IMAM KHOMEINI INTERNATIONAL UNIVERSITY
IJGPB
Iranian Journal of Genetics and Plant Breeding
Print ISSN : 2251-9610
Online ISSN : 2676-346X

Identification of key genes involved in heat stress response in *Brassica napus* L.: reconstruction of gene networks, hub genes, and promoter analysis

Nina Makvandi[1], Abozar Ghorbani[2]*, Mahsa Rostami[2], Atefe Rostami[3], Ali Akbar Ghasemi-Soloklui[2]

[1]Department of Agricultural Biotechnology, Payame Noor University (PNU), Isfahan, Iran.
[2]Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Karaj, Iran.
[3]Department of Biology, Faculty of Science, University of Zanjan, Zanjan, Iran.
[*]Corresponding author, 0000-0002-2183-0033. Email: Abghorbany@aeoi.org.ir.

ABSTRACT INFO

Research Paper

Received: 18 May 2023

Accepted: 06 Aug 2023

ABSTRACT

Brassica napus is a versatile crop with oil and protein-rich seeds, used in food, industry, medicine, and animal feed. However, heat stress limits its productivity, making it essential to identify genes and pathways involved in stress response. We analyzed differentially expressed genes (DEGs) in *B. napus* under heat stress using bioinformatics tools to identify key genes and pathways. Firstly, DEGs were analyzed for gene interactions using the STRING database and visualized using Cytoscape. To identify key genes involved in heat stress response in *B .napus* L., we employed the CytoHubba tool for hub gene identification. Additionally, we conducted Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses to gain insights into the functional roles and potential biological pathways associated with these genes. We also used MEME Suite to analyze the promoter regions of hub genes. Our results showed decreased activity of the b6-f complex, a key component of the electron transport chain, under heat stress. We also identified significantly enriched calcium transporter ATPase and heat shock protein family (HSP20). KEGG and cluster analyses highlighted the importance of membrane lipids, galactose metabolism, and protein processing in the endoplasmic reticulum in stress and signal transduction. Our study provided key genes, including transcription factors and chaperones for developing heat-resistant plants via genetic modification. However, these promising results were obtained through rigorous bioinformatics analysis and require further validation using experimental approaches, such as gene editing, phenotypic characterization, and field trials.

Key words: Biological networks, *Brassica napus*, Heat stress, Hub genes, Promoter analysis, Protein-protein interactions.

How to cite this article:
Makvandi N., Ghorbani A., Rostami M., Rostami A., and Ghasemi-Soloklui A. A. (2022). Identification of key genes involved in heat stress response in *Brassica napus* L.: reconstruction of gene networks, hub genes, and promoter analysis. *Iranian Journal of Genetics and Plant Breeding,* 11(1): 71-86.
DOI: 10.30479/IJGPB.2023.18795.1340

INTRODUCTION

Brassica napus L. (rapeseed) is an amphidiploid species derived from the two diploid species *B. rapa* L. and *B. oleracea* L. (Rahaman *et al.*, 2018). The research about the genetics of *B. napus* as an amphidiploid species derived from *B. rapa* and *B. oleracea* helps to emphasize the significance of this crop's characteristics, heterosis-driven advantages, and its potential for targeted breeding programs aimed at developing superior varieties with improved traits for sustainable agriculture. Approximately 70 million tons (MT, yield) of rapeseed are cultivated annually worldwide, spanning across 66 countries. Rapeseed is the second largest oil crop in terms of yield production after soybean, surpassing other oil crops such as sunflower, peanut, and cottonseed (Raboanatahiry *et al.*, 2021). This crop is highly valued for its high protein content and healthy fatty acid compounds. High temperatures during the storage of rapeseed seeds reduce germination. Analysis of the transcriptome of seeds treated with different temperatures has identified key genes and important heat response pathways that help improve heat tolerance in rapeseed (Gao *et al*., 2021). In recent years, the demand for these oilseeds has increased, leading to the expansion of cultivation to drier areas, which has increased the vulnerability of this crop to adverse weather conditions (Lohani *et al.*, 2021). In dry areas, rapeseed plants face prolonged periods of drought stress, high temperatures, and increased occurrence of heat waves. Additionally, soil salinity and water scarcity further exacerbate the vulnerability of rapeseed crops to adverse weather conditions. These factors collectively contribute to decreased yields and increased susceptibility to various physiological disorders, including accelerated flowering and reduced seed set (Wu *et al*., 2018).

Climate models predict that the global average temperature will continue to rise (Mackay, 2008). This temperature increase poses a serious threat to agricultural products, with heat stress being one of the non-living stressors that limit the production of many crops. High temperatures have been shown to disrupt several vital physiological and biochemical processes in plants, thereby compromising their growth and development. Heat stress can lead to alterations in respiration, photosynthesis, and germination, among other processes (Ray *et al*., 2015; Liu *et al*., 2015). Increased respiration rates under heat stress conditions can result in accelerated metabolic activity, leading to energy depletion and cellular damage. Similarly, heat stress often inhibits photosynthesis, reducing the efficiency of carbon assimilation and impairing plant growth and productivity. Furthermore, high temperatures can adversely affect seed germination, resulting in poor establishment and reduced crop yields. These physiological changes not only limit crop production but also pose challenges to plant adaptation and survival in a changing climate (Ferguson *et al*., 2021). Also, High temperatures affect plant reproduction, adaptation, and physiological processes (Hall, 2002), and can significantly reduce respiration, photosynthesis, and germination (Wahid *et al.*, 2007). The temperature suitable for rapeseed growth is between 15 and 20 °C, and a temperature above 27 °C can cause pod death and pollen sterility (Gan *et al*., 2004; Young *et al.*, 2004). Each 1 °C increase above the suitable range for growth during pod regulation can result in a 10% yield reduction (Rahaman *et al*., 2018). The study of responses of canola to drought, heat and combined stress showed that seed yield was reduced by 85.3% under the heat treatment and to a lesser extent under the drought treatment (31%), and seed oil content decreased by 52% in plants exposed to heat (Elferjani and Soolanayakanahally, 2018).

The process of tolerance to heat stress in plants is complex and involves several biological pathways, possibly related to unsaturated lipids, gene expression, translation, and protein stability (Rao *et al.*, 1992). One of the well-established mechanisms involved in heat stress response is the induction of heat shock proteins (HSPs). These proteins act as molecular chaperones and play a crucial role in protein folding, preventing protein aggregation, and maintaining cellular homeostasis under stress conditions. Also, heat stress can lead to changes in the fluidity and integrity of cellular membranes, which can affect various cellular processes. Besides, Heat stress can lead to the production of ROS, including superoxide radicals, hydrogen peroxide, and singlet oxygen, which can cause oxidative damage to cellular components (Fortunato *et al*., 2023). Expression data from several plant species indicate that high temperatures affect approximately 2% of the plant genome in different tissue types and growth stages (Qu *et al.*, 2013). Heat stress can disrupt reproductive processes, ultimately leading to a decrease in seed production. Elevated temperatures during flowering can negatively impact pollen viability and pollen tube growth, leading to reduced fertilization and subsequent seed set. Furthermore, different tissues may exhibit varying degrees of susceptibility to high temperatures. For example, heat stress can lead to cellular damage in leaf tissues, disrupting photosynthetic processes and impairing overall plant productivity. Heat-induced cellular damage may

manifest as leaf chlorosis, necrosis, or even cell death, which can further compromise plant growth and development (Hassan *et al*., 2021). Also, numerous research studies have revealed the negative impact of severe temperature changes on plants during crucial developmental stages. By delving into the specific models of tissue and understanding the components and mechanisms of heat stress response, we can pave the way for a better understanding of thermo-tolerance regulation in vital crop species (Kourani *et al.*, 2022).

To understand the biological networks underlying heat stress tolerance, a change in approach has occurred in the study of genome projects, with researchers focusing on interactive networks rather than individual genes (Javadi *et al.*, 2021). High-quality global quantification of molecular components and their interactions is essential for understanding biological networks, and hub genes (the most connected genes in the network) predict the biological functions of these networks. This approach provides new insights into the hierarchical organization of genes in different aspects and their response to stress (Ko and Brandizzi, 2020). Analyzing interactive networks allows us to capture the emergent properties and system-level behavior that cannot be fully elucidated by studying individual genes in isolation. This approach enables us to identify key regulators, hubs, or central nodes that have a significant impact on the overall system. Also, analyzing interactive networks allows us to decipher transcriptional cascades and signaling pathways by examining the connections and interactions between genes (Serin *et al*., 2016). In this study, we performed gene expression profiling of *B. napus* seeds under heat stress using the RNA-Seq method and determined genes with differential expression (DEGs) by transcriptome data analysis (Gao *et al*., 2021). Using the DEGs from the previous study, we performed bioinformatic analysis to investigate genes involved in heat stress, and conducted additional analyses such as Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and promoter analysis, with particular attention to hub genes that play an essential role in creating tolerance under stress conditions.

MATERIALS AND METHODS

Data Collection of Genes in Response to Heat Stress

In a previous study (Gao *et al*., 2021), differentially expressed genes (DEGs) were determined from the analysis of transcriptome data of *Brassica napus* seeds under heat stress. For RNA-Seq analysis, a total of 12 samples were utilized, including a control group and three treatment groups, each with three biological replicates. The extraction of total RNA was performed from the seeds. These genes (579 genes), with fold changes ranging from +6.57 to -4.26, were used for systems biology analyses and gene networks to identify critical genes and pathways involved in heat stress. Gene expression levels were assessed using the FPKM method. The DESeq2 R package was employed to compare gene expression between heat stress and control conditions. A p-value<0.05 and a $|\log2(\text{fold change})| \geq 1$ were used as the criteria to determine significant differences in gene expression between the two conditions in Gao *et al*. (2021) study.

PPI Networks and Hub Analysis

The DEGs obtained under heat stress were analyzed using the web-based application STRING ver.10 (http://string-db.org) with a minimum interaction score of 0.15 (low confidence), and a PPI list was generated. Choosing a lower confidence score threshold means that more edges will be included in the network, even if they have weaker support in the underlying data. This can be useful for exploring potential interactions between genes that may not be well-studied or have low expression levels. In the case of the *Brassica napus* plant, which may have less well-characterized interactions compared to model organisms like Arabidopsis, including edges with lower confidence scores may help identify novel regulatory relationships that could be further investigated. The PPI export file from STRING was imported into Cytoscape (version 3.9.1) for PPI and network analysis. The CytoHubba plugin software (version 0.1) was used to determine the protein hub and its subnetwork. Four computational algorithms of CytoHubba, including MCC, DEGREE, DMNC, and MNC, were used to identify and classify hub genes (Ghorbani *et al*., 2023). The subnetworks depicting the hub genes and their interactions were presented MCC algorithm identifies hub genes based on the concept of cliques in a PPI network. It calculates the maximal clique centrality score for each gene, which reflects its importance within highly interconnected regions. Genes with higher MCC scores are considered potential hub genes. The advantage of MCC is that it considers the connectivity of genes within dense clusters, emphasizing their significance in network organization. The degree algorithm calculates the number of direct interactions that each gene has in the PPI network. Hub genes are often characterized by having a higher number of connections or degrees compared to other genes. The degree algorithm is widely used due to its simplicity and ability to identify

highly connected genes. DMNC identifies hub genes by integrating information from multiple gene co-expression networks. It calculates the centrality of each gene in multiple networks and selects genes with consistently high centrality scores as hub genes. DMNC provides a more robust approach by considering the consensus across multiple networks, which helps to filter out the noise and identify reliable hub genes. The MNC algorithm aims to identify densely connected gene modules or clusters within a PPI network. It identifies hub genes as the key regulators within these modules by assessing their positions and connectivity. MNC focuses on identifying important genes that act as central players within specific functional modules (Chin *et al*., 2014).

Gene Ontology and Pathway Enrichment Analysis of the Subnetwork

To classify the major biochemical pathways of hub genes and their subnetwork, gene accession numbers of the subnetwork were imported into the STRING database and the analysis and functional enrichment section after drawing network were used. Molecular functions (MF), cellular components (CC), and biological processes (BP) results were extracted for gene ontology (GO) classification. For enrichment analysis, KEGG pathways using STRING were also extracted.

Cluster Analysis of the Subnetwork

The subnetwork of hub genes was clustered using CytoCluster (version 2.1.0) with the Identifying Protein Complex Algorithm (IPCA) (with a threshold of 10) for cluster analysis. The top clusters' KEGG pathways were then obtained from the STRING databases (Ghorbani *et al*., 2023). Clusters represent subsets of genes that share similar expression profiles, suggesting potential functional associations (Gralinska *et al*., 2022). The identification of such clusters enables us to gain insights into the coordinated regulation of genes involved in the heat stress response in *Brassica napus* L.

Promoter Motif Analysis of Hub Genes

Promoter motif analysis is a widely used approach in molecular biology and genomics research to understand the regulatory mechanisms underlying gene expression (Boeva, 2016). By conducting promoter motif analysis, we aim to uncover potential cis-elements or regulatory motifs that may be associated with the heat stress response. These motifs can act as binding sites for TFs involved in the transcriptional regulation of heat stress-responsive genes. To extract 1000 bp upstream flanking regions (UFRs) of hub genes and identify the motifs in the upstream sequence, Biomart Ensemble Web Services (https://asia.ensembl.org/info/data/biomart/index.html) were used. The MEME Suite (version 5.4.1) (https://meme.nbcr.net/meme/intro.html) was used with standard parameters, except for the threshold p-value<0.01 (Bailey *et al*., 2009) to identify cis-elements. The Tomtom tool (version 5.4.1) (http://meme-suite.org/tools/tomtom) (Gupta *et al*., 2007) was used to remove additional motifs and identify the role of each identified motif using the GOMo tool (http://meme-suite.org/tools/gomo) (Buske *et al*., 2010). Biomart Ensemble Web Services facilitated the retrieval of accurate and up-to-date gene annotation information, which is essential for downstream analyses. The MEME Suite allowed us to discover overrepresented motifs within the extracted UFRs. Tomtom enabled us to compare the identified motifs against a comprehensive database of known motifs, providing insights into potential TF binding partners. Lastly, GOMo allowed us to assess the functional enrichment and potential regulatory role of the identified motifs.

RESULTS AND DISCUSSION

PPI networks and the hub analysis

In this study, we aimed to identify differentially expressed genes (DEGs) in *B*. *napus* under heat stress and investigate the interaction of hub genes through protein-protein interaction (PPI) networks. Using transcriptional data analysis, we identified a total of 442 significant DEGs that were both up-and down-regulated. We further analyzed these genes using the web-based application STRING, which generated a PPI network consisting of 100 nodes and 264 edges. We then used Cytoscape to visualize the gene network, where a fold change from +6.57 to -4.26 was used to represent the nodes (Figure 1).

To investigate the interaction of hub genes in the network, we used the computational algorithm CytoHubba to draw a subnetwork (Figure 2). This subnetwork consisted of 52 nodes and 205 edges, and the hub genes were represented with different colors around the network. Hub analysis led to the identification of 10 genes with the most interactions (Table 1).

Among these hub genes, two genes encoding subunits of calcium-transporting ATPase, BnaA01g24360D and BnaC01g31340, were found to be up-regulated (Table 1). This finding is of great significance since calcium-transporting ATPase has been implicated in signal transduction in response to

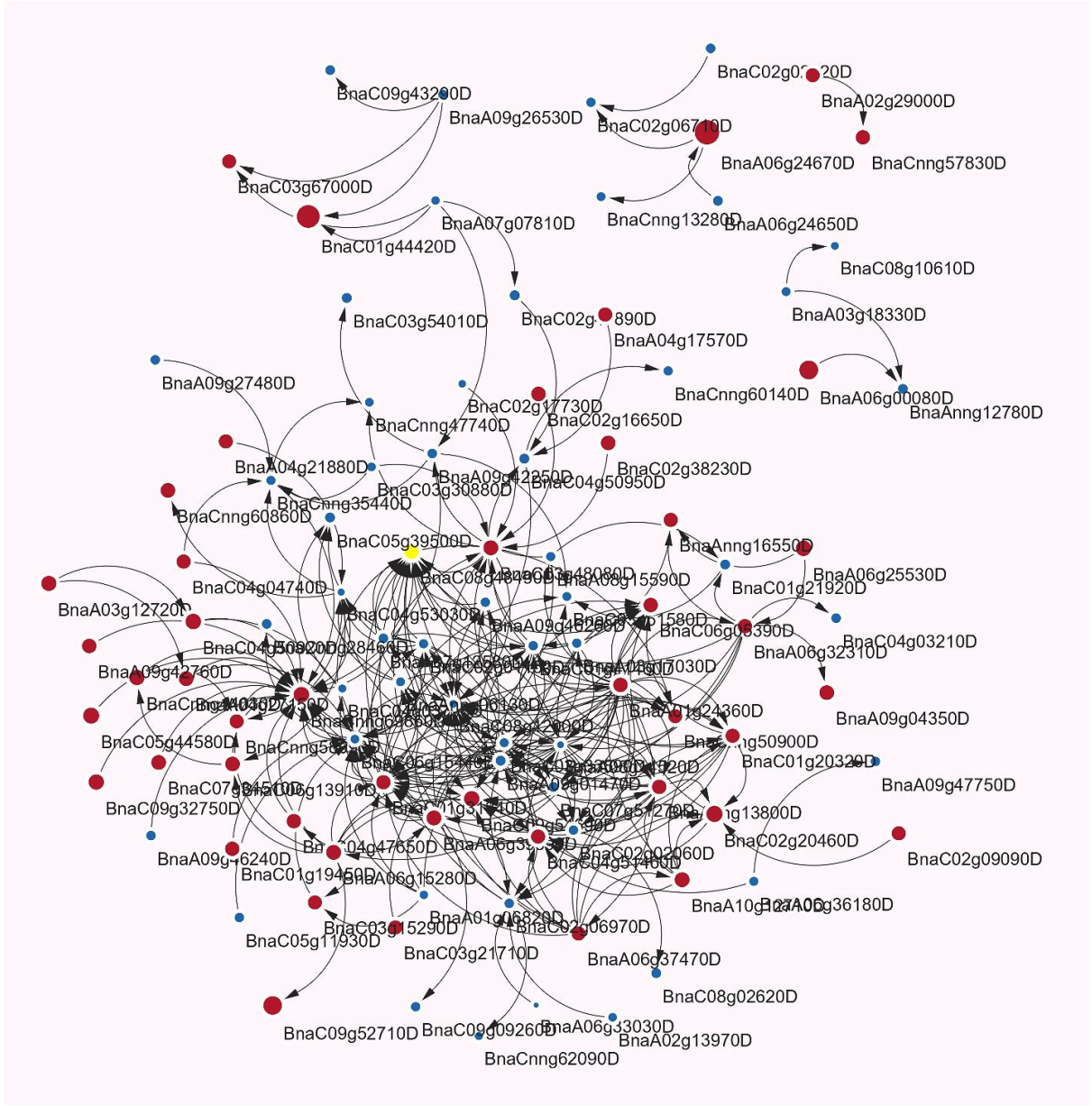

Figure 1. Network of the overexpressed genes of *Brassica napus* seeds under heat stress plus their known neighbors based on data using Cytoscape software. Larger nodes have a higher fold change than smaller ones. Red circles are up-regulated genes and blue circles are down-regulated genes.

heat stress in other plant species, such as hard fescue (Wang *et al*., 2018), switchgrass (Li *et al*., 2013), pollen, pistils of *B. napus* (Lohani *et al*., 2021), and tea plants (Wang *et al*., 2019). Preconditioning of tobacco (*Nicotiana plumbaginifolia*) seedlings with calcium ions or ethylene glycol-bis-N,N,N′,N′-tetraacetic acid resulted in increased or decreased thermos-tolerance, respectively, compared to untreated seedlings. These findings imply a potential role of cytosolic Ca2+ in the transduction of heat-shock (HS) signals. It can be inferred from these results that changes in cellular calcium levels play a role in signal transduction during heat stress, and the transient increase in calcium ions can be a response to environmental stimuli, including biotic and abiotic stresses (Gong *et al*., 1998).

In this study, three genes were identified, namely BnaC01g20320D, BnaAnng13800D, and BnaA10g12710D, which encode the small heat shock protein (HSP20) family, and were found to be up-regulated under heat stress conditions (Table 1). Small HSPs is recognized as one of the most vital components of the response to heat stress conditions (Waters, 2013), and they play an essential role in reducing heat stress by restoring natural protein synthesis. HSP20 genes

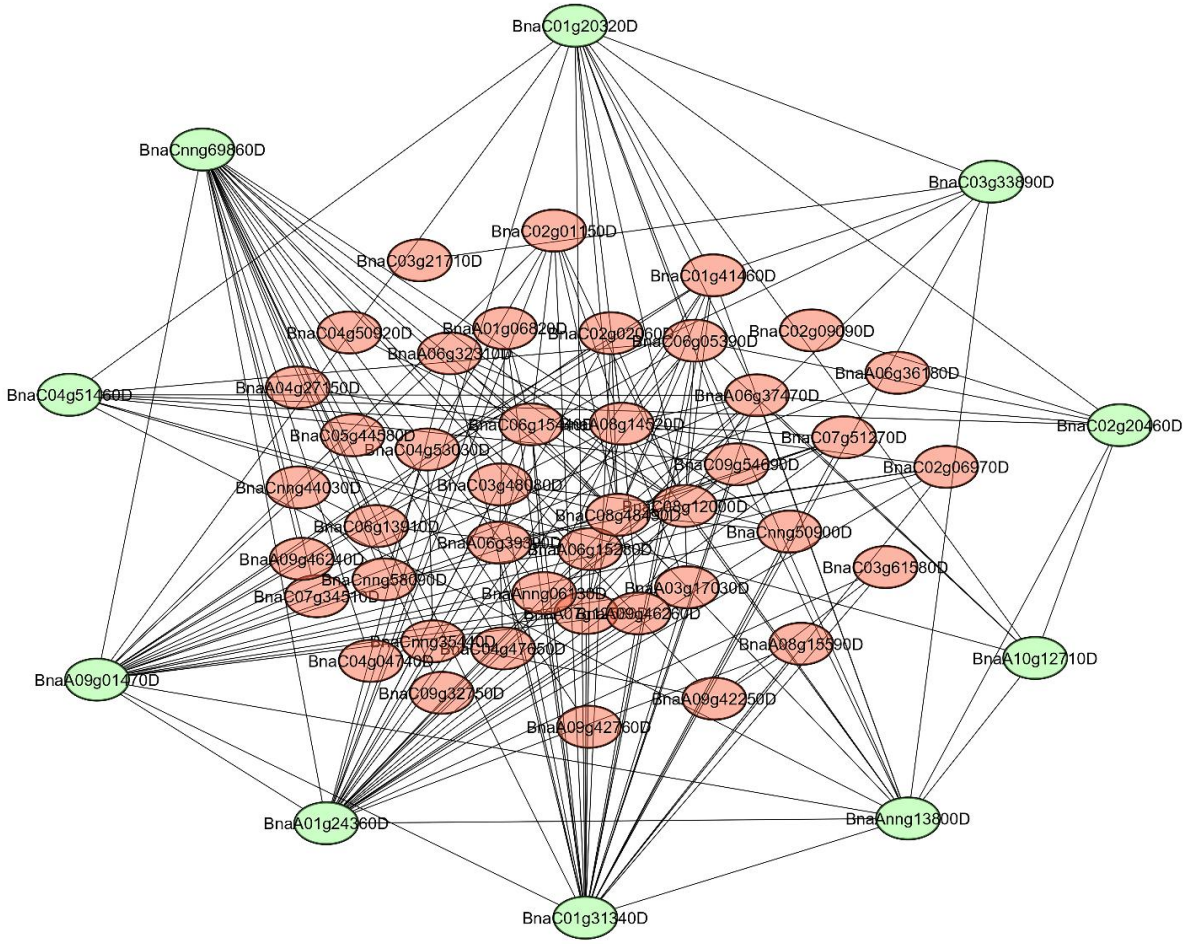

Figure 2. Subnetwork of the overexpressed genes in *Brassica napus* seeds under heat stress plus their known neighbors using the CytoHubba App.

have been studied in numerous plant species such as Arabidopsis (Scharf *et al*., 2001), rice (Ouyang *et al*., 2009), pepper (Guo *et al*., 2015), and tomato (Yu *et al*., 2016). Expression pattern analysis of HSF and six HSP family genes was performed by using expression values from different tissues and heat treatments in *B. rapa*, showing their intricate interactions in response to heat stress (Yu *et al*., 2022).

The HSP20 family in Arabidopsis exhibited the highest expression pattern among other protein groups and was introduced as a factor for multiple growths (Swindell *et al*., 2007). In rice, 19 small HSP genes were induced by high temperature, and HSPs in chloroplasts played a significant role in protecting photosynthesis under heat-stress conditions (Heckathorn *et al*., 1998). Additionally, it was observed that overexpression of HSP17.7 in transgenic rice plants that were under high temperature, contributed to drought tolerance in addition to heat tolerance (Murakami *et al*., 2004).

Lohani *et al*. (2021) reported that in *B. napus* pollen, all small HSPs and chaperones were up-regulated by heat stress, and most of them were also up-regulated in the heat-stressed pistil. These results suggest that the ability of pollen and pistil to activate mechanisms to respond to heat stress increases with the accumulation of HSPs and chaperones (Bokszczanin *et al*., 2013; Fragkostefanakis *et al*., 2015). Six small HSPs were also differentially expressed in *B. napus* seeds under heat stress, and most of them were upregulated (Gao *et al*., 2021)(Gao *et al*., 2021). Most of the StHSP20 genes in potatoes were sensitive to heat stress and did not show a decrease in expression (Zhao *et al*., 2018). Heat shock proteins of 20 gene families in tomato (*Solanum lycopersicum*) was characterized through the integration of gene structure, chromosome location, phylogenetic relationship, and expression profile, that showed SlHSP20 gene expression increase significantly at high temperatures in susceptible plants (Yu *et al*., 2016). Guo *et al*. (2015) showed that heat

Table 1. Ranking of hub genes identified in *Brassica napus* seeds under heat stress by using CytoHubba computational algorithms, including MCC, Degree, MNCC, and MNC.

Gene ID	Method	Rank	Foldchange	Gene description
BnaA09g01470D	MCC, MNC, Degree	1,3,4	-1.04	Cytochorome b6-f complex iron-sulfur subunit; photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions Component of the cytochrome b6-f complex, which mediates electron transfer between.
BnaA01g24360D	MCC, MNC, Degree	4,1,1	1.21	Calcium-transporting ATPase; This magnesium-dependent enzyme catalyzes the hydrolysis of ATP coupled with the transport of calcium
BnaC01g31340D	MNC, Degree	1,1	1.41	Calcium-transporting ATPase; This magnesium-dependent enzyme catalyzes the hydrolysis of ATP coupled with the transport of calcium.
BnaCnng69860D	MNC, Degree	4,3	1.76	BnaCnng69860D protein
BnaC01g20320D	MCC	2	1.06	BnaC01g20320D protein; Belongs to the small heat shock protein (HSP20) family.
BnaAnng13800D	MCC	2	1.20	BnaAnng13800D protein; Belongs to the small heat shock protein (HSP20) family.
BnaC02g20460D	DMNC	1	2.05	BnaC02g20460D protein
BnaA10g12710D	DMNC	1	1.46	BnaA10g12710D protein; Belongs to the small heat shock protein (HSP20) family.
BnaC04g51460D	DMNC	3	1.04	Hexosyltransferase; Belongs to the glycosyltransferase 8 family
BnaC03g33890D	DMNC	4	-1.01	BnaC03g33890D protein

stress affects *SlHSP20* and *CaHSP20* genes more in susceptible plants than in resistant ones. Moreover, the relationship of HSP20 with lipid molecules to regulate fluidity suggests that stress stimuli lead to the activation of signal transduction pathways (Sung *et al*., 2003).

In this study, several hub genes were identified, including BnaA09g1470, which encodes the iron-sulfur subunit of the cytochrome b6-f complex. This protein was down-regulated under heat stress and plays a critical role in facilitating electron transfer between photosystems, cyclic electron flow around photosystem I, and state change (Malone *et al.*, 2021). It has been previously reported that membrane proteins, including those involved in photosynthesis, signal transduction, and protein modification, are decreased under heat stress (Wang *et al*., 2017). Similarly, PETB and PETC, encoding the cytochrome b6f complex, were significantly decreased in *Populus tomentosa* Carr under heat stress, leading to suppression of photosynthesis and a negative impact on PSII repair (Ren *et al*., 2019). Additionally, the iron-sulfur subunit of the cytochrome b6-f complex, chloroplast genes encoding ferredoxin-NADP reductase, and PsbP-like protein were also significantly downregulated in hybrid rice after 24 h of heat stress (Wang *et al*., 2020). Heat stress affects electron transport, NADPH and ATP synthesis, photosynthetic carbon cycle, light-harvesting system, and assimilate utilization, which, if damaged, can lead to a reduction in photosynthetic capacity (Sharkey and Zhang, 2010; Song *et al*., 2014). Disruption of the cytochrome b6f complex can lead to excess energy and accumulation of ROS (Schöttler *et al*., 2007).

Another hub gene identified in this study was BnaC04g51460D, which encodes the enzyme hexosyltransferase belonging to the glycosyltransferase family. This enzyme is responsible for transferring hexoses such as galactose, glucose, and fucose from activated donor molecules to specific receptor molecules, and the synthesis of disaccharides, oligosaccharides, and polysaccharides. Dawood *et al*.

(2020) reported that the level of hexosyltransferase was up-regulated in wheat and barley under heat stress, indicating the importance of this enzyme in response to heat stress. Furthermore, hexosyltransferase was also up-regulated in young leaves of poplar (Georgii *et al*., 2019). It has been suggested that plants that maintain high carbohydrate content under heat stress are more heat tolerant (Triboï *et al*., 2003).

Gene Ontology and pathway enrichment analysis of hub genes

The results of the gene ontology and pathway enrichment analysis are presented in Figures 3 to 6. The biological processes (BP) category revealed significant enrichment in the response to heat (Figure 3), while the molecular function (MF) category showed protein self-association (Figure 4) as a significant term. The cellular component (CC) category revealed chloroplast nucleoid, cytoplasm, and cellular anatomical unit as the most frequent terms (Figure 5). These results indicate that chloroplasts are the most affected organelles by heat stress, leading to reduced photosynthetic efficiency, redox imbalance, and possibly cell death.

One of the important proteins involved in protecting Photosystem II during heat stress is HSP21. This protein is present only in chloroplast and has been described in many plant species and plays a crucial role in maintaining chloroplast protein content (Zhong *et al*., 2013). The role of HSP21 in regulating heat

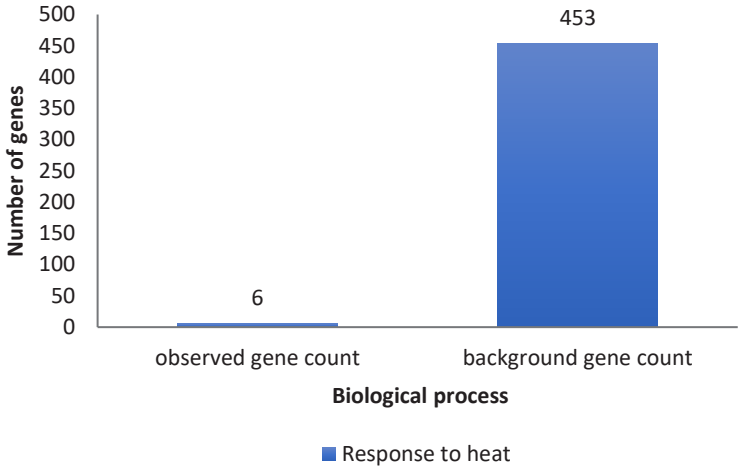

Figure 3. Gene Ontology enrichment analysis (Biological Process) of detected hub genes in *Brassica napus* seeds under heat stress by Cytohubba app with STRING ver.10 (http://string-db.org).

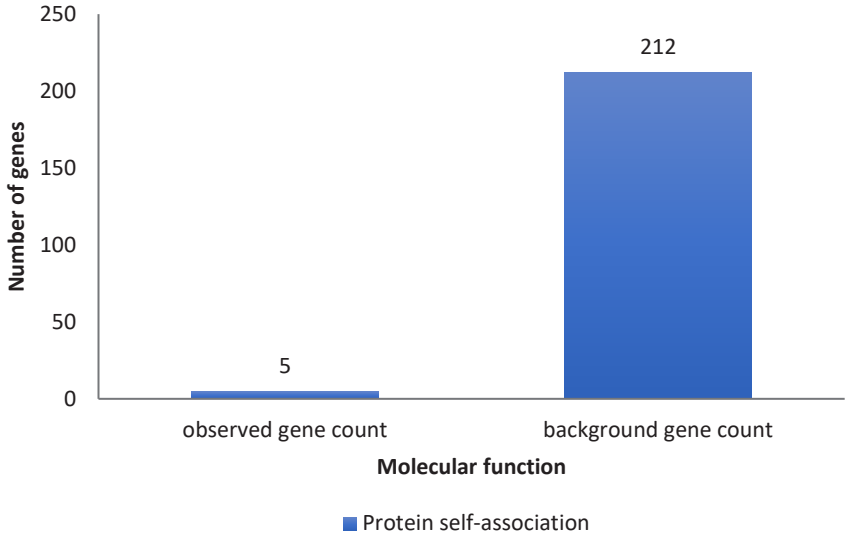

Figure 4. Gene Ontology enrichment analysis (Molecular function) of detected hub genes in *Brassica napus* seeds under heat stress by Cytohubba app with STRING ver.10 (http://string-db.org).

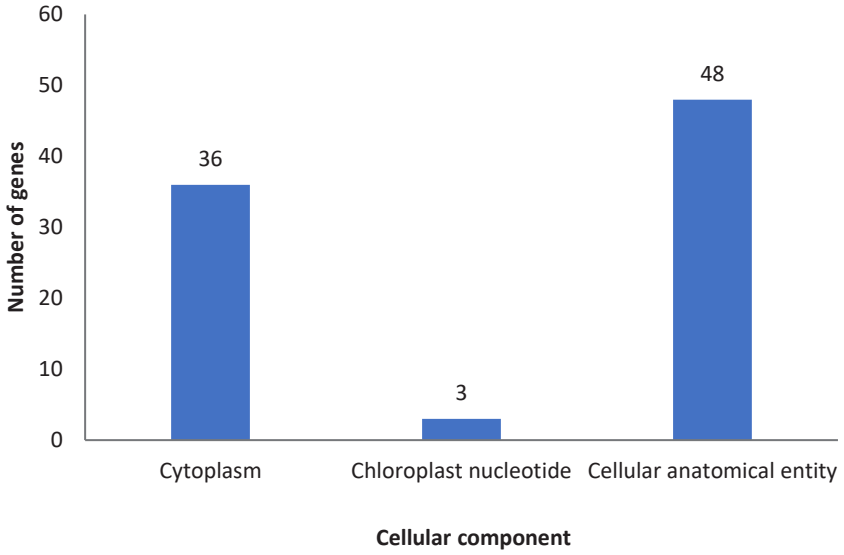

Figure 5. Gene Ontology enrichment analysis (Cellular Component) of detected hub genes in *Brassica napus* seeds under heat stress by Cytohubba app with STRING ver.10 (http://string-db.org).

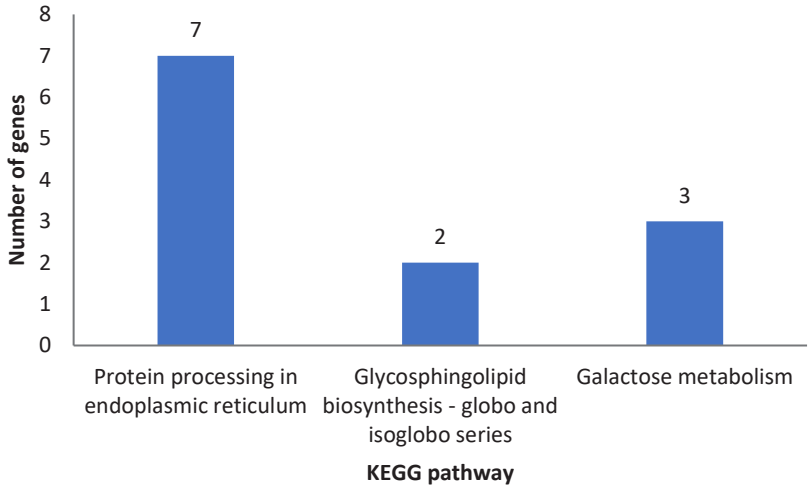

Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of detected hub genes in *Brassica napus* seeds under heat stress by Cytohubba app with STRING ver.10 (http://string.db.org).

stress has been demonstrated through its involvement in retrograde signaling in response to heat stress, and chloroplast ribosomal protein S1 (RPS1) has been identified as a heat-responsive protein required for the initiation of this signaling (Yu *et al*., 2012).

The MF terms revealed significant enrichment in protein self-association, which is related to protein binding and protein self-binding under heat-stress conditions. Heat stress leads to protein misfolding and aggregation, which can inactivate many proteins, but some remain unchanged and do not lose their function (Wallace *et al*., 2015). The folding and aggregation of endogenous proteins are thought to be part of a self-regulatory process that allows the cells to adapt to stress conditions. When cells are subjected to heat shock, protein synthesis slows down, and a transcriptional program known as the heat shock response is initiated, leading to a reduction in the toxicity of the accumulation of misfolded endogenous proteins (Verghese *et al*., 2012).

Finally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to identify important biochemical pathways of hub genes under heat stress conditions using the STRING database. The major metabolic pathways identified were comprised of glycosphingolipid biosynthesis, galactose

Table 2. Summary of the clusters (rank 1 to 4) resulting from the cluster analysis of the subnetwork overexpressed genes in *Brassica napus* seeds under heat stress plus their known neighbors using the CytoCluster App.

Cluster	Rank	Nodes	Edges	KEGG pathway
1	1	16	72	Glycosphingolipid biosynthesis - globo and isoglobo series Protein processing in endoplasmic reticulum Sphingolipid metabolism Galactose metabolism Glycerolipid metabolism
2	2	10	31	Glycosphingolipid biosynthesis - globo and isoglobo series Galactose metabolism Glycerolipid metabolism Sphingolipid metabolism
3	3	10	30	Protein processing in the endoplasmic reticulum
4	4	10	32	Protein processing in the endoplasmic reticulum

metabolism, and protein processing in the endoplasmic reticulum (Figure 6). Glycosphingolipids are a group of membrane lipids that modulate membrane protein function, contribute to cell-cell communication, and exert important cellular functions (Ryckman *et al*., 2020). The enrichment of terms related to glycosphingolipid biosynthesis suggests that this pathway may contribute to heat stress adaptation by modulating membrane composition and properties, thus influencing various cellular functions. The enrichment of terms related to galactose metabolism suggests its involvement in the heat stress response of *B. napus*. Galactose metabolism is interconnected with other metabolic pathways and can influence energy production, carbohydrate partitioning, and stress signaling. The activity of enzymes involved in galactose metabolism and starch and sucrose metabolism resulted in the accumulation of soluble sugars in leaves of *Rhazya stricta* under heat stress, which may serve as osmolytes to maintain cell turgor and protect membranes and proteins from damage caused by various abiotic stresses (Obaid *et al*., 2016). Galactose and cellular protein metabolic processes were also up-regulated in rice under heat stress and were exclusively associated with a prolonged heat stress response (Jung *et al*., 2012). Exploring the role of galactose metabolism in the context of heat stress can provide insights into the metabolic adjustments required for plant survival under high-temperature conditions. In summary, our results provide insights into the molecular mechanisms of heat stress response in plants, particularly with regard to the involvement of chloroplasts, protein self-association, and important biochemical pathways. These findings may contribute to the development of stress-tolerant crops and have potential implications for food security.

Cluster analysis of the network

In this study, we employed the CytoCluster tool which incorporates six clustering algorithms. Specifically, we utilized the IPCA algorithm, which is based on density to identify dense subgraphs in PPI networks. The weight of each edge was calculated based on the shared nodes of common neighbors, while the weight of each node was calculated as the sum of the weights of its intersecting edges (Li *et al*., 2017). Cluster analysis of biological networks is a valuable technique to identify functional molecules and predict protein biomarkers (Pizzuti and Rombo, 2014).

As depicted in Table 2, four clusters were obtained. Clusters 1 and 2 share common metabolic pathways including glycosphingolipid biosynthesis, sphingolipid metabolism, glycerolipid metabolism, and galactose metabolism. These pathways involve three significant groups of membrane lipids, namely, glycosphingolipids, sphingolipids, and glycerolipids. Membrane lipids have been shown to influence protein aggregation and proteostasis, playing important roles in maintaining cellular homeostasis under stress conditions (Rütgers *et al*., 2017). Besides, sphingolipids are unique in their ability to function as both structural components and signaling molecules, playing a crucial role in protein aggregation in lipid rafts associated with signal transduction in response to biotic and abiotic stresses (Gault *et al*., 2010).

Under various biological stresses, plants develop extracellular signal processing mechanisms, including second messengers, to adapt to these conditions. Second messengers modulate the physiological response of the plant under stress conditions, and membrane lipids such as phosphoinositide, phosphatidic

acid, sphingolipids, and glysophospholipids have been identified as substrates for second messenger production. They can transiently recruit target proteins to the membrane and influence protein combination, activity, and gene expression, thus acting as platforms for signal transduction (Rodas-Junco *et al*., 2021). Also, Glycosphingolipids can act as chaperones, stabilizing proteins and preventing their aggregation, or they can facilitate the formation of membrane microdomains that promote protein aggregation (Wennekes *et al*., 2009; Niu and Xiang, 2018). Therefore, identifying differentially expressed genes involved in glycosphingolipid metabolism suggests their potential roles in modulating protein aggregation in response to heat stress. Signal transduction networks are crucial for coordinating biochemical, physiological, and genetic responses under stress conditions, and second messenger components are referred to as master regulators as they modulate key downstream regulatory components under stress conditions (Hou *et al*., 2016).

Clusters 3 and 4 share a common metabolic pathway, i.e., protein processing in the endoplasmic reticulum (ER). Around one-third of protein production and folding occurs in this organelle, and this process must be tightly regulated. Newly synthesized polypeptides enter the lumen of ER and are folded into their three-dimensional shape with the assistance of chaperones. Folded proteins leave the ER and reach their target through the secretory pathway. Eventually, these polypeptides are transported to the cytosol where they are degraded by the endoplasmic reticulum machinery (ERAD). ERAD degrades toxic proteins via the ubiquitin/proteasome system, which includes an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and 26S proteasome removal system. On the other hand, in the cytosol, the cytosolic protein response (CPR) is activated, leading to an increase in the expression of HPS-encoding genes (Singh *et al*., 2021). The CPR, also known as the heat shock response, is an essential mechanism for cells to adapt to heat stress. This response involves the transcriptional activation of HSPs that act as molecular chaperones, assisting in protein folding, preventing aggregation, and promoting protein refolding under stressful conditions. HSPs, including HSP70 and HSP90, play a critical role in cellular protein homeostasis and are involved in the protection against protein misfolding and aggregation induced by heat stress (Lohani *et al*., 2022). The ER is also considered the most important organelle involved in calcium homeostasis since the processes of folding and glycosylation occurs in the presence of calcium-dependent enzymes (Bravo *et al*., 2013). Furthermore, during heat stress, the ER faces increased demands, which can disrupt protein folding and lead to the accumulation of misfolded or unfolded proteins. To counteract this, cells activate the unfolded protein response (UPR), a signaling pathway aimed at restoring ER homeostasis. One of the key outcomes of UPR activation is the upregulation of ERAD components, which are responsible for the targeted degradation of misfolded or unwanted proteins. These include E3 ubiquitin ligases, such as Hrd1 and Doa10, which facilitate the recognition, ubiquitination, and subsequent proteasomal degradation of ERAD substrates (Sun *et al*., 2021).

Promoter motif analysis of hub genes

To identify motifs and cis-regulatory elements (CREs), we extracted a 1000 bp fragment from the upstream region of hub genes using Ensemble Biomart (Kinsella *et al*., 2011). We analyzed the extracted upstream flanking region (UFR) sequences with Tomtom to identify significant motifs and then used GOMO to further analyze the selected motifs. We discovered eighteen significant motifs ranging in length from 8 to 28 nt.

The promoter analysis revealed that HAT5, SPL8, AHL12, AHL20, AHL25, TSO1, TCX2, ZHD3, ATHB-40, DOF5.8, CDF5, ATHB-23, ZHD9, ZHD1, ZHD6, SOL1, TCX6, and ZHD10 were the most abundant transcription factor families that bind to the promoters of our hub genes. The specific groups of transcription factors found in this plant plays a crucial role in both plant development and its ability to adapt to external environmental stressors. For example, genes belonging to the HD-Zip family are involved in a wide range of vital biological processes, including pigment synthesis, regulation of the cell cycle, response to defense stress, etc (Yin *et al*., 2023).

GOMO analysis of the identified motifs showed several interesting biological functions (Table 3). The Gene Ontology analysis revealed that these motifs are involved in the endomembrane system, transcription factor activity, transcriptional regulation, nucleus, plasma membrane, DNA-dependent kinase activity, pseudouridine synthase activity, and auxin-mediated signal transduction, as well as responses to abscisic acid stimulus, ethylene stimulus, salt stress, water deprivation, and wounding.

Among all motifs, the most common pathways for the response to heat stress for cellular component (CC) were the endomembrane system, molecular function

Table 3. The conserved motifs found in promoters of hub genes in *Brassica napus* seeds under heat stress by MEME suite analysis using the GOMo tool.

Motif	TFs	Logo	Top 5 Specific Predictions
MA0008.3(HAT5)	HAT5		CC endomembrane system
MA0578.1(SPL8)	SPL8		MF transcription factor activity BP regulation of transcription
MA0932.1 (AHL12)	AHL12		MF transcription factor activity CC endomembrane system BP regulation of transcription
MA0933.1(AHL20)	AHL20		MF transcription factor activity CC endomembrane system BP regulation of transcription
MA0934.1 (AHL25)	AHL25		MF transcription factor activity CC endomembrane system BP regulation of transcription
MA1161.1 (TSO1)	TSO1		MF transcription factor activity
MA1162.1 (TCX2)	TCX2		CC endomembrane system
MA1213.2(ZHD3)	ZHD3		CC endomembrane system MF transcription factor activity MF carboxylesterase activity
MA1214.1(ATHB-40)	ATHB-40		CC endomembrane system MF transcription factor activity
MA1267.1(DOF5.8)	DOF5.8		MF transcription factor activity CC nucleus CC plasma membrane BP regulation of transcription, DNA-dependent
MA1268.1(CDF5)	CDF5		MF transcription factor activity CC plasma membrane CC nucleus BP regulation of transcription, DNA-dependent MF kinase activity
MA1327.2(ATHB-23)	ATHB-23		MF transcription factor activity CC endomembrane system BP regulation of transcription
MA1328.1 (ZHD9)	ZHD9		MF transcription factor activity CC plasma membrane CC endomembrane system
MA1329.2 (ZHD1)	ZHD1		MF transcription factor activity CC endomembrane system BP regulation of transcription
MA1330.1(ZHD6)	ZHD6		MF transcription factor activity CC endomembrane system BP regulation of transcription

Table 3 (Continued). The conserved motifs found in promoters of hub genes in *Brassica napus* seeds under heat stress by MEME suite analysis using the GOMo tool.

Motif	TFs	Logo	Top 5 Specific Predictions
MA1379.1 (SOL1)	SOL1		MF transcription factor activity CC endomembrane system MF pseudouridine synthase activity BP auxin-mediated signaling pathway
MA1380.1(TCX6)	**TCX6**		MF transcription factor activity BP response to ethylene stimulus BP response to abscisic acid stimulus BP response to salt stress BP response to water deprivation
MA1807.1 (ZHD10)	ZHD10		MF transcription factor activity CC endomembrane system BP regulation of transcription BP response to wounding

(MF), the activity of transcription factors, and biological process (BP), the regulation of transcription. The inner membrane structure includes major components of the endoplasmic reticulum membrane system, Golgi body, vesicle, cell membrane, and nuclear envelope, which are involved in transport within the cell. The perception of heat stress is caused by the lipids of the cell membrane, which leads to signal transduction. The inner membrane system likely aids in signal transduction and increases the production of proteins such as HSP to enhance resistance to heat stress. Our study suggests a possible link between the identified CRE genes and heat stress. However, further studies are needed to explore the relationship between these genes and CREs under heat-stress conditions and to develop heat-resistant plants.

Overall, our findings contribute to a better understanding of the regulatory mechanisms involved in heat stress response, which could be useful in developing strategies to improve crop yield under heat stress conditions.

CONCLUSION

Our study aimed to identify DEGs in *B. napus* seeds under heat stress and to investigate the interaction of hub genes through PPI networks. Through transcriptional data analysis, we identified 442 significant DEGs, which were further analyzed using the web-based application STRING to generate a PPI network. Using the CytoHubba algorithm, we identified 10 hub genes, including two genes encoding subunits of calcium-transporting ATPase, three genes encoding small HSP20 family proteins, and one gene encoding the iron-sulfur subunit of the cytochrome b6-f complex. Our study showed that changes in cellular calcium levels play a role in signal transduction during heat stress, and the transient increase in calcium ions can be a response to environmental stimuli, including biotic and abiotic stresses. Moreover, the small HSP20 family proteins were identified as one of the most vital components of the response to heat stress conditions, reducing heat stress by restoring natural protein synthesis. Finally, we identified the downregulation of the iron-sulfur subunit of the cytochrome b6-f complex under heat stress, which plays a critical role in facilitating electron transfer between photosystems, cyclic electron flow around photosystem I, and state change. The identification of hub genes and their interactions through PPI networks provides insight into the molecular mechanisms underlying heat stress responses in *B. napus* seeds. Our findings provide a foundation for further research into the molecular mechanisms underlying the response of *B. napus* to heat stress and for the development of strategies for enhancing heat tolerance in this economically important crop.

Compliance with Ethical Standards

The authors declare that they have no conflict of interest. The research reported here did not involve experimentation with human participants or animals.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Nuclear

Science and Technology Research Institute (NSTRI) for their invaluable support throughout this research project.

REFERENCES

Bailey T. L., Boden M., Buske F. A., Frith M., Grant C. E., Clementi L., and Noble W. S. (2009). MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Research*, 37(suppl_2): W202-W208.

Boeva V. (2016). Analysis of genomic sequence motifs for deciphering transcription factor binding and transcriptional regulation in eukaryotic cells. *Frontiers in Genetics*, 7: 24.

Bokszczanin K. L., Consortium S. P. N., and Fragkostefanakis S. (2013). Perspectives on deciphering mechanisms underlying plant heat stress response and thermotolerance. *Frontiers in Plant Science*, 4: 315.

Bravo R., Parra V., Gatica D., Rodriguez A. E., Torrealba N., Paredes F., and Jaimovich E. (2013). Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. *International Review of Cell and Molecular Biology*, 301: 215-290.

Buske F. A., Bodén M., Bauer D. C., and Bailey T. L. (2010). Assigning roles to DNA regulatory motifs using comparative genomics. *Bioinformatics*, 26(7): 860-866

Chin C. H., Chen S. H., Wu H. H., Ho C. W., Ko M. T., and Lin C. Y. (2014). CytoHubba: Identifying hub objects and sub-networks from complex interactome. *BMC Systems Biology*, 8(4): 1-7.

Dawood M. F., Moursi Y. S., Amro A., Baenziger P. S., and Sallam A. (2020). Investigation of heat-induced changes in the grain yield and grains metabolites, with molecular insights on the candidate genes in barley. *Agronomy*, 10(11): 1730.

Elferjani R., and Soolanayakanahally R. (2018). Canola responses to drought, heat, and combined stress: shared and specific effects on carbon assimilation, seed yield, and oil composition. *Frontiers in Plant Science*, 9: 1224.

Ferguson J. N., Tidy A. C., Murchie E. H., and Wilson Z. A. (2021). The potential of resilient carbon dynamics for stabilizing crop reproductive development and productivity during heat stress. *Plant, Cell & Environment*, 44(7): 2066-2089.

Fragkostefanakis S., Roeth S., Schleiff E., and Scharf K. D. (2015). Prospects of engineering thermotolerance in crops through modulation of heat stress transcription factor and heat shock protein networks. *Plant, Cell & Environment*, 38(9): 1881-1895.

Fortunato S., Lasorella C., Dipierro N., Vita F., and de Pinto M. C. (2023). Redox signaling in plant heat stress response. *Antioxidants*, 12(3): 605.

Gan Y., Angadi S., Cutforth H., Potts D., Angadi V., and McDonald C. (2004). Canola and mustard response to short periods of temperature and water stress at different developmental stages. *Canadian Journal of Plant Science*, 84(3): 697-704.

Gao G., Hu J., Zhang X., Zhang F., Li M., and Wu X. (2021). Transcriptome analysis reveals genes expression pattern of seed response to heat stress in *Brassica napus* L. *Oil Crop Science*, 6(2): 87-96.

Gault C. R., Obeid L. M., and Hannun Y. A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. *Advances in Experimental Medicine and Biology*, 688: 1-23.

Georgii E., Kugler K., Pfeifer M., Vanzo E., Block K., Domagalska M. A., and Reinhardt R. (2019). The systems architecture of molecular memory in poplar after abiotic stress. *The Plant Cell*, 31(2): 346-367.

Ghorbani A., Rostami M., and Izadpanah K. (2023). Gene network modeling and pathway analysis of maize transcriptomes in response to Maize Iranian mosaic virus. *Genomics*, 115(3): 110618.

Gong M., van der Luit A. H., Knight M. R., and Trewavas A. J. (1998). Heat-shock-induced changes in intracellular Ca2+ level in tobacco seedlings in relation to thermotolerance. *Plant Physiology*, 116(1): 429-437.

Gralinska E., Kohl C., Fadakar B. S., and Vingron M. (2022). Visualizing cluster-specific genes from single-cell transcriptomics data using association plots. *Journal of Molecular Biology*, 434(11): 167525.

Guo M., Liu J.-H., Lu J.-P., Zhai Y.-F., Wang H., Gong Z.-H., and Lu M.-H. (2015). Genome-wide analysis of the CaHsp20 gene family in pepper: comprehensive sequence and expression profile analysis under heat stress. *Frontiers in Plant Science*, 6: 806.

Gupta S., Stamatoyannopoulos J. A., Bailey T. L., and Noble W. S. (2007). Quantifying similarity between motifs. *Genome Biology*, 8(2): 1-9.

Hall J. á. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, 53(366): 1-11.

Hassan M. U., Chattha M. U., Khan I., Chattha M. B., Barbanti L., Aamer M., and Aslam M. T. (2021). Heat stress in cultivated plants: Nature, impact, mechanisms, and mitigation strategies—A review. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, 155(2): 211-234.

Heckathorn S. A., Downs C. A., Sharkey T. D., and Coleman J. S. (1998). The small, methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. *Plant Physiology*, 116(1): 439-444.

Hou Q., Ufer G., and Bartels D. (2016). Lipid signalling in plant responses to abiotic stress. *Plant, Cell & Environment*, 39(5): 1029-1048.

Javadi S. M., Shobbar Z.-S., Ebrahimi A., and Shahbazi M. (2021). New insights on key genes involved in drought stress response of barley: gene networks reconstruction, hub, and promoter analysis. *Journal of Genetic Engineering and Biotechnology*, 19(1): 1-12.

Jung K.-H., Ko H.-J., Nguyen M. X., Kim S.-R., Ronald P., and An G. (2012). Genome-wide identification and analysis of early heat stress responsive genes in rice. *Journal of Plant Biology*, 55(6): 458-468.

Kinsella R. J., Kähäri A., Haider S., Zamora J., Proctor

G., Spudich G., and Kerhornou A. (2011). Ensembl BioMarts: a hub for data retrieval across taxonomic space. *Database*, 2011: bar030.

Ko D. K., and Brandizzi F. (2020). Network-based approaches for understanding gene regulation and function in plants. *The Plant Journal*, 104(2): 302-317.

Kourani M., Mohareb F., Rezwan F. I., Anastasiadi M., and Hammond J. P. (2022). Genetic and physiological responses to heat stress in *Brassica napus*. *Frontiers in Plant Science*, 13: 832147.

Li M., Li D., Tang Y., Wu F., and Wang J. (2017). CytoCluster: a cytoscape plugin for cluster analysis and visualization of biological networks. *International Journal of Molecular Sciences*, 18(9): 1880.

Li Y.-F., Wang Y., Tang Y., Kakani V. G., and Mahalingam R. (2013). Transcriptome analysis of heat stress response in switchgrass (*Panicum virgatum* L.). *BMC Plant Biology*, 13(1): 1-12.

Liu J., Feng L., Li J., and He Z. (2015). Genetic and epigenetic control of plant heat responses. *Frontiers in Plant Science*, 6: 267.

Lohani N., Singh M. B., and Bhalla P. L. (2021). RNA-seq highlights molecular events associated with impaired pollen-pistil interactions following short-term heat stress in *Brassica napus*. *Frontiers in Plant Science*, 11: 622748.

Lohani N., Singh M. B., and Bhalla P. L. (2022). Rapid transcriptional reprogramming associated with heat stress-induced unfolded protein response in developing *Brassica napus* anthers. *Frontiers in Plant Science*, 13: 905674.

Mackay A. (2008). Climate change 2007: impacts, adaptation and vulnerability. Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change. *Journal of Environmental Quality*, 37(6): 2407.

Malone L. A., Proctor M. S., Hitchcock A., Hunter C. N., and Johnson M. P. (2021). Cytochrome b6f–Orchestrator of photosynthetic electron transfer. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1862(5): 148380.

Murakami T., Matsuba S., Funatsuki H., Kawaguchi K., Saruyama H., Tanida M., and Sato Y. (2004). Over-expression of a small heat shock protein, sHSP17. 7, confers both heat tolerance and UV-B resistance to rice plants. *Molecular Breeding*, 13(2): 165-175.

Niu Y., and Xiang Y. (2018). An overview of biomembrane functions in plant responses to high-temperature stress. *Frontiers in Plant Science*, 9: 915.

Obaid A. Y., Sabir J. S., Atef A., Liu X., Edris S., El-Domyati F. M., and Al-Kordy M. A. (2016). Analysis of transcriptional response to heat stress in *Rhazya stricta*. *BMC Plant Biology*, 16(1): 1-18.

Ouyang Y., Chen J., Xie W., Wang L., and Zhang Q. (2009). Comprehensive sequence and expression profile analysis of Hsp20 gene family in rice. *Plant Molecular Biology*, 70(3): 341-357.

Pizzuti C., and Rombo S. E. (2014). Algorithms and tools for protein–protein interaction networks clustering, with a special focus on population-based stochastic methods. *Bioinformatics*, 30(10): 1343-1352.

Qu A.-L., Ding Y.-F., Jiang Q., and Zhu C. (2013). Molecular mechanisms of the plant heat stress response. *Biochemical and Biophysical Research Communications*, 432(2): 203-207.

Raboanatahiry N., Li H., Yu L., and Li M. (2021). Rapeseed (*Brassica napus*): Processing, utilization, and genetic improvement. *Agronomy*, 11(9): 1776.

Rahaman M., Mamidi S., and Rahman M. (2018). Genome-wide association study of heat stress-tolerance traits in spring-type *Brassica napus* L. under controlled conditions. *The Crop Journal*, 6(2): 115-125.

Rao G., Jain A., and Shivanna K. (1992). Effects of high temperature stress on Brassica pollen: viability, germination and ability to set fruits and seeds. *Annals of Botany*, 69(3): 193-198.

Ray D. K., Gerber J. S., MacDonald G. K., and West P. C. (2015). Climate variation explains a third of global crop yield variability. *Nature Communications*, 6(1): 1-9.

Ren S., Ma K., Lu Z., Chen G., Cui J., Tong P., and Jin B. (2019). Transcriptomic and metabolomic analysis of the heat-stress response of *Populus tomentosa* Carr. *Forests*, 10(5): 383.

Rodas-Junco B. A., Racagni-Di-Palma G. E., Canul-Chan M., Usorach J., and Hernández-Sotomayor S. T. (2021). Link between lipid second messengers and osmotic stress in plants. *International Journal of Molecular Sciences*, 22(5): 2658.

Ryckman A. E., Brockhausen I., and Walia J. S. (2020). Metabolism of glycosphingolipids and their role in the pathophysiology of lysosomal storage disorders. *International Journal of Molecular Sciences*, 21(18): 6881.

Rütgers M., Muranaka L. S., Schulz-Raffelt M., Thoms S., Schurig J., Willmund F., and Schroda M. (2017). Not changes in membrane fluidity but proteotoxic stress triggers heat shock protein expression in *Chlamydomonas reinhardtii*. *Plant, Cell & Environment*, 40(12): 2987-3001.

Scharf K.-D., Siddique M., and Vierling E. (2001). The expanding family of *Arabidopsis thaliana* small heat stress proteins and a new family of proteins containing α-crystallin domains (Acd proteins). *Cell Stress & Chaperones*, 6(3): 225.

Schöttler M. A., Flügel C., Thiele W., and Bock R. (2007). Knock-out of the plastid-encoded PetL subunit results in reduced stability and accelerated leaf age-dependent loss of the cytochrome b6f complex. *Journal of Biological Chemistry*, 282(2): 976-985.

Serin E. A., Nijveen H., Hilhorst H. W., and Ligterink W. (2016). Learning from co-expression networks: possibilities and challenges. *Frontiers in Plant Science*, 7: 444.

Sharkey T. D., and Zhang R. (2010). High temperature effects on electron and proton circuits of photosynthesis. *Journal of Integrative Plant Biology*, 52(8): 712-722.

Singh M. B., Lohani N., and Bhalla P. L. (2021). The role

of endoplasmic reticulum stress response in pollen development and heat stress tolerance. *Frontiers in Plant Science*, 12: 661062.

Song Y., Chen Q., Ci D., Shao X., and Zhang D. (2014). Effects of high temperature on photosynthesis and related gene expression in poplar. *BMC Plant Biology*, 14(1): 1-20.

Sun J. L., Li J. Y., Wang M. J., Song Z. T., and Liu J. X. (2021). Protein quality control in plant organelles: current progress and future perspectives. *Molecular Plant*, 14(1): 95-114.

Sung D.-Y., Kaplan F., Lee K.-J., and Guy C. L. (2003). Acquired tolerance to temperature extremes. *Trends in Plant Science*, 8(4): 179-187.

Swindell W., Huebner M., and Weber A. (2007). Plastic and adaptive gene expression patterns associated with temperature stress in *Arabidopsis thaliana*. *Heredity*, 99(2): 143-150.

Triboï E., Martre P., and Triboï-Blondel A. M. (2003). Environmentally-induced changes in protein composition in developing grains of wheat are related to changes in total protein content. *Journal of Experimental Botany*, 54(388): 1731-1742.

Verghese J., Abrams J., Wang Y., and Morano K. A. (2012). Biology of the heat shock response and protein chaperones: budding yeast (*Saccharomyces cerevisiae*) as a model system. *Microbiology and Molecular Biology Reviews*, 76(2): 115-158.

Wahid A., Gelani S., Ashraf M., and Foolad M. R. (2007). Heat tolerance in plants: an overview. *Environmental and Experimental Botany*, 61(3): 199-223.

Wallace E. W., Kear-Scott J. L., Pilipenko E. V., Schwartz M. H., Laskowski P. R., Rojek A. E., and Franks A. M. (2015). Reversible, specific, active aggregates of endogenous proteins assemble upon heat stress. *Cell*, 162(6): 1286-1298.

Wang J., Juliani H. R., Jespersen D., and Huang B. (2017). Differential profiles of membrane proteins, fatty acids, and sterols associated with genetic variations in heat tolerance for a perennial grass species, hard fescue (*Festuca Trachyphylla*). *Environmental and Experimental Botany*, 140: 65-75.

Wang J., Yuan B., Xu Y., and Huang B. (2018). Differential responses of amino acids and soluble proteins to heat stress associated with genetic variations in heat tolerance for hard fescue. *Journal of the American Society for Horticultural Science*, 143(1): 45-55.

Wang M., Zhang X., Li Q., Chen X., and Li X. (2019). Comparative transcriptome analysis to elucidate the enhanced thermotolerance of tea plants (*Camellia sinensis*) treated with exogenous calcium. *Planta*, 249(3): 775-786.

Waters E. R. (2013). The evolution, function, structure, and expression of the plant sHSPs. *Journal of Experimental Botany*, 64(2): 391-403.

Wennekes T., van den Berg R. J., Boot R. G., van der Marel G. A., Overkleeft H. S., and Aerts J. M. (2009). Glycosphingolipids—nature, function, and pharmacological modulation. *Angewandte Chemie International Edition*, 48(47): 8848-8869.

Wu W., Ma B. L., and Whalen J. K. (2018). Enhancing rapeseed tolerance to heat and drought stresses in a changing climate: perspectives for stress adaptation from root system architecture. *Advances in Agronomy*, 151: 87-157.

Yin L., Sun Y., Chen X., Liu J., Feng K., Luo D., and Liu L. (2023). Genome-wide analysis of the HD-Zip gene family in chinese cabbage (*Brassica rapa* subsp. pekinensis) and the expression pattern at high temperatures and in carotenoids regulation. *Agronomy*, 13(5): 1324.

Young L. W., Wilen R. W., and Bonham-Smith P. C. (2004). High temperature stress of *Brassica napus* during flowering reduces micro-and megagametophyte fertility, induces fruit abortion, and disrupts seed production. *Journal of Experimental Botany*, 55(396): 485-495.

Yu H.-D., Yang X.-F., Chen S.-T., Wang Y.-T., Li J.-K., Shen Q., and Guo F.-Q. (2012). Downregulation of chloroplast RPS1 negatively modulates nuclear heat-responsive expression of HsfA2 and its target genes in Arabidopsis. *PLoS Genetics*, 8(5): e1002669.

Yu J., Cheng Y., Feng K., Ruan M., Ye Q., Wang R., and Yang Y. (2016). Genome-wide identification and expression profiling of tomato Hsp20 gene family in response to biotic and abiotic stresses. *Frontiers in Plant Science*, 7: 1215.

Yu T., Bai Y., Liu Z., Wang Z., Yang Q., Wu T., and Li Q. (2022). Large-scale analyses of heat shock transcription factors and database construction based on whole-genome genes in horticultural and representative plants. *Horticulture Research*, 9: uhac035.

Zhao P., Wang D., Wang R., Kong N., Zhang C., Yang C., and Chen Q. (2018). Genome-wide analysis of the potato Hsp20 gene family: identification, genomic organization and expression profiles in response to heat stress. *BMC Genomics*, 19(1): 1-13.

Zhong L., Zhou W., Wang H., Ding S., Lu Q., Wen X., and Lu C. (2013). Chloroplast small heat shock protein HSP21 interacts with plastid nucleoid protein pTAC5 and is essential for chloroplast development in Arabidopsis under heat stress. *The Plant Cell*, 25(8): 2925-2943.