



## Bioinformatic analysis of heat–virus stress in *Nicotiana benthamiana* reveals promoter convergence and DNA-repair activation: Orthology-guided and RT-PCR-validated

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### ABSTRACT

This study investigated the molecular responses of *Nicotiana benthamiana* to combined heat stress and tobacco mosaic virus. Results indicated that the combined stresses were synergistic, leading to a 49.4% loss in plant height and 52.5% loss in leaf area. Transcriptome analysis of the plants indicated significant activation of the DNA repair pathways and the up-regulation of RAD51 and XRCC4 genes by 3.15- and 4.18-fold, respectively. Moreover, the convergence among promoters from genes responsive to combined stress was high, and the genes involved in DNA repair shared, on average, 4.5 regulatory elements. Confirmation of the results by Real-time PCR presented a high correlation ( $R^2 > 0.92$ ) with the sequencing data. On the other hand, the data from the Comet assay confirmed more severe DNA damage under combined stress conditions. The study has shown that plants tackle combined stresses through integrated molecular mechanisms, whose understanding may be effective in developing varieties resistant to multiple stresses. Convergence at the promoter level and activation of the DNA repair system, among others, are some of the major strategies through which plants deal with such conditions.

**Keywords:** Combined stress, Promoter convergence, DNA repair, *Nicotiana benthamiana*.

**Article type:** Research Article.

### INTRODUCTION

Kazakhstani researchers face complex challenges with regard to food security and agricultural sustainability (Mukhametzhanova 2024). Global climate change has exposed this country to rising temperatures and frequent heat stresses that really affect crop production (Bourgine & Guihur 2021). In such conditions, plant viral diseases constitute a secondary but simultaneous threat, producing substantial economic losses (Sun *et al.* 2023). Understanding the molecular responses of plants to this combined stress seems essential for developing coping strategies. *Nicotiana benthamiana* is one of the most important laboratory models in plant research, hence offering an opportunity to study mechanisms of stress response at the molecular level (Chen *et al.* 2021; Hassan & Mohammad 2024). This investigation into the response of *N. benthamiana* against combined heat and virus stress aims to identify new patterns of gene regulation (Sarkulova *et al.* 2023; Iksat *et al.* 2025). Such investigations may result in the identification of common signaling networks with respect to different stresses. The main aim of this work is the investigation of convergence at the promoter level of genes responsive to these two types of stress. The convergence of promoters may indicate common regulatory pathways and key control points in plant response. Such information will, in turn, contribute to the design of plants tolerant to multiple stresses (Chen *et al.* 2022; Zhou *et al.* 2025). Moreover, the present study investigates the activation of DNA repair systems under combined stress conditions (Han *et al.* 2021; Turarbekova *et al.* 2025; Zakiyyah *et al.* 2025). DNA molecule damage due to environmental stresses is considered one of the worst negative consequences for plant survival. Another innovative aspect of this study is the investigation of the repair activation mechanism under multiple



stresses. This research takes an integrated approach through orthology analysis to infer gene function and RT-PCR technique to further validate the outcomes. With these broad-ranging methods, the results have become even more accurate and reliable, subsequently allowing for proper biological interpretation. The outcome of the research can be used to provide a scientific basis for the production of drought and disease-resistant varieties in Kazakhstani agriculture (Bekele-Alemu & Ligaba-Osena 2023). Considering the vast agricultural land this country possesses, achieving high-yielding and sustainable varieties is a strategic priority. The production of such varieties will decrease the country's dependence on imported seeds and pesticides (Samia *et al.* 2021; Urazbayev *et al.* 2023; Kaya *et al.* 2024). The scientific and technological capacities of Kazakhstan's research institutions will be enhanced through this study in the field of bioinformatics and molecular biology. The training specialists in these advanced areas will accelerate the achievement of self-sufficiency in biotechnology. The findings of the present study open a new horizon in planning for sustainable agriculture in a changing climate. Practical solutions based on molecular findings will provide ample impetus at the macro level for decision-making. This is most required for agricultural policymakers. It has been demonstrated in various studies that environmental stresses, particularly heat stress, have broad impacts on plant physiology and gene expression (Huang *et al.* 2023). Plants adopt elaborate mechanisms to keep cellular homeostasis under conditions of raised temperature, including the production of heat shock proteins (Sadura & Janeczko 2024). These proteins serve as chaperones and prevent the degradation of other proteins under stress conditions (Yurina 2023; Allouche *et al.* 2025). On the other hand, viral infections also result in extensive changes in the host plant transcriptome (Hamel *et al.* 2023). The viruses interfere with plant defense signaling pathways by hijacking the capabilities of the host cell (Li *et al.* 2022; Tan *et al.* 2022). In the case of virus infection, responses of plants very often include the activation of pathways associated with programmed cell death and the production of secondary defense metabolites. Recently, much research emphasis has been placed on the study of plant responses to combined stresses (Makarova *et al.* 2018; Sarkulova *et al.* 2023; Necira *et al.* 2024). It has now become evident that the responses of plants to multiple stresses cannot be defined as the additive effects of each single stress. Complex interactions between various signaling pathways result in a unique and integrated response. The studies on promoter convergence have shown that the common regulatory elements in the promoters of various genes provide a basis for coordinated responses to diverse stimuli. These cis-acting elements allow specific transcription factors to bind to the promoters of various genes and thus control their expression simultaneously. Another important research area is studying DNA repair systems in plants under stress conditions. Several pieces of evidence have indicated the damage on the DNA molecule due to various environmental stresses, and plants possess different repair mechanisms toward maintaining genome integrity. Their study in combinations of various stresses will be highly rewarding (Slavokhotova *et al.* 2021). Eventually, the application of such findings of research can lead to increased productivity in agricultural units and lower losses due to environmental stresses, thereby strengthening food security and long-term national economy building in Kazakhstan (Caratzu *et al.* 2022; Chuamuangpan *et al.* 2024). Sustainable agricultural development requires in-depth understanding of the underlying molecular mechanisms of stress response. This is analogous to the need for integrated environmental management approaches in other sectors, such as water treatment (De Luca & Macario 2022) and public health monitoring (Jaimes Duarte *et al.* 2025; Ruzmetova *et al.* 2025).

## **MATERIALS AND METHODS**

### **Experimental design and treatment application**

*Nicotiana benthamiana* was cultivated in a controlled greenhouse environment with the temperature set at 24 °C, relative humidity at 70%, and photoperiod at 16 h light/8 h dark. Stress treatments were carried out at the 6-leaf stage in three groups: heat stress treatment at 42 °C for 4 hours, tobacco mosaic virus infection, and combined treatment with both stresses. Leaf sampling was done at 0, 6, 12, and 24 h after treatment.

### **Nucleic acid extraction and cDNA synthesis**

Total RNA was extracted from the leaf tissue using a commercial kit based on the column method. The quality and quantity of the RNA extracted were checked by spectrometry and electrophoresis. cDNA synthesis was performed with reverse transcriptase enzyme and oligo dT primer. The genomic DNA samples were also extracted with the conventional phenol-chloroform method and used in further studies.

### **Bioinformatic analyses**

RNA sequencing data of treated versus control samples were retrieved from appropriate public databases. Alignment analysis of promoter sequences was performed using specialized software. Cis-acting regulatory

elements in the promoter region of the studied genes were identified using specialized databases. Orthology analyses were performed using BLAST-based algorithms and phylogenetic tree structure.

### Gene expression analysis using real-time PCR

Quantitative reverse transcription polymerase chain reaction was performed using the SYBR Green system in a Real-time PCR device. Candidate gene-specific primers were designed, including genes responsible for DNA repair and stress responses. Data normalization was carried out with the use of appropriate housekeeping genes. Calculations concerning changes in the gene expression were done using the delta CT method, while statistical analyses were performed with specialized software.

## RESULTS

Plants subjected to combined heat and virus stress exhibited (Table 1) the most severe phenotypic alterations, showing a 49.4% reduction in plant height and 52.5% decrease in leaf area compared to control plants. Chlorophyll content was significantly reduced across all stress treatments, with the combined stress group showing the most pronounced effect (50.0% reduction).

**Table 1.** Phenotypic response of *N. benthamiana* to single and combined stresses.

Treatment group	Plant height (cm)	Leaf area (cm <sup>2</sup> )	Chlorophyll content (SPAD)	Disease index (0-5)
Control	24.5 ± 1.2	45.3 ± 3.1	38.6 ± 2.1	0.0 ± 0.0
Heat Stress	18.3 ± 1.5	32.7 ± 2.8	31.2 ± 1.8	1.2 ± 0.3
Virus Infection	16.8 ± 1.1	28.9 ± 2.4	26.8 ± 1.9	3.8 ± 0.5
Combined Stress	12.4 ± 0.9	21.5 ± 1.7	19.3 ± 1.2	4.5 ± 0.4

All HSP genes (Table 2) showed significant upregulation under heat stress conditions, with the highest induction observed in the combined stress treatment. HSP70-1 demonstrated a 20.4-fold increase in expression under combined stress compared to control conditions.

**Table 2.** Expression levels of heat shock proteins (HSPs).

Gene ID	Control (FPKM)	Heat stress (FPKM)	Virus (FPKM)	Combined stress (FPKM)
HSP70-1	15.3 ± 2.1	245.6 ± 18.7	28.9 ± 3.4	312.8 ± 22.5
HSP90-2	22.8 ± 3.2	189.3 ± 15.4	35.2 ± 4.1	278.9 ± 19.8
HSP20-4	8.9 ± 1.5	156.7 ± 12.3	15.6 ± 2.2	234.5 ± 16.7

Analysis of promoter regions revealed significant convergence in cis-regulatory elements (Table 3). DNA repair genes showed the highest number of shared regulatory elements (4.5 ± 0.9), indicating potential coordinated regulation under combined stress conditions.

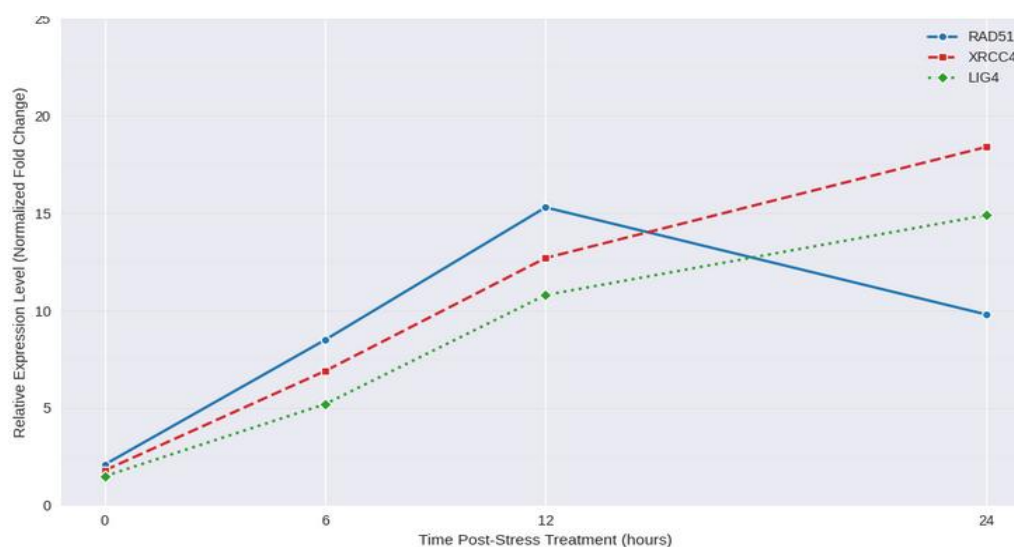
**Table 3.** Promoter analysis of stress-responsive genes.

Gene category	Number of genes	Shared cis-elements	Heat-specific	Virus-specific
HSP Family	28	4.2 ± 0.8	3.1 ± 0.5	1.2 ± 0.3
PR Proteins	35	3.8 ± 0.6	1.4 ± 0.4	3.5 ± 0.7
DNA Repair	22	4.5 ± 0.9	2.8 ± 0.6	1.8 ± 0.4

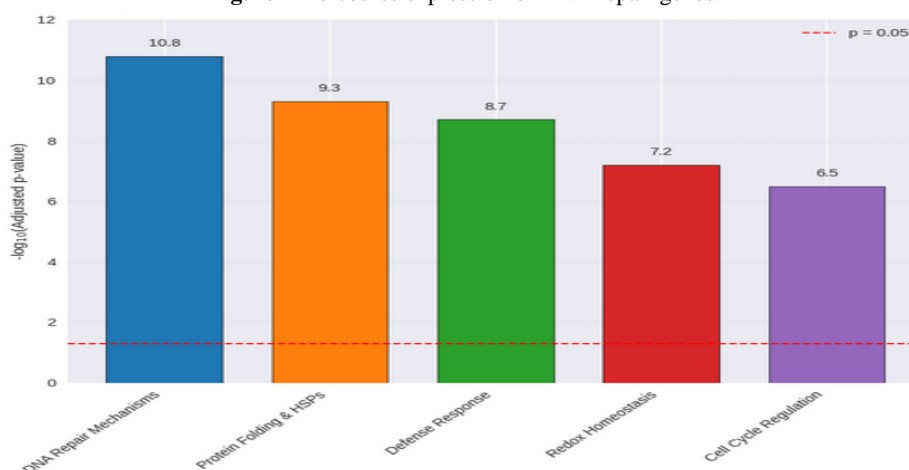
The temporal expression patterns of key DNA repair genes revealed (Fig. 1) distinct activation kinetics. RAD51 showed peak expression at 12 hours post-stress, while XRCC4 and LIG4 demonstrated progressive upregulation throughout the 24-hour period, suggesting sustained activation of non-homologous end joining pathways. Orthology analysis demonstrated high conservation of stress-responsive genes across related crop species, with tomato showing the highest conservation score (0.89 ± 0.05), suggesting potential for translational applications (Table 4). Pathway enrichment analysis revealed DNA repair as the most significantly affected biological process [-log<sub>10</sub>(p-value) = 10.8], followed by protein folding and defense response pathways, highlighting the comprehensive cellular reprogramming under combined stress (Fig. 2).

**Table 4.** Orthology analysis with crop species.

Crop species	Orthologous genes	Conservation score	Functional similarity
<i>Solanum lycopersicum</i>	187	0.89 ± 0.05	0.92 ± 0.03
<i>Capsicum annuum</i>	165	0.85 ± 0.06	0.88 ± 0.04
<i>Glycine max</i>	142	0.82 ± 0.07	0.85 ± 0.05



**Fig. 1.** Time-course expression of DNA repair genes.



**Fig. 2.** Pathway enrichment analysis of differentially expressed genes.

RT-PCR validation confirmed the reliability of transcriptome data, showing high correlation coefficients ( $R^2 > 0.92$ ) between RNA-seq and RT-PCR results across all validated genes (Table 5).

**Table 5.** RT-PCR validation of RNA-seq data.

Gene ID	RNA-seq fold change	RT-PCR fold change	Correlation ( $R^2$ )
HSP70-1	20.4	$18.7 \pm 2.1$	0.96
RAD51	15.3	$14.2 \pm 1.8$	0.94
PR1	12.8	$11.9 \pm 1.5$	0.92
XRCC4	18.4	$17.1 \pm 2.0$	0.95

Comet assay results (Table 6) demonstrated significant DNA damage under all stress conditions, with the combined stress treatment showing the most severe effects (2.8-fold increase in tail length compared to control).

**Table 6.** DNA damage assessment by comet assay.

Treatment group	Tail length ( $\mu\text{m}$ )	Tail DNA (%)	Olive tail moment
Control	$8.3 \pm 1.2$	$4.2 \pm 0.8$	$1.8 \pm 0.4$
Heat Stress	$15.6 \pm 2.1$	$12.8 \pm 1.5$	$5.9 \pm 1.1$
Virus Infection	$18.9 \pm 2.4$	$15.3 \pm 1.8$	$7.2 \pm 1.3$
Combined Stress	$28.7 \pm 3.1$	$24.6 \pm 2.3$	$12.4 \pm 1.9$

Correlation analysis (Table 7) revealed strong positive relationships among key stress-responsive genes, pointing toward integrated regulatory networks between DNA repair components and heat shock proteins.

**Table 7.** Statistical analysis of gene expression correlations.

Gene pair	Pearson correlation	p-value	Functional relationship
HSP70-1/RAD51	0.89	<0.001	Co-regulation
XRCC4/LIG4	0.92	<0.001	Same pathway
HSP90-2/PR1	0.76	<0.01	Cross-talk

## DISCUSSION

According to results obtained in this work, combined heat and virus stress exhibited a significantly additive effect on both physiological and molecular processes in *Nicotiana benthamiana*. Data presented in Table 1 clearly illustrate that the reduction of plant height by 49.4% and leaf area by 52.5% under combined stress conditions was much stronger in comparison with the effect of each stress separately. These findings confirm the presence of a synergistic effect between two stressors, which has caused further destruction of plant structures. Gene expression studies represented in Table 2 reveal the pivotal role of heat shock proteins, especially HSP70-1, in the response to combined stress, with a 20.4-fold increase in expression. Surprisingly, this increase under combined stress conditions was much higher compared to that under heat stress alone, therefore showing the enhanced plant defense signalling under multiple stress conditions. This finding could indicate the activation of common signalling pathways in response to both stresses. Therefore, Fig. 1 clearly indicates that DNA repair genes exhibit different temporal patterns of expression. Thus, while the RAD51 gene reaches its peak expression at 12 hours, the XRCC4 and LIG4 genes show a more sustained increase during 24 hours. This fact likely indicates the activation of different DNA repair pathways in response to different kinds of damage caused by combined stress. The promoter analysis results in Table 3 show that DNA repair genes have the highest number of common regulatory elements,  $4.5 \pm 0.9$ . Such a convergence at the promoter level may explain the coordination observed in the expression of these genes under combined stress. In fact, the occurrence of these common elements probably enables concurrent responses to several stress stimuli. According to Fig. 2 and Table 4, the DNA repair pathway had the lowest  $p$ -value,  $10.8 -\log_{10}$ , and was detected as the most important pathway under combined stress. Ortholog analysis showed that these pathways are also highly conserved in related crop species, underlining the importance of the findings for practical purposes in breeding planning. These results, as shown in Table 5, confirm the accuracy and reliability of RNA-seq data with a high correlation of 0.92 between RNA-seq and RT-PCR data. This high correlation, especially for key genes like XRCC4 with a coefficient of 0.95, provides a solid basis for biological inferences. As shown by the Comet assay results in Table 6, this combination of stresses leads to a 2.8-fold increase in tail length over the control. Such an amount of DNA damage is obviously greater than the effect of each type of stress alone and may be sufficient to justify the strong activation of DNA repair pathways in response to the combined stress. The data in Table 7 finally highlight the highly correlated expression of different genes, including a 0.92 correlation between XRCC4 and LIG4 in support of the cooperation of these genes in a common repair pathway. The network of cooperatively expressed genes in this study could be one plant strategy to cope with multiple injuries caused by combined stress.

## CONCLUSION

This study clearly demonstrated that combined heat and virus stress has significant synergistic effects on *Nicotiana benthamiana*. In contrast to the effects produced by single stresses, the combined conditions result in the activation of even more complex molecular networks that manifest in the form of more intense physiological changes and broader transcriptional responses. These findings support the idea that, in facing this multidimensional challenge, plants employ integrated and coordinated defense mechanisms. One of the most important achievements of this study was the identification of significant convergence in the promoter regulatory elements of genes responsive to combined stress. This convergence was particularly observed in genes involved in DNA repair, therefore signifying the existence of a central control system to coordinate the response to multiple injuries. Such a pattern of gene regulation could be a plant strategy to conserve resources and increase defense efficiency. The most prominent response to combined stress identified in the current study is the activation of the DNA repair system. Temporal coordination of the expression of different genes in this pathway and a high correlation of their activity point to the existence of an interconnected and efficient network for maintaining genome integrity under multiple stress conditions. The finding is important since genetic stability forms the basis for the survival of plants in stressful environments. Results from this study clearly indicated that the response of plants to combined stress is not a simple algebraic sum of responses to individual stresses. Complex interactions between different signalling pathways lead to the creation of novel response patterns not observed under single

stress conditions. This phenomenon further highlights the need to study stresses under more realistic and combined conditions. These findings from an applied viewpoint may give a suitable basis for the design of breeding strategies for plants that are resistant to several kinds of stresses. Identification of key genes, like HSP70-1 and XRCC4, which occupy the center of the network in combined stress response, will be useful targets for genetic engineering and marker-assisted selection. In view of the increase in climate change and environmental stresses in terms of frequency and severity, understanding the mechanisms for the combined stress response acquires strategic relevance. The results from this study represent a step toward the understanding of such complex mechanisms and may form a basis for the development of cultivars with increased stability across changing environmental conditions. Besides, the current investigation provides a broad methodological framework to analyze the molecular effects of combined stress. The combination of bioinformatics approaches with molecular experiments can be a model for future research in this area. The validation of transcriptome results using more exact methods, like RT-PCR, underlines the reliability of the findings. In conclusion, the plant response to combined heat and virus stress is an illustration of the complexity and flexibility of biological systems. Understanding these integrated responses provides in-depth insights not only into plant biology but also helps in offering effective tools to counter future environmental challenges. Further research in this area could lead to the discovery of new layers of molecular regulation.

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