulum* (P-value = 4.97E-06, FDR = 8.44E-05), highlighting the importance of protein folding and quality control under heat stress. For downregulated genes, cellular component (CC) enrichment was most prominent for *nucleosome* (Count = 9, FDR = 4.67E-13), while molecular function terms included *structural constituent of chromatin* (Count = 9, FDR = 5.83E-12), *protein heterodimerization activity* (Count = 9, FDR = 1.42E-10), microtubule binding (Count = 7, FDR = 3.27E-06), and DNA binding (Count = 12, FDR = 2.51E-05). These results suggest that heat stress leads to suppression of chromatin organization, DNA-related processes, and cytoskeleton functions, while activating protein stability and calcium-mediated signaling pathways.

**Table 1.** Eight transcription factor (TF) genes significantly differentially expressed in cucumber leaves under 42°C heat stress.

Ensemble ID	TF family	Description	LogFC	FDR
Csa_1G524660	GARP	transcription factor PCL1	5.665101	1.52E-53
Csa_2G359980	B3	B3 domain-containing protein	-4.87162	1.59E-15
Csa_3G183960	MYB	protein REVEILLE 6	4.858061	2.10E-50
Csa_3G731130	Trihelix	trihelix transcription factor GT-3b	4.048846	8.28E-12
Csa_3G736740	MYB	protein REVEILLE 8	-4.81563	4.49E-17
Csa_4G193250	NAC	NAC domain-containing protein 87	4.422278	4.61E-09
Csa_6G363560	WRKY	probable WRKY transcription factor 50	5.10763	2.50E-11
Csa_6G517310	HSF	heat stress transcription factor B-2a	4.280192	4.42E-13

In addition to HSF-dependent pathways, previous studies have shown that heat stress responses can involve transcription factor pathways independent of HSFs. For example, in Arabidopsis, the circadian-associated transcription factors REVEILLE 4 and 8 (RVE4/8) activate HSF-independent regulatory pathways under heat stress (Blair *et al.*, 2019). Although direct information on PCL1's role in cucumber heat stress is lacking, considering its role in circadian regulation and evidence from Arabidopsis, PCL1 may regulate heat stress responses through circadian-related pathways. Further temporal expression and protein interaction studies are needed to verify this. REVEILLE 6 (RVE6) and REVEILLE 8 (RVE8), members of the MYB-like transcription factor family, play key roles in regulating plant circadian rhythms. Along with RVE4, they activate the expression of afternoon and evening phase genes and partly overlap in function (Hughes *et al.*, 2024). The circadian clock helps plants time their responses to environmental cues like heat. When temperature rises, RVE6 and RVE8 ensure timely activation of genes that prepare the plant for heat stress, enhancing tolerance. The expression of ZmGT-3b is rapidly induced by light, and its promoter has several cis-elements related to light and biotic/abiotic stress responses, such as W-box, MBS, ABRE, and TGACG motifs. Functional studies using ZmGT-3b knockdown mutants showed reduced photosynthesis-related gene expression and increased resistance to pathogens such as *Fusarium graminearum*, indicating its role in balancing growth and defense under normal conditions (Zhang *et al.*, 2021). In this study, increased expression of this gene under heat stress supports its involvement in abiotic stress responses, consistent with the presence of stress-related cis-elements in its promoter. In our study, three key genes (NAC domain-containing protein 87, probable WRKY transcription factor 50, and heat stress transcription factor B-2a) showed significant upregulation under heat stress, aligning with previous reports of their roles in abiotic stress responses, including heat (Surabhi and Badajena, 2020). Additionally, the B3 domain-containing protein likely acts as a negative regulator in heat stress response. Its reduced expression under heat may relieve repression, enabling activation of stress-responsive genes. However, further studies are required to fully understand its mechanism, including identifying its target genes, interaction networks, and effects of its overexpression or knockout on heat tolerance in cucumber. The results indicate that many key transcription factors involved in cucumber's heat stress response are connected to the plant's circadian clock. Factors like REVEILLE 6, REVEILLE 8, and PCL1, known for their role in regulating the circadian rhythm, help control the timing of heat-responsive gene expression. This means the plant uses its internal clock to activate protective genes at the correct times, improving its ability to cope with high temperatures. Therefore, cucumber's heat stress response likely involves a combination of circadian-regulated pathways along with the classical heat shock factor pathways, allowing the plant to respond precisely and efficiently to heat stress. This insight aligns with prior evidence from Arabidopsis showing circadian-regulated heat stress pathways operating alongside classical heat shock factor-mediated responses (Blair *et al.*, 2019). Thus, a functional circadian system is fundamental for an efficient and energy-saving adaptation to heat stress. Functional analysis using knockout or overexpression lines in cucumber will provide valuable insights into the precise roles of these transcription factors in heat stress. Moreover, examining allelic variation among different cucumber populations and correlation with heat tolerance can help identify beneficial variants for breeding programs.

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