Mariam Kourani

EVALUATING TRANSCRIPTOMIC AND METABOLOMIC ADAPTATIONS TO HEAT STRESS IN *BRASSICA NAPUS* (OILSEED RAPE)

Faculty of Engineering and Applied Sciences

PhD in Environment and Agrifood

PhD

Academic Year: 2020- 2024

Supervisor: Prof. Fady Mohareb

Associate Supervisors: Prof. John Hammond, Dr Maria Anastasiadi,

January, 2025

Faculty of Engineering and Applied Sciences

PhD in Environment and Agrifood

PhD

Academic Year 2020 - 2024

Mariam Kourani

EVALUATING TRANSCRIPTOMIC AND METABOLOMIC ADAPTATIONS TO HEAT STRESS IN *BRASSICA NAPUS* (OILSEED RAPE)

Supervisor: Prof. Fady Mohareb

Associate Supervisors: Prof. John Hammond, Dr Maria Anastasiadi

January 2025

© Cranfield University 2025. All rights reserved. No part of this publication may be reproduced without the written permission of the copyright owner.

Academic integrity declaration

I declare that:

- the thesis submitted has been written by me alone.
- the thesis submitted has not been previously submitted to this university or any other.
- that all content, including primary and/or secondary data, is true to the best of my knowledge.
- that all quotations and references have been duly acknowledged according to the requirements of academic research.

I understand that to knowingly submit work in violation of the above statement will be considered by examiners as academic misconduct.

Abstract

Oilseed rape (*Brassica napus*) is an essential oilseed crop, accounting for approximately 12% of the world's vegetable oil production. Its ability to adapt to various climates, has made it one of the predominant oil crops in Europe. Previous research on Brassica species has shown a negative relationship between heat stress and seed yield and quality. To date, only few studies have addressed the impact of heat stress on the transcriptome and metabolome of *B. napus* especially during the flowering stage and the underlying cellular mechanisms are still poorly understood. To address this, combining genetic studies with different omics approaches will provide the means to explore the intricate responses arising following exposure to heat stress and accomplish a system-level understanding of how heat stress alters growth and metabolism of oilseed rape.

This thesis presents (i) a comprehensive review of the impact of heat stress on the physiology and genetics of *B. napus* with a focus on the reproductive stages, along with the mechanisms involved in heat stress response. (ii) A heat treatment experiment simulating a heatwave during the flowering stage of *B. napus*, which caused a significant transcriptional and metabolic changes in buds and leaves, including the down-regulation of many respiratory pathways. RNA-seq analysis showed that heat stress resulted in the differential expression of many genes involved in key biological processes such as heat shock, regulation of cellular processes, transcription factors, cell wall remodelling, sugars and secondary metabolites transport and metabolism. At the metabolomic level, heat stress increased the content of carbohydrates (glucose, fructose and sucrose) and aliphatic glucosinolates (gluconapin, progoitrin) in the leaves but decreased the content of the indolic glucosinolate (glucobrassicin). Additionally, heat treatment also reduced yield and altered oil composition. (iii) This thesis also presents a Cytoscape plugin to visualise genes-TFs interactions in a user-friendly environment. Using the plugin, the top DEGs identified in the heat treatment

experiment were found to interact with a wide range of TFs, with members of the MYB, NAC, and WRKY families showing the highest number of gene interactions.

Overall, these results revealed important components of heat stress response and tolerance mechanisms in *B. napus*. The identified genes and metabolites could form the theoretical basis for potential biotechnological applications and a genetic resource for further studies towards the identification and development of high-yield and nutritious varieties that can cope with the heat predicted by the global climate change.

Keywords: Heatwaves, reproduction, RNA-Seq, metabolic pathways, tolerance, sugars, glucosinolates, transcription factors, Cytoscape

Acknowledgements

First and foremost, I would like to give my deepest gratitude to Almighty God for His continuous blessings and guidance through the journey of my PhD.

My sincerest thanks to my lead supervisor Prof. Fady Mohareb for his academic guidance and mentorship, since I joined Cranfield University for the MSc course. His approachable and supportive nature over the last 6 years have helped me to achieve this milestone, something I never thought I could reach.

I extend my thanks to my co-supervisor Dr Maria Anastasiadi for her support especially during challenging metabolomics lab work and analysis, where with her expertise and guidance, always made sure that I achieve good quality results.

Additionally, I give my thanks to my co-supervisor Prof. John Hammond from the University of Reading. I am incredibly fortunate to have had the opportunity to work with him and learn from his expertise which is sufficient to drive any project into success. Beyond his professional guidance, his kindness and exceptional manners have made this journey a very positive experience.

Further, I would like to thank my reviewer Prof. Andrew Thompson for his invaluable feedback during the annual reviews, which helped me get deeper understandings of the project's findings. I am also grateful to Dr Corentin Molitor for his technical guidance during my MSc and the early stages of my PhD project.

I would also like to thank all the members of the Bioinformatics team (Dr Tomasz Kurowski, Dr Alexey Larionov, Dr Samuel Hibdige, Dr Kevin De Castro Cogle, Zahra Karimi, John Pearce, Sophie Dodd and Shintaro Kinoshita), with whom I had a great time socialising, and my dear friend Dr Mennatullah Shehata whose constant encouragement and sincere prayers during her time in Cranfield and afterward meant a lot to me.

Likewise, I am equally appreciative to the laboratory technicians Emma Collings and Monika Jodkowska from the Plant Science Group at Cranfield University and

Mariana Madruga Kruger from the University of Reading for their invaluable laboratory training and support.

Finally, my heartfelt gratitude goes to my family and friends who their patience, encouragement and love made it possible for me to complete this PhD.

This PhD work is dedicated to all the blessed souls who left this world in the path of seeking justice and truthfulness. May their memory live on in the pursuit of a fairer and more just world.

Table of Contents

Academic integrity declaration i
Acknowledgements iv
List of Figures ix
List of Tables xv
List of Abbreviations xvi
1 Introduction 20
1.1 Aims and Objectives 21
1.1.1 Investigating the impact of heat stress on seed weight, oil content and total biomass under simulated field conditions 21
1.1.2 Identifying the impact of heat stress on the plant metabolites 21
1.1.3 Identifying key differentially expressed genes involved in plant heat stress response 22
1.1.4 Implementing an interactive visualisation tool to display a network of interactions between heat stress biomarkers and transcription factors 22
1.2 Thesis Structure 22
2 Genetic and Physiological Responses to Heat Stress in *Brassica napus* 25
2.1 Our Changing Climate and the Potential Impacts on Crop Productivity 27
2.2 Physiological Impact of Heat Stress at Different Developmental Stages 29
2.2.1 Impact of heat stress on seedling and vegetative growth 29
2.2.2 Impact of heat stress on reproduction 31
2.2.3 Impact of heat stress on postharvest seed storage 38
2.3 Plant Response Mechanisms to Heat Stress 39
2.3.1 Phytohormones 44
2.3.2 Transcription factors 46
2.3.3 Glucosinolates 48
2.4 Impact of Polyploidy and Alternative Splicing on Heat Stress 49
2.5 Dynamics of Epigenetic Modifications under Heat Stress 52
2.5.1 DNA methylation 52
2.5.2 Histone modifications 54
2.5.3 Non-coding RNAs 56
2.6 Systems Biology Approaches in *Brassica napus* under Heat Stress 58
2.7 Conclusion 63
3 Prolonged heat stress in *Brassica napus* during flowering negatively impacts yield and alters glucosinolate and sugars metabolism 65
3.1 Introduction 66
3.2 Materials and Methods 69
3.2.1 Plant materials and physiological parameters 69

3.2.2 Heatwave experimental design 69
3.2.3 Sample preparation for transcriptomic analysis 71
3.2.4 RNA extraction and sequencing 71
3.2.5 Differential expression and cluster analysis 72
3.2.6 Gene Ontology and KEGG pathway enrichment analysis of DEGs . 73
3.2.7 Validation of RNA-seq results by real-time quantitative PCR (RT-qPCR) 73
3.2.8 Sample preparation for metabolomic analysis 74
3.2.9 Glucosinolates extraction and analysis 74
3.2.10 Sugars content extraction and analysis 75
3.2.11 Near-Infra Red Spectroscopy (NIRS) scanning 76
3.2.12 Statistical analysis 76
3.3 Results 77
3.3.1 Prolonged heat treatment reduced yield and altered *B. napus* oil composition 77
3.3.2 Differential gene expression identifies distinct patterns of expression in *B. napus* under heat stress and recovery 79
3.3.3 Validation of RNA-seq data by quantitative real-time PCR 96
3.3.4 Functional annotation of DEGs identifies key pathways responding to heat treatment and recovery 97
3.4 Discussion 117
3.4.1 Heat stress resulted in down-regulation of many genes involved in multiple functional and metabolic processes at 2 DAT 118
3.4.2 DEGs showed enrichment of pathways related to respiratory metabolism 119
3.4.3 Heat stress altered GSL metabolism during treatment 120
3.4.4 Heat stress altered the expression of sulphur assimilation and transport genes 124
3.4.5 Heat stress altered sugars metabolism during treatment and recovery periods 124
3.5 Conclusion 125
4 NetViewer: A Cytoscape plugin for TF-gene network extension and enhancement 128
4.1 Introduction 128
4.2 Methods 130
4.2.1 Input files 130
4.2.2 Network display workflow 130
4.2.3 Regulatory interaction network file for the TFs 132
4.2.4 Network extension 132
4.2.5 Case study 133

4.3 Results and Discussion 135
5 General Discussion 150
5.1 Limitations 157
6 Concluding Remarks and Future Perspectives 158
6.1 Conclusion 158
6.2 Future Perspectives 158
REFERENCES 161
Appendix A 193
Supplementary Figures and Tables 193
Appendix B 216
NetViewer Workflow 216

List of Figures

Figure 2.1 Physiological impact of heat stress at different developmental stages of *B. napus*. Heat stress negatively impacts all stages of plant development including vegetation, floral transition, post-flowering and organogenesis. RWC: Relative water content, FA: Fatty acids, ABA: Abscisic acid, GA: Gibberellic acid. 30

Figure 2.2 Major flowering time regulators in *B. napus*. Flowering is regulated by the expression of a set of floral integrator genes to activate the meristem identity genes causing the transition from vegetative to a floral meristem. Me3: Trimethylation, H3K4: Histone 3 Lysine 4, H3K36: Histone 3 Lysine 36, EFS: Early Flowering in Short Days, BnaFRI: *B. napus* homologues of *FRI*, *FLC*: Flowering Locus C, *FT*: Flowering Locus T, *SOC1*: Suppressor of Overexpression of CONSTANS 1, CO: CONSTANS, *LFY*: *LEAFY*, *AP1*: *APETALA1, SEP3: SEPALLATA3, FUL: FRUITFULL*. 33

Figure 2.3 Regulatory and signalling events involved in plant heat stress response. Following initial stress perception, the generated signals induce the expression of thermotolerance-related genes through a complex network of events involved in the stress response. UPR: Unfolded protein response, Ca^{2+}: Calcium, ER: Endoplasmic reticulum, H2A.Z: Histone variant, ETH: Ethylene, SA: Salicylic acid, JA: Jasmonic acid, CTK: Cytokinin, BR: Brassinosteroids, GA: Gibberellic acid, ABA: Abscisic acid, PYL: Pyrabactin resistance 1-like gene, ABF: ABA-responsive element binding factor, DWF4: A BR biosynthetic *dwarf 4* gene, CBL: Calcineurin B-like , CIPKs: CBL-interacting protein kinases, WRKY: TF containing WRKY-protein domain, MADS-box TF containing MADS-box protein domain, bZIP: Basic Leucine Zipper TF, NAC: TF containing N-terminal NAC domain, AP2/ERF: APETALA2/Ethylene Responsive Factor, MYB: Myeloblastosis viral oncogene homolog, DOF: DNA-binding with one finger, HSP: Heat shock protein, VIP1: VirE2-interacting protein 1, HSFA1: Heat shock factor A1, BnTR1: *B. napus* thermal resistance 1 gene. 41

Figure 3.1 Maximum daily temperature (°C) recorded at Heathrow airport during May 2017, May 2018, May 2019 and May 2020. 70

Figure 3.2 Heat treatment experimental design. To mimic a heatwave, the temperature in the heat treatment cabinet was increased gradually from 20 °C to 30 °C between 9:00 h and 12:00 h. The temperature was held at 30 °C until 22:00 h (B), before it was dropped gradually and maintained at 24 °C until 8:00 h of the next day (B). This cycle of gradual increase (day) and decrease (night) of temperature was held for the next 5 days (C). On the 6th day of treatment, the temperature was gradually decreased to 20 °C/14 °C day/night cycle (D) and held for a recovery period of 7 days (E). 71

Figure 3.3 *B. napus* flowers and buds at 5 DAT & 3 DOR respectively. 77

Figure 3.4 Above ground dry weight and seed yield per plant at maturity. Significant differences between heat-treated and control plants are denoted by (*) for p-value ≤ 0.05. 78

Figure 3.5 Fatty acid content in seeds per plant. Seeds from heat-treated plants (yellow) exhibited significant decrease in linoleic acid, linolenic acid and palmitic acid concentrations, but an increase in oleic acid and stearic acid concentrations compared to seeds from control plants (blue). Significant differences between heat-treated and control plants are denoted by (*) for p-value ≤ 0.05 or (**) for p-value ≤ 0.01 or (***) for p-value ≤ 0.001. 79

Figure 3.6 Principal component analysis of the control and heat treatment samples: Samples distribution in PC1 and PC2. 80

Figure 3.7 K-means cluster analysis of DEGs in *B. napus* during and after heat treatment. K-means clustering was applied to 9933 DEGs with log2FC ≥ 0.5 using the “kmeans” function in R package “stats” (v. 3.2.2), where k represents a pre-specified number of clusters. Six groups of genes were classified, with N the number of genes in each cluster, and the solid red lines in each cluster indicate the mean in differential expression. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. 96

Figure 3.8 Correlation analysis of the results obtained from RNA-seq and qt-PCR: *SS5* exhibited similar expression profiles between RT-qPCR and RNA-seq data with correlation coefficients of r=0.86. 97

Figure 3.9 Correlation analysis of the results obtained from RNA-seq and qt-PCR: *HSP20* exhibited similar expression profiles between RT-qPCR and RNA-seq data with correlation coefficients of r=0.63. 97

Figure 3.10 Enriched GO terms at 0 DAT, as identified by GO enrichment analysis..... 98

Figure 3.11 Enriched GO terms at 1 DAT, as identified by GO enrichment analysis..... 99

Figure 3.12 Enriched GO terms at 2 DAT, as identified by GO enrichment analysis..... 99

Figure 3.13 Enriched GO terms at 1 DOR, as identified by GO enrichment analysis..... 100

Figure 3.14 Enriched GO terms at 7 DOR, as identified by GO enrichment analysis..... 100

Figure 3.15 Enzyme code distribution at different timepoints as identified by GO enrichment analysis. 101

Figure 3.16 The top enriched KEGG pathways and their corresponding number of genes at 2 DAT at p-value ≤ 0.05. The x-axis represents the enrichment factor. The y-axis represents KEGG pathways. The circle represents the number of genes mapped to pathways and the red to green bar indicates the significance. 102

Figure 3.17 KEGG pathway for Glycolysis at 2 DAT. *Blue denotes down-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated. 103

Figure 3.18 KEGG pathway for Citrate cycle at 2 DAT. *Blue denotes down-regulated genes, red denotes up-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.... 104

Figure 3.19 Heatmap showing differential expression of genes involved in the regulation or biosynthesis of aliphatic GSL in *B. napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. 105

Figure 3.20 Heatmap showing differential expression of genes involved in the regulation or biosynthesis of indolic GSL in *B. napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. 106

Figure 3.21 Heatmap showing differential expression of genes involved in the assimilation and transport of sulphur in *B. napus*. Twenty transcripts were down-regulated in response to heat at either 1 or 2 DAT or both. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. 107

Figure 3.22 Leaf progoitrin (PRO) concentrations in ng/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). GSL concentrations were quantified using LC-MS. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, or (**) for p-value ≤ 0.01..... 109

Figure 3.23 Leaf gluconapin (GNA) concentration in ng/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). GSL concentrations were quantified using LC-MS. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, (**) for p-value ≤ 0.01, or (***) for p-value ≤ 0.001. 110

Figure 3.24 Leaf glucobrassicin (GBS) concentrations in ng/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). GSL concentrations were quantified using LC-MS. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, (**) for p-value ≤ 0.01 or (****) for p-value ≤ 0.0001. 111

Figure 3.25 Heatmap showing differential expression of *SWEET* genes involved in sugars transport in *Brassica napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. 112

Figure 3.26 Heatmap showing differential expression of *ERD-6 like* genes involved in sugars transport in *B. napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under

control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. 113

Figure 3.27 Leaf glucose concentration in mg/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30°C/24°C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). Concentration was quantified using HPLC. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, or (**) for p-value ≤ 0.01. 115

Figure 3.28 Leaf fructose concentration in mg/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). Concentration was quantified using HPLC. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, or (**) for p-value ≤ 0.01. 116

Figure 3.29 Leaf sucrose concentration in mg/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at timepoints 0-5 (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). Concentration was quantified using HPLC. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. 117

Figure 3.30 Three major biosynthesis pathways of the GSL in Brassicaceae. Outline of the pathways which can be divided into three parts; (1) amino acid chain elongation; (2) formation of the core structure of GSLs; (3) side chain modification. Blue bold indicates biosynthetic genes involved in each step. The names in parentheses denote common names (Seo & Kim, 2017).. 123

Figure 4.1 NetViewer functional workflow. Blue colour represents the steps that must be performed before launching Cytoscape. Grey colour represents the

steps within NetViewer, and pink colour highlights information messages provided by NetViewer..... 131

Figure 4.2 NetViewer workflow..... 133

Figure 4.3 Extended network of interactions between top DEGs (grey nodes) and TFs (Blue nodes). The blue edges represent the interactions between the genes and TFs..... 134

Figure 4.4 Extended network of interactions between top DEGs (grey nodes) and TFs (Blue nodes). As an example, the network also highlights in orange the interactions between one transcription factor (HSF6) and its interacting genes along with the edges connecting them..... 135

Figure 4.5 Transcription factor AtMYC2-genes interactions generated network. Green node (AtMYC2) and grey nodes (genes). Histogram bars represent expression levels in average transcript per million values (TPM) in response to heat treatment. Each timepoint is represented by two bars (blue: control condition, red: heat stress). The timepoints are as follows, from left to right: 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR..... 138

Figure 4.6 Transcription factor AtMYC2-genes interactions generated network. Green node (AtMYC2), orange nodes (genes encoding TFs) and grey nodes (other genes). Histogram bars represent expression levels in average TPM in response to heat treatment. Each timepoint is represented by two bars (blue: control condition, red: heat stress). The timepoints are as follows, from left to right: 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR. 140

Figure 4.7 Multiple TFs regulate AtMYC2-regulated genes. Green node (AtMYC2), blue nodes (TFs) and grey nodes (genes). Histogram bars represent expression levels in average TPM in response to heat treatment. Each timepoint is represented by two bars (blue: control condition, red: heat stress). The timepoints are as follows, from left to right: 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR..... 142

List of Tables

Table 2.1 Heat stress responsive genes identified in *B. napus* and related species. 42

Table 3.1 Log2FC of top 30 up-regulated genes at 1 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)..... 82

Table 3.2 Log2FC of top 30 down-regulated genes at 1 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast) 85

Table 3.3 Log2FC of top 30 up-regulated genes at 2 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast) 89

Table 3.4 Log2FC of top 30 down-regulated genes at 2 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast) 92

Table 4.1 Number of genes interacting with each TFs 136

Table 4.2 AtMYC2 genes network table. C0, C1, C2, C6 & C12 represent TPM values at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively of control conditions. HS0, HS1, HS2, HS6 & HS12 represent TPM values at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively of heat stress conditions..... 143

Table 4.3 Differential expression data of JAZ transcripts in log2FC. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)..... 148

List of Abbreviations

A_{sat}	Saturating Light
ABA	Abscisic acid
ATP	Adenosine Triphosphate
AGT	Alanine-Glyoxylate Aminotransferase
AS	Alternative Splicing
ANOVA	Analysis of Variance
AP1	APETALA1
AP2/EREBPs	APETALA2/Ethylene Responsive Element Binding Proteins
AP2/ERF	APETALA2/Ethylene Responsive Factor
ABR1	Arabidopsis Abscisic acid Repressor 1
ATNTT2	Aryl Hydrocarbon Receptor Nuclear Translocator 2
ATPS	ATP Sulphurylase
bHLH	Basic Helix-loop-Helix
bZIP	Basic Leucine Zipper
BnTR1	*Brassica napus* Thermal Resistance gene 1
BR	Brassinosteroids
CBLs	Calcineurin B-like proteins
ClpB1	Caseinolytic Peptidase B protein
CIPKs	CBL-interacting protein kinases
ChIP	Chromatin Immunoprecipitation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CO	CONSTANS transcription factor
Cas9	CRISPR-associated protein 9
CRISPRa	CRISPR-mediated gene activation
C18:2	Linoleic acid
C18:3	Linolenic acid
DAS	Days After Sowing
DAT	Days after Treatment
DOR	Days of Recovery
DREB	Dehydration Responsive Element Binding
DME	DEMETER family of DNA repair mechanism
DNA	Deoxyribonucleic acid
dCAPS	Derived Cleaved Amplified Polymorphic Sequences
DE	Differentially Expressed
DEGs	Differentially Expressed Genes
DOF	DNA-binding with one finger
E	Transpiration
EFS	Early Flowering in Short Days

ERD	Early Responsive to Dehydration
EHD1	EH domain-containing protein 1
ETC	Electron Transport Chain
ER	Endoplasmic Reticulum
ELSD HPLC	Evaporative Light Scattering Detector HPLC
FDR	False Discovery Rate
FA	Fatty acids
FLC	Flowering Locus C
FT	Flowering locus T
FTi	Flowering time
FUL	FRUITFULL gene
GO	Gene Ontology
GFF	General Feature Format
GWAS	Genome-Wide Association Study
GEBV	Genomic Estimated Breeding Value
GA	Gibberellic acid
GBS	Glucobrassicin
GNA	Gluconapin
G6PDH	Glucose-6-Phosphate Dehydrogenase
GSL	Glucosinolates
GLYI	Glyoxalase I
GC	Guanine and Cytosine
HSF	Heat Shock Factor
HSP	Heat Shock Protein
HT	Heat Treatment
HPLC	High Performance Liquid Chromatography
H3K27me3	Histone H3 Lysine-27 Tri-methylation
HMT	Homocysteine Methyltransferase
IR	Intron Retention
IIL1	Isopropyl malate isomerase large subunit 1
JA	Jasmonates
JAZ	Jasmonate-Zim Domain
KEGG	Kyoto Encyclopedia of Genes and Genomes
LHY	Late Elongated Hypocotyl
LFY	LEAFY
LC-MS	Liquid Chromatography-Mass Spectrometry
Log2FC	Log2 Fold Change
lincRNAs	long intergenic noncoding RNAs
lncNATs	long noncoding natural antisense transcripts
lncRNAs	long non-coding RNAs
Met	Meteorological
miRNAs	micro-RNAs
MYB	Myeloblastosis
MOPS	3-(N-Morpholino) Propanesulfonic acid

NCBI	National Center for Biotechnology Information
NIRS	Near-Infra Red Spectroscopy
NIT2	Nitrilase 2
OPPP	Oxidative Phase of Pentose Phosphate Pathway
PPP	Pentose Phosphate Pathway
PGM	Phosphoglucomutase
PHD	Plant Homeodomain
PRC2	Polycomb Repressive Complex
PTGS	Post-Transcriptional Gene Silencing
PCA	Principle Component Analysis
PRO	Progoitrin
PRT6	Proteolysis 6
QTRAP LC-MS	Quadrupole Trap LC-MS
qtPCR	Quantitative Polymerase Chain Reaction
QTLs	Quantitative Trait Loci
ROS	Reactive Oxygen Species
rt-qPCR	Real time-Quantitative Polymerase Chain Reaction
RINGv	Really Interesting New Gene Variant
RAV	Related to ABI3 (Abscisic acid Insensitive 3) and VP1 (Viviparous1)
RWC	Relative Water Content
Rpm	Revolutions per Minute
RNA	Ribonucleic acid
RSEM	RNA-Seq by Expectation-Maximization
SABP3	Salicylic cid-Binding Protein 3
SCIEX	Scientific Export
SAGs	Senescence-Associated Genes
SEP3	SEPALLATA3
SSR	Simple Sequence Repeat
SNP	Single Nucleotide Polymorphism
siRNAs	small interfering-RNAs
sRNAs	small RNAs
SlMAPK3	*Solanum lycopersicum* Mitogen-activated protein kinase 3
STAR	Spliced Transcripts Alignment to a Reference
SLU7	Splicing factor synergistic lethal with U5 snRNA 7
SDH	Succinate Dehydrogenase
SPS	Sucrose Phosphate Synthase
SS5	Sucrose Synthase 5
SUC	Sucrose Transporter

SWEET	Sugars Will Eventually be Exported Transporter
SMXL8	Suppressor of MAX2-like 8
SOC1	Suppressor of Overexpression of CONSTANS 1
sHSP	Small Heat Shock Protein
TGMS	Thermosensitive Genic Male Sterility
TPM	Transcript per Million
TF	Transcription Factor
TGS	Transcriptional Gene Silencing
TK	Transketolase
UPR	Unfolded protein response
USDA	United States Department of Agriculture
UDP	Uridine Diphosphate
WGBS	Whole-Genome Bisulfite Sequencing
6PGDH	6-Phosphogluconate Dehydrogenase

1 Introduction

Various climate models have forecasted a global temperature increase of 1–4 °C by the end of the 21st century (Driedonks et al., 2017). Along with more frequent heatwaves, this temperature rise is expected to create unfavourable conditions that could adversely impact both crop yield and quality (Castroverde & Dina, 2021). Studies showed that high temperature stress could have deleterious effects on plants especially if it occurred during the reproductive stages, often leading to impaired growth and reduced productivity (Zhang et al., 2017; Lamaoui et al., 2018). Moreover, high temperature can induce irreversible structural and physiological changes in the floral organs, leading to premature senescence (Lohani et al., 2020; Jagadish et al., 2021).

Brassica napus, like many other temperate field crops, is sensitive to high temperature stress, especially during reproduction (Angadi et al., 2000; Koscielny et al., 2018). Research from different Brassica species have found negative relationships between heat stress and seed yield and quality (Yu et al., 2014). It has been estimated that for every 1 °C rise above the optimal temperature range for growth and development during *B. napus* pod setting, yield decreases by 10% (Nuttall et al., 1992). In a recent study, Jedličková et al (2023) found that heat stress triggered cellular damages and induced the up-regulation of genes involved in the photosynthesis and plant hormone signalling pathways.

To cope with such conditions, plants have evolved interconnected regulatory pathways including various molecular and biochemical mechanisms to resist and adapt to heat stress (Wahid et al., 2007; Hasanuzzaman et al., 2013). These mechanisms involve activating of various signalling cascades that ultimately result in the expression of heat stress-responsive genes, and accumulation of different metabolites (Mittler et al., 2012; Ikram et al., 2022). Therefore, understanding the mechanisms involved in the heat stress response and in particular, the specific genes and metabolites involved in this response will be beneficial for developing stress-tolerant varieties. Despite recent progress in studying the effects of various biotic and abiotic stress factors within different Brassica species, a comprehensive analysis of how heat stress impacts *B. napus*

during its yield determining reproductive stages remains limited. Therefore, this thesis will focus on providing insights into the effect of a future warmer climate on *B. napus* during reproduction. In particular, this thesis will address the transcriptomic and metabolomic responses to heat stress under simulated field conditions.

1.1 Aims and Objectives

The aim of this thesis is to provide a system-level molecular and metabolic understanding of the impact of heatwaves on yield, quality and tolerance mechanisms in *B. napus.* The ultimate goal is to identify key metabolites and genes, along with their underlying biochemical and molecular mechanisms, involved in the plant's response and adaptation to heat stress. To achieve this, the following objectives are addressed:

1.1.1 Investigating the impact of heat stress on seed weight, oil content and total biomass under simulated field conditions

To investigate the impact of heat stress on *B. napus* plant, a heat treatment experiment simulating heatwave episode was conducted where *B. napus* plants were exposed to high temperature treatment at the flowering stage (day/night: 30/24°C- treatment vs 20/14°C-control) for a period of 6 days, followed by 7 days of recovery. After the plants completed their lifecycle, seed yields and total aboveground were examined under heat treatment as compared to control. Furthermore, seed samples were analysed for moisture, oil, and protein concentrations as well as for fatty acid composition of the oil.

1.1.2 Identifying the impact of heat stress on the plant metabolites

To identify the impact of heat stress on the plant metabolites and in specific on glucosinolates (GSL) and sugars, the concentration of two aliphatic glucosinolates (progoitrin and gluconapin), one indole GSL (glucobrassicin) and sugars (glucose, fructose and sucrose) were quantified from *B. napus* leaves.

1.1.3 Identifying key differentially expressed genes involved in plant heat stress response

Through transcriptome profiling using RNA-Seq, followed by qtPCR for selected genes, differential gene expression analysis was performed using DESEQ2 pipeline followed by Gene Ontology (GO) and KEGG pathway analysis. The significantly enriched GO terms and biological pathways in heat treated samples as compared to control at different timepoints were identified to determine heat stress-related pathways.

1.1.4 Implementing an interactive visualisation tool to display a network of interactions between heat stress biomarkers and transcription factors

To explore the interactions between differentially expressed genes (DEGs) and transcription factors (TFs) that play a crucial role in modulating gene expression and various signal transduction pathways using Cytoscape software, a purpose-build Cytoscape plugin, NetViewer, has been developed. It was designed to automate data input and file conversion steps necessary for the visualisation task by Cytoscape and ultimately making the gene network functionality more accessible to non-technical users. Moreover, once the network is displayed, the plugin allows the user to enhance the network through adding more layers of information to the nodes such as gene description, GO terms or GO IDs.

1.2 Thesis Structure

This thesis is comprised of five chapters, of which three have been written in a manuscript format. A brief description of each chapter is provided:

Chapter 1: This Chapter is an introductory chapter to the thesis and includes its aims, objectives, and thesis structure.

Chapter 2: This Chapter is a comprehensive literature review. It summarises the latest studies on the impact of heat stress on *B. napus* with a focus on the reproductive stages and identifies the knowledge gaps existing within the last 10 years of research. This has been achieved through highlighting the impact of heat stress on the physiology and molecular biology of different growth and

developmental stages of *B. napus*, along with the underlying mechanisms involved in mediating heat stress response with a focus on phytohormones, transcription factors and glucosinolates. This review also investigated the role of polyploidy, alternative splicing, and different epigenetic mechanisms involved in heat stress response. Additionally, this review illustrated the exploitation of different systems biology approaches in studying the impact of heat stress on different genetic and physiological properties of *B. napus*.

This review paper was published in the Frontiers in Plant Science, section Plant Abiotic Stress, volume 13-2022, published on April 5th, 2022. DOI: 10.3389/fpls.2022.832147. The thesis version was updated with relevant recent studies to reflect an up-to-date literature in the context of the impact of heat stress on *B. napus.*

Chapter 3: This Chapter presents findings from a heat treatment experiment designed to investigate the transcriptomic and metabolomic responses to heat stress in *B. napus* plants under simulated field conditions. The chapter describes the results from the transcriptomic profiling using RNA-Seq to identify key DEGs and pathways under heat stress, and the metabolomic analysis using HPLC and LC-MS methods to detect changes in sugars and glucosinolates concentration in leaves in response to heat treatment. Similar findings from *Arabidopsis thaliana* and other related species were also discussed in the context of heat stress, allowing a more holistic picture of the stress response system in *B. napus* and other related species. This Chapter has been published in the Frontiers in Plant Science, section Functional and Applied Plant Genomics as a research paper titled "Prolonged heat stress in *Brassica napus* during flowering negatively impacts yield and alters glucosinolates and sugars metabolism", section Functional and Applied Plant Genomics on May 9th, 2025. DOI: 10.3389/fpls.2025.1507338.

Chapter 4: This Chapter describes NetViewer, a Cytoscape plugin designed to facilitate the visualisation of interactions between DEGs and their regulatory TFs. The chapter presents an introduction about the crucial roles of transcription factors in regulating gene expression and states the purpose of the plugin. The

methods section specifies the required input and the workflow, while the results feature a case study to model interactions between a list of DEGs identified in the heat treatment experiment described in Chapter 3 and TFs. Interactions with transcription factor AtMYC2 were selected for further exploration.

Chapter 5: This Chapter provides an overall discussion and summary of the key findings of the thesis. It also highlights the possible implementations of these findings with examples extracted from the results. Additionally, it states the limitations that were encountered during the thesis project.

Chapter 6: This Chapter presents a conclusion for this thesis and provides insights into possible future work that could improve the predictions of how heat stress will impact future yield and quality.

2 Genetic and Physiological Responses to Heat Stress in *Brassica napus*

Mariam Kourani[1] , Fady Mohareb[1*,] Faisal I. Rezwan[1†] , Maria Anastasiadi[1] and John P. Hammond[2*]

[1] Bioinformatics Group, Cranfield University, Cranfield, United Kingdom,

[2] School of Agriculture, Policy and Development, University of Reading, Reading, United Kingdom

[†] Present address: Faisal I. Rezwan, Department of Computer Science, Aberystwyth University, Aberystwyth, United Kingdom

Abstract

Given the current rise in global temperatures, heat stress has become a major abiotic challenge affecting the growth and development of various crops and reducing their productivity. *Brassica napus*, the second largest source of vegetable oil worldwide, experiences a drastic reduction in seed yield and quality in response to heat.

This review outlines the latest research that explores the genetic and physiological impact of heat stress on different developmental stages of *B. napus* with a special attention to the reproductive stages of floral progression, organogenesis, and post flowering. Several studies have shown that extreme temperature fluctuations during these crucial periods have detrimental effects on the plant and often leading to impaired growth and reduced seed production. The underlying mechanisms of heat stress adaptations and associated key regulatory genes are discussed. Furthermore, an overview and the implications of the polyploidy nature of *B. napus* and the regulatory role of alternative splicing in forming a priming-induced heat-stress memory are presented.

New insights into the dynamics of epigenetic modifications during heat stress are discussed. Interestingly, while such studies are scarce in *B. napus*, opposite trends in expression of key genetic and epigenetic components have been

identified in different species and in cultivars within the same species under various abiotic stresses, suggesting a complex role of these genes and their regulation in heat stress tolerance mechanisms. Additionally, omics-based studies are discussed with emphasis on the transcriptome, proteome and metabolome of *B. napus,* to gain a systems level understanding of how heat stress alters its yield and quality traits. The combination of omics approaches has revealed crucial interactions and regulatory networks taking part in the complex machinery of heat stress tolerance.

We identify key knowledge gaps regarding the impact of heat stress on *B. napus* during its yield determining reproductive stages, where in-depth analysis of this subject is still needed. A deeper knowledge of heat stress response components and mechanisms in tissue specific models would serve as a stepping-stone to gaining insights into the regulation of thermotolerance that takes place in this important crop species and support future breeding of heat tolerant crops.

Keywords: *Brassica napus*, heat stress, flowering, alternative splicing, epigenetic modifications

2.1 Our Changing Climate and the Potential Impacts on Crop Productivity

Over the last century, global warming has become a major environmental challenge triggered by anthropogenic emissions of greenhouse gases driving the continuous rise in ambient temperature (Pachauri et al., 2014; Zandalinas et al., 2021). Furthermore, the climate projections for 2100 suggest an increase in the number of drought affected areas by 50% (Battisti and Naylor, 2009). The combination of these environmental changes has the potential to destabilise plant growth and development and could result in drastic yield reductions (Lamaoui et al., 2018; Magno et al., 2022). Although the impact of elevated CO_2 on crops is still under debate with potential benefits on photosynthesis, Frenck et al (2011) showed that rising CO_2 could not compensate for the negative impact on yield caused by high temperature stress.

On a global scale, environmental climate change is aggravating different abiotic stresses, including heat and water deficit across many biomes. Plants, being sessile organisms, cannot escape their environment and must endure a wide variety of stresses (Saraswat et al., 2017). Although plant stress responses are dynamic and encompass a complex crosstalk between different molecular and cellular interactions to establish physiological and morphological adaptations (Krasensky & Jonak, 2012), temperature exceeding the threshold of adaptation will substantially influence plant survivability (Hasanuzzaman et al., 2014). In particular, this affects cellular functioning, which in turn, hinders plant growth and development and weakens resilience to biotic stresses (Ahmed et al., 2020).

Several studies have demonstrated the effect of heat stress, often in combination with drought or other stresses, on crop production and its impact on plant metabolism, reproduction, and physiology. These studies have reported an extensive reduction in yield of various crops (Frenck et al., 2011; Hasanuzzaman et al., 2014). Lesk et al (2016) studied the influence of extreme weather disasters on global crop production and found that, between 1964 and 2007, 1.82 and 1.19 billion metric tons of cereals were lost due to droughts and extreme heat respectively. Using four different process-based crop models, Zhao et al (2017)

demonstrated that for each 1°C rise in global mean temperature would cause a reduction in the annual production of wheat, rice, maize and soybean. In addition to its impact on yield, the increase in climate temperature has also been found to negatively impact the quality of oil in oilseed crops such as soybean, oilseed rape and sunflower, where a negative correlation between temperature and the proportion of essential fatty acids such as C18:2 and C18:3 was reported (Werteker et al., 2010; Namazkar et al., 2016; Magno et al., 2021; Magno et al., 2022; Magno et al. , 2023). Thus, the effect of climate change is inevitably impeding vital components of human diet and nutrition.

Oilseed rape (*Brassica napus* L.) is the second-largest source of oilseeds after soybean worldwide (USDA, 2025). Given its eco-physiological adaptations to temperate climates, it is currently considered the predominant oil crop in Europe (Namazkar et al., 2016; Sun et al., 2017). *B. napus* belongs to the genus *Brassica* from the mustard Family Brassicaceae (Liu et al., 2018a), where selection has given rise to several agronomically important lines such as oilseed rape, rutabaga, fodder rape, and kale morphotypes (Chalhoub et al., 2014). These are cultivated globally for vegetables, good quality oil, fodder, and bio-diesel industries (Chalhoub et al., 2014; Pokharel et al., 2020). As a cool-season crop, Brassica crops can be extremely sensitive to increasing temperatures, which significantly affect its production (Yu et al., 2014; Ahmed et al., 2020). Though studies in different Brassica species have found negative relationships between heat stress and seed yield and quality, the underlying mechanisms are still not well understood and require further investigation (Yu et al., 2014). Thus, to increase resilience against adverse environmental growth conditions and to maintain a stable oil supply to the global population, understanding and improving heat tolerance in *B. napus* is critical.

The acclimation mechanisms of plants to heat stress are complex and mediated by the activation of different physiological, cellular, and metabolic processes depending on their developmental stage (Zandalinas et al., 2018). These mechanisms normally encompass a combination of stress avoidance and tolerance (thermotolerance) approaches that vary with genotypes (Chaves et al.,

2002; Kashyap et al., 2022). At a molecular level, the abundance in transcriptomic data facilitates the analysis of the expression of genes that play regulatory roles in different stages and tissues of plant growth and development under various stress conditions. However, the challenge lies in linking this information with the relevant biological processes taking place during stress (Chaves et al., 2009). So, uncovering the controlling mechanisms for heat tolerance is therefore imperative for successful breeding programs and future production sustainability.

In this review, we outline the latest research exploring heat stress responses in *B. napus*, focusing on the physiological impact at different developmental stages along with their underlying mechanisms. We further explore new insights into the dynamics of epigenetic changes and systems biology approaches of this plant species under heat stress. However, due to the scarcity of information specific to *B. napus*, many of the reviewed findings stem from research on the model organism *A. thaliana* and other Brassica species.

2.2 Physiological Impact of Heat Stress at Different Developmental Stages

2.2.1 Impact of heat stress on seedling and vegetative growth

As thermotolerance is regulated both developmentally and tissue-specifically, addressing the impact of high temperature on different developmental stages is imperative to produce heat-tolerant cultivars capable of coping with heat stress (Figure 2.1). Different optimum temperatures also differ considerably among species or genotypes (Angadi et al., 2000; Baux et al., 2013).

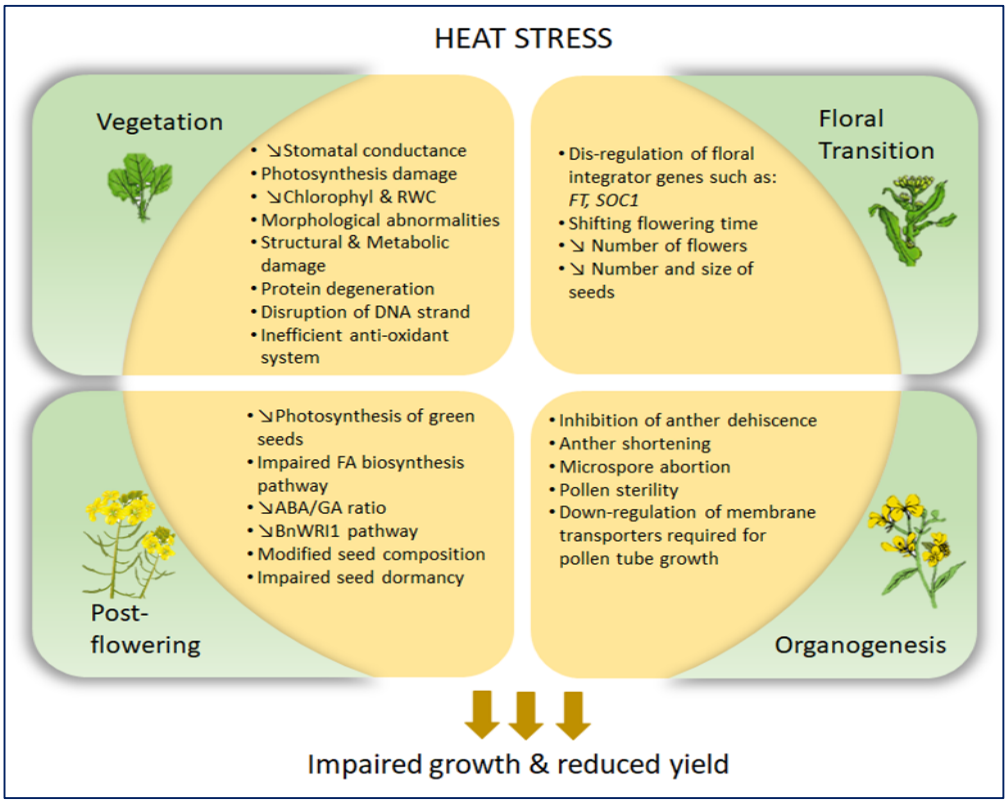

Figure 2.1 Physiological impact of heat stress at different developmental stages of *B. napus*. Heat stress negatively impacts all stages of plant development including vegetation, floral transition, post-flowering and organogenesis. RWC: Relative water content, FA: Fatty acids, ABA: Abscisic acid, GA: Gibberellic acid.

In general, heat stress takes place when soil and ambient temperature is beyond a plants' growth and photosynthetic temperature range such that, permanent harm occurs. A plants performance under heat stress is also influenced by the concomitant presence of other types of stress, such as drought (Frenck et al., 2011).

Under heatwave conditions during early vegetative growth, well-watered *B. napus* experiences an increase in saturating light (A_{sat}), transpiration (E) and in the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) (Dikšaitytė et al., 2019). The increase in ambient temperature enhances photosynthesis performance and increases above-ground growth. However, in the presence of drought, the combined stress conditions lead to severely impaired photosynthesis, which is accompanied by a profound decrease in stomatal conductance (Dikšaitytė et al., 2019). To avoid overheating, plants open their stomata and increase transpiration, thus preventing leaf damage. In contrast,

drought triggers stomatal closure as a conservative mechanism to protect plants from excessive water loss (Elferjani & Soolanayakanahally, 2018). During the flowering stage, *B. napus* responds to combined heat and drought stress by a rapid closure of their stomata. This avoids further loss of water through transpiration at the expense of evaporative cooling. Consequently, this results in an excessive increase in leaf temperature and more photosynthetic damage (Elferjani & Soolanayakanahally, 2018). This paradox requires plants to maintain a balance between avoiding overheating and minimising water loss (Zandalinas et al., 2018).

Additionally, impairment of chlorophyll biosynthesis and disruption of the biochemical reactions of photosystems were exhibited in ten-day-old *B. napus* seedlings subjected to 38°C (Hasanuzzaman et al., 2014). This was manifested through a significant reduction in chlorophyll and leaf relative water content as well as through an inefficient antioxidant defence system (Hasanuzzaman et al., 2014). During heat stress, the physiological changes that occur at the level of photosynthesis and transpiration alter the synthesis and usage of assimilates, which in turn translate into morphological abnormalities in the floral organs (Echer et al., 2014).

At the molecular level, heat stress can either induce structural damage through altering membrane proteins and stability or metabolic damage through disrupting enzymatic activities and production of toxic metabolites (Mittler et al., 2012). In *A. thaliana*, heat stress induced reactive oxygen species (ROS) production and subsequently a higher lipid peroxidation and membrane injury (Rocco et al., 2013). Moreover, protein degradation, pigment bleaching and disruption of DNA strands are also consequences of heat stress (Mathur & Jajoo, 2014). Therefore, heat stress during vegetative and seedling growth works as an induction point for a cascade of events that can impact on reproductive organs later in development and consequently lead to reduced productivity.

2.2.2 Impact of heat stress on reproduction

Since the developmental progression of plants from seed to seed is bound to the success of sexual reproduction, susceptibility to heat stress at this stage can have

deleterious implications on yield potential. Plants exhibit higher sensitivity to extreme temperature fluctuations during the reproductive stage of development than during their vegetative growth, often leading to impaired growth and reduced seed production (Zhang et al., 2017; Lamaoui et al., 2018), especially during meiosis and fertilization (Zhang et al., 2017), with the male reproductive organs being the most vulnerable (Giorno et al., 2013). This sensitivity fluctuates within different reproductive processes, such as: pollen development, pollen tube growth, pollen/pistil interactions, fertilization and embryo development (Erickson & Markhart, 2002; Young et al., 2004; Baron et al., 2012; Giorno et al., 2013; Zhang et al., 2017). The after-effects of heat stress during reproduction influence plant physiology in three main areas: reduction in the number of flowers at pre-anthesis, reduction in flower fertility, and poor pods and seeds development (Morrison & Stewart, 2002; Ibrahim et al., 2024). Nevertheless, this has been poorly studied and specific responses at each of these processes remain elusive. Similarly, in *A. thaliana* only a limited number of reports addressed the effect of heat stress on its reproductive development (Antoun and Ouellet, 2013, Guan et al., 2014, Bac-Molenaar et al., 2015; Chao et al., 2017). Under short-term heat exposure, a genome-wide association (GWA) mapping approach in *A. thaliana* revealed different QTLs associated with heat response at pre- and post- anthesis, suggesting that these genetic regulators are developmental stage-specific (Bac-Molenaar et al., 2015). Moreover, when *A. thaliana* was exposed to 37 °C for 24 hours, it exhibited a severe growth impairment of stamen filaments which prevented pollination (Baron et al., 2012). At the structural level, heat stress caused abnormalities in the reproductive organs including vacuolation, collapse within microspores and abnormally shaped pollen sacs, incapable of accumulating carbohydrates (Baron et al., 2012). In spring wheat, pre-anthesis associated events such as gametogenesis and gamete development appeared highly sensitive to high-temperature treatment as heat stress resulted in reduced floret fertility and consequently a significant reduction in seed number and weight (Bheemanahalli et al., 2019).

2.2.2.1 Floral progression

In agriculture, flowering is a key prerequisite stage for the transition from the vegetative phase to reproduction. It is regulated by the expression of a set of floral integrator genes such as *Flowering Locus T (FT)*, to activate the meristem identity genes *LEAFY (LFY), APETALA1 (AP1), SEPALLATA3 (SEP3)* and *FRUITFULL (FUL)*, causing the transition from vegetative to a floral meristem (Blümel et al., 2015). Under different stress conditions, plants utilise different mechanisms to alter flowering time (FTi) as a strategy to ensure the success of reproduction (Figure 2.2; Simpson, 2004; Ko et al., 2010; Blümel et al., 2015; Kazan & Lyons, 2016). However, these mechanisms are known to impact crop productivity (Kazan & Lyons, 2016).

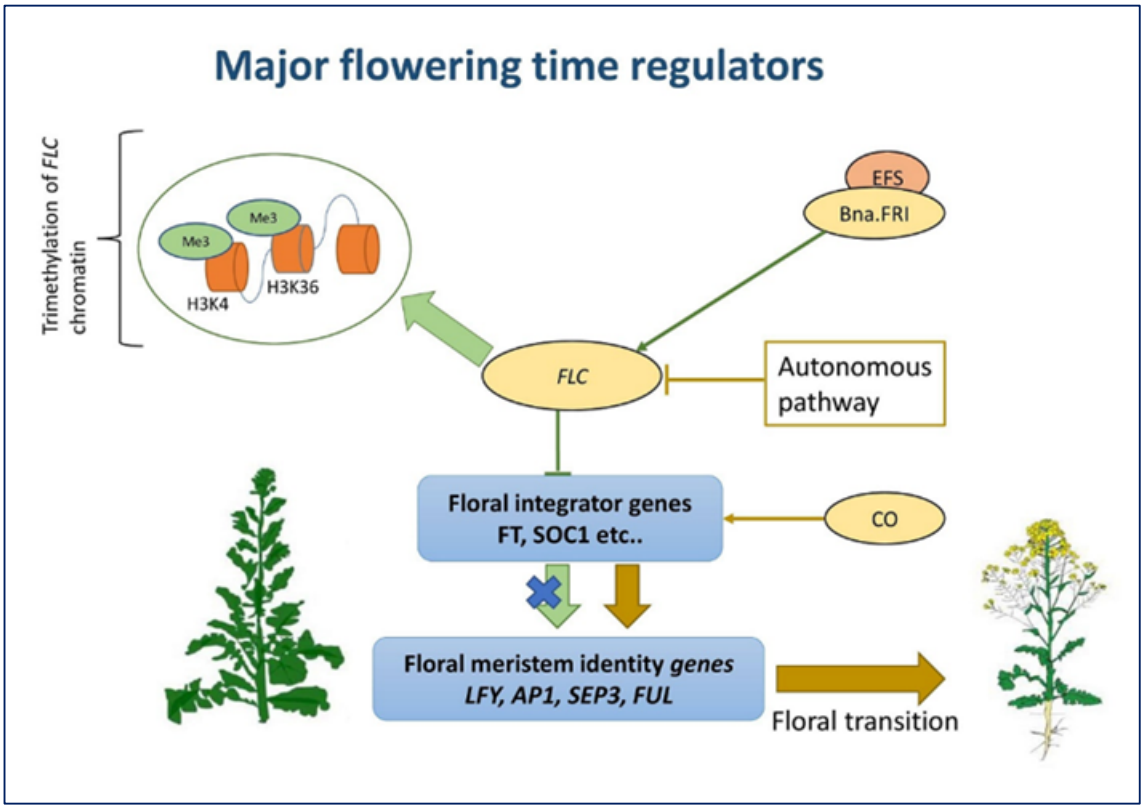

Figure 2.2 Major flowering time regulators in *B. napus*. Flowering is regulated by the expression of a set of floral integrator genes to activate the meristem identity genes causing the transition from vegetative to a floral meristem. Me3: Trimethylation, H3K4: Histone 3 Lysine 4, H3K36: Histone 3 Lysine 36, EFS: Early Flowering in Short Days, BnaFRI: *B. napus* homologues of *FRI*, *FLC*: Flowering Locus C, *FT*: Flowering Locus T, *SOC1*: Suppressor of Overexpression of CONSTANS 1, CO: CONSTANS, *LFY*: *LEAFY*, *AP1*: *APETALA1, SEP3: SEPALLATA3, FUL: FRUITFULL*.

During flowering, energy demand spikes due to different processes, such as: formation of flowers, nitrogen use efficiency and reduced photosynthesis (Schiessl, 2020). Such crucial physiological period has been found sensitive to various biotic and abiotic stress and particularly to heat, which has a pronounced effect on the timing of flowering, depending on its duration, frequency, and intensity (Lohani et al., 2020b). In a split-plot experiment, different genotypes of *B. napus* were exposed to high-temperature stress (31 °C/14 °C - day/night) during flowering across two sowing periods (Koscielny et al., 2018). Results showed a reduction in yield by 55% in the heat treatment during winter and by 41% during the subsequent autumn experimental run (Koscielny et al., 2018). At different developmental stages, severe damage to reproductive organs of three Brassica species (*B. juncea* L., *B. napus* L., *B. rapa* L.) occurred when exposed to 35 °C/15° C for 7 days resulting in yield reductions (Angadi et al., 2000). It was noted that yield was affected by heat stress at flowering more than at pod development, indicating a vital developmental threshold passed by pods rendering them more heat tolerant. Interestingly, among the three studied Brassica species, only *B. napus* could not recover completely after heat stress was removed, producing abnormal, plump, and short pods (Angadi et al., 2000). A reduction in seed yield was observed following 29.5 °C temperature treatment during flowering, primarily due to the reduction in the number of flowers and in the number and size of seeds produced per flower (Morrison & Stewart, 2002).

During flower opening in rice, air temperature is crucial in determining the fate of reproduction (Bheemanahalli et al., 2017) and the resulting earlier or delayed flowering can negatively impact crop growth and quality. In a recent study carried out by Pokharel et al. (2020), high night temperature stress-induced a shift in *B. napus* peak flower opening time into earlier and cooler morning hours, suggesting an adaptation towards the heat escape response, yet this was accompanied by a significant reduction in yield. Whilst *B. napus* is not strictly dependent on pollinators, it has been shown that pollinators enhance seed yield and quality (Zou et al. 2017). So, shifting flowering time drives the flower opening out of sync with pollinator activity, and could potentially lead to reductions in yield.

Although FTi regulator genes and their associated networks have been well characterised in *A. thaliana*, the conservation of these genes in other Brassica species indicates that their regulation takes place in the same way as in this model system. However, environmental adaptation has led to the evolution of new FTi regulator genes with no functional equivalence in *A. thaliana* (Blümel et al, 2015). Moreover, some FTi gene copy numbers were found to either change or lose functionality over evolutionary periods resulting in sub-functionalisation (Schiessl, 2020). As a result, it is difficult to functionally characterise crop FTi regulators and pathways based on inferences from this model species. This was manifested in a study by Del Olmo et al. (2019) when *B. rapa* delayed flowering was associated with reduced *BraA.FT.a* mRNA levels upon high-temperature treatment. High levels of H2A.Z were also found to occupy the *BraA.FT.a* locus, which in turn affected chromatin conformation and hindered its accessibility. The same conserved chromatin regulatory mechanism in *A. thaliana* showed an opposite trend and resulted in accelerated flowering (del Olmo et al., 2019). This brings attention to the fact that thermosensory pathways perform differently in different crops to alter FTi regulators.

Considering the scarcity of studies addressing the impact of increased ambient temperature on FTi-related signalling pathways in *B. napus*, the continuous rise in global temperature will need to drive future research to explore these pathways and the underlying regulators under heat stress. Such studies will help identify key regulators, direct genetic modifications to candidate genes involved in these pathways and to provide more reliable data for breeding programmes to deliver heat stress tolerant cultivars.

2.2.2.2 Organogenesis

The next stage after floral transition is the differentiation of floral organs or organogenesis. In *B. napus* as in other flowering plants, gametogenesis and reproduction are highly vulnerable to high-temperature stress (Angadi et al., 2000; Young et al., 2004). From inhibition of anther dehiscence and anther shortening to reducing pollen germinability and viability (Rahaman et al., 2018), the impact of heat stress on different reproductive organs has been reported in

various species, such as: wheat, soybean, Brassicas and tomato (Giorno et al., 2013; Draeger & Moore, 2017; Djanaguiraman et al., 2019). Moreover, heat stress during microsporogenesis leads either to microspore abortion or pollen sterility (Lohani et al., 2020b). The variety of affected species suggests the existence of common processes involved in heat stress-related infertility (Kim et al., 2001; Rang et al., 2011; Sage et al., 2015) and yield loss (Morrison & Stewart, 2002; Frenck et al., 2011; Zhao et al., 2017). However, in *B. napus*, such processes are still not very well studied.

In *A. thaliana*, during anther development, heat stress causes male sterility in a stage-specific manner (Kim et al., 2001). Disruption of male meiotic processes was associated with abnormal pollen development and morphological analysis with a failure in the separation of pollen mother cells and a subsequent microspore differentiation (Kim et al., 2001). Heat stress also caused a dramatic down-regulation in membrane transporters required for pollen tube growth such as K^+ and carbohydrate co-transporters, which would adversely impact pollination (Poidevin et al., 2020). Moreover, in *B. napus*, anthers exposed to high temperature triggered an immediate heat stress response through the rapid activation of the ER-UPR signalling pathway mediated by ER membrane-anchored transcription factor (TF) (Lohani et al., 2022). To discover the effect of heat stress after flowering initiation in *B. napus*, Young et al. (2004) reported that following heat exposure at 35 °C, both micro and megagametophytes were found thermosensitive and resulted in a significant reduction in pollen viability and germinability as well as in seed development. An increase in temperature was also reported to induce sterility and lead to a decrease in fertility rate, pod-setting ratio and seed number per pod (Zhang et al, 2012). Similarly, in a comparative transcriptomic analysis, Tang et al (2019) found that genes encoding HSPs, skeleton proteins, GTPase and calmodulin were potentially involved in the mechanism of thermosensitive genic male sterility (TGMS) under high temperature inducement. Moreover, genes encoding plant hormone signaling pathways, (auxin, gibberellins, jasmonic acid, abscisic acid, brassinosteroid signalling), along with some well-known TFs (MADS, NFY, HSF, MYB/C and

WRKY) were also found involved in the regulation of TGMS in the flowers grown under 25°C (Tang et al., 2019).

Although many studies have reported that male reproductive organs are more sensitive to heat stress than female reproductive organs, changes in the female reproductive organs following heat stress have also been documented (Hedhly, 2011). Additionally, according to the identity of the cell, the female gametophyte possesses a unique and differentially mediated response to heat stress (Ambastha & Leshem, 2020). These findings suggest that temperature plays a vital role in affecting the fertility of pollen and the morphology of the flower reproductive organs.

2.2.2.3 Post-flowering

The thermotolerance of seed setting rate and filling, are also crucial in determining grain yield and composition. During later reproductive growth, the biosynthesis and deposition of oils and proteins in seeds take place at the seed filling stage. Hence, it is imperative to strictly regulate this stage for the generation of high-quality and balanced oil (Brunel-Muguet et al., 2015). Photosynthesis in *B. napus* green seeds and pods induces the formation of the necessary components of the fatty acid biosynthesis pathway (Huang et al., 2019). This pathway was found to be regulated by the transcription factor BnWRI1 (Wu et al., 2014). In a recent study, heat stress suppressed oil deposition in developing seeds and impaired fatty acid biosynthesis where the inhibition of the *de novo* fatty acid biosynthesis pathway was associated with the inhibition of photosynthesis and down-regulation of many genes in the BnWRI1 pathway (Huang et al., 2019). These findings have highlighted the crucial function of BnWRI1 as a key regulator within the intricate regulatory systems of heat stress and biosynthesis of storage compounds. Brunel-Muguet et al. (2015) also showed that high temperature during seed development modified seed composition and impaired the acquisition of seed dormancy. This was accompanied by a decrease in abscisic acid/Gibberellic acid (ABA/GA) ratio, which plays a role in controlling seed composition and storage capacity (Brunel-Mugugt et al., 2015). Similarly, the total lipids and all the fatty acids studied were

significantly lower in *B. napus* seed oil when temperature was raised from 19°C to 24°C, resulting in imbalanced proportions of essential fatty acids (Namazkar et al., 2016). Canales et al. (2023) also found that post-flowering temperature increase negatively affected seed yield and number, producing genotype-specific responses (Canales et al., 2023).

Interestingly, not only high day temperature was found to impact seed quality and yield, but also high night temperature was reported to have detrimental effect on yield and seed fatty acid composition in *B. napus*. During flowering and silique-filling stages. Pokharel et al. (2020) reported that high night temperature caused significant alterations in the reproductive organs and led to a substantial reduction in seed yield and number of siliques, which was further explained by the thermal impairment of photosynthetic assimilation enzymes. Likewise, exposure of susceptible *B. napus* cultivars to high night temperature stress between flowering and seed filling stages also induced a profound reduction in total fatty acids and in fatty acid composition in seeds (Zhou et al., 2018). It is worth noting that the magnitude of heat stress and the cardinal temperature unequivocally contribute to the degree of impact on each crop species (Mittler et al., 2012).

In summary, high-temperature stress induces adverse effects on different processes and organs during *B. napus* reproduction. From floral transition to seed filling, such environmental threats will eventually translate into unfavourable agronomic and quality traits and ultimately challenge the production of seed oil with strong nutritional values. Knowledge about heat stress during these stages is limited and more insight on this topic will help scientists and breeders to design strategies to protect this important crop species from the impact of the stress potentially caused by climate change presently and in the future.

2.2.3 Impact of heat stress on postharvest seed storage

During postharvest storage, high-temperature stress has also been found to impact the safety and nutritional qualities of stored seeds (Krasucki et al., 2002). Brassica seeds are rich in bioactive compounds that exhibit antioxidant action and can play a vital role in reducing blood cholesterol levels (Ratnayake & Daun,

2004). In *B. napus* seeds, the concentrations of phytosterols can be twice that of sunflower and soybean oils (Vlahakis & Hazebroek, 2000), and the content of phenolic compounds can be 10-fold greater than in seeds of other crops (Siger et al., 2018). The abundance of such important biologically active compounds is sensitive to storage conditions. High temperature and moisture content were associated with the deterioration of seeds and degradation of their biochemical contents (Gawrysiak-Witulska et al., 2012). After 18 days of storage at 25°C and 30°C, the amount of phytosterols decreased significantly, reaching 24% and 58% less in *B. napus* seeds with a moisture content of 15.5% (Gawrysiak-Witulska et al., 2012). Under similar storage conditions, tochopherols dropped by 14.4% while plastochromanol-8 loss ranged from 4 to 24% (Gawrysiak-Witulska et al., 2011). These natural antioxidants play a crucial role in determining lipid stability and inhibition of auto-oxidation in stored seeds (Olejnik et al., 1997). As a result, the degradation of such compounds not only alters the quality of oil but can also lead to the formation of oxidized derivatives with cytotoxic properties (Rudzinska et al., 2009). To investigate the effect of inappropriate storage conditions (13.5% moisture content and 25°C temperature) on seed quality in *B. napus*, Siger et al. (2018) reported an intense growth of fungi along with a decrease in Sinapic acid derivatives, the main phenolic compounds in *B. napus* (Siger et al., 2018). The development of fungal growth not only reduces the time of safe storage but also causes a risk of seed deterioration and mycotoxin contamination posing a serious threat to human and animal health (Siger et al., 2018). Furthermore, fungal growth and respiration could also result in the production of additional heat and moisture in the seeds (Mills, 1996), which further exacerbates the problem of temperature stress and microbial infestation.

2.3 Plant Response Mechanisms to Heat Stress

Plants respond to environmental adversities, such as changes in ambient temperature through a cascade of signalling pathways, resulting in cellular readjustments at the transcriptome, epigenome, proteome and metabolome levels. Eventually, these adjustments provide the required balance through multiple adaptive strategies to ensure survival under the new conditions. Such

intricate machinery involves an interconnected crosstalk between different components of the cell, initiated by stress perception, carried out through stress signalling and ultimately end with the expression of a specific set of downstream stress-induced genes that result in a phenotypic response to the stress (Table 2.1).

Different sensors are involved in the initial stress perception (Lamaoui et al., 2018; Baillo et al., 2019), and these include plasma membrane mediated calcium flux, endoplasmic reticulum (ER) and cytosolic unfolded protein response (UPR) sensors, and decreased histone occupancy in the nuclei (Mittler et al., 2012). The generated signals are then integrated through a network of multiple events that includes calcium fluxes, calmodulin, phosphatases, transcriptional regulators, hormones, and protein kinases such as CDPKs, MAPKs (Figure 2.3) (Verma et al., 2016; Lohani et al., 2020a). Hence, the complexity of heat response lies in the broad range of pathways involved, which appear to be tissue and developmental stage specific (Zhang et al., 2017).

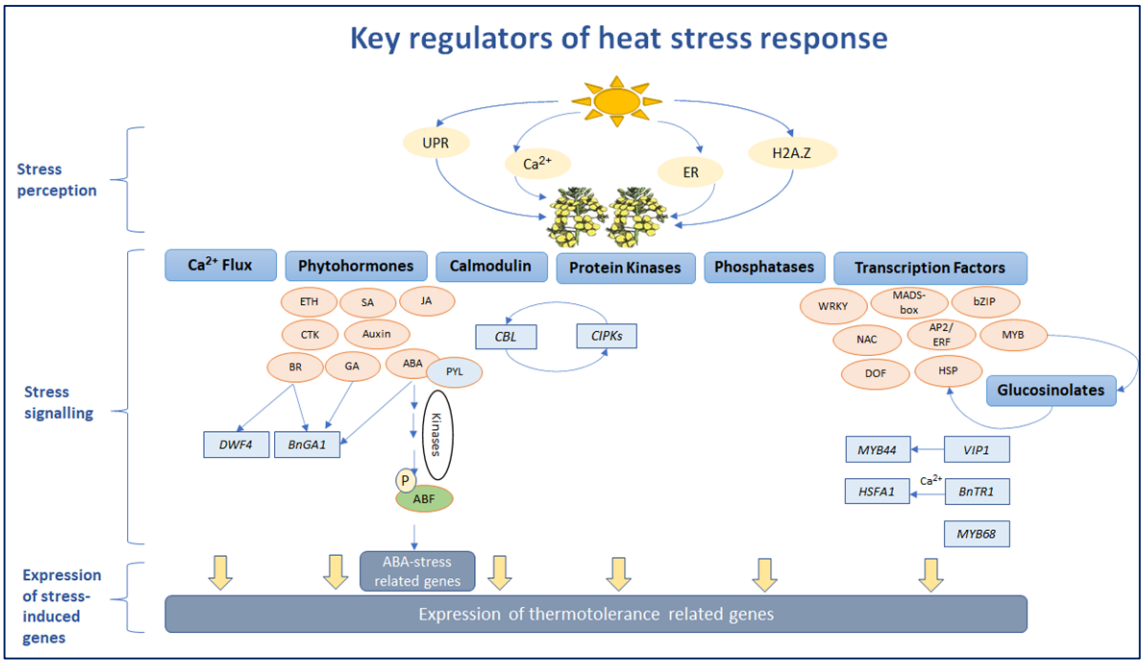

Figure 2.3 Regulatory and signalling events involved in plant heat stress response. Following initial stress perception, the generated signals induce the expression of thermotolerance-related genes through a complex network of events involved in the stress response. UPR: Unfolded protein response, Ca^{2+}: Calcium, ER: Endoplasmic reticulum, H2A.Z: Histone variant, ETH: Ethylene, SA: Salicylic acid, JA: Jasmonic acid, CTK: Cytokinin, BR: Brassinosteroids, GA: Gibberellic acid, ABA: Abscisic acid, PYL: Pyrabactin resistance 1-like gene, ABF: ABA-responsive element binding factor, DWF4: A BR biosynthetic *dwarf 4* gene, CBL: Calcineurin B-like , CIPKs: CBL-interacting protein kinases, WRKY: TF containing WRKY-protein domain, MADS-box TF containing MADS-box protein domain, bZIP: Basic Leucine Zipper TF, NAC: TF containing N-terminal NAC domain, AP2/ERF: APETALA2/Ethylene Responsive Factor, MYB: Myeloblastosis viral oncogene homolog, DOF: DNA-binding with one finger, HSP: Heat shock protein, VIP1: VirE2-interacting protein 1, HSFA1: Heat shock factor A1, BnTR1: *B. napus* thermal resistance 1 gene.

Table 2.1 Heat stress responsive genes identified in *B. napus* and related species.

Gene/ Gene Family	**Expression***	**Function**	**Species**	**Reference**
BnaDRMc	Up-regulation	DNA methylase & de-methylases	*B. napus*	Fan *et al*, 2020
BnaDNMT2	Up-regulation			
BnaDRMa	Up-regulation			
BnaDRMg	Up-regulation			
BnaDRMh	Up-regulation			
BnaROS1a	Down-regulation			
BnaCMTa	Down-regulation			
BnaMETs	Down-regulation			
BnaHsf15/16 (subclass A2)	Down-regulation	Heat shock transcription factors	*B. napus*	Zhu *et al*, 2017
BnaHsf47 (subclass B1)	Down-regulation			
BnaHsf50 (subclass B2A)	Down-regulation			
BnaHsf21/22 (subclass A4A)	Up-regulation			
BnaHsf46 (subclass B1)	Up-regulation			
BnaHsf17/18 (subclass A3)	Up-regulation			
BnaHsf43 (subclass A8)	Up-regulation			
PYR1-3	Up-regulation	ABA receptor PYR/PYL (pyrabactin resistance 1-like) family	*B. napus*	Di *et al*, 2018
PYR1-4	Down-regulation			
PYL1-2	Up-regulation			
PYL2-2	Down-regulation			
PYL3-1	Up-regulation			
PYL4-2	Up-regulation			
PYL4-6	Down-regulation			
PYL5-4	Up-regulation			
PYL6-1	Up-regulation			
PYL7-2	Up-regulation			
PYL8-5	Down-regulation			
PYL8-6	Down-regulation			

Gene/ Gene Family	Expression*	Function	Species	Reference
PYL 9-1	Down-regulation			
PYL 9-2	Up-regulation			
BraA.FT.a	Down-regulation	Flowering time regulator gene	*B. rapa*	del Olmo *et al*., 2019
ABA2/4 (in cauline leaves & inflorescence meristems)	Up-regulation	ABA biosynthesis genes	*A. thaliana*	Baron *et al*., 2012
NCED2/5/6 (in cauline leaves)	Down-regulation			
NCED5/6 (in developing siliques)	Up-regulation			
NCED3 (in cauline leaves)	Down-regulation			
NCED3 (in inflorescence meristems & developing siliques)	Up-regulation			
CYP707A1/4 (in cauline leaves)	Up-regulation	ABA catabolism		
CYP707A2/3 (in cauline leaves)	Down-regulation			
ABCG25/40 (in cauline leaves & inflorescence meristems)	Down-regulation	ABA transport & homeostasis		
UGT71B6 (in cauline leaves & inflorescence meristems)	Down-regulation			
AtBG1 (in cauline leaves)	Up-regulation			
AtBG1 (in inflorescence meristems)	Down-regulation			
BnGA1	Down-regulation	A putative G protein α subunit (Gα)	*B. napus*	Gao *et al*., 2010
B. napus thermal resistance gene 1 (*BnTR1*)	Up-regulation	Membrane-bound RINGv (Really Interesting New Gene Variant) protein with E3 ligase activity	*B. napus*	Liu *et al*., 2014
BrMYB28	Up-regulation	Transcription factors	*B. rapa*	Justen & Fritz, 2013

Gene/ Gene Family	Expression*	Function	Species	Reference
BrMYB34	Up-regulation			
Hsp90	Up-regulation	Transcription factor	*A. thaliana*	Ludwig-Müller & Krishna, 2000
BnaCBL1/9	Up-regulation	Ca2+ sensors and regulators of CBL-interacting protein kinases	*B. napus*	Zhang *et al*., 2014a
BnaCBL10	Down-regulation			
BnaCIPK3/6	Up-regulation			
BnaCIPK1/11/12/26	Down-regulation			
BnGLYI-3	Up-regulation	Glyoxalase System	*B. napus*	Yan *et al.,* 2016
BnWRI1	Up-regulation	De novo fatty acid biosynthesis pathway	*B. napus*	Huang *et al.*, 2019

*Expression: The state of the gene under heat stress, which could be up-regulated or down-regulated.

2.3.1 Phytohormones

One of the principal regulators of abiotic stress responses are phytohormones, which encompass several compounds, such as: abscisic acid (ABA), brassinosteroids (BR), salicylic acid, Jasmonates (JA), ethylene, auxins, cytokinins, and gibberellins (Verma et al., 2016). These phytohormones have essential roles in protecting plants against environmental stress (Lohani et al., 2020a). The intricate signalling role of these compounds is carried out either directly or through crosstalk between interacting arrays of various secondary messengers (Verma et al., 2016). During biotic and abiotic stress responses, ABA plays a powerful role in mediating plant adaptation to stress (Baron et al., 2012). In *A. thaliana*, temperature stress induced the differential expression of several ABA biosynthesis genes (*ABA1, ABA2, ABA4, AAO3, NCED3*) in an organ-specific manner (Baron et al., 2012). Moreover, in *B. napus,* Di et al. (2018) identified 14 differentially expressed (DE) pyrabactin resistance 1-like (PYL) genes, which play core roles in ABA signalling networks, at different time points of heat treatment. *BnPYR1-3, BnPYL1-2, BnPYL3-1, BnPYL4-2, BnPYL5-4,*

BnPYL6-1, BnPYL7-2, and BnPYL 9-2 were up-regulated while *BnPYR1-4, BnPYL2-2, BnPYL4-6, BnPYL8-5, BnPYL8-6, BnPYL9-1* were down-regulated. Also, some *BnPYL* genes such as *BnPYR1-3, BnPYL1-2*, and *BnPYL7-2* had similar expression patterns under drought, salinity, and heat treatments suggesting that these genes might be important candidates for improving tolerance to abiotic stress (Di et al., 2018). In addition to its role during abiotic stress response, tissue ABA concentrations were also found to influence seed components during grain filling (Chandrasekaran et al., 2014). Under high-temperature conditions, changes in ABA content in seeds were involved in the modification of downstream expression of genes associated with the biosynthesis of seed storage compounds in castor beans (Chandrasekaran et al., 2014). Thus, the quality determining characteristics of seeds such as seed germination, vigour and storability can be negatively affected by the impact of heat stress through inducing changes in the concentration of important hormonal signalling molecules (Gao et al., 2010).

Although many studies addressed the role of ABA as a major stress-responsive hormone, the role of other stress hormones has also been studied in response to various abiotic stress factors. For example, high concentrations of ABA and BR and low concentrations of gibberellic acid 3 (GA3) significantly induced *BnGA1* expression, a gene that encodes a putative G protein α subunit (Gα) in *B. napus* (Gao et al., 2010). *BnGA1* was initially found strongly expressed at the bolting and fruiting stages. However, different abiotic stresses prompted different expression patterns with an apparent low expression in response to heat. This reduction in transcription could be attributed to one of the physiological events in decelerating cellular processes under temperature stress (Gao et al., 2010). BR were also found to enhance seedling tolerance to drought and cold stress and counterbalance the inhibitory impact of salt stress on seed germination in *A. thaliana* and *B. napus* (Kagale et al., 2007). Transgenic *B. napus* overexpressing the *A. thaliana* BR biosynthetic gene *DWF4* exhibited increased seed yield and enhanced stress response to heat and drought (Sahni et al., 2016). At higher concentrations, stress hormones such as JA inhibit growth as a strategy to mobilize the metabolic reservoir between growth and stress response processes

(Heinrich et al., 2013). The overexpression of BR biosynthetic gene *DWF4* induced a down-regulation of several JA biosynthesis and signalling genes, suggesting a mechanism played by BR to balance the inhibitory effect of JA on growth (Sahni et al., 2016). Furthermore, analysis of global transcription profiles after heat stress showed diverse expression patterns between different hormone signalling pathways. While genes encoding proteins involved in the biosynthesis of ethylene and GAs were all reduced, genes encoding auxin synthesis, binding and transport were all induced in seeds (Yu et al., 2014). These studies suggest a complex interaction among different phytohormones, through switching the activities of certain genes as needed depending on the stress situation. Thereby, functioning as key regulators to achieve a state of homeostasis and maintain a balance between stress tolerance and plant productivity.

2.3.2 Transcription factors

Different TF families have been identified in plants such as AP2/ERF, MYB, HSP, bZIP, NAC, DOF, MADS-box, etc. (Ghorbani et al. 2020). In a recent study, Wang et al. (2018) identified 2,167 TFs belonging to five different families in *B. napus* with 7.6% identified as novel and unique to *B. napus*. Interestingly, among the DEG identified in response to different abiotic stresses, 70% were responsive to heat after 12 hours of treatment and these include: 62 BnAP2/EREBPs, 32 BnbZIPs, 61 BnMYBs, 40 BnNACs and 27 BnWRKYs (Wang et al., 2018).

During plant heat stress, the heat shock response is a conserved response during which heat shock transcription factors (HSF) regulate heat shock proteins (HSP) through recognising and binding to conserved palindromic motifs in the promoter region of heat-stress responsive genes (Scharf et al., 2012). As a result, HSP bind to denatured proteins and inhibit their aggregation, thus maintaining protein homeostasis and eventually thermotolerance (Su & Li, 2008). Unlike the limited number of HSF encoding genes in yeast and animals, plants possess large and diverse HSF gene families with complex species-specific functions (Scharf et al., 2012). There are 21 HSF genes in the *A. thaliana* genome (Nover et al., 2001)**,** 25 HSF genes in the rice genome (Chauhan et al., 2011), and 56 HSF genes in the wheat genome (Xue et al., 2014). Interestingly, 64 HSF encoding genes were

discovered in *B. napus*, rendering it the largest HSF family in plants identified so far (Zhu X. et al., 2017). The diversity of HSF genes in plants has likely arisen from whole-genome duplications events that took place during evolution (Chalhoub et al., 2014). In the case of *B. napus*, it is believed that the allopolyploidy process contributes significantly to the expansion of its HSF gene families, a key element for its adaptation and survivability under different environmental conditions (Zhu X. et al., 2017).

An example of a novel yet conserved gene that plays a key role in the thermal resistance in *B. napus* is *Brassica napus thermal resistance gene 1* (*BnTR1*). BnTR1 belongs to a new type of membrane-bound RINGv (Really Interesting New Gene Variant) protein with E3 ligase activity and was found to control the expression of HSFs related genes such as *HSFA1a* through the regulation of cytosolic Ca^{2+} concentration under heat stress, thus enhancing thermotolerance (Liu et al., 2014). It was also noticed that *BnTR1* was able to increase heat tolerance in *Oryza sativa* without producing any defective growth phenotypes as seen with other thermal-resistant genes, such as: *DREB1A*, *AtHSFA3* and *BhHSF1* (Yoshida et al., 2008; Hong et al., 2009; Zhu et al., 2009). Liu et al (2014) also found that the expression of *BnTR1* increased the yield of rice suggesting its role in alleviating adverse environmental impacts on the crop through functioning as a master regulator of heat stress-responsive genes.

Other heat-responsive markers have also been identified in *B. napus* heat stress tolerance mechanisms. MYB genes were concomitantly reprogrammed and induced in the silique wall and seeds of *B. napus* following exposure to heat stress (Shamloo-Dashtpagerdi et al., 2018). After constructing a gene regulatory network of key stress regulatory genes that respond to drought and salt tolerance in *B. napus*, comparative genomic analysis from *A. thaliana* and *Eutrema salsugineum* and their *B. napus* homologs showed that transcription factor *MYB44* and its direct regulator *VIP1* had the largest number of connectivity and achieved the highest centrality values among the studied genes, implying its pivotal role in the regulatory network and ultimately in the plant stress tolerance system (Shamloo-Dashtpagerdi et al., 2018). At the physiological level, TFs also play important roles in plant growth and development. In *A. thaliana*, the ectopic

expression of transcription factor *AtMYB68* in *B. napus* following severe heat stress at flowering enhanced pollen viability and led to significant improvement in yield (Deng et al., 2020). These findings show the key role of TFs in the reproductive heat tolerance system pertained by *A. thaliana* and other related species (Yu et al., 2014) and altogether suggest a conserved stress response with key resistance factors in different tissues and plants (Yu et al., 2014; Huang et al., 2019).

2.3.3 Glucosinolates

The involvement of TFs in modulating glucosinolates (GSL) concentrations under heat stress has also been reported. GSL are secondary metabolites found almost exclusively in the Brassicaceae (Fahey et al., 2001). Although they are known to be present in all parts of the plant, their concentration varies with different tissues and developmental stages (Fieldsend & Milford, 1994). While seeds are considered the major compartment for their storage, their synthesis takes place after flowering in the leaves and the reproductive tissues such as the silique wall (Nour-Eldin et al., 2012). Some GSL play protective roles in the plant stress defence mechanisms against biotic and abiotic stress (Ludwig-Müller & Krishna, 2000; Martínez-Ballesta et al., 2013). Elevated temperature was found to increase GSL concentrations in *B. rapa*, with a noticeable involvement of BrMYB28 and BrMYB34 transcription factors in this process (Justen & Fritz, 2013). In *A. thaliana*, mutants deficient in GSL metabolism experienced reduced expression of cytoplasmic Hsp90 and less thermostability under elevated temperature (Ludwig-Müller & Krishna, 2000), whilst the supply of two exogenous GSL hydrolysis products increased the expression of HSPs and improved thermotolerance (Hara et al., 2013). Conversely, after heat treatment of 20-day-old siliques of *B. napus*, Yu et al. (2014) reported a dramatic decrease in the expression of several transcripts involved in GSL metabolism such as those involved in the indolic and benzenic pathways, suggesting the suppression of GSL biosynthesis in the silique wall due to heat stress. This shows that down-regulation of such pathways under stressful conditions could act as a protective measure to conserve energy (Yu et al., 2014), but also to optimise redistribution

of GSL (Martínez-Ballesta et al., 2013), ensuring survival under adverse environmental conditions. Such opposite trends in GSL concentrations in response to heat treatment and the involvement of distinct signalling molecules, such as TFs, reveal a complex stress response powered by various factors such as the intensity and duration of stress as well as the plant developmental stage. As little is known about the biological functions of GSL metabolism under heat stress, there is a need for more research to further understand the auxiliary roles of individual compounds in modulating the plant physiological status. Thus, revealing the interconnectivity between different components and pathways involved in an effective heat tolerance system in *B. napus.*

2.4 Impact of Polyploidy and Alternative Splicing on Heat Stress

Polyploidy or whole-genome duplication (WGD) is one of the major forces in plant evolution (Soltis et al., 2014). The resulting speciation and biodiversity have influenced the structure and evolution of plant genomics, giving rise to highly dynamic genomes with broad gene expression changes (Wang et al, 2019a). Most of the world crops have undergone polyploidy including wheat, cotton, tobacco, oats, potato and coffee. A recent allopolyploidy event took place around 7,500 years ago between ancestors of *Brassica oleracea* and *Brassica rapa* resulting in the formation of the highly adaptive species *B. napus*. This in addition to earlier polyploidisation events, has caused an aggregate of 72x genome multiplication since the origin of angiosperms (Chalhoub et al., 2014). These evolutionary events have led to the acquisition of strong and valuable agronomic traits, giving these species the ability to adapt and survive different climatic conditions. For example, one copy of *Flowering Locus C (FLC)*, a key adaptive gene controlling vernalisation and photoperiod responses, exists in *A. thaliana*, while four copies exist in *B. rapa* and *B. oleracea* and nine or more in *B. napus* (Chalhoub et al., 2014).

There has been an increasing interest in studying the expression patterns of such duplicated genes. In polyploidy, duplicated genes may also be differentially spliced by the action of alternative splicing (AS) (Wang et al, 2019a). This

mechanism is characterised by the excision of introns and the production of differential exon combinations. The resulting genetic modifications alter transcript abundance and gene expression and eventually produce variant proteins (Liu et al., 2018b). During plant growth and development, AS plays key roles in different biological processes and most importantly, in response to biotic and abiotic stress (Guo et al., 2020; Wang et al., 2023b). It is becoming increasingly evident that AS is not a by-product of stress but a regulatory part of the larger stress response (Kannan et al., 2018). Heat stress was found to induce an increase in AS events in genes that are associated with abiotic stress response such as those relating to protein folding (Chang et al., 2014; Kannan et al., 2018). In a recent study, analysis of differential AS landscape among A, B & D wheat subgenomes under salt treatment found that homologous genes displayed differential AS responses under salt stress and more AS events were identified in the salt-sensitive cultivar than in the salt-tolerant one. Interestingly, specific AS events were found induced at different time points of salt stress (Guo et al., 2020). When multiple stresses were assessed, combined heat and drought stresses (HD) induced specific AS events compared with those induced by individual stresses. Upon comparison of differentially spliced genes (DSGs) and differentially expressed genes (DEGs), 40% of DSGs were overlapping with DEGs under heat stress and HD conditions (Liu et al., 2018b). Additionally, Wang et al. (2023b) found that the linseed response to the repeated drought treatment was coregulated by both transcription and AS. These findings demonstrate the coordination between AS regulation and transcriptional regulation to orchestrate an effective stress response and ultimately contribute to abiotic stress tolerance. Similarly, in *B. napus*, RNA-Seq of plants subjected to cold, heat, and drought stress treatments also showed variation in expression and AS levels within the duplicated genes, with an overall A_T subgenome biases in gene expression and C_T subgenome biases in the extent of AS (Lee & Adams, 2020). Saminathan et al. (2015) hypothesized that polyploidy can influence AS, resulting in transcriptome modifications. In two independently synthesized lines of *B. napus*, alterations in AS events after polyploidy were reported (Zhou et al., 2011). Also, in response to heat, the number and type of AS events in the two related species *A. thaliana*

and the highly thermotolerant polyploid *Boechera depauperate* varied significantly, suggesting a role for polyploidy in this variance. Moreover, this also demonstrates that AS responses to heat stress are species-specific (Kannan et al., 2018). In summary, the divergence in gene expression and AS patterns within the homologous genes may contribute to gene functional evolution which explains the flexibility of polyploids when responding to different abiotic stress (Zhang et al., 2010; Lee & Adams, 2020).

To further explore the role of AS in heat stress, Ling et al. (2018) investigated the existence of a “splicing memory” which is thought to be a key factor for achieving thermotolerance in *A. thaliana*. As intron retention (IR) is the main AS form present in plants, the study aimed to investigate whether primed plants would preserve the same levels of these events during the recovery and memory-establishment phases and whether their response would differ from the response of non-primed plants after a recurrent exposure to heat. It was observed that heat-stressed plants accumulated unprocessed transcripts through splicing repression with marked levels of IR events, which eventually decreased to normal levels during recovery. In the second heat exposure, primed plants exhibited a very distinct response from non–primed plants. Primed plants remembered to undergo splicing and correctly processed transcripts similar to control plants under normal conditions. As a result, primed plants maintained a “splicing memory” that can carry out correct splicing and produce the necessary transcripts and proteins needed for the growth and development of plants after the cessation of stress, and subsequently ensuring the survivability of plants following another stress event (Ling et al., 2018). This further supports the role of AS as an integral component in forming a priming-induced heat-stress memory in *A. thaliana* (Sanyal et al., 2018). Further elucidation of this mechanism is needed to explore whether the discovered splicing-linked stress memory can be inherited across generations, or its function is limited somatically within an individual generation. Taken together, the polyploidy nature of *B. napus* and the regulatory role of AS in response to abiotic stress entail a further exploration of these evolutionary mechanisms under heat stress aiming to unveil novel species-specific interactions and to provide more understanding on its impact on thermotolerance,

and more broadly, how polyploidy can impact the organismal response to heat stress.

2.5 Dynamics of Epigenetic Modifications under Heat Stress

In plants, high throughput technologies such as Whole-Genome Bisulfite Sequencing (WGBS) and Chromatin Immunoprecipitation (ChIP), and more recently nanopore technology, provide the opportunity to study epigenetic modifications in species and genotypes at genome-wide levels (Laird, 2010). Different types of epigenetic mechanisms exist and the most common are DNA methylation, histone modifications and small RNAs. Along with its vital role in maintaining genome integrity, increasing evidence indicates that epigenetic mechanisms in plants play a substantial part in modulating gene expression under different environmental conditions, including salinity, drought, extreme temperatures and exposure to heavy metals (Gao et al., 2014; Fan et al., 2020; Ueda & Seki, 2020; Talarico et al., 2024).

2.5.1 DNA methylation

In higher plants, 30-50% of all cytosine residues of the nuclear DNA are methylated (Gao et al., 2014), and DNA methylation occurs in all three sequence contexts: the symmetric CG and CHG, and the asymmetric CHH, where H stands for A, C or T (Li et al., 2016; Klungland & Robertson, 2017). Despite the availability of epigenetic research in *A. thaliana*, these studies are still very limited in Brassica crops. Different methylation levels can produce different profiles of gene expression and transcription, giving rise to different phenotypic responses (Sun et al., 2022). In plants, genome methylation status is maintained by the dynamic function of DNA methyltransferase and demethylase enzymes. These enzymes are classified into different categories depending on their protein structure and function (Liu et al., 2018a). Fan et al. (2020) identified 22 methylase genes and six demethylase genes through studying two varieties of *B. napus* (YN171 and 93275). In addition to the variations in methylation patterns detected at different sequence locations (gene regions and up/downstream regions) and contexts (CHG, CHH & CG), analysis of gene expression in 22 tissues across

different developmental stages showed that these genes have different regulatory roles in plant growth and development.

Several reports have demonstrated that plant adaptations to different abiotic stresses are associated with differences in methylation levels (Marconi et al., 2013; Dhar et al., 2014; Liu et al., 2017; Li et al., 2023; Zhang et al., 2023). In *B. napus*, a large set of genes were associated with changes in cytosine methylation in response to heat treatment. More specifically, this variation was prominent at the genotype level where more DNA methylation occurred in the heat-sensitive cultivar and more DNA demethylation occurred in the heat-tolerant one (Gao et al., 2014). The expression level of DNA methyltransferase and demethylase enzymes changed significantly in response to salt and heat stress, indicating that methylation of some genes is essential for the response to abiotic stress in plants (Fan et al., 2020). Similarly, position- and context-dependent methylation variations were also seen in *B. rapa* under heat stress. Analysis of differential methylation and differential gene expression found that lowly expressed genes were enriched in genes with high methylation levels and vice versa, except for mCG methylation in exon regions. Interestingly, different sets of differentially methylated DEGs were involved at the early and late stages of heat treatment (Liu et al., 2018a).

During the reproductive stages, more complex relationships between DNA methylation and gene expression have also been elucidated. During the two pollen pathways (gametophytic development and pollen embryogenesis) and in response to heat treatment, *in vitro*-cultured microspores of *B. napus* changed their gametophytic developmental pathway towards embryogenesis through an epigenetic reprogramming control. This was associated with a decrease in global DNA methylation and activation of cell proliferation, while DNA methylation increased with pollen and embryo differentiation in a cell-type-specific manner (Solís et al., 2012). On the contrary, Li et al. (2016) reported that short term heat shock treatment induced a decrease in global DNA methylation in cultured microspores of *B. napus,* suggesting a lack of trend in the changes of DNA methylation under heat stress in different species or cell types.

2.5.2 Histone modifications

Histone modifications, including methylation, demethylation, and acetylation, are recognised as ubiquitous epigenetic marks that play a vital role in regulating chromatin structure and modulating gene expression (Xu et al., 2017; Nunez-Vazquez et al., 2023). Other posttranslational modifications (PTMs), such as phosphorylation and ubiquitination have also been identified in transcriptional regulation in *A. thaliana* (Füßl et al., 2018). The presence or absence of methylation on the lysine and arginine residues in histones affects their interaction with reader proteins and results in chromatin conformational changes. Eventually, these changes lead to either transcriptional activation or repression (Teperino et al., 2010). In plants, two Arg methylation sites (H3R17 and H4R3) and five Lys methylation sites (H3K4, H3K9, H3K27, H3K36, and H4K20) have been identified (Liu et al., 2010). In *A. thaliana*, histone methylation involves both repressive (H4R3me2, H3K9me2/3, and H3K27me3) and active modifications (H4R3me2, H3K4me3, and H3K36me2/3 (Wang et al., 2016). In contrast to methylation, histone acetylation marks neutralise basic charges in histones, thus weakening histone/DNA interactions and making the DNA more accessible for the transcriptional machinery (Allis & Jenuwein, 2016). In *A. thaliana*, studies reported an increase in histone H3 acetylation levels in response to drought stress which in turn, altered the expression of stress-responsive genes (Nunez-Vazquez et al., 2022). Histone modifications involve a complex network of epigenetic modifiers that serve to recognize, add or remove chemical moieties on histone tails or core domains (Xu et al., 2017). In response to stress, the combined activity of writers, readers and erasers regulate the level and type of histone modifications. Because tissue-specific expression is essential for stress response, these modifiers need to be recruited to specific loci for their participation. Various elements, such as TFs and long non-coding RNAs have been proposed to act as recruiters of these regulators into specific chromatin sites (Deng et al., 2018). However, their role is still poorly explored in *B. napus*.

Recent studies have highlighted the role of histone modifications and their modifiers in response to abiotic stress. Alam et al. (2019) reported 18 *B. rapa* PHD (Plant homeodomain) finger genes as highly responsive to drought and salt

treatments. As “epigenome readers”, PHD finger proteins can bind specifically to several histone modifications and confer activation or repression of the corresponding gene. Interestingly, some of these genes were found to be induced by more than 18-fold and others displayed different expression patterns at different time points of the treatments (Alam et al., 2019). In another study, 15 paralogous pairs of histone methyltransferase and demethylase genes also showed high variation in their expression profile in response to heat and cold stress where dynamic variations also took place in specific tissues and treatments, suggesting a complex role of these genes as robust candidates in the stress tolerance mechanisms of *B. rapa* (Liu et al, 2019b).

Adding to this complexity, epigenetic regulators were found to interact with other stress-responsive elements to promote tolerance (Liu et al, 2019a). To explore this relationship, Liu and colleagues investigated the role of Polycomb Repressive Complex 2 (PRC2) in regulating ABA stress response at a genome level (Liu et al., 2019a). PRC2 is one of the major families of Polycomb group (PcG) factors that functions to induce a state of chromatin inactivation. Its core enzymes are responsible for histone H3 lysine-27 tri-methylation (H3K27me3), which is one of the major epigenetic marks that causes gene silencing (Liu et al., 2019a). In *A. thaliana* more than 20% of genes are covered by H3K27me3 in one given organ (Lafos et al., 2011) and around 60% of protein-coding genes are silenced by H3K27me3 in different cell types (You et al., 2017). Therefore, H3K27me3 is considered a hallmark of gene repression, but its exact mechanism of action is not fully understood. H3K27me3 was found to preferentially target ABA-induced senescence-associated genes (SAGs) (Liu et al., 2019a). ABA-induced senescence allows for the recycling and redistributing of nutrients to new growing organs and seeds, thus boosting the plant’s fitness and tolerance against abiotic stress (Zhao et al., 2016). In their study, Liu and colleagues found that mutants of PRC2 enzymes were hypersensitive to ABA. Accordingly, SAGs were derepressed and appeared highly induced in comparison to the wild type. This indicates that PRC2-mediated H3K27me3 plays a key role in attenuating the ABA response through buffering the expression of ABA-induced genes. Hence, fine tuning of ABA-induced senescence to protect the plant from oversensitive

response (Liu et al., 2019a). The role of PRC2 in regulating stress responses through modification of histones were also previously explored in different abiotic conditions such as drought, cold and salt where H3K27me3 modified the promotors of major stress response genes (Kwon et al., 2009).

2.5.3 Non-coding RNAs

Another component of the epigenetic machinery thought to play a role in the response to abiotic stress are non-coding RNAs, which are involved in the regulation of gene expression, along with other roles in different biological processes (Zhang et al., 2014b). The short (20-24 nucleotides) small RNAs (sRNAs) and the long (more than 200 nucleotides) non–coding RNAs (lncRNAs) have no or limited discernible protein-coding functionalities.

sRNAs include micro-RNAs (miRNAs) which are the most thoroughly characterized, and small interfering RNAs (siRNAs) (Bartel, 2009). sRNAs are known to affect gene expression via two main mechanisms: transcriptional (TGS) and posttranscriptional gene silencing (PTGS). To date, several studies have identified both conserved and novel miRNAs from various model plants such as *A. thaliana* and rice in response to different abiotic stress (Begum, 2022), yet there is limited knowledge about the small RNA population in *B. napus* where only a small number of miRNAs have been identified and their role in response to heat stress is largely unknown (Jian et al., 2016). In a recent study, miRNAs mediated thermotolerance through the action of miR160 which altered the expression of *HSPs* and improved seed germination and seedling survival under heat stress in *A. thaliana* (Lin et al., 2018). Additionally, five conserved miRNAs families along with four novel ones were found responsive to heat stress and resulted in either positive or negative regulation of their putative genes in *B. rapa* (Yu et al., 2012). Using computational prediction methods, Xie et al. (2007) were the first to study miRNAs in *B. napus* where they detected five miRNAs in response to auxin, cadmium stress and phosphate starvation. However, this study was carried out prior to the availability of a reference sequence which explains the small number of miRNAs detected by their approach. Subsequently, a set of DE miRNAs in response to sulphate deficiency and cadmium stress have

been identified (Huang et al., 2010). High-throughput sequencing revealed a diverse and complex set of core miRNAs in *B. napus* that were involved in seed imbibition response to salt and drought stresses, suggesting the role of miRNAs in response to stressful conditions during seed germination (Jian et al., 2016). In *B. napus*, the regulatory role of miRNAs has been explored and detected in response to different abiotic stresses, yet little or no information is available in response to heat stress. Hence, more studies are warranted on this aspect to gain insight about their role in thermotolerance.

LncRNAs were found to function as precursors of small RNAs (Joshi et al., 2016). They are grouped according to their genomic positions and orientations as follows: long intergenic noncoding RNAs (lincRNAs), long noncoding natural antisense transcripts (lncNATs), long intronic noncoding RNAs, and sense lncRNAs. In plants, lncRNAs research is still in its infancy with only a few studies showing their role in plant development and adaptation to abiotic stress (Jha et al., 2020; Bhogireddy et al., 2023). Nonetheless, lncRNAs have been found highly expressed in a tissue-specific or stage-specific manner, with some having preferential expression in the reproductive stages as well (Zhang et al., 2014b). There is increasing evidence about the role of lncRNAs as 'biological regulators' for various processes including developmental, biotic and abiotic stress in animals and plants (Chekanova, 2015). To explore the current understanding of the participatory role of lncRNAs in the tolerance response to different abiotic stress conditions in plants, Jha et al. (2020) reviewed several studies that identified lncRNAs under drought, heat, cold, nutrient deficiency and metal toxicity. As a result, a plethora of DE heat stress lncRNAs and their corresponding heat stress-responsive target genes were reported from different crops. Song et al. (2016) identified 9,687 novel lncRNAs in *B*. *rapa* in response to heat treatment using RNA-seq. Likewise, two up-regulated lncRNAs (TalnRNA27 and TalnRNA5), which are miRNA precursors, were up-regulated in wheat in response to heat stress (Xin et al., 2010). During pollen development and fertilisation, a systematic analysis in *B. rapa* revealed that 47 *cis*-acting lncRNAs and 451 *trans*-acting lncRNAs were highly co-expressed with their target genes (Huang et al., 2018). Furthermore, a co-expression network of *B. rapa* depicted

210 DEGs, 4 miRNAs and 33 lncRNAs under heat stress, where the biological functions identified through GO enrichment analysis revealed terms such as “BR mediated signalling pathway”, “response to stimulus” and “response to heat”, suggesting their contribution in the heat response through these signalling pathways (Wang et al., 2019b). At the seedling stage, two genotypes of *B. napus* exhibiting different sensitivity to drought were exposed to drought stress and rehydration treatment. Genome-wide analysis showed that the up and down-regulated mRNAs co-expressed with lncRNAs participated in different metabolic pathways and were involved in different regulatory mechanisms, which suggested the existence of divergent mechanisms to modulate the response to drought stress and re-watering treatment (Tan et al., 2020). These findings show that lncRNAs in plants could form an additional layer of complexity to other stress response mechanisms during abiotic stress events. In *B. napus*, only a few studies were found addressing the roles of lncRNAs in response to certain stress conditions such as pathogens (Joshi et al., 2016), cadmium (Feng et al., 2016) and drought stress (Tan et al., 2020), while to the best of our knowledge, no studies have investigated the role of lncRNAs in responses to heat stress in this species. There is a significant knowledge gap concerning the roles of different epigenetic mechanisms in response to heat stress in *B. napus*. Owing to its complexity and poorly understood mechanisms, a deeper knowledge of these intricate epigenetic components would serve as a stepping-stone to gain insights into the genomic regulation behind heat stress-mediated responses in this important crop species.

2.6 Systems Biology Approaches in *Brassica napus* under Heat Stress

Omics approaches are now routinely used in helping to understand plant responses and adaptations to different biotic and abiotic conditions. However, their exploitation in *B. napus* is limited and the knowledge of the impact of heat stress on its yield determining reproductive stages is still fragmentary. Nevertheless, the availability of reference genomes in Brassica species has contributed to major improvements in understanding the genetics of these crops.

This has created a foundation for identifying possible interactions that link genotypes and phenotypes. Subsequently, emerging genomics and transcriptomics studies have resulted in the development of transgenic cultivars with potentially stronger agronomic properties than their non-transgenic cultivars (Raza et al., 2021). In *B. napus* and other related species, several genomic projects have been conducted to study the effect of different abiotic stresses on different genetic and physiological properties. Using GWAS, numerous studies examined genomic regions and quantitative trait loci (QTL) controlling various traits that are affected by heat stress (Bac-Molenaar et al., 2015; Rahaman et al., 2018). Others identified TFs that play regulatory roles in abiotic stress responses including heat (Yoshida et al., 2008; He et al., 2016; P. Wang et al., 2018; Liang et al., 2019; Ghorbani et al., 2020) and some looked at gene families that are involved in controlling different hormonal signalling networks during heat stress (Justen & Fritz, 2013; Di et al., 2018).

At the transcript level, global transcriptome profiling allows for dynamic quantification of gene expression and their isoforms at different developmental stages in different tissues (Wang et al., 2009). For instance, combined RNA-seq and qRT-PCR identified and analysed the expression patterns of 64 genes encoding BnaHSF proteins in the *B. napus* genome across different tissues and under heat, drought and high CO_2 stresses (Zhu et al., 2017). Similarly, organ-specific expression was detected using transcription profiling in *B. napus* siliques at the seed-filling stage, where some genes were preferentially expressed in heat-stressed silique walls or seeds and others were involved in specific biological functions of each tissue, such as GSL metabolism in silique wall and flavonoid synthesis in seeds (Yu et al., 2014). Furthermore, a transcriptional analysis of three different cultivars of *B. napus* (two heat-sensitive and one heat-tolerant) showed that all three cultivars shared a common transcriptional response with the down- regulation of cell metabolism and up-regulation of genes linked to heat stress, protein folding and HSPs binding (Jedličková et al., 2023).

Examining the impact of high night temperature on the fatty acid composition of *B. napus* seeds, in-depth RNA-seq analysis revealed up-regulated GA signalling

which was also associated with active expression of genes involved in fatty acid catabolism and glyoxylate metabolism pathways (Zhou et al., 2018). Furthermore, a genome-wide analysis allowed the identification and functional analysis of 321 putative AP2/ERF TFs belonging to five major subfamilies, including DREB, ERF, AP2, RAV, and BnSoloist in *B. napus* (Owji et al., 2017). Gene expression analysis using RNA-seq revealed a high expression of AP2/ERF genes in the roots as compared to other organs. Gene ontology annotation, regulatory elements identification, and functional association network showed that most of these TFs are responsive to different abiotic stresses such as drought, salinity, and cold and play significant roles in the developmental processes, particularly organ speciation and embryogenesis (Owji et al., 2017).

Plant responses to heat are depicted by many quantitative factors such as time, intensity and duration of stress. At the molecular level, these responses result in profound structural and metabolic modifications, ranging from disruption of membrane and cytoskeleton stability to metabolic alterations of enzyme activity and accumulation of toxic metabolites such as ROS. Although genomic and transcriptomic studies using RNA-seq have offered revolutionary approaches to interpret genomic data, these data do not always reflect the exact proteomic and metabolic state as gene products are also influenced by other processes such as post-translational and co-translational modifications. Hence, integration of metabolic and proteomic approaches will further help to better understand the events taking place at the molecular level (Rocco et al., 2013). Recently, omics approaches have been fine-tuned to elucidate the abiotic stress-related genes and develop tolerant cultivars that are capable to withstand current climatic and environmental changes (Raza et al., 2021). For example, studying the proteome response of a crop species during high-temperature stress will help to reveal the proteins and their corresponding biological mechanisms responding to these stresses. Moreover, as proteins act as direct effectors of phenotype, this proximity allows the prediction of affected phenotypes and hence, improving the breeding outcomes (Kosová et al., 2015). Although the proteome comprises of all protein components in a living organism, this set of proteins varies considerably depending on the developmental stage, growth conditions, tissue and cell type,

giving rise to infinite proteomes within one organism (Kosová et al., 2015). Therefore, to gain a comprehensive insight into the protein profiles and pathways that are associated with heat, extensive research needs to be carried out not just at the organismal level but also looking at specific developmental stages and growth conditions.

Although numerous proteomic studies addressing the impact of various abiotic stresses in model species and various crops have been reported and reviewed (Benešová et al., 2012; Hossain et al., 2012; Kosová et al., 2013; Li et al., 2013), there is very limited knowledge to date on the proteome of *B. napus* under heat stress. The existing studies have identified proteins that are thought to be involved in thermotolerance, redox homeostasis, carbohydrate metabolism and protein synthesis and degradation (Lee et al., 2007; Neilson et al., 2010). In *A. thaliana*, analysis of the protein repertoire in the rosette leaves after exposing seedlings to short-term heat stress revealed differentially represented proteins that grouped into two main broad classes including energy and metabolism and stress response proteins (Rocco et al., 2013). In the first proteomic report in *B. napus* in response to heat stress, Ismaili et al. (2015) observed an increase in the abundance of ascorbate peroxidase protein and its corresponding mRNA transcript, suggesting its role in short-term high-temperature stress response. Also, the up-regulation of proteins involved in energy and metabolism is thought to have utilised most of the nutritive reserves in the seedlings, resulting in a noticeable reduction in growth as a response to heat (Ismaili et al., 2015). Similarly, the first proteome analysis of *B. napus* response to drought stress also reported a reduction in growth that was associated with decreased abundance of HSP 70 and tubulin beta-2 proteins in the drought-sensitive and hybrid F1 lines (Mohammadi et al., 2012). Using yeast two-hybrid assay, the interactions among various calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) were demonstrated in *B. napus* under several abiotic stresses (Zhang et al., 2014a). Furthermore, expression levels of six selected *BnaCBLs* and 12 *BnaCIPKs* genes in response to salt, drought, cold, heat, and ABA showed that the two gene families were responsive to multiple stimuli, suggesting that the CBL-CIPK network in *B. napus* may be a common link for a variety of signalling

pathways (Zhang et al., 2014a). Yan et al. (2016) used a comparative proteomic approach to screen for thermotolerance genes in seeds of *B. napus*. As a result, a *B. napus GLYI* gene was identified. GLYI is a member of glyoxalase system that plays a role in methylglyoxal detoxification during carbohydrate and lipid metabolism. Two-dimensional gel analyses showed that the abundance of BnGLYI-3 protein in thermotolerant seeds significantly increased in response to heat stress, suggesting its role in inducing thermotolerance to high temperature stress in *B. napus* seeds (Yan et al., 2016).

Along with proteomics and other omics approaches, metabolomics has emerged as an exciting scientific discipline where cellular metabolites are profiled to reveal connections among genes, phenotypes and other cellular biomarkers in different biological species (Raza, 2020). The metabolome is the total pool of metabolites present in an organism (Razzaq et al., 2019). Through metabolomics studies, it is now possible to identify and measure primary and secondary metabolites to characterize genetic or environmental variations in plants (Razzaq et al., 2019). Under heat stress conditions, metabolomics provides a systems biology approach to understand complex metabolic networks and enhance thermotolerance and can be a useful tool in distinguishing between genotypes with different heat sensitivity and understanding how changes in the transcriptome and proteome manifest themselves at the cellular and tissue levels. Using comparative metabolomics, Amjadi et al. (2024) showed that heat stress increased the susceptibility to *Leptosphaeria maculans* (blackleg disease causing-agent) through down-regulation of signal transduction pathway components and deduction in signalling metabolites (Amjadi et al., 2024). Moreover, Koscielny et al. (2018) identified 25 metabolic markers that discriminated between the heat-tolerant and -susceptible genotypes of *B. napus* during the reproduction stage under heat stress, through an untargeted metabolic assessment using Gas Chromatography-Mass Spectrometry. The latter finding demonstrates how genetic variations exhibited by different genotypes are translated into distinct agronomic traits that eventually allow its survivability under suboptimal conditions. In addition, metabolome information collected from stressed plants has also helped define the severity and timing of heat stress

(Koscielny et al., 2018). In another metabolomic study, numerous unknown potential regulatory relationships involved in glucosinolate metabolism were identified through a doubled-haploid mapping population of *B. napus*. Feng et al. (2012) demonstrated the impact of genetic variation on the biochemical pathways through combining genetic and metabolic analysis of different GSL components in the leaves and seeds. Subsequently, this has resulted in the identification of 105 metabolite QTLs and the construction of an advanced metabolic network for the GSL composition in both leaves and seeds (Feng et al., 2012). Integration of metabolomic approaches with other omics tools has therefore the potential to reveal crucial interactions and regulatory networks between genes, proteins, and many essential regulatory components, where different responses meet to form a complex machinery of plant stress tolerance.

2.7 Conclusion

Despite current advances in exploring the impact of different biotic and abiotic stress factors within different Brassica species, this review has identified several knowledge gaps regarding the impact of heat stress on *B. napus* during its yield determining reproductive stages, where in-depth analysis of this subject is still needed. The opposite trends in expression of key genetic and epigenetic components identified in different species and in cultivars within the same species under various abiotic stresses call for further research in this field. Moreover, variations in intensity, time and duration of heat stress have been found to generate modified responses in plants. Therefore, to understand its impact on *B. napus* crops in specific geographical areas, regional studies on local cultivars should take place to simulate field conditions specific to these areas. To date, only a few studies have addressed the impact of heat stress on the metabolome and proteome of *B. napus*, and the underline cellular mechanisms are still poorly understood. On this account, combining genetic studies with different omics approaches will provide the means to explore the intricate responses arising following exposure to heat stress and accomplish a system-level understanding of how heat stress alters the yield and quality of the *B. napus* crop. It is expected that the findings of these studies will help breeders to produce cultivars with

strong thermotolerance traits and to mitigate the detrimental effect of global warming on agriculture through contributing to sustainable food production and security.

Author Contributions:

FM, FR, MA, and JH conceived the research. MK undertook the review of the literature, prepared the figures, and drafted the manuscript. All authors contributed to and revised the manuscript, given the opportunity to comment, and approved the final version of the manuscript.

3 Prolonged heat stress in *Brassica napus* during flowering negatively impacts yield and alters glucosinolate and sugars metabolism

Mariam Kourani[1], Maria Anastasiadi[1], John P. Hammond[2] and Fady Mohareb[1]*

[1] Bioinformatics Group, Cranfield University, Cranfield, United Kingdom,

[2] School of Agriculture, Policy and Development, University of Reading, Reading, United Kingdom

Abstract

Oilseed rape (*Brassica napus* L.), one of the most important sources of vegetable oil worldwide, is adversely impacted by heat wave-induced temperature stress especially during its yield determining reproductive stages. However, the underlying molecular and biochemical mechanisms are still poorly understood.

In this study, the transcriptomic and metabolomic responses to heat stress were investigated in *B. napus* plants after exposure to a gradual increase in temperature reaching 30 °C in the day and 24 °C at night for a period of 6 days. High Performance Liquid Chromatography and Liquid Chromatography-Mass Spectrometry were used to quantify the content of carbohydrates and glucosinolates respectively. Results showed that heat stress reduced yield and altered oil composition. Heat stress also increased the content of carbohydrate (glucose, fructose and sucrose) and aliphatic glucosinolates (gluconapin, progoitrin) in the leaves but decreased the content of the indolic glucosinolate (glucobrassicin).

RNA-Seq analysis of flower buds showed a total of 1892, 3253, 4553 genes were differentially expressed at 0, 1 & 2 DAT (Days after Treatment) and 4165 and 1713 at 1 & 7 DOR (Days of Recovery) respectively. Heat treatment resulted in down- regulation of genes involved in respiratory metabolism namely Glycolysis, Pentose Phosphate Pathway, Citrate Cycle and Oxidative Phosphorylation especially after 48 hrs of heat stress. Other down-regulated genes mapped to sugars transporters, nitrogen transport and storage, cell wall modification and

methylation. In contrast, up-regulated genes mapped to small heat shock proteins and other heat shock factors that play important roles in thermotolerance. Furthermore, two genes were chosen from the pathways involved in the heat stress response to further examine their expression using real time RT-qPCR.

Taken together, the results of this study demonstrated that the changes in carbohydrates and glucosinolates metabolism and transport under heat stress is one of the response mechanisms employed by the plant under heat stress conditions. Moreover, the transcriptomic data will be useful for further understanding the molecular mechanisms of *B. napus* plant tolerance to heat stress and provide a basis for breeding heat-tolerant varieties.

3.1 Introduction

Over the last decade, climate change has led to more extreme climatic events, impacting crop productivity and threatening global food security (Ismaili et al., 2015; Lamaoui et al., 2018). These extreme events include periods of high temperature stress in the form of heatwaves (Goel et al., 2023), broadly defined as periods of excessively high temperature as compared to the local climate (Dikšaitytė et al., 2019). Previous studies have shown that heat stress can be detrimental during reproductive development due to the damage caused to plant organs and cellular structures (Hedhly 2011; Hinojosa et al., 2019, Goel et al., 2023). During reproduction, high temperature can induce irreversible structural and physiological changes in both male and female floral organs, leading to premature senescence (Lohani et al., 2020; Jagadish et al., 2021). High temperature stress can also decrease chlorophyll synthesis and disrupt photosynthesis and respiration, further reducing the yield potential of crop plants (Hasanuzzaman et al., 2014; Zandalinas et al., 2016). Thus, understanding the impact of heatwaves on crops and particularly during reproductive stages is essential to the development of varieties that can withstand these periods of elevated temperatures.

Traditionally, most heat stress experiments involve a heat shock, where plants are subjected to a high temperature within a very short time (10–15 °C above their optimal temperature, from several minutes to a few hours (Wahid et al,

2007). As a result, plant responses to heat shock have been well studied (Huang et al., 2019; Wang et al., 2020), but relatively little information exists on the responses to heatwaves, especially at the transcriptomic and metabolomic levels (Jin et al., 2011; Glaubitz et al., 2017).

In plants, heat shock reduces photosynthesis and respiratory metabolism, and increases antioxidant activity (Huang et al., 2019; Wang et al., 2020). Heat shock also negatively affects plant growth and productivity (Ismaili et al., 2015). While these experiments revealed the regulatory mechanisms in response to sudden heat stress (Seth et al, 2021; Ikram et al., 2022; Li et al., 2022), they do not fully represent the impact of temperature changes in the field during heatwave conditions. As a result, several studies employed prolonged warming experiments to study heat stress (Jin et al., 2011; Way & Yamori 2014; Glaubitz et al., 2017). In *A. thaliana*, studies showed that plants exhibit different response patterns to prolonged warming (7 days) as compared to heat shocks (Jin et al., 2011; Wang et al., 2020). While prolonged warming led to a reduction in stomatal conductance, heat shock increased transpiration. Under both heat shock and prolonged heat stress there was an induction of antioxidant enzymes in *A. thaliana*, however, these were significantly higher under the heat shock treatment compared to the prolonged heat treatment (Wang et al., 2020). *A. thaliana* plants grown at 28/23 °C (elevated by 5 °C from ambient temperature) suffered from significant reduction in life span, total biomass, total weight of seeds, and leaf concentration of starch, chlorophyll, and proline (Jin et al., 2011). In rice, under prolonged elevated night temperature, transcriptomic analysis showed changes in cell wall, abscisic acid (ABA) signalling and secondary metabolism related genes, while metabolite profiles revealed a highly activated Citrate Cycle and enzyme activities (Glaubitz et al., 2017).

Oilseed rape (*Brassica napus* L.) is the second-largest source of oilseeds after soybean and the third-largest source of vegetable oil worldwide (USDA, 2025). Cultivation and breeding practices have resulted in numerous genetically diverse lines with strong agronomic and adaptation traits (Sun et al., 2017). As a result, *B. napus* is widely cultivated around the world for food, biofuel and animal feed

(Namazkar et al., 2016). Like other major temperate field crops, *B. napus* can be extremely sensitive to high temperature stress, especially if it occurs during flowering or seed development, threatening yields and quality (Angadi et al., 2000; Koscielny et al., 2018; Kourani et al., 2022). Studies in different Brassica species have found negative relationships between heat stress and seed yield and quality (Yu et al., 2014). For example, under elevated temperature scenarios, four *B. napus* cultivars experienced a significant reduction in seed biomass, resulting in a 58% decrease in the oil yield, and 77% decrease in the essential fatty acid C18:3-ω3 (Namazkar et al., 2016). Heat stress also reduced oil seed content and impaired carbohydrate incorporation into triacylglycerols in *B. napus* (Huang et al., 2019). Additionally, impairment of chlorophyll biosynthesis and disruption of the biochemical reactions of photosystems were exhibited in 10-day-old *B. napus* seedlings subjected to 38 °C (Hasanuzzaman et al., 2014). This was manifested through a significant reduction in chlorophyll and leaf relative water content as well as through an inefficient antioxidant defence system (Hasanuzzaman et al., 2014). Despite current advances in studying the effect of heat on different Brassica species, the impact of heatwaves on *B. napus,* especially during its yield determining reproductive stages, is still poorly understood (Kourani et al., 2022).

To address this, we investigated the transcriptomic and metabolomic responses to heat stress in *B. napus* under simulated field conditions. To achieve this, a heat treatment experiment simulating heatwave episode during the flowering stage of *B. napus* cv. Westar was conducted in a controlled environment. Global transcriptome profiling using RNA-Seq was employed to identify differentially expressed genes (DEGs) in flower buds of heat stressed plants (30 °C/24 °C day/night for a period of 6 days). This was complemented with High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) analyses to detect changes in sugars and glucosinolates (GSL) concentration in leaves in response to the heat treatment. Therefore, this study aims to provide insights into the impact of a future warmer climate on the important oil crop species *B. napus* during its reproductive stages.

3.2 Materials and Methods

3.2.1 Plant materials and physiological parameters

Seeds of spring oilseed rape (*Brassica napus* L., cv. Westar) were surface-sterilized and sown into seed trays filled with a seed potting mix (Clover Peat, Dungannon, Northern Ireland). Trays were then placed in polyethylene tunnels for four weeks at the Crops and Environment Laboratory, University of Reading, to germinate. Twenty-eight days after sowing (DAS), the plants were transplanted into 3L pots containing a peat based potting mix (Clover Peat).

At green bud stage (38 DAS), the plants were moved to two controlled environment chambers (Fitotron, Weiss Technik (UK) Ltd), for a period of one week to adjust to the new environment before the start of the heat experiment. Each cabinet contained 16 plants and was maintained under control conditions of 20 °C/14 °C day/night and a photoperiod of 16/8-hr light/dark.

3.2.2 Heatwave experimental design

Since the flowering stage of winter sown *B. napus* occurs during May in the UK, an analysis of daily weather temperatures during this developmentally crucial period was carried out using the UK Meteorological (Met) Office weather data obtained from the Met office (Met office n.d.) - Heathrow station - and from WeatherOnline (WeatherOnline n.d.) platforms. The Met office Heathrow site was selected due to its strategic location near London, meaning that it provides data relevant to a densely populated and economically important region.

The data showed an increase in the frequency of high temperature fluctuations during May, ranging between 26 °C and 28 °C over several days (Figure 3.1). Given the continuous rise in global temperature, it is expected that future heatwaves would increase in severity and duration (Seneviratne et al., 2012). Based on these data, the current study temperature simulates a heat stress event through a temperature increase to 30°C/24 °C day/night for a period of 6 days and 20 °C/14 °C day/night for control. Forty-five DAS, the temperature in one cabinet was kept under control conditions (20 °C/14 °C) and denoted as control cabinet, while the second cabinet was set to a gradual increase in temperature

and denoted as heat treatment (HT) cabinet. To mimic a heatwave, the temperature in the HT cabinet was increased gradually from 20 °C to 30 °C between 9:00 h and 12:00 h in 3 steps: 20 °C at 9:00 h, 24 °C at 10:00 h, 28 °C at 11:00 h, and 30 °C at 12:00 h. The temperature was held at 30 °C until 22:00 h (Figure 3.2B), before it was dropped gradually and maintained at 24 °C until 8:00 h of the next day. This cycle of gradual increase (day) and decrease (night) of temperature was held for the next 5 days (Figure 3.2C). On the 6th day of treatment, the temperature was gradually decreased to 20 °C/14 °C day/night cycle (Figure 3.2D) and held for a recovery period of 7 days (Figure 3.2E). During heat treatment, the plants were frequently irrigated to avoid drought.

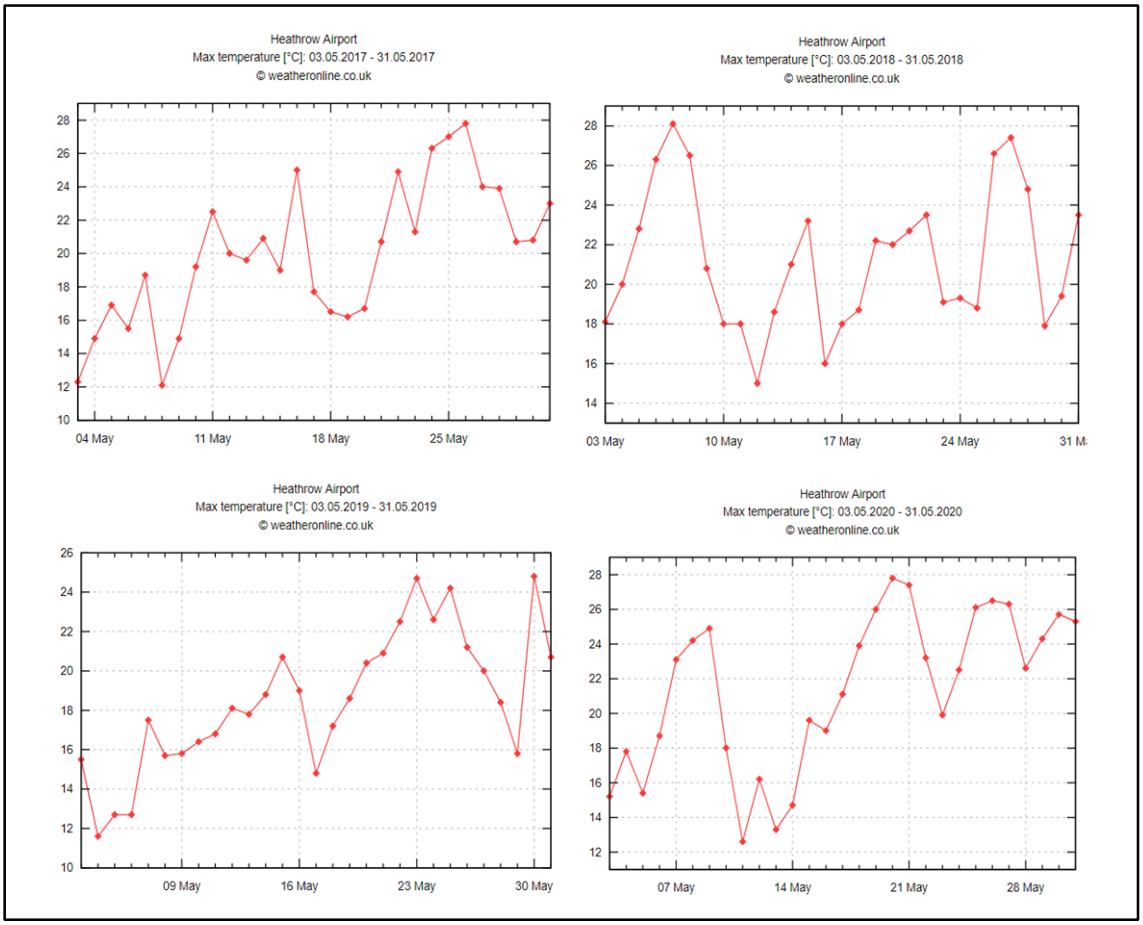

Figure 3.1 Maximum daily temperature (°C) recorded at Heathrow airport during May 2017, May 2018, May 2019 and May 2020.

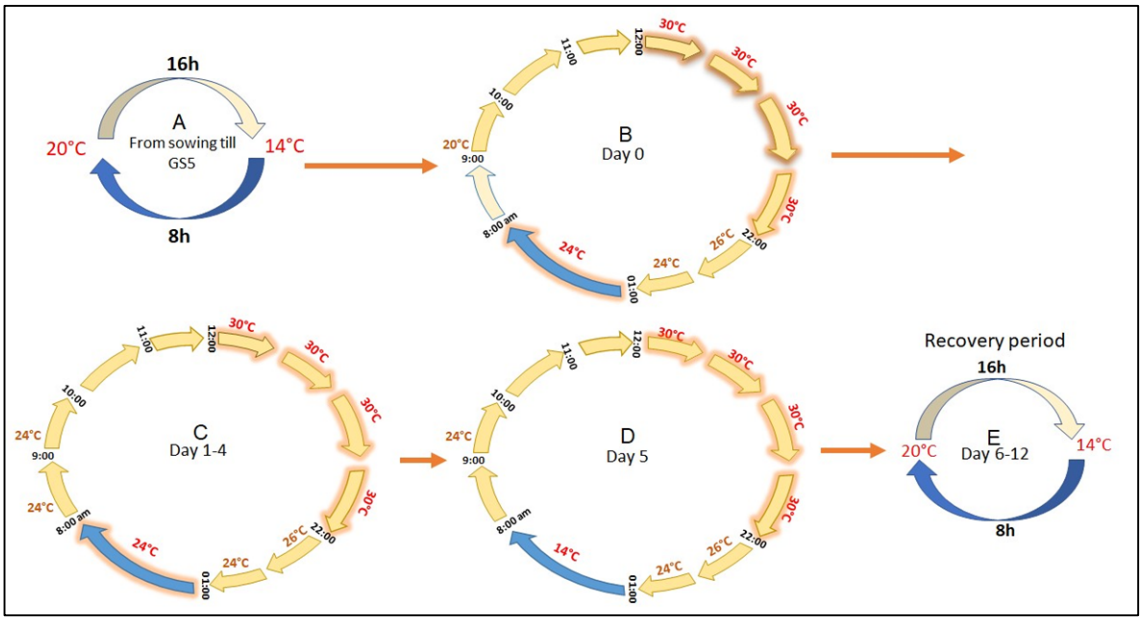

Figure 3.2 Heat treatment experimental design. To mimic a heatwave, the temperature in the heat treatment cabinet was increased gradually from 20 °C to 30 °C between 9:00 h and 12:00 h. The temperature was held at 30 °C until 22:00 h (B), before it was dropped gradually and maintained at 24 °C until 8:00 h of the next day (B). This cycle of gradual increase (day) and decrease (night) of temperature was held for the next 5 days (C). On the 6th day of treatment, the temperature was gradually decreased to 20 °C/14 °C day/night cycle (D) and held for a recovery period of 7 days (E).

3.2.3 Sample preparation for transcriptomic analysis

Five biological replicates from each treatment condition were collected at 0, 1, 2 DAT (Days after Treatment) and 1 & 7 DOR (Days of Recovery) of the experiment. Each replicate was made up of three individual plants. Three flower buds were collected per plant (nine buds per replicate). All samples were immediately snap-frozen in liquid nitrogen before they were stored at -80°C. Bud samples were ground into fine powder using pestle and mortar, with the frequent addition of liquid nitrogen to stop enzymatic reactions. The ground material was then stored at -80°C until RNA extraction.

3.2.4 RNA extraction and sequencing

Total RNA from, bud samples was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) in accordance with the manufacturer's protocol and treated with genomic DNAse using DNASE 70-On Column DNase I Digestion set (Sigma-Aldrich) to eliminate DNA contamination. RNA quantification was estimated on both NanoDrop 2000 (Thermo Scientific) and Qubit 2.0 fluorometer

(Invitrogen, USA) and its quality was evaluated on 1% (w/v) denaturing formaldehyde agarose gel (MOPS). Samples were shipped on dry-ice to Novogene Europe, where llumina-based RNA sequencing was performed on the Illumina Novaseq™ 6,000 (150bp paired-ends reads) platform.

3.2.5 Differential expression and cluster analysis

Raw sequence reads were assessed for quality using FastQC tools (v0.11.5) (Andrews 2012). The mean sequence lengths were 150 bp and the mean sequence GC content was 43%. The mean quality scores in each base position were higher than 36 and the mean quality scores per sequence were 36. As a result, sequence trimming was not necessary.

STAR (Spliced Transcripts Alignment to a Reference) software (v2.7.10a) was used to map the clean reads to the *B. napus* cv. Westar v0 reference genome (Song et al., 2020), and the percentage of aligned reads was calculated by using the flagstat command from samtools on each alignment file generated by STAR aligner (Figure S1). To estimate transcript abundance, RSEM (RNA-Seq by Expectation-Maximization) software (v1.2.18) was used in three steps to prepare the reference, calculate expression and generate the count matrices. Firstly, rsem-prepare-reference script was used with '–gff3' option to extract reference sequences from the genome and '—star' option to build STAR indices. Next, rsem-calculate-expression script was used with '--paired-end' option to align input reads against the reference transcriptome with STAR and calculated expression values using the alignments. Finally, rsem-generate-data-matrix script was used to generate the count matrices from expression results files.

For differential expression analysis, DESeq2 (v1.41.10) was used in R environment (Love et al., 2014) with default parameters. A pre-filtering step was performed to keep transcripts that have a minimum count of 10 reads in a minimum of four samples. To identify transcripts that were significantly differentially expressed (DE) by heat stress (HT) as compared to control (C), the two treatment conditions (HT versus C) were contrasted in pairwise comparisons. Thresholds of $|\log2(\text{fold change})| \geq 0.5$ and adjusted P-value < 0.05 (using the

Benjamini and Hochberg method) were considered to identify significantly DE transcripts between the two treatment conditions.

Significantly DEGs in each contrast were further analysed using K-mean clustering based on the kmeans() function in R (distance: Euclidean), with log2 fold change (log2FC) as the input.

3.2.6 Gene Ontology and KEGG pathway enrichment analysis of DEGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted on the DEGs using OmicsBox (BioBam Bioinformatics, 2019). The significantly enriched GO terms and biological pathways in HT samples as compared to control at different timepoints were identified to determine heat stress-related functions and pathways. The analysis was carried out using Fisher Exact statistical test with FDR adjustment cut-off <0.05. The background dataset consisted of all *B. napus* cv. Westar identifiers present in the assembly's annotation file (Song et al., 2020).

3.2.7 Validation of RNA-seq results by real-time quantitative PCR (RT-qPCR)

Genes encoding sucrose synthase 5 (*SS5*) and heat shock protein 20 (*HSP20*) were chosen to further examine their expression in real time using RT-qPCR. sucrose synthase is one of the crucial enzymes in the conversion of sucrose to UDP-glucose (Coleman et al., 2006) and HSP20 is one of the small heat shock proteins (sHSP). In response to heat treatment, RNA-seq analysis showed that both genes were DE at 2 DAT. Moreover, studies have also reported their involvement in the heat stress response (Sun et al., 2020; Li et al., 2022). The relative expression of each gene was measured at five timepoints during heat treatment and recovery. *SS5* and *HSP20* were not analysed at 1 DAT as they

exhibited similar expression profile at 0 & 1 DAT, therefore one timepoint was selected (

Table **S2**).

Total RNA used for transcriptomic sequencing was used for cDNA synthesis. First, cDNA was synthesized from 1 µg total RNA by Invitrogen SuperScript IV VILO Master Mix kit according to the manufacturer's instructions (ThermoFisher Scientific). The RT-qPCR reaction volume was 20 µL consisting of 3 µL cDNA, 10 µL SYBR Premix Ex Taq II (Takara, China), 1 µL (200 nM final concentration) of 4 µM of each primer (forward and reverse) (Table S3) and 5 µL RNAse-free water. Three technical and three biological replicates for each sample were measured with the following protocol: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s, followed by dissociation curve analysis ramping from 60°C to 95 °C with a ramp rate of 0.3 °C s^{-1} on an ABI StepOne Plus RT-qPCR platform (Applied Biosystems, USA). *BnaActin* was used as housekeeping gene to normalize the data. The relative expression level of all selected genes at each timepoint was calculated using the $2^{-\Delta\Delta CT}$ method of the StepOne software (v2.2.2).

3.2.8 Sample preparation for metabolomic analysis

Five biological replicates from each treatment condition were collected at 0, 1, 2, 5 DAT and 1, 3 & 7 DOR of the heat treatment experiment. Each replicate was made up of three youngest fully expanded leaves collected from three plants. All leaf samples were immediately snap frozen in liquid nitrogen before being stored at -80°C until further analysis. Frozen leaf samples were freeze-dried for five days, then ground into a fine powder using a Precellys 24 lysis & homogenization (Stretton Scientific, Alfreton, UK) and stored at -40 °C in sealed sample bags until extraction and analysis.

3.2.9 Glucosinolates extraction and analysis

GSL extraction was carried out as per the protocol of Bell et al. (2015) as follows: 40 mg of ground leaf powder was heated in a dry block at 75 °C for 2 min. This step was done as a precautionary measure to inactivate as much myrosinase enzyme as possible before extraction (Pasini et al., 2012). Afterwards, 1 mL of preheated (70 % v/v) methanol (70 °C) was added to each sample and placed in a water bath for 20 min at 70 °C. Samples were then centrifuged for 5 min (6000 rpm at 18 °C) to collect the loose material into a pellet. The supernatant was then transferred into fresh labelled Eppendorf tube and stored at -80 °C until further analysis. Before LC-MS analysis, the samples were filtered using 0.25 µm filter discs, diluted in 50% methanol at a 1:4 ratio (dilution factor =5) and spiked with a 100 µL of the internal standard sinigrin (100 ng mL^{-1}). The GSL concentrations were analysed by SCIEX QTRAP 6500+ LC-MS.

For LC separation, a Waters Acquity BEH C18 column (particle size 1.7µ, 2.1 x 50 mm) with a Security VanGuard system from Waters (UK) was used. The mobile phase consisted of water with 0.1 % formic acid (A) and methanol with 0.1 % formic acid (B). The gradient started at 5 % B and was raised to 90 % B in 3.5 min, held at 90 % B for 0.5 min and re-equilibrated at 5 % B for 1 min. The total time of the gradient program was 5 min. The flow rate was 0.4 mL min^{-1} with a column temperature of 60 °C. A 1 µL aliquot of sample was injected for analysis.

The LC system was interfaced with a SCIEX QTRAP 6500+ mass spectrometer equipped with an IonDrive Turbo V ion source. Multiquant software was used to control sample acquisition and data analysis. The QTRAP 6500+ mass spectrometer was adjusted and calibrated according to the manufacturer's recommendations. For quantification, an external standard calibration curve was prepared using a series of standard samples containing the following GSL: Gluconapin (GNA), progoitrin (PRO) & glucobrassicin (GBS), with concentrations: 1000 ng mL^{-1}, 500 ng mL^{-1}, 250 ng mL^{-1}, 100 ng mL^{-1}, 50 ng mL^{-1}, 10 ng mL^{-1}, and 1 ng mL^{-1}. Two-way mixed ANOVA in R was performed at 0.05 significance level to calculate the significance variation in concentration under heat treatment as compared to control.

3.2.10 Sugars content extraction and analysis

The extraction of soluble sugars (glucose, fructose, and sucrose) was performed as follows: 50 mg of ground leaf powder was extracted with 1 mL of 62.5 % methanol: 37.5% HPLC-grade water (v/v) at 55 °C over a period of 15 min in a shaking water bath and vortexed every 5 min. Afterwards, the samples were allowed to cool for 2 min and then centrifuged at 13000 rpm for 10 min and filtered using 0.25 µm filter discs. The supernatant was then transferred to a clean labelled Eppendorf tube and stored at -80 °C until further analysis. Before HPLC analysis, the samples were diluted in HPLC-grade water in a 1:2 ratio (dilution factor = 3). Chromatographic separation of sugars was performed using HPLC (Agilent 1260, Infinity Series) equipped with an Evaporative Light Scattering Detector (ELSD) system and an Asahipak NH2P-50 4E column (250 × 4.6 mm; Shodex, Tokyo, Japan) in an isocratic elution mode. The mobile phase consisted of 75 % acetonitrile: 25 % water at a flow rate of 1 mL min^{-1}, a column temperature of 40 °C and an injection volume of 20 µL. The analysis had a total run time of 25 min. For quantification, an external standard calibration curve was prepared using a series of standard samples containing glucose, fructose and sucrose with concentrations: 0.025 mg mL^{-1}, 0.05 mg mL^{-1}, 0.1 mg mL^{-1}, 0.5 mg mL^{-1} and 1 mg mL^{-1}.

3.2.11 Near-Infra Red Spectroscopy (NIRS) scanning

Following the treatments, the plants were allowed to complete their lifecycle and total aboveground dry weight and seed yields were collected from individual plants. Seed samples were analysed with a DA 7250 NIR analyzer (PerkinElmer, Beaconsfield, UK). The seed samples were analysed for moisture, oil and protein concentrations as well as for fatty acid composition of the oil.

3.2.12 Statistical analysis

Two-way mixed ANOVA was performed at 0.05 significance level to calculate the statistically significant differences between the means of sugars and GSL concentrations under heat treatment as compared to control. The assumption of two-way ANOVA was checked through normality test, homogeneity of variance

and Levene test. Then, for a significant two-way interaction between treatment and timepoint, the following analysis were conducted: The simple main effects of treatment on sugars concentration were examined at each timepoint, using Bonferroni-adjusted p-values for multiple comparisons. This was followed by pairwise comparisons between treatment levels (heat stress vs. control) at each timepoint to determine whether the mean sugars concentrations differed significantly between heat stress and control (Table S8 - Table S13). In addition, NIRS data were analysed for the investigated components using a t-test. All statistical analyses were performed using R.

3.3 Results

3.3.1 Prolonged heat treatment reduced yield and altered *B. napus* oil composition

Heat treatment significantly altered plant growth and appearance, with visible physiological changes to the flowers and buds (Figure 3.3). At 5 DAT, flowers displayed smaller and paler petals compared to plants growing under control conditions. At 3 DOR, many buds suffered from abscission, appeared brown and shrunken in size on plants subjected to the heat treatment. At maturity, total seed weight, but not biomass, was significantly ($P<0.05$) lower in heat treated plants compared to control plants (Figure 3.4). In terms of oil quality, heat treatment significantly decreased linoleic acid, linolenic acid and palmitic acid concentration, but increased oleic acid and stearic acid concentration in seeds compared to control plants (Figure 3.5).

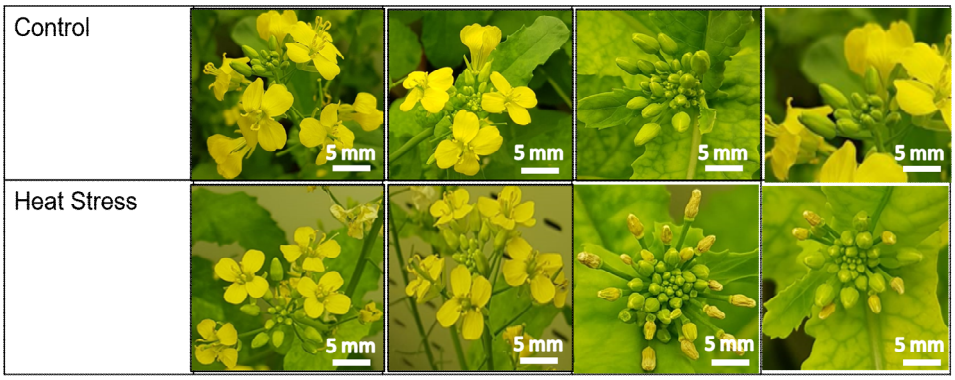

Figure 3.3 *B. napus* flowers and buds at 5 DAT & 3 DOR respectively.

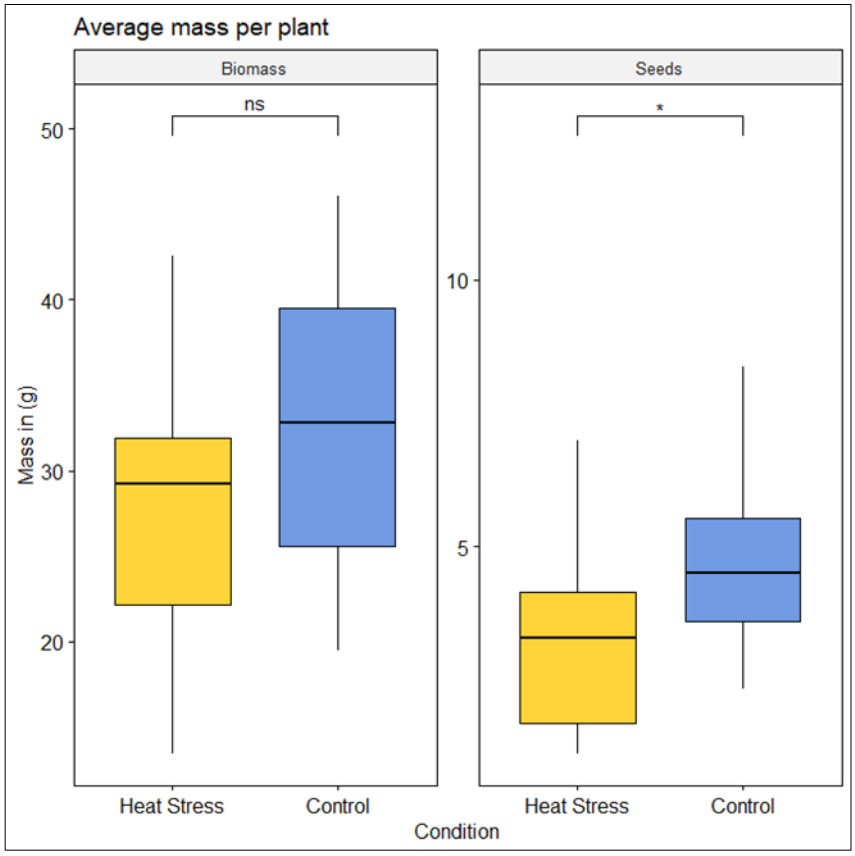

Figure 3.4 Above ground dry weight and seed yield per plant at maturity. Significant differences between heat-treated and control plants are denoted by (*) for p-value ≤ 0.05.

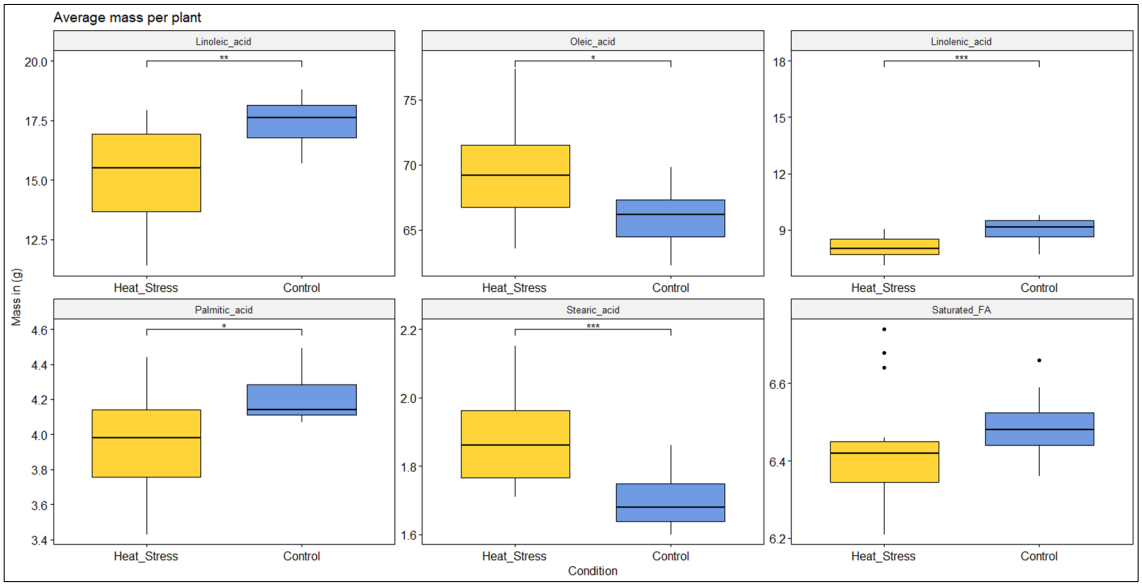

Figure 3.5 Fatty acid content in seeds per plant. Seeds from heat-treated plants (yellow) exhibited significant decrease in linoleic acid, linolenic acid and palmitic acid concentrations, but an increase in oleic acid and stearic acid concentrations compared to seeds from control plants (blue). Significant differences between heat-treated and control plants are denoted by (*) for p-value ≤ 0.05 or (**) for p-value ≤ 0.01 or (***) for p-value ≤ 0.001.

3.3.2 Differential gene expression identifies distinct patterns of expression in *B. napus* under heat stress and recovery

Libraries were constructed and sequenced on the Illumina NovaSeq platform. An average of 47 million reads were generated per sample after low quality reads had filtered out (Table S1). Following this cleaning step, around 90%-95.49% total reads per sample were mapped to the *B. napus* cv. Westar v0 reference genome (Figure S1) (Song et al., 2020). Principal Component Analysis (PCA) was used to analyse the samples which drove the group separation (Figure 3.6). PC1 and PC2 captured 48.9% and 8.2% of the total variance of the samples respectively. The analysis showed that, both control and treatment samples clustered near each other at 7 DOR which demonstrated that heat treated plants might have started to return to normal after 7 days of recovery.

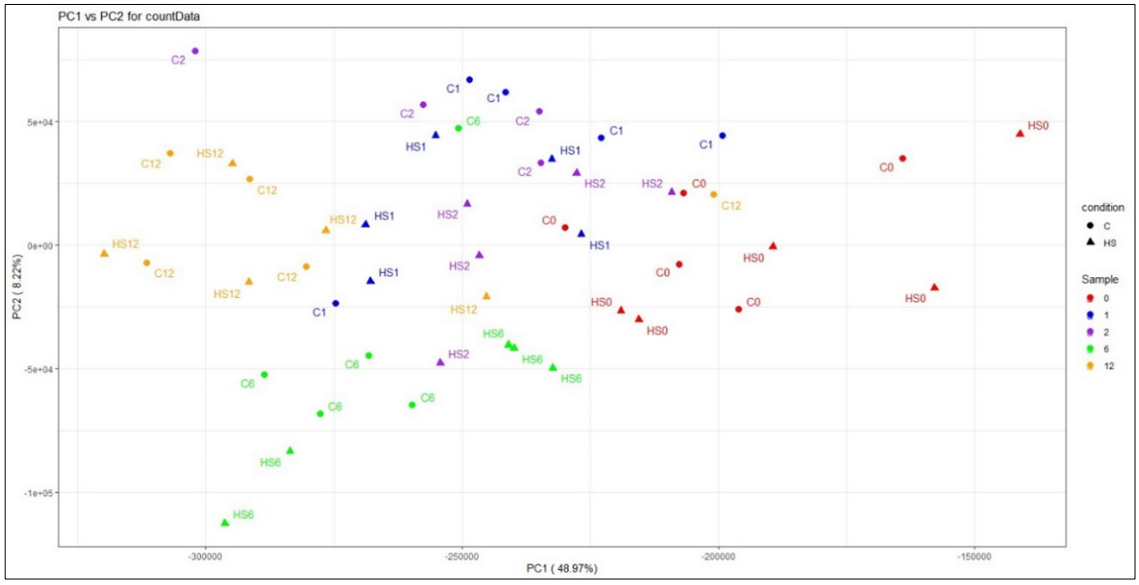

Figure 3.6 Principal component analysis of the control and heat treatment samples: Samples distribution in PC1 and PC2.

To understand the effect of heat stress on gene expression in *B. napus*, DEGs were identified in a pairwise comparison between heat treatment and control conditions at five timepoints during and after heat treatment. A total of 1892, 3253, 4553, 4165 and 1713 genes were DE at days 0, 1, 2 DAT and 1 & 7 DOR respectively (Figure S3).

The analysis of the top DEGs after 24 hours of heat treatment revealed that several of the most up-regulated transcripts were associated with sHSP20 and other heat shock-related factors, including elongation factor 1-beta 1-like, chaperone protein ClpB1, and zinc finger protein. In addition, the analysis also identified transcripts involved in regulatory roles in plant abiotic stress defence, such as ATP-dependent DNA helicase *CHR12* (Mohapatra et al., 2023), developmentally regulated G-protein (Wu et al., 2018), pre-mRNA-processing factor *SLU7* (Cui & Xiong, 2015), and pyridoxal 5'-phosphate synthase (Hanson et al., 2016) (Table 3.1).

Conversely, the top down-regulated genes included transcripts associated with sugars and auxin transporters, such as *SWEET4* and auxin:hydrogen symporters, as well as nitrogen transport and storage components like asparagine synthetase. Transcripts involved in cell wall modification, including endo-1,4-beta-xylanase, peroxidase, and pectinesterase/pectinesterase

inhibitors, were also prominent. In addition, there were transcripts mapped to enzymes involved in methylation, such as homocysteine s-methyltransferase, as well as those linked to protein biosynthesis and catabolism, like glutamyl-tRNA(Gln) amidotransferase subunit A (Pujol et al., 2008), and transcription factors, including homeobox-leucine zipper protein. Furthermore, the analysis identified transcripts related to the respiratory pathway, such as alanine-glyoxylate aminotransferase (*AGT*) (Liepman et al., 2019), along with regulatory components involved in the plant stress response, including serine/threonine protein phosphatase (Farkas et al., 2007) and strictosidine synthase (Wang et al., 2023a) (

Table 3.2).

Similarly, after 48 hours of heat treatment, many of the identified transcripts remained among the top DE, both up and down-regulated, highlighting the significant effect of heat stress on the functions performed by these enzymes and proteins (Table 3.3 and Table 3.4).

Table 3.1 Log2FC of top 30 up-regulated genes at 1 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)

Gene	HS0vsC0	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Gene description
Bnascaffold2770G0000100WE	NA*	19.39	22.95	-22.29	NA	Probable ATP-dependent DNA helicase CHR12; K11647 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2/4 [EC:3.6.4.-]
BnaC03G0236200WE	10.026	10.83	11.02	5.90	NA	HSP22; 22.0 kDa heat shock protein; K13993 HSP20 family protein
BnaA06G0312000WE	7.11	7.78	6.90	NA	NA	23.6 kDa heat shock protein, mitochondrial-like; K13993 HSP20 family protein
BnaA01G0068500WE	7.69	7.31	6.12	6.23	NA	23.6 kDa heat shock protein, mitochondrial-like; K13993 HSP20 family protein
BnaC06G0410600WE	5.13	6.54	7.80	4.71	1.57	Developmentally-regulated G-protein 2; K06944 uncharacterized protein
BnaC02G0043400WE	7.05	5.56	6.30	4.49	NA	17.6 kDa class II heat shock protein; K13993 HSP20 family protein
BnaC05G0190700WE	4.42	5.55	5.20	3.39	NA	P23; uncharacterized protein OsI_027940-like; K15730 cytosolic prostaglandin-E synthase [EC:5.3.99.3]
BnaA06G0177800WE	6.46	5.33	4.96	5.62	NA	BcHSP, BcHSP17.6; 17.4 kDa class I heat shock protein;

						K13993 HSP20 family protein
BnaA09G0429400WE	4.48	5.32	5.65	4.46	NA	P23; uncharacterized protein OsI_027940-like; K15730 cytosolic prostaglandin-E synthase [EC:5.3.99.3]
BnaA10G0226400WE	4.75	5.14	4.92	3.19	NA	17.6 kDa class II heat shock protein-like; K13993 HSP20 family protein
BnaA08G0073800WE	4.06	4.74	4.64	3.27	2.13	Zinc finger protein ZPR1-like; K06874 zinc finger protein
Bnascaffold3043G0000200WE	4.53	4.42	4.78	3.69	NA	17.6 kDa class II heat shock protein; K13993 HSP20 family protein (A)
BnaC09G0508300WE	4.00	4.34	3.70	2.13	NA	Elongation factor 1-beta 1-like; K03232 elongation factor 1-beta
BnaC08G0070600WE	3.19	4.27	3.78	2.48	1.29	Zinc finger protein ZPR1-like; K06874 zinc finger protein
BnaC06G0424500WE	3.25	3.72	3.33	2.49	NA	Chaperone protein ClpB1; K03695 ATP-dependent Clp protease ATP-binding subunit ClpB
BnaA03G0160300WE	NA	3.433512	2.66	NA	NA	Potassium transporter 1; K03549 KUP system potassium uptake protein
BnaC06G0380500WE	NA	3.28	5.34	3.86	5.64	Hypothetical protein; K17803 methyltransferase OMS1, mitochondrial [EC:2.1.1.-]
BnaC01G0386900WE	3.47	3.17	3.31	2.84	NA	Probable mediator of RNA polymerase II transcription subunit 37c; K03283 heat shock 70kDa protein 1/8
BnaA07G0329100WE	3.51	3.11	3.11	3.09	NA	Developmentally regulated G-protein 2; K06944 uncharacterized protein
BnaA07G0341000WE	2.98	2.99	2.76	2.65	NA	Chaperone protein ClpB1; K03695 ATP-dependent Clp protease ATP-

						binding subunit ClpB
BnaC04G0385500WE	2.89	2.92	3.32	2.29	NA	40S ribosomal protein S9-2; K02997 small subunit ribosomal protein S9e
BnaA01G0210500WE	2.32	2.83	2.12	2.74	1.25	Pre-mRNA-splicing factor SLU7-like; K12819 pre-mRNA-processing factor SLU7
BnaA02G0000800WE	NA	2.83	10.64	NA	11.21	Pyridoxal biosynthesis protein PDX1.3; K06215 pyridoxal 5'-phosphate synthase pdxS subunit [EC:4.3.3.6]
BnaA05G0117900WE	2.46	2.79	2.82	1.65	NA	Heat shock 70 kDa protein 8-like; K09489 heat shock 70kDa protein 4
BnaA03G0022900WE	1.93	2.70	1.92	2.25	NA	Transcription factor bHLH100-like; K18486 heart-and neural crest derivatives-expressed protein 2
BnaA07G0306600WE	1.93	2.70	1.92	2.25	NA	Transcription factor bHLH100-like; K18486 heart-and neural crest derivatives-expressed protein 2
BnaA05G0366100WE	NA	2.67	4.27	NA	NA	Chaperonin 60 subunit beta 2, chloroplastic; K04077 chaperonin GroEL
BnaA03G0344900WE	2.25	2.67	2.54	1.34	NA	Chaperonin 60 subunit beta 2, chloroplastic-like; K04077 chaperonin GroEL
BnaA09G0044200WE	2.42	2.46	2.98	1.30	NA	Beta-D-xylosidase 1; K15920 beta-D-xylosidase 4 [EC:3.2.1.37]
BnaA04G0180700WE	2.21	2.41	2.79	2.84	NA	17.6 kDa class I heat shock protein 2-like; K13993 HSP20 family protein

Table 3.2 Log2FC of top 30 down-regulated genes at 1 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)

Gene	HS0vsC0	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Gene description
BnaA01G0299900WE	NA*	-25.04	-24.76	NA	NA	Probable ATP-dependent DNA helicase CHR12; K11647 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2/4 [EC:3.6.4.-]
BnaA02G0390800WE	NA	-24.10	NA	NA	NA	50S ribosomal protein L22-like; K02890 large subunit ribosomal protein L22
Bnascaffold390G0000100WE	NA	-22.84	23.50	NA	NA	BTB/POZ and MATH domain-containing protein 2-like; K10523 speckle-type POZ protein
BnaA09G0548300WE	NA	-21.05	NA	NA	NA	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform-like; K03456 serine/threonine-protein phosphatase 2A regulatory subunit A
Bnascaffold2041G0000500WE	NA	-10.50	NA	NA	-9.63	Solute carrier family 2, facilitated glucose transporter member 8-like; K08145 MFS transporter, SP family, solute carrier family 2 (facilitated glucose transporter), member 8
BnaC03G0531800WE	NA	-10.02	NA	NA	NA	Asparagine synthetase [glutamine-hydrolyzing]; K01953 asparagine synthase

						(glutamine-hydrolysing) [EC:6.3.5.4]
BnaA05G0410300WE	NA	-7.40	8.06	NA	NA	Cell division control protein 48-like; K08900 mitochondrial chaperone BCS1 (A)
BnaC03G0161100WE	NA	-5.64	-7.23	-6.53	NA	Bidirectional sugars transporter SWEET9; K15382 solute carrier family 50 (sugars transporter)
BnaA08G0122000WE	NA	-5.09	NA	NA	NA	Endo-1,4-beta-xylanase C-like; K01181 endo-1,4-beta-xylanase [EC:3.2.1.8]
BnaC06G0349400WE	NA	-4.83	NA	NA	NA	Peptidase, putative (EC:3.4.21.25); K18443 golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1
BnaA05G0285000WE	NA	-4.25	-3.68	-4.24	NA	Homocysteine S-methyltransferase 3; K00547 homocysteine S-methyltransferase [EC:2.1.1.10]
BnaC02G0269700WE	NA	-3.97	NA	NA	NA	Strictosidine synthase 1-like; K01757 strictosidine synthase [EC:4.3.3.2]
BnaC03G0440400WE	NA	-3.43	NA	NA	NA	Geranylgeranyl diphosphate reductase, chloroplastic; K10960 geranylgeranyl reductase [EC:1.3.1.83]
BnaA08G0177000WE	NA	-3.41	NA	NA	NA	Glutamyl-tRNA(Gln) amidotransferase subunit A; K02433 aspartyl-tRNA(Asn)/glutamyl-tRNA(Gln) amidotransferase subunit A [EC:6.3.5.6 6.3.5.7]
BnaA03G0270900WE	NA	-3.26	-3.36	-3.13	NA	Uncharacterized LOC103858630; K14488 SAUR family protein
BnaC05G0336600WE	-2.85	-3.01	-3.35	-2.71	NA	Homocysteine S-methyltransferase 3-like; K00547

						homocysteine S-methyltransferase [EC:2.1.1.10]
BnaC02G0072900WE	NA	-2.95	-3.60	-3.89	NA	Homeobox-leucine zipper protein HDG9-like; K09338 homeobox-leucine zipper protein (A)
BnaA03G0328400WE	-3.61	-2.90	-3.15	-3.01	NA	Alanine--glyoxylate aminotransferase 2 homolog 3, mitochondrial; K00827 alanine-glyoxylate transaminase / (R)-3-amino-2-methylpropionate-pyruvate transaminase [EC:2.6.1.44 2.6.1.40]
BnaA05G0069600WE	-2.44	-2.83	-2.23	-1.87	NA	Ribonucleoprotein At2g37220, chloroplastic-like; K11294 nucleolin
BnaC01G0410600WE	NA	-2.82	-4.13	-3.26	NA	Cytosolic sulfotransferase 11-like; K01016 estrone sulfotransferase [EC:2.8.2.4]
BnaC04G0129500WE	-2.72	-2.78	NA	-2.45	NA	Peroxidase 19; K00430 peroxidase [EC:1.11.1.7]
BnaA06G0144700WE	-2.07	-2.73	-2.42	-2.29	NA	Auxin:hydrogen symporter, putative; K07088 uncharacterized protein
BnaC07G0304700WE	-1.57	-2.70	-3.10	-1.91	NA	Bidirectional sugars transporter SWEET4-like; K15382 solute carrier family 50 (sugars transporter)
BnaC03G0244200WE	NA	-2.70	-2.35	-3.11	NA	Auxin-responsive protein SAUR36-like; K14488 SAUR family protein
BnaC04G0003100WE	-3.12	-2.66	NA	NA	NA	Probable pectinesterase/pectinesterase inhibitor 20; K01051 pectinesterase [EC:3.1.1.11]
BnaA04G0096200WE	NA	-2.55	-2.11	NA	NA	Proline dehydrogenase 2, mitochondrial; K00318 proline dehydrogenase [EC:1.5.-.-]

BnaC02G0359700WE	-2.66	-2.53	-3.28	NA	NA	BAHD acyltransferase DCR; K19747 BAHD acyltransferase [EC:2.3.1.-]
BnaA05G0341600WE	NA	-2.43	-2.45	NA	NA	AAEL008905-PA; K14966 host cell factor
BnaC03G0583300WE	NA	-2.37	NA	NA	NA	Glutamyl-tRNA(Gln) amidotransferase subunit A; K02433 aspartyl-tRNA(Asn)/glutamyl-tRNA(Gln) amidotransferase subunit A [EC:6.3.5.6 6.3.5.7]
BnaC05G0172700WE	-2.12	-2.34	-2.52	-2.03	NA	Auxin:hydrogen symporter, putative; K07088 uncharacterized protein

Table 3.3 Log2FC of top 30 up-regulated genes at 2 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)

Gene	HS0vsC	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Gene Description
BnaC06G0468700WE	NA*	NA	24.45	-23.10	NA	Sister chromatid cohesion protein PDS5 homolog A; K11267 sister chromatid cohesion protein PDS5
BnaC02G0000100WE	NA	NA	23.93	NA	24.17	Uncharacterized LOC104816269; K11978 E3 ubiquitin-protein ligase UBR3 [EC:2.3.2.27]
Bnascaffold390G0000100WE	NA	-22.84	23.50	NA	NA	BTB/POZ and MATH domain-containing protein 2-like; K10523 speckle-type POZ protein
Bnascaffold2770G0000100WE	NA	19.39	22.95	-22.29	NA	Probable ATP-dependent DNA helicase CHR12; K11647 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2/4 [EC:3.6.4.-]
BnaC02G0211000WE	NA	NA	21.93	-21.60	NA	Putative caffeoyl-CoA O-methyltransferase At1g67980; K00588 caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]
BnaA02G0169400WE	NA	NA	21.93	NA	NA	Putative caffeoyl-CoA O-methyltransferase At1g67980; K00588 caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]
BnaA05G0284500WE	NA	NA	21.52	NA	NA	Hypothetical protein; K00128 aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
BnaC03G0236200WE	10.02	10.83	11.02	5.90	NA	HSP22; 22.0 kDa heat shock protein;

						K13993 HSP20 family protein
BnaA02G0000800WE	NA	2.83	10.64	NA	11.21154	Pyridoxal biosynthesis protein PDX1.3; K06215 pyridoxal 5'-phosphate synthase pdxS subunit [EC:4.3.3.6]
Bnascaffold1692G0000200WE	NA	NA	10.20	NA	NA	Nucleolar protein 56-like; K14564 nucleolar protein 56
BnaA05G0410300WE	NA	-7.40	8.06	NA	NA	Cell division control protein 48-like; K08900 mitochondrial chaperone BCS1
BnaA03G0518500WE	NA	NA	7.94	NA	NA	Spermidine coumaroyl CoA acyltransferase-like; K15400 omega-hydroxypalmitate O-feruloyl transferase [EC:2.3.1.188]
BnaC06G0410600WE	5.13	6.54	7.80	4.71	1.57	Developmentally regulated G-protein 2; K06944 uncharacterized protein
BnaA05G0186500WE	NA	NA	7.73	NA	NA	Aldehyde dehydrogenase family 3 member H1-like; K00128 aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
BnaA06G0312000WE	7.11	7.78	6.90	NA	NA	23.6 kDa heat shock protein, mitochondrial-like; K13993 HSP20 family protein
BnaC02G0043400WE	7.05	5.56	6.30	4.49	NA	17.6 kDa class II heat shock protein; K13993 HSP20 family protein
BnaA01G0068500WE	7.69	7.31	6.12	6.23	NA	23.6 kDa heat shock protein, mitochondrial-like; K13993 HSP20 family protein
BnaA09G0429400WE	4.48	5.32	5.65	4.46	NA	P23; uncharacterized protein OsI_027940-like; K15730 cytosolic prostaglandin-E synthase [EC:5.3.99.3]
BnaC06G0380500WE	NA	3.28	5.34	3.86	5.64	Hypothetical protein; K17803 methyltransferase

						OMS1, mitochondrial [EC:2.1.1.-]
BnaC05G0190700WE	4.42	5.55	5.20	3.39	NA	P23; uncharacterized protein OsI_027940-like; K15730 cytosolic prostaglandin-E synthase [EC:5.3.99.3]
BnaC03G0524800WE	5.40	NA	5.08	2.72	NA	17.6 kDa class I heat shock protein 3-like; K13993 HSP20 family protein
BnaA06G0177800WE	6.46	5.33	4.96	5.62	NA	BcHSP, BcHSP17.6; 17.4 kDa class I heat shock protein; K13993 HSP20 family protein
BnaA10G0226400WE	4.75	5.14	4.92	3.19	NA	17.6 kDa class II heat shock protein-like; K13993 HSP20 family protein
Bnascaffold3043G0000200WE	4.53	4.42	4.78	3.69	NA	17.6 kDa class II heat shock protein; K13993 HSP20 family protein (A)
BnaA04G0104000WE	6.42	NA	4.72	5.49	NA	40S ribosomal protein S9-2-like; K02997 small subunit ribosomal protein S9e (A)
BnaA08G0073800WE	4.06	4.74	4.64	3.27	2.13	Zinc finger protein ZPR1-like; K06874 zinc finger protein
BnaC03G0057000WE	6.37	NA	4.49	6.20	NA	18.1 kDa class I heat shock protein-like; K13993 HSP20 family protein
BnaA05G0366100WE	NA	2.67	4.27	NA	NA	Chaperonin 60 subunit beta 2, chloroplastic; K04077 chaperonin GroEL
BnaA04G0089500WE	2.93	NA	4.26	NA	NA	15.7 kDa heat shock protein, peroxisomal; K13993 HSP20 family protein
BnaC08G0285900WE	NA	NA	4.23	NA	NA	Aquaporin PIP2-1-like; K09872 aquaporin PIP

Table 3.4 Log2FC of top 30 down-regulated genes at 2 DAT, as obtained by DESeq2 pipeline. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)

Gene	HS0vsC0	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Gene Description
BnaA01G0299900WE	NA*	-25.04	-24.76	NA	NA	Probable ATP-dependent DNA helicase CHR12; K11647 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2/4 [EC:3.6.4.-]
BnaC06G0162800WE	23.16	NA	-22.40	NA	NA	Hypothetical protein; K10352 myosin heavy chain
BnaC03G0161100WE	NA	-5.64	-7.23	-6.53	NA	Bidirectional sugars transporter SWEET9; K15382 solute carrier family 50 (sugars transporter)
BnaA08G0139800WE	NA	NA	-4.68	NA	NA	Geranylgeranyl diphosphate reductase, chloroplastic; K10960 geranylgeranyl reductase [EC:1.3.1.83]
BnaA10G0021500WE	NA	NA	-4.38	NA	NA	Secretory carrier-associated membrane protein 2-like; K19995 secretory carrier-associated membrane protein
BnaA03G0204300WE	NA	NA	-4.18	NA	NA	Bra000116, SWEET9; bidirectional sugars transporter SWEET9; K15382 solute carrier family 50 (sugars transporter)
BnaC01G0410600WE	NA	-2.82	-4.13	-3.26	NA	Cytosolic sulfotransferase 11-like; K01016 estrone sulfotransferase [EC:2.8.2.4]
BnaC05G0020800WE	NA	NA	-4.11	-4.93	NA	Secretory carrier-associated

						membrane protein 2; K19995 secretory carrier-associated membrane protein
BnaC03G0671900WE	NA	NA	-3.96	-2.74	NA	Geranylgeranyl diphosphate reductase, chloroplastic; K10960 geranylgeranyl reductase [EC:1.3.1.83]
BnaC09G0341100WE	NA	NA	-3.93	-3.46	NA	Flavone 3'-O-methyltransferase 1-like; K13066 caffeic acid 3-O-methyltransferase [EC:2.1.1.68]
BnaC08G0470200WE	NA	NA	-3.76	NA	NA	Probable fructose-bisphosphate aldolase 3, chloroplastic; K01623 fructose-bisphosphate aldolase, class I [EC:4.1.2.13]
BnaA06G0424600WE	NA	NA	-3.72	NA	NA	1,8-cineole synthase 1, chloroplastic-like; K07385 1,8-cineole synthase [EC:4.2.3.108]
BnaA03G0462900WE	NA	NA	-3.70	-5.34	NA	CYP71A13; K11868 indoleacetaldoxime dehydratase [EC:4.99.1.6]
BnaC09G0263300WE	NA	NA	-3.69	NA	NA	auxin-responsive protein SAUR36-like; K14488 SAUR family protein (A)
BnaA05G0285000WE	NA	-4.25	-3.68	-4.24	NA	Homocysteine S-methyltransferase 3; K00547 homocysteine S-methyltransferase [EC:2.1.1.10]
BnaC02G0072900WE	NA	-2.95	-3.60	-3.89	NA	Homeobox-leucine zipper protein HDG9-like; K09338 homeobox-leucine zipper protein
BnaC06G0215000WE	-5.08	NA	-3.48	NA	NA	Probable mitochondrial chaperone BCS1-B; K08900 mitochondrial chaperone BCS1

BnaA05G0348800WE	NA	NA	-3.43	-3.97	NA	NAC domain-containing protein 89-like; K12581 CCR4-NOT transcription complex subunit 7/8 (A)
BnaA03G0270900WE	NA	-3.26	-3.36	-3.13	NA	uncharacterized LOC103858630; K14488 SAUR family protein (A)
BnaC05G0336600WE	-2.85	-3.01	-3.35	-2.71	NA	Homocysteine S-methyltransferase 3-like; K00547 homocysteine S-methyltransferase [EC:2.1.1.10]
BnaA02G0003400WE	-2.32	NA	-3.32	NA	NA	ATP-dependent zinc metalloprotease FTSH 10, mitochondrial; K08956 AFG3 family protein [EC:3.4.24.-] (A)
BnaA08G0141600WE	NA	NA	-3.31	-6.37	NA	Venom phosphodiesterase 2-like; K01513 ectonucleotide pyrophosphatase/ phosphodiesterase family member 1/3 [EC:3.1.4.1 3.6.1.9]
BnaC02G0359700WE	-2.66	-2.53	-3.28	NA	NA	BAHD acyltransferase DCR; K19747 BAHD acyltransferase [EC:2.3.1.-]
BnaA03G0328400WE	-3.61	-2.90	-3.15	-3.01	NA	Alanine--glyoxylate aminotransferase 2 homolog 3, mitochondrial; K00827 alanine-glyoxylate transaminase / (R)-3-amino-2-methylpropionate-pyruvate transaminase [EC:2.6.1.44 2.6.1.40]
BnaC07G0304700WE	-1.57	-2.70	-3.10	-1.91	NA	Bidirectional sugars transporter SWEET4-like; K15382 solute carrier family 50 (sugars transporter)
BnaC06G0297300WE	NA	NA	-3.06	NA	NA	Uncharacterized LOC101250545;

						K11426 SET and MYND domain-containing protein
BnaC03G0258400WE	NA	NA	-2.99	NA	NA	Glutathione S-transferase F3-like; K00799 glutathione S-transferase [EC:2.5.1.18]
BnaC04G0492800WE	NA	-1.58	-2.95	NA	NA	BrNIT1, NIT-T2; nitrilase 2-like; K01501 nitrilase [EC:3.5.5.1]
BnaC01G0191600WE	NA	NA	-2.95	-5.16	NA	Caffeic acid 3-O-methyltransferase-like; K13066 caffeic acid 3-O-methyltransferase [EC:2.1.1.68]
BnaA10G0150800WE	NA	-2.02	-2.86	NA	NA	Laccase-17-like; K05909 laccase [EC:1.10.3.2]

To identify trends and clusters in the expression of genes across the time course of the experiment, K-means clustering was applied to 9933 DE genes with logFC ≥ |0.5|. The algorithm assigned each gene into one of the clusters based on the Euclidean distance between the gene and the cluster mean. Six distinctive clusters based on expression changes across the five timepoints were identified (Figure 3.7). Clusters 1 & 3 (475 & 607 genes) showed opposite expression patterns towards the end of the heat treatment and the start of the recovery phase. In cluster 1, the expression profile of genes decreased at 1 DOR (HS6vsC6), before returning to normal levels at 7 DOR (HS12vsC12), while in cluster 3, a sharp increase in expression was observed at 1 DOR (HS6vsC6), which returned to normal levels at 7 DOR (HS12vsC12).

In clusters 2 & 5 (3470 & 1333 genes), genes were down-regulated along the course of the experiment, but more pronounced during the heat treatment phase especially in cluster 5, which exhibited a greater decease in expression. Both clusters 4 & 6 (398 & 3518 genes) showed increased expression during the treatment, before it started to decline towards the end, reaching normal levels at 7 DOR (HS12vsC12).

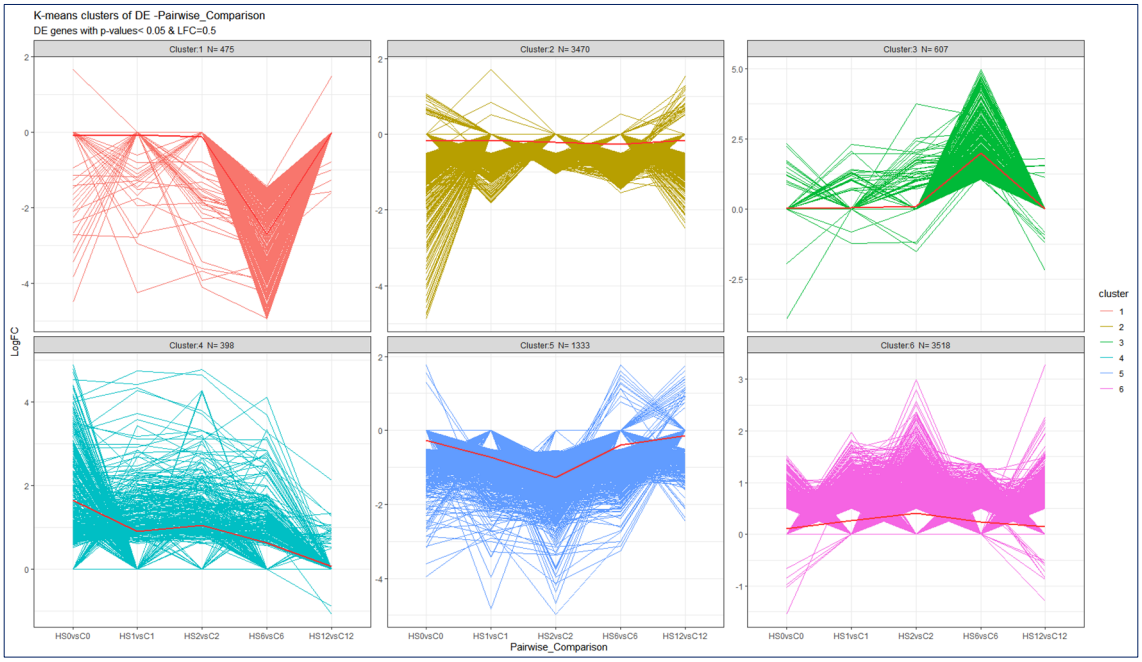

Figure 3.7 K-means cluster analysis of DEGs in *B. napus* during and after heat treatment. K-means clustering was applied to 9933 DEGs with log2FC ≥ 0.5 using the "kmeans" function in R package "stats" (v. 3.2.2), where k represents a pre-specified number of clusters. Six groups of genes were classified, with N the number of genes in each cluster, and the solid red lines in each cluster indicate the mean in differential expression. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

3.3.3 Validation of RNA-seq data by quantitative real-time PCR

To verify the reliability of RNA sequencing results, two genes with diverse expression profiles, including up-regulated or down-regulated at different timepoints of the experiment, were selected for real-time qPCR to measure expression levels. As a result, *SS5* (sucrose synthase 5) and *HSP20* (heat shock protein 20), exhibited similar expression profiles between RT-qPCR and RNA-seq data (Figure 3.8, Figure 3.9 &

Table S4), with correlation coefficients of r=0.86, and r=0.68 respectively.

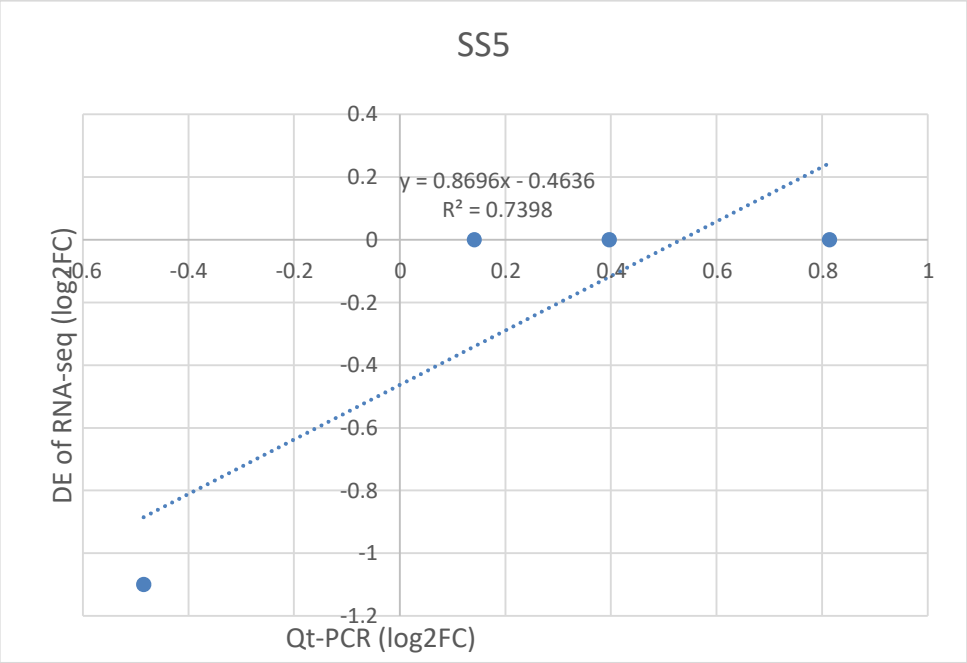

Figure 3.8 Correlation analysis of the results obtained from RNA-seq and qt-PCR: *SS5* exhibited similar expression profiles between RT-qPCR and RNA-seq data with correlation coefficients of r=0.86.

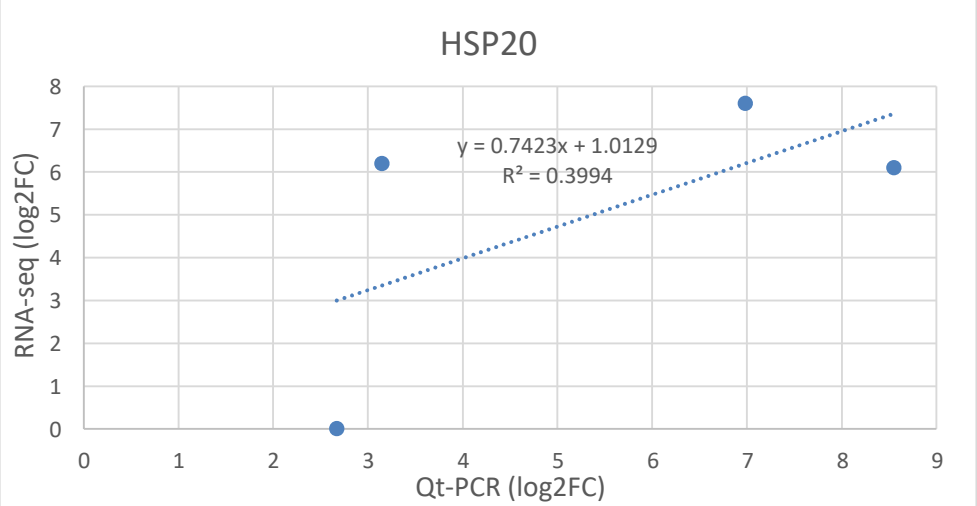

Figure 3.9 Correlation analysis of the results obtained from RNA-seq and qt-PCR: *HSP20* exhibited similar expression profiles between RT-qPCR and RNA-seq data with correlation coefficients of r=0.63.

3.3.4 Functional annotation of DEGs identifies key pathways responding to heat treatment and recovery

Gene ontology (GO) enrichment analysis of the DEGs was performed to identify enriched GO terms. Terms such as “binding”, “catalytic activity”, “metabolic

process", "hydrolase activity" and "ion binding" were among the predominant enriched terms at all timepoints with more transcripts mapped to these terms during heat treatment than during recovery (Figure 3.10-Figure 3.14). Oxidoreductase, hydrolase and transferase were among the top identified enzymes (Figure 3.15). These results suggest that high temperature activates several metabolic processes through the expression of numerous enzymes involved in alleviating the impact of heat stress not only during the stress period but also during recovery.

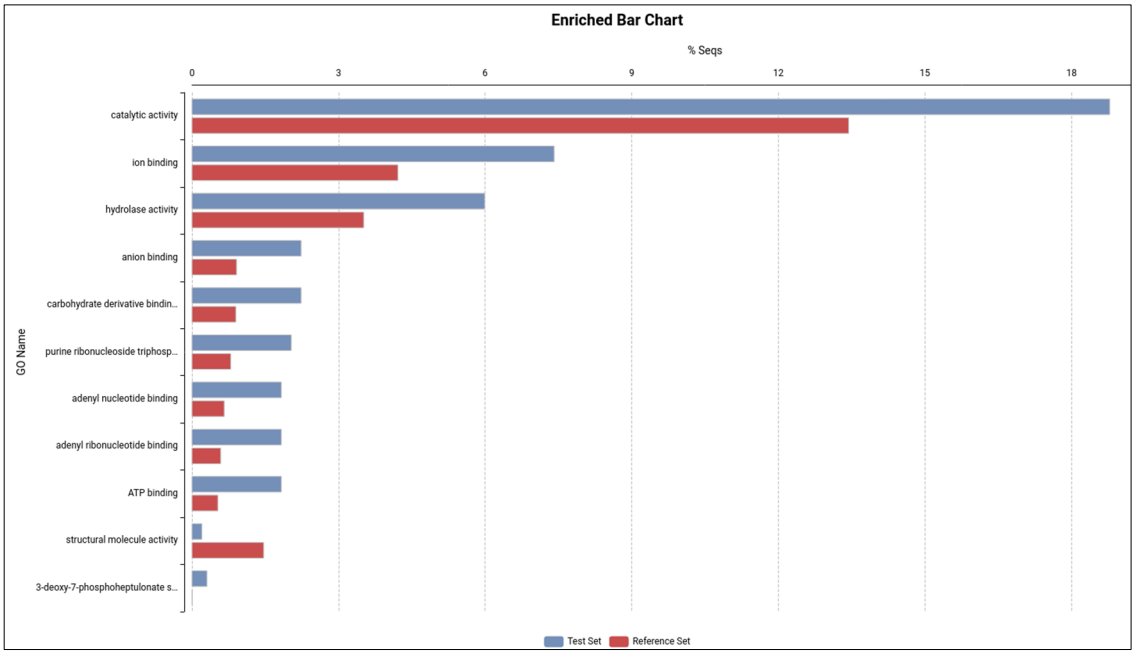

Figure 3.10 Enriched GO terms at 0 DAT, as identified by GO enrichment analysis.

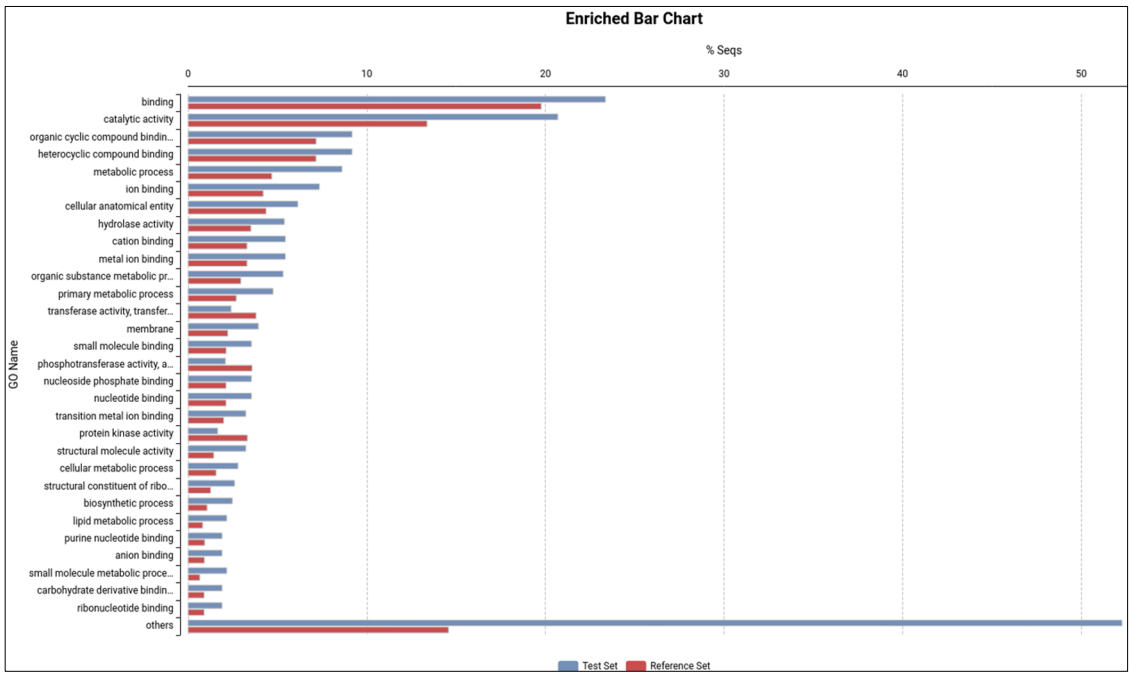

Figure 3.11 Enriched GO terms at 1 DAT, as identified by GO enrichment analysis.

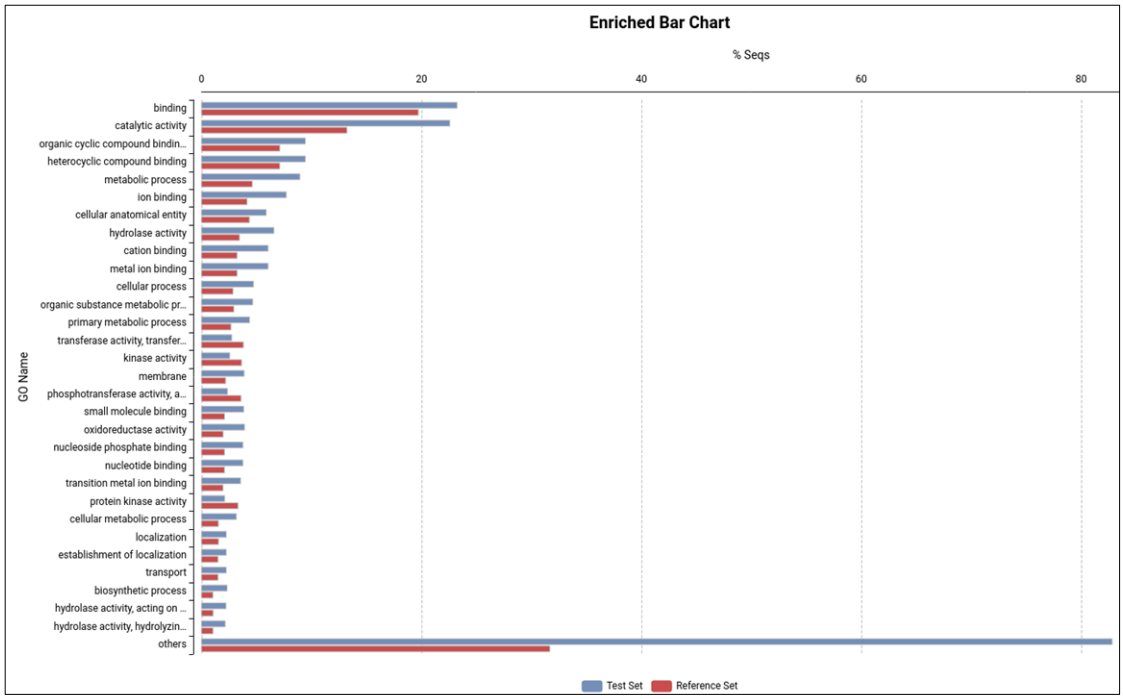

Figure 3.12 Enriched GO terms at 2 DAT, as identified by GO enrichment analysis.

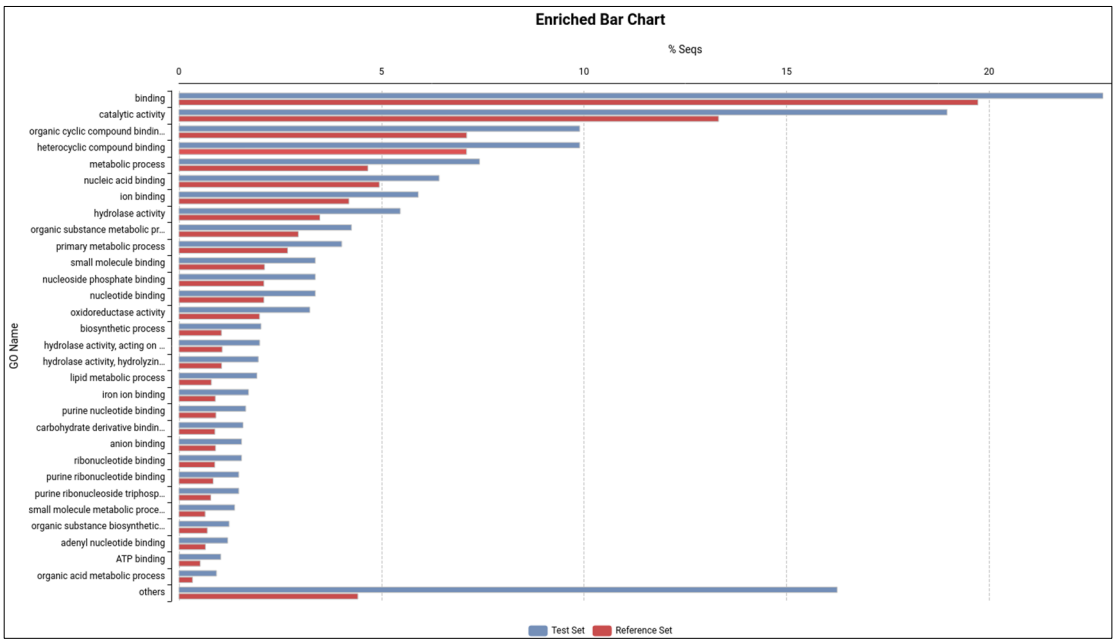

Figure 3.13 Enriched GO terms at 1 DOR, as identified by GO enrichment analysis.

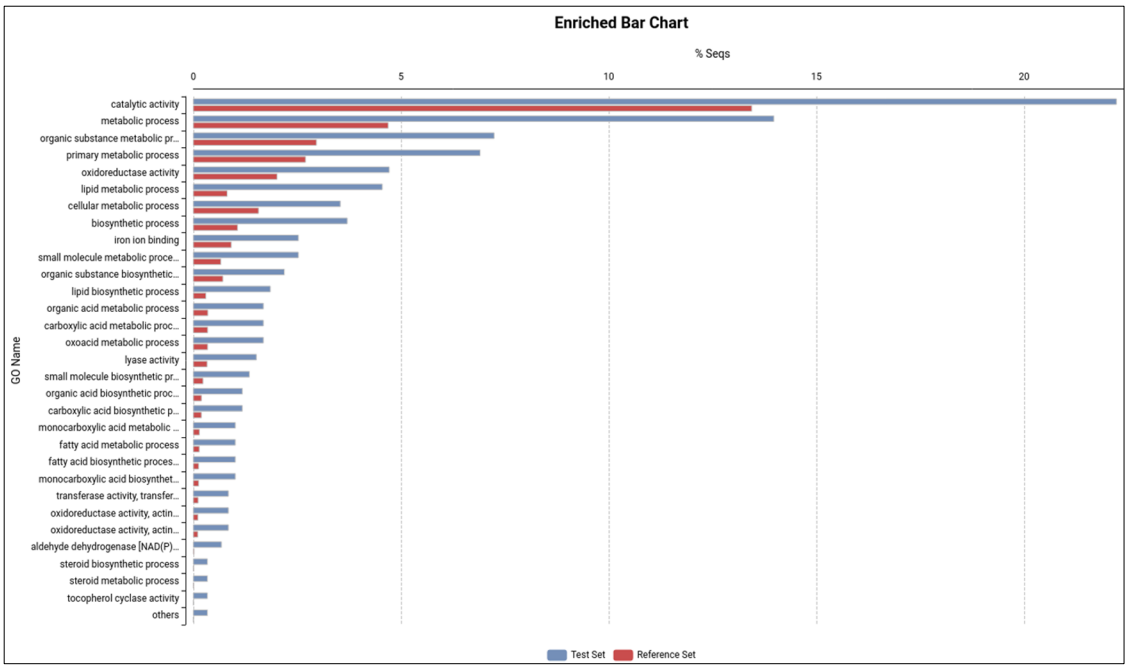

Figure 3.14 Enriched GO terms at 7 DOR, as identified by GO enrichment analysis.

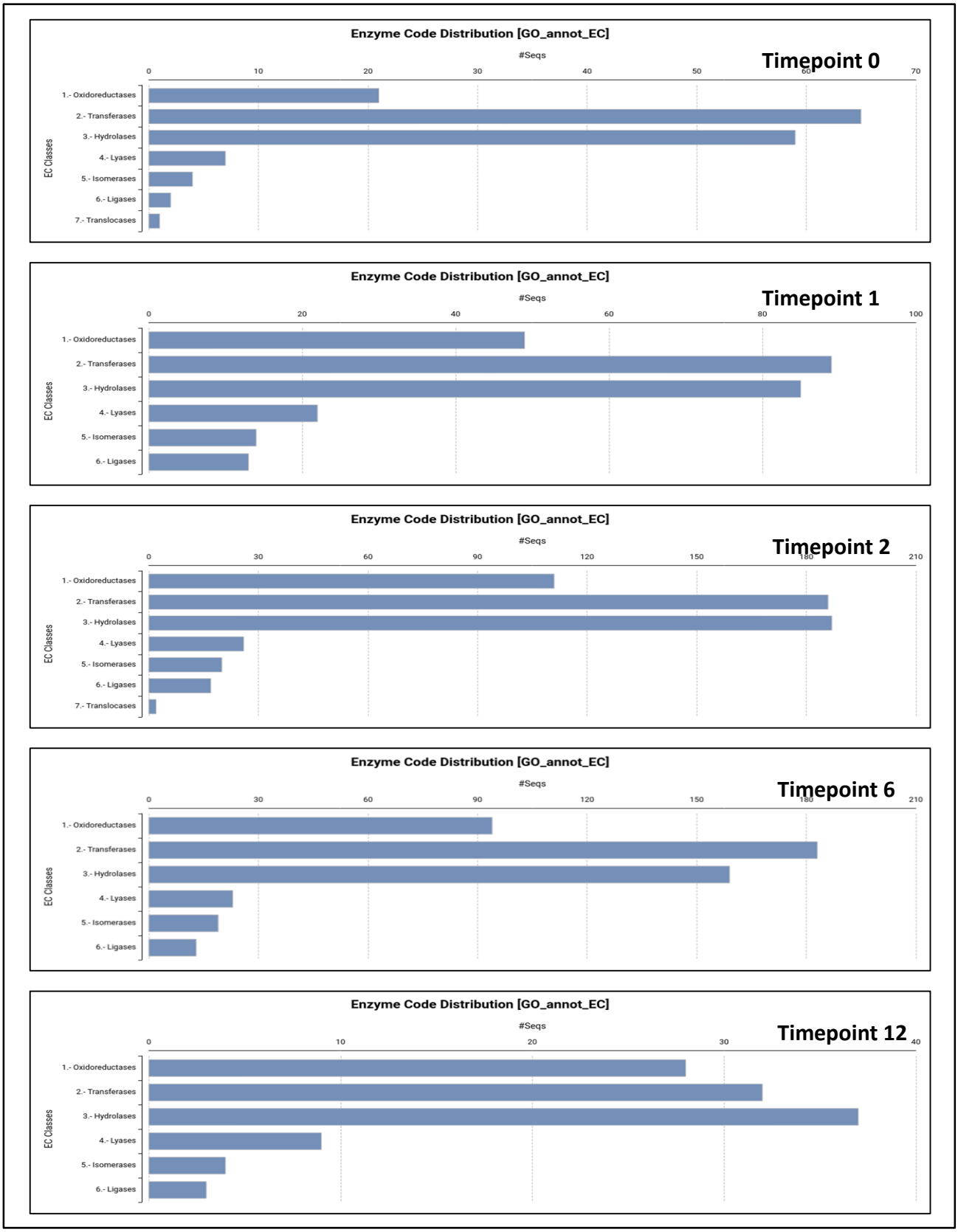

Figure 3.15 Enzyme code distribution at different timepoints as identified by GO enrichment analysis.

Based on KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis (Kanehisa & Goto, 2000), pathways related to respiratory metabolism, namely Glycolysis and Pentose phosphate pathway were enriched in all heat treatment comparisons at all timepoints, while Citrate cycle and Oxidative phosphorylation were enriched at 2 DAT and 1 DOR respectively (Figure 3.16 & Figure S5-Figure S7). Other related pathways were also enriched along the course of the treatment and during recovery period, and these include one Carbon pool by folate (enriched at 1 & 2 DAT and 1 DOR), Carbon fixation in Photosynthetic organisms, Cysteine and methionine metabolism (enriched at 1 & 2 DAT), Glycerolipid and Tryptophan metabolism (enriched at 2 DAT), Fatty acid biosynthesis and Pyruvate metabolism (enriched at 1 DOR) and Fructose and mannose metabolism (enriched at 7 DOR).

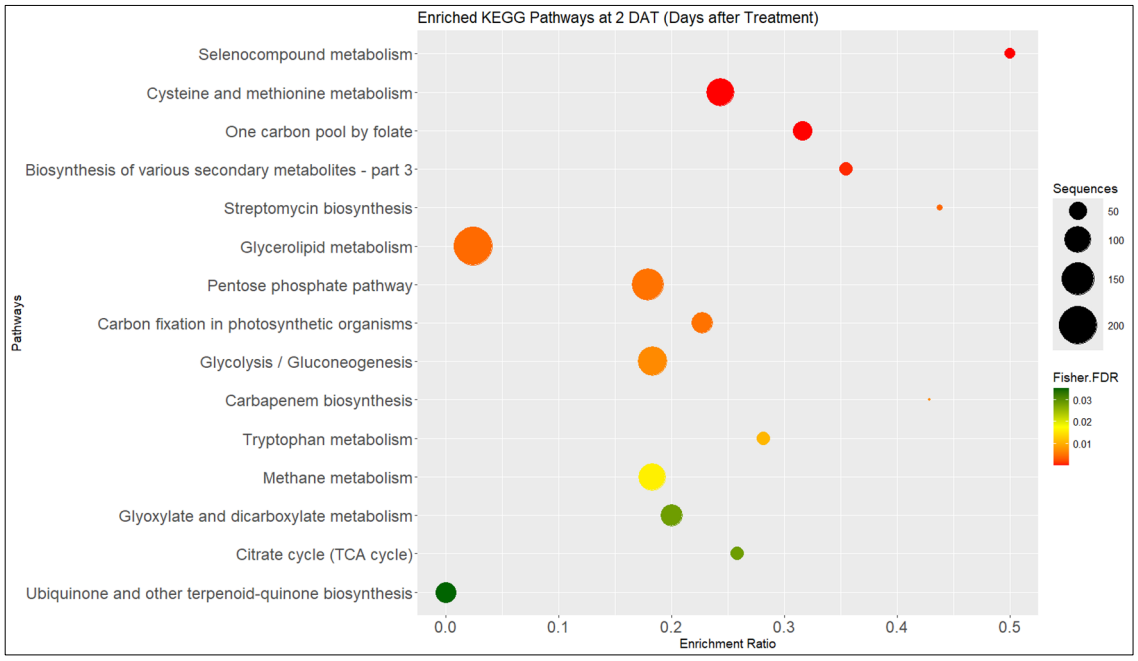

Figure 3.16 The top enriched KEGG pathways and their corresponding number of genes at 2 DAT at p-value ≤ 0.05. The x-axis represents the enrichment factor. The y-axis represents KEGG pathways. The circle represents the number of genes mapped to pathways and the red to green bar indicates the significance.

In the present study, most of the genes involved in these pathways were responsive to high temperature stress, with their expression declining with the onset of heat treatment, especially after 48 hours (Figure 3.17, Figure 3.18 & Figure S8-Figure S13).

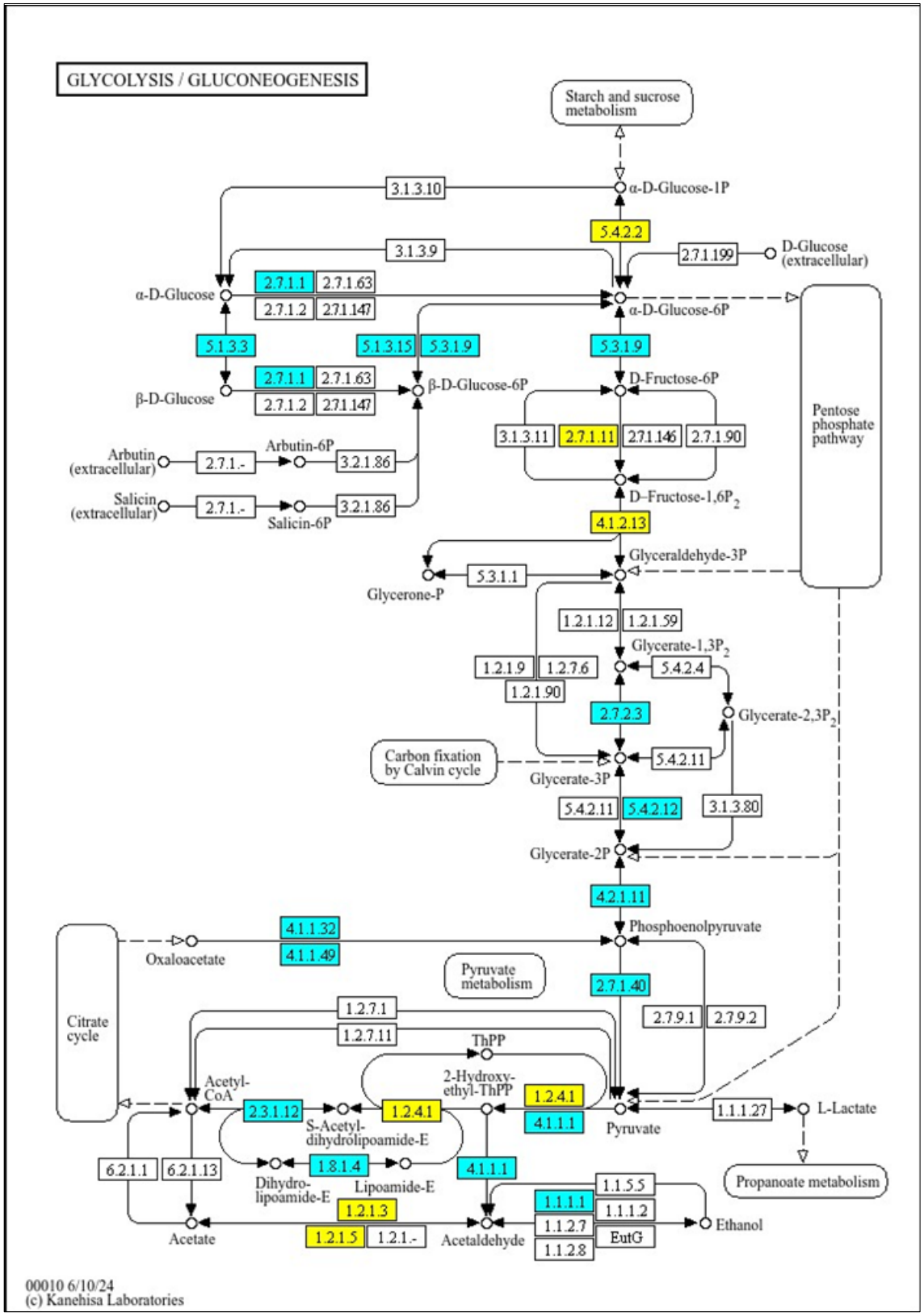

Figure 3.17 KEGG pathway for Glycolysis at 2 DAT. *Blue denotes down-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

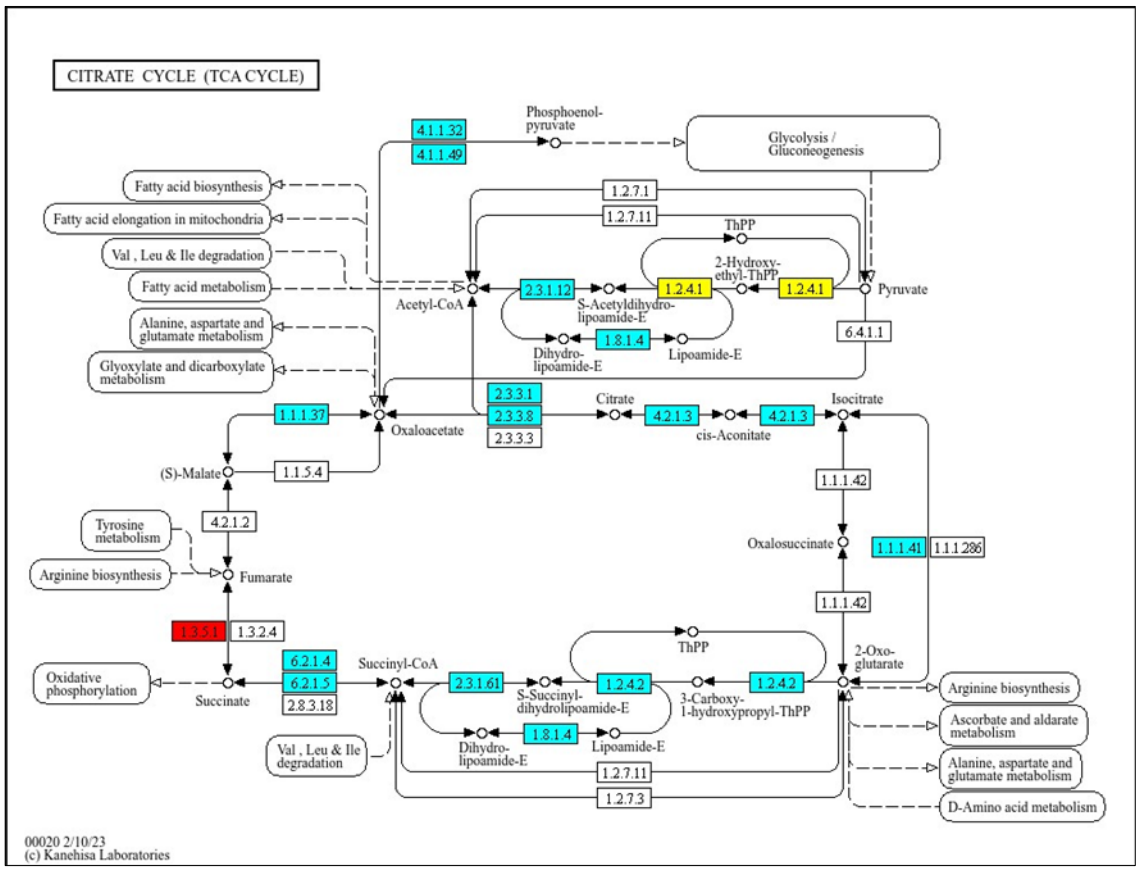

Figure 3.18 KEGG pathway for Citrate cycle at 2 DAT. *Blue denotes down-regulated genes, red denotes up-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

3.3.4.1 Heat treatment down regulated the transcript levels of most aliphatic and indolic GSLs synthetic genes

Analysis of the aliphatic GSL synthetic and regulatory genes showed that heat treatment had a significant effect on the expression of 18 genes at 2 DAT (Figure 3.19). Thirteen genes encoding different GSL synthetic genes including different UDP glycosyltransferases and glutathione s-transferases were down-regulated while five genes encoding GSL related transcription factors were up-regulated. At 1 DOR, which marks 24 hours of recovery, nine genes that were not DE under heat treatment were found up-regulated. These genes include different cytochrome P450 genes, transcription factors, branched-chain aminotransferase 4.2 and isopropylmalate dehydrogenase 2.

Analysis of the indolic GSL synthetic and regulatory genes showed that heat treatment altered the expression of 16 and 23 out of 44 genes at 1 and 2 DAT respectively (Figure 3.20). These genes include sulfotransferases, cytochrome

P450 genes, UDP glycosyltransferases, glutathione s-transferases and transcription factors. After one week recovery (7 DOR), most of the DEGs identified during heat treatment were no longer DE. This is similar to what was observed with the genes encoding aliphatic GSL synthetic enzymes. Interestingly, one transcript encoding UDP glycosyltransferase 74E2-like remained down-regulated after one week of recovery.

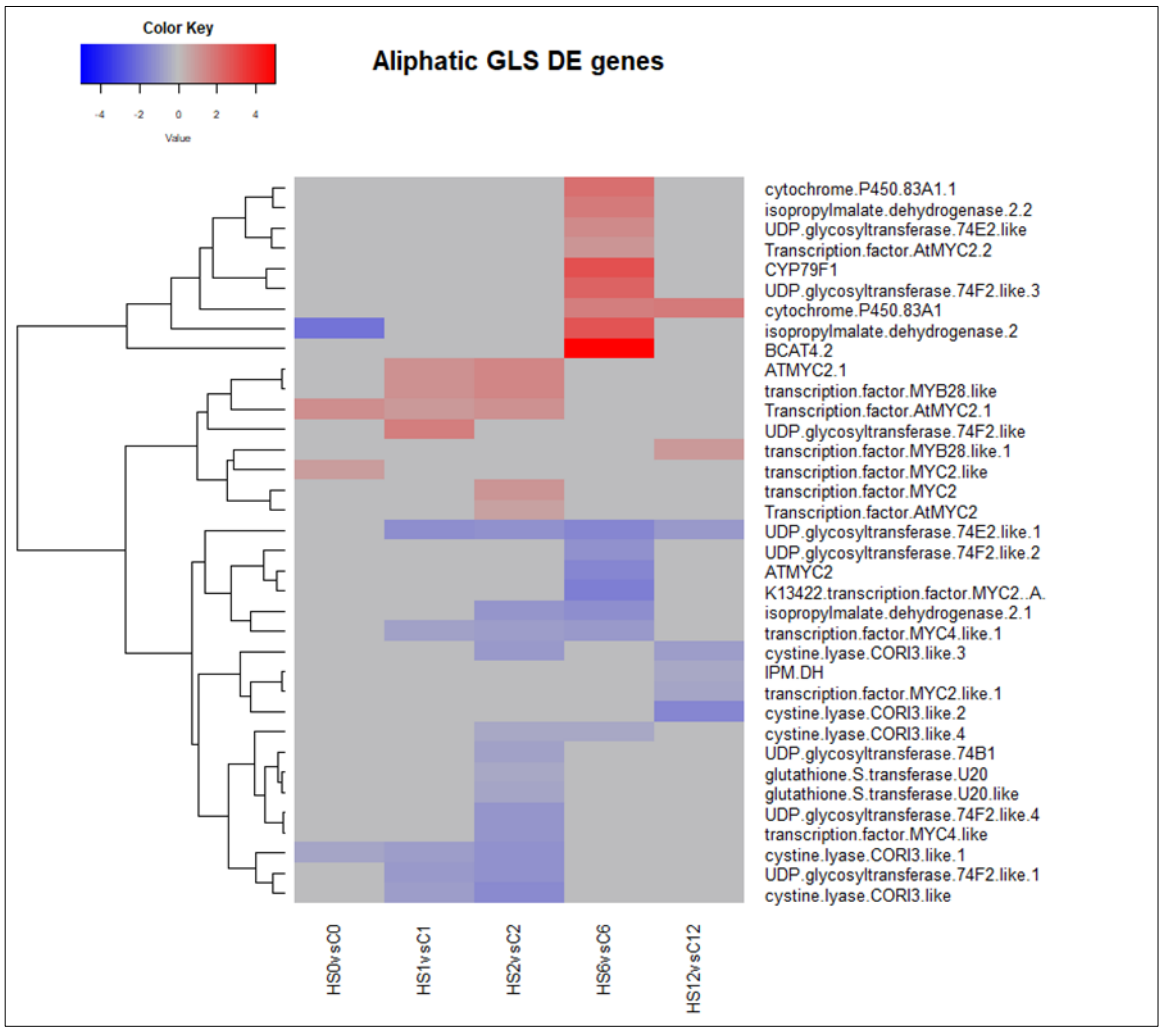

Figure 3.19 Heatmap showing differential expression of genes involved in the regulation or biosynthesis of aliphatic GSL in *B. napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

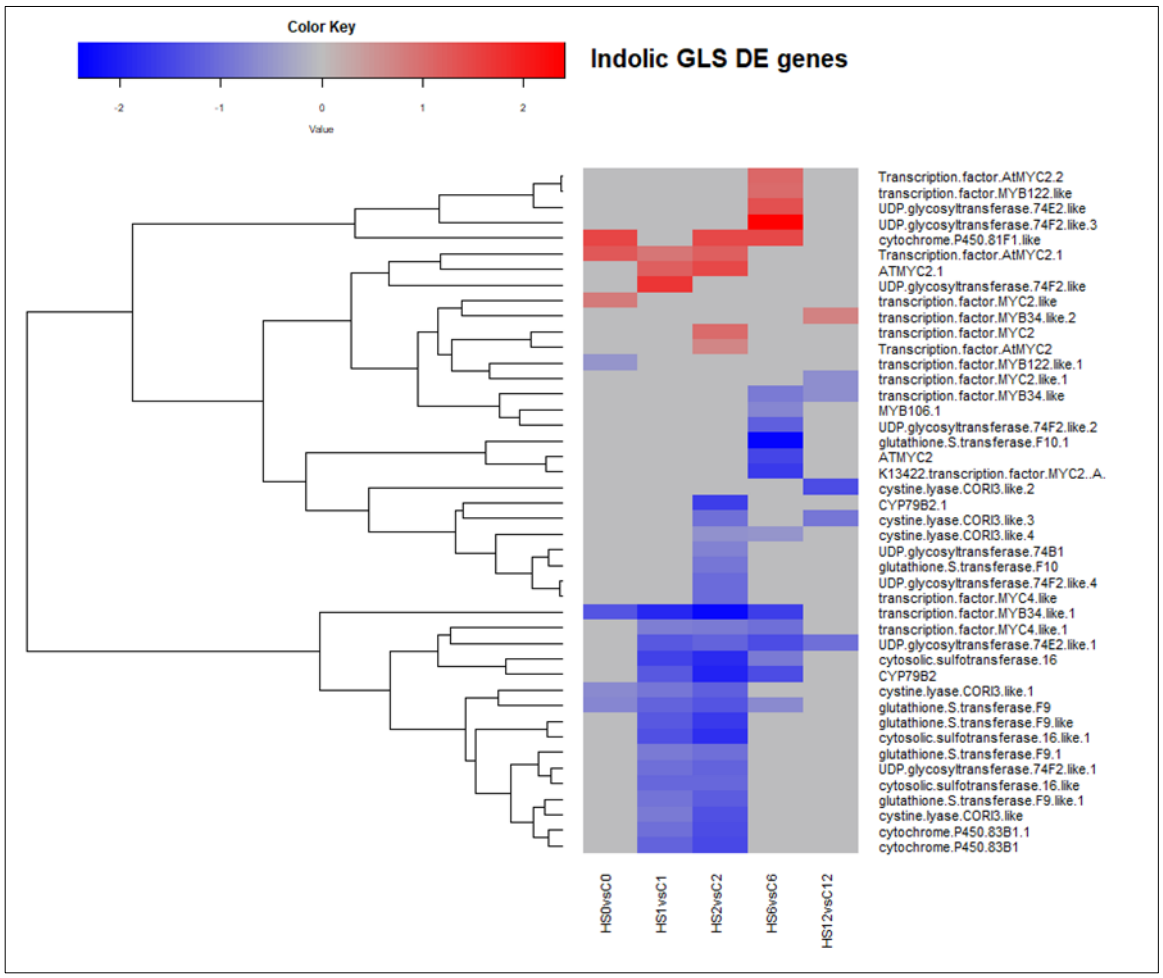

Figure 3.20 Heatmap showing differential expression of genes involved in the regulation or biosynthesis of indolic GSL in *B. napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

3.3.4.2 Heat treatment down-regulated the transcript levels of most sulphur assimilation and transport genes

Analysis of the genes encoding sulphur assimilation and transport during heat treatment showed that 20 transcripts were down-regulated in response to heat at either 1 or 2 DAT or both (Figure 3.21). One transcript encoding a sulphate transporter was up-regulated at 1 & 2 DAT, while two transcripts encoding adenylyl sulphate kinase was up-regulated at 2 DAT only. Interestingly, after the end of heat treatment, only five transcripts remained down-regulated at 1 DOR,

and two transcripts encoding adenylyl sulphate kinase and ATP sulfurylase were up-regulated at 7 DOR.

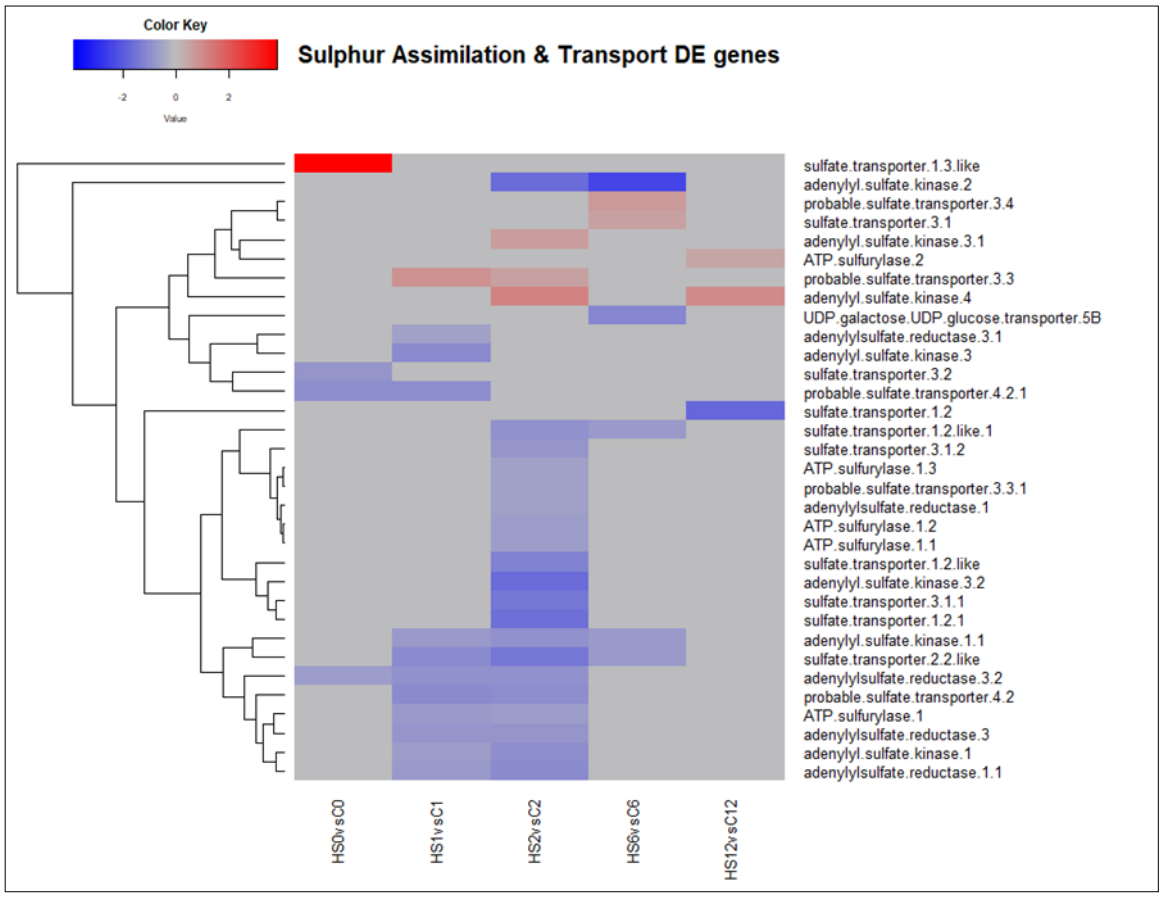

Figure 3.21 Heatmap showing differential expression of genes involved in the assimilation and transport of sulphur in *B. napus*. Twenty transcripts were down-regulated in response to heat at either 1 or 2 DAT or both. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

3.3.4.3 Metabolic analysis revealed altered leaf concentration of aliphatic and indolic GSL in response to heat treatment

Given the changes observed in the abundance of transcripts associated with GSL metabolism and sulphur assimilation and transport, the concentration of two aliphatic GSL progoitrin (PRO) and gluconapin (GNA), and one indole GSL glucobrassicin (GBS) were quantified using LC-MS. Their role in response to different abiotic stress factors has been previously reported (Ljubej et al., 2021; Jasper et al., 2020). Two-way mixed ANOVA pairwise comparisons showed a gradual increase in both PRO and GNA concentration in response to heat (Figure 3.22 and Figure 3.23). This increase was significant at 2 DAT. After 5 days of the heat treatment, the concentration of GSL started to decline but remained higher in heat treated plants than in the leaves of control plants. In contrast, the concentration GBS decreased in response to heat treatment (Figure 3.24). During recovery, the concentration of GBS continued to decrease with the difference being significant at 1 & 3 DOR. After one week of recovery, GBS concentration in heat treated plants remained lower than that of the control.

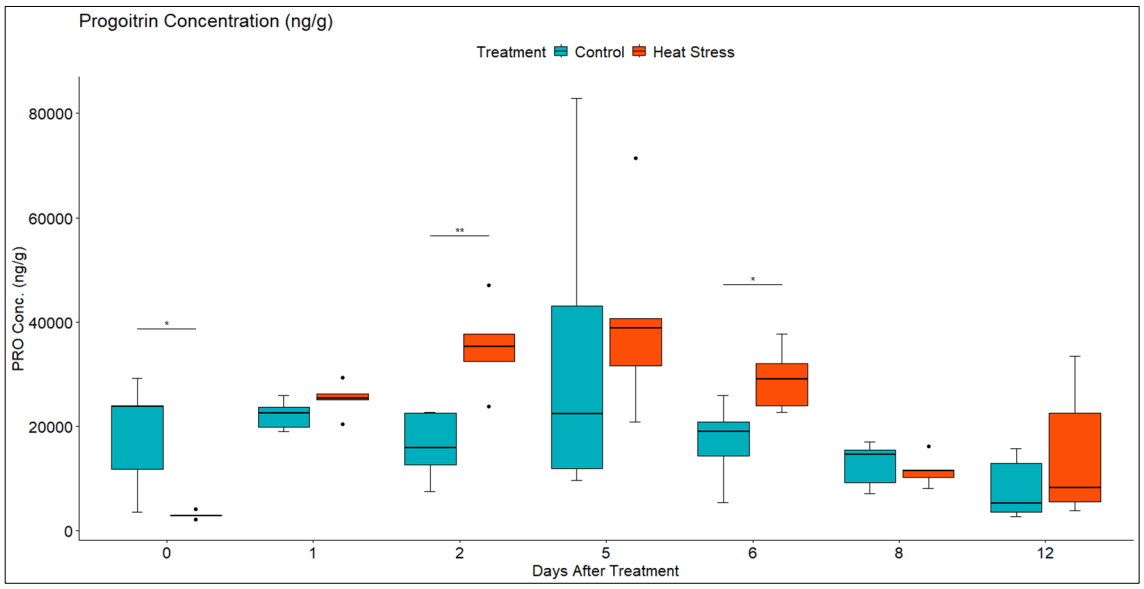

Figure 3.22 Leaf progoitrin (PRO) concentrations in ng/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). GSL concentrations were quantified using LC-MS. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, or (**) for p-value ≤ 0.01.

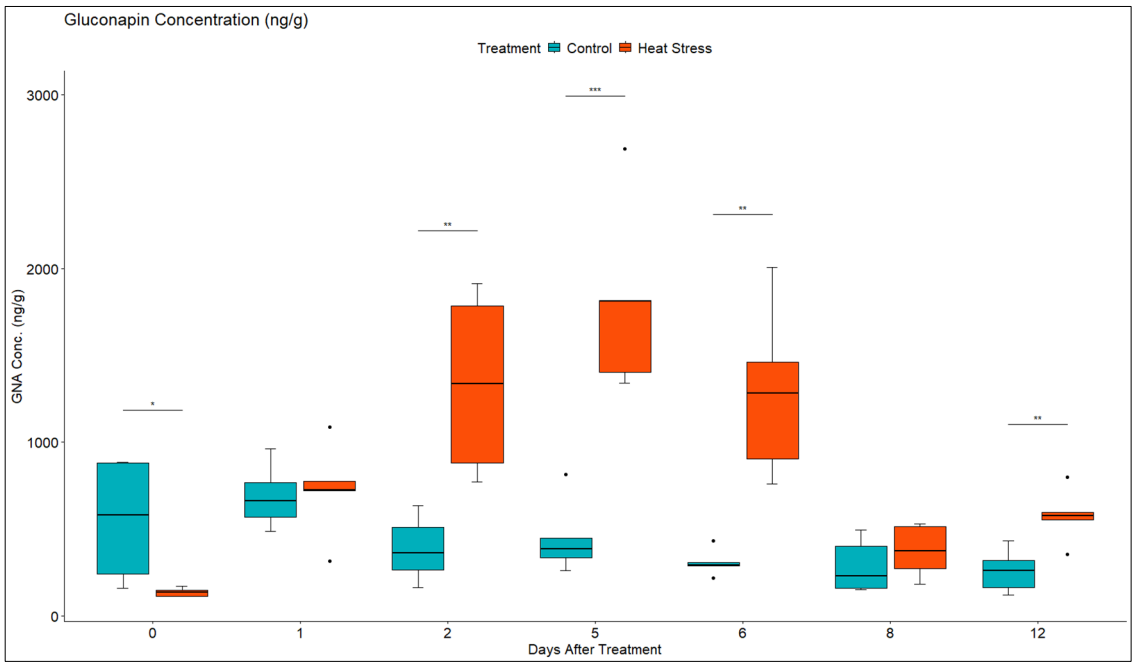

Figure 3.23 Leaf gluconapin (GNA) concentration in ng/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). GSL concentrations were quantified using LC-MS. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, (**) for p-value ≤ 0.01, or (***) for p-value ≤ 0.001.

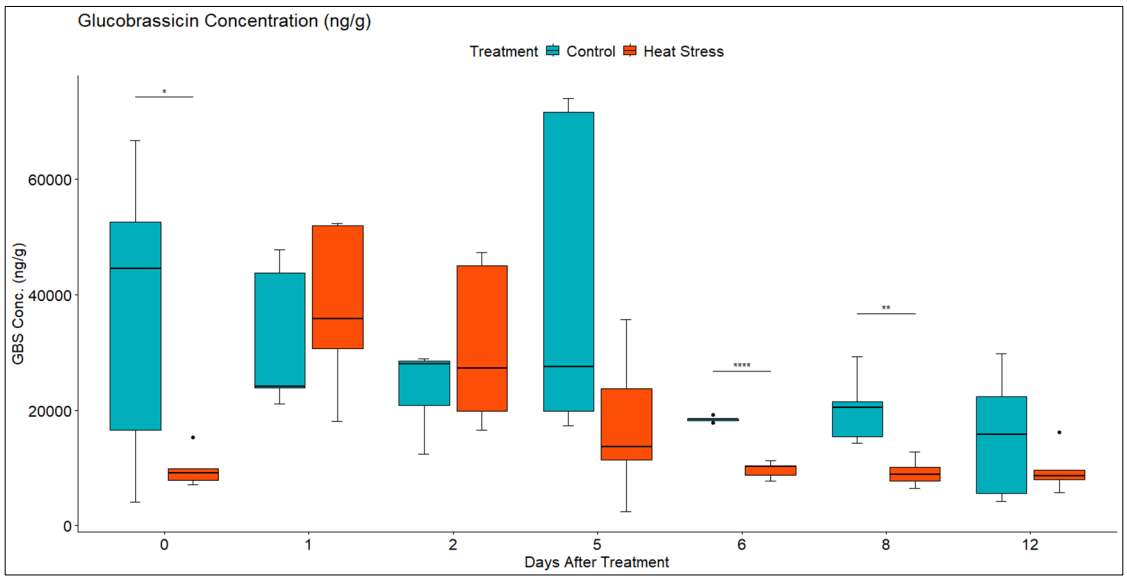

Figure 3.24 Leaf glucobrassicin (GBS) concentrations in ng/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). GSL concentrations were quantified using LC-MS. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25th and 75th percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, (**) for p-value ≤ 0.01 or (****) for p-value ≤ 0.0001.

3.3.4.4 Heat treatment down-regulated the transcript levels of most sugars transporter genes

To investigate the effect of heat stress on sugars transport and metabolism, analysis of genes encoding the SWEET and ERD6-like sugars transporters were undertaken. Compared to the control plants, heat stress significantly altered the expression of 21 *SWEET* genes with log2FC ranged between -5 and 5 (Figure 3.25). These genes belonged to the phylogenetic clades II and III. Members of these clades predominantly transport hexose and sucrose respectively. Nine of these transcripts were down-regulated at 2 DAT, while two were up-regulated. Interestingly, out of the 13 genes that were DE at 1 DOR, five up-regulated genes were not DE during heat stress (were not affected by heat treatment) and became up-regulated after the removal of stress.

Analysis of genes encoding the tonoplastic glucose symporters showed that 11 transcripts mapped to different ERD6-like genes (Figure 3.26). At 2 DAT, five were down-regulated and two were up-regulated.

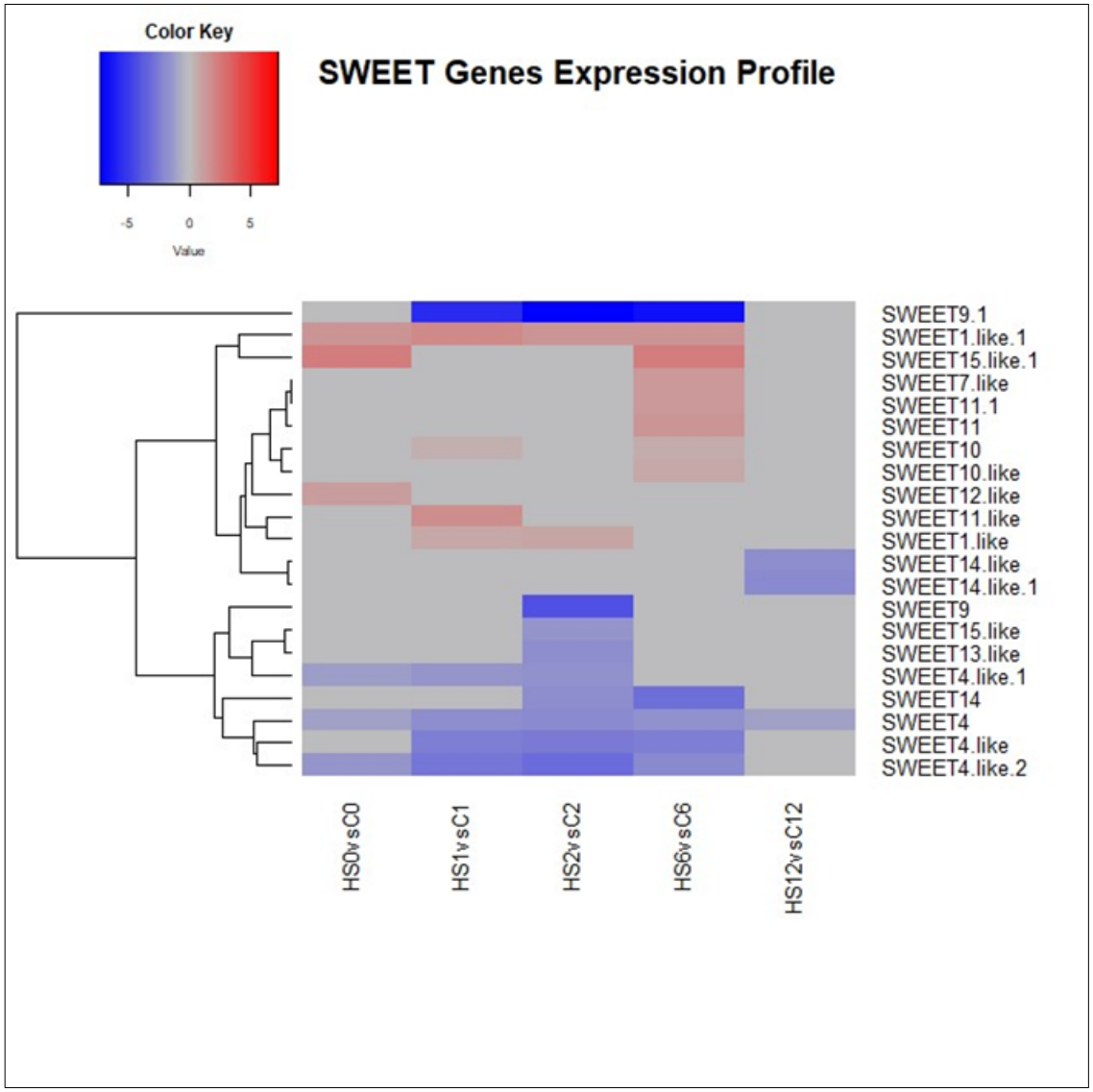

Figure 3.25 Heatmap showing differential expression of *SWEET* genes involved in sugars transport in *Brassica napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

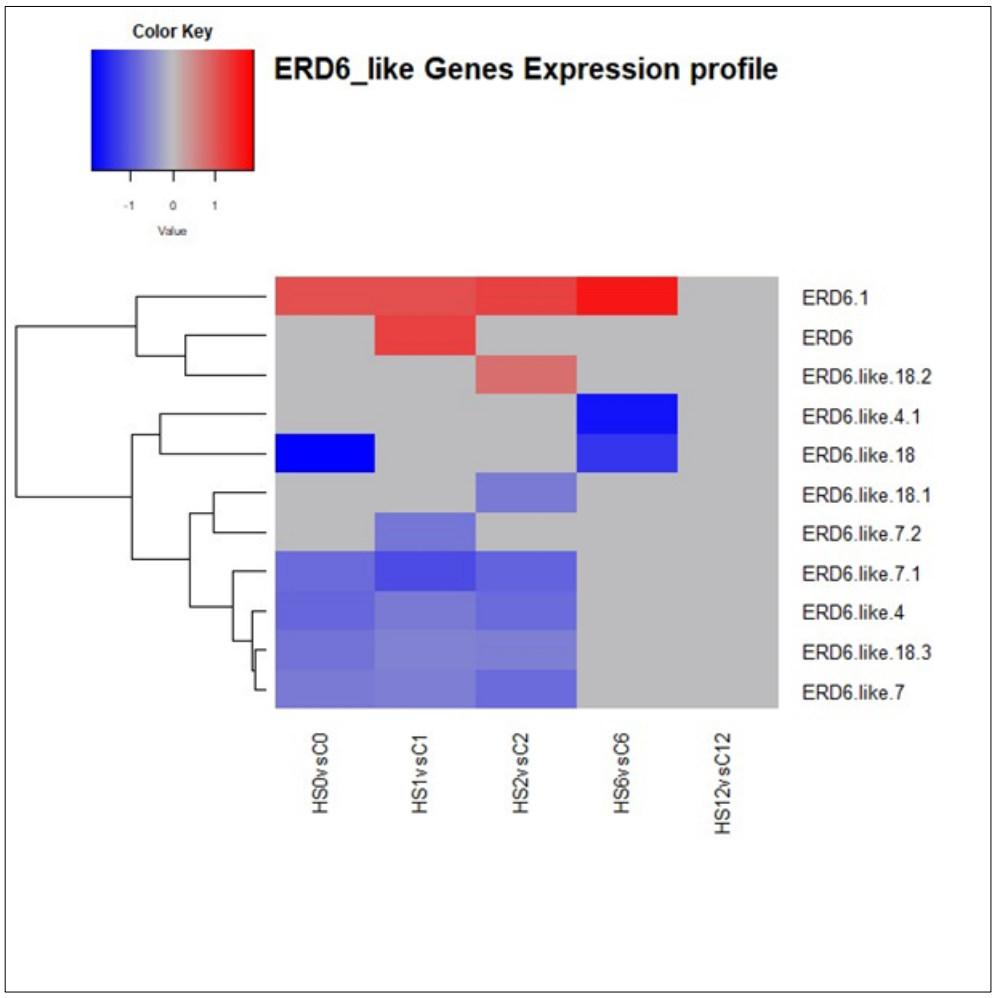

Figure 3.26 Heatmap showing differential expression of *ERD-6 like* genes involved in sugars transport in *B. napus*. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

In addition, one transcript mapping to a facilitated glucose transporter member 8-like showed a Log2FC of -9.2 at 1 DAT, and transcripts encoding sucrose transporters (SUCs) were found up-regulated at 1 DAT & 1 DOR (Figure S14). In addition to their significant role in phloem loading and unloading, these transporters are also involved in sugars influx into the cytosol. Furthermore, the present results showed that the expression of transcripts mapping to the sucrose synthesizing enzyme, sucrose phosphate synthase (SPS) increased during heat

treatment (Table S5), while the expression of sucrose catalysing enzymes, namely sucrose synthase and cell wall invertase significantly decreased (

Table S6).

3.3.4.5 Metabolic analysis revealed an increase in leaf concentration of sugars in response to heat treatment

To investigate the effect of heat stress on the sugars concentration in the leaves of *B. napus*, the concentration of fructose, glucose and sucrose were quantified using HPLC. Under control growth conditions, leaf fructose, glucose and sucrose concentration increased slowly as the plants grew (Figure 3.27-Figure 3.29). In contrast, in the heat-treated plants, the leaf concentration of individual sugars increased. Two-way mixed ANOVA pairwise comparisons showed that the change in sugars concentration was significant during both heat treatment and recovery for glucose and fructose.

After the removal of heat stress, the leaf concentration of sucrose in the treated plants decreased gradually until it reached a lower concentration than that of the control plants at 3 DOR. In contrast, leaf fructose and glucose concentration showed a slight decrease initially before they increased again significantly at 3 DOR. After 7 days of recovery, the leaf concentration of fructose, glucose and sucrose dropped substantially, reaching lower concentration than that of the control plants.

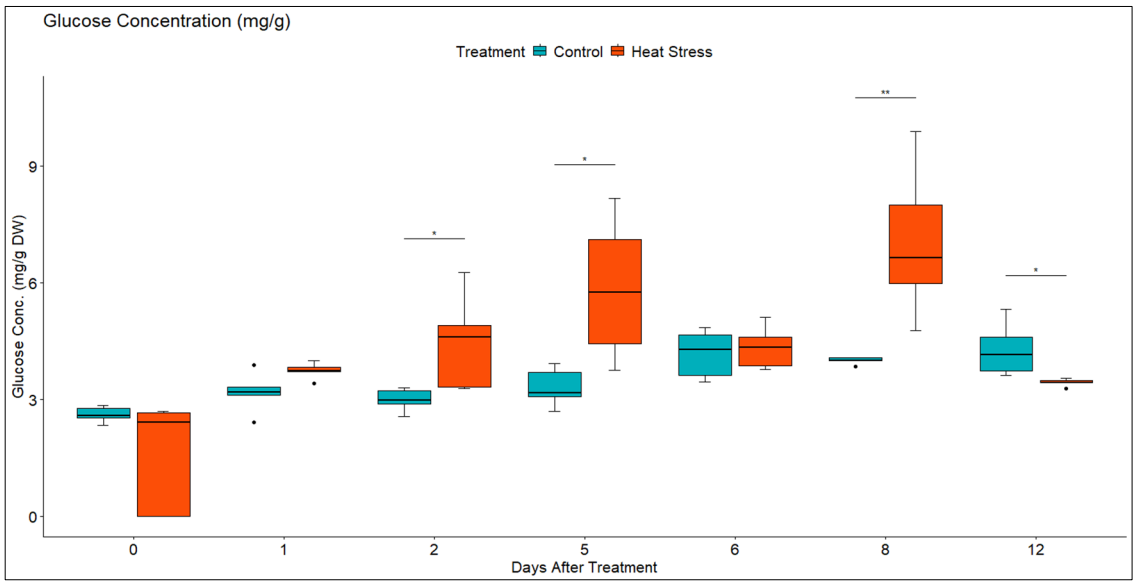

Figure 3.27 Leaf glucose concentration in mg/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30°C/24°C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). Concentration was quantified using HPLC. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25^{th} and 75^{th} percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, or (**) for p-value ≤ 0.01.

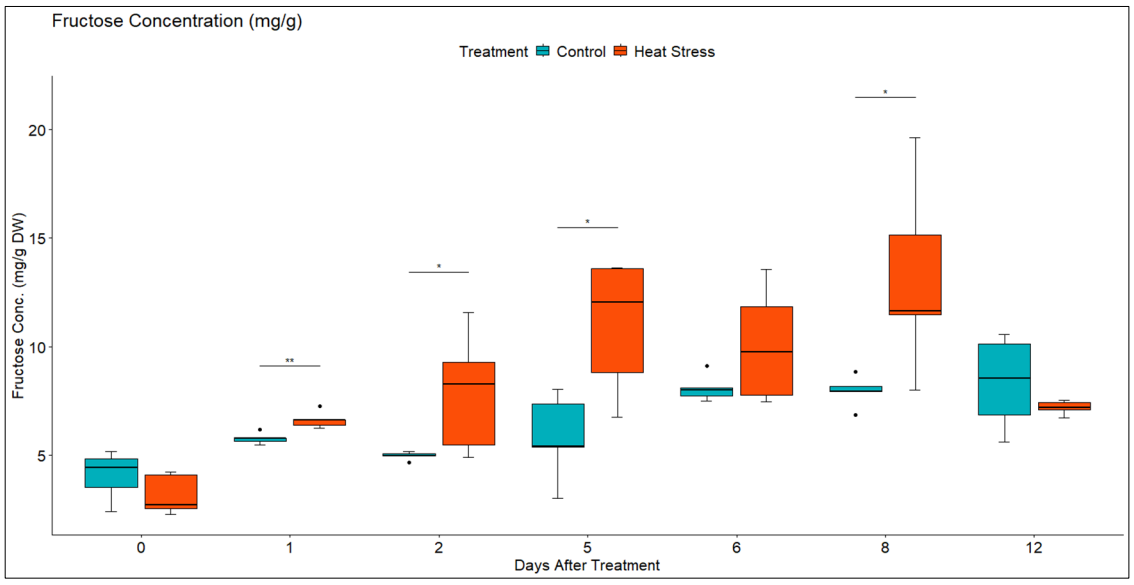

Figure 3.28 Leaf fructose concentration in mg/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). Concentration was quantified using HPLC. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25^{th} and 75^{th} percentiles (the box) and the min and max values (whiskers), with outliers as dots. Significant differences between heat treated and control plants at each timepoint are denoted by (*) for p-value ≤ 0.05, or (**) for p-value ≤ 0.01.

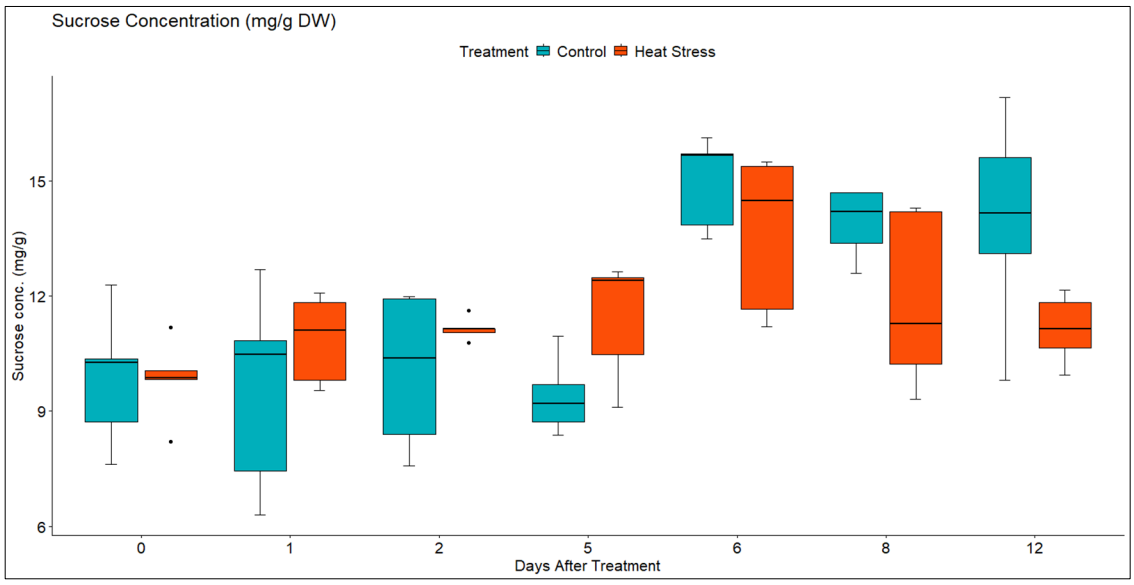

Figure 3.29 Leaf sucrose concentration in mg/g of dry weight (DW) in *B. napus* plants subjected to heat treatment (30 °C/24 °C day/night) at timepoints 0-5 (red) or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints (blue). Concentration was quantified using HPLC. Data were analysed using two-way mixed ANOVA pairwise comparisons. Each box plot represents data from 5 replicates, showing the median (horizontal bar), 25^{th} and 75^{th} percentiles (the box) and the min and max values (whiskers), with outliers as dots.

3.4 Discussion

To investigate the effect of prolonged high temperatures (heatwaves) on the growth and adaptability of *B. napus,* especially during its yield-determining reproductive stages, heat treatment experiment was designed in a way that mimic field temperature fluctuations. In the present study, *B. napus* plants were exposed to a gradual increase in temperature for a six-day period and samples were collected at different timepoints during treatment and recovery. The results showed a prevailing effect on the plants both physiologically and metabolically. Heat treatment negatively impacted seed weight and total plant biomass (Figure 3.4), consistent with similar negative impacts documented in *B. napus* and in other plants such as, *A. thaliana* and wheat (Aksouh-Harradj et al., 2006; Zhang et al., 2017; Bheemanahalli et al., 2019). This suggests that heat stress during flowering irreversibly damages vital processes leading to reduced productivity. NIRS showed significant effects of heat treatment on fatty acid concentrations in seeds in response to heat (Figure 3.5). Several studies also observed heat-induced alteration in lipid composition (Namazkar et al., 2016; Zhou et al., 2018;

Zoong Lwe et al., 2021; Magno et al., 2023). In *B. napus*, high night temperature has led to overexpression of genes involved in fatty acid catabolism (Zhou et al., 2018). Furthermore, different heat tolerant and susceptible genotypes exhibited different heat-induced changes in their lipid content (Narayanan et al., 2016; Narayanan et al., 2020). Because lipids and proteins are the major constituents of biological membranes, maintaining cellular homeostasis depends mainly on the dynamic nature of lipid composition (Zheng et al., 2011). This suggests an essential role of cellular membrane lipid remodelling under high temperature stress. Additionally, under periods of reduced carbon availability in plants, the conversion of lipids to organic acids provides an additional source of energy, thus contributing to the plants' acclimation process (Yuenyong et al., 2019).

At the metabolomic level, heat treatment significantly altered the concentration of GSL and sugars and led to the differential expression of many genes involved in their synthesis, metabolism and transport. GSL and sugars are among the major secondary metabolites naturally occurring in Brassica species (Ayaz et al., 2006; Rao et al., 2021). Their primary site of synthesis occurs mainly in the leaves, from where they are transported to other parts of the plant (Pacheco-Sangerman et al., 2022; Ren et al., 2022). While reports on the leaf sugars concentrations are common (Ren et al., 2022; Zafar et al., 2022; Dellero et al., 2024), GSL have been thoroughly investigated in seeds (Wang et al., 2019c; Tang et al., 2023; Jhingan et al., 2023), with fewer studies focusing on their presence in leaves (Rhee et al., 2020).

In this study, the concentrations of GSL and sugars were quantified from *B. napus* leaves, and their roles in heat stress response were investigated.

3.4.1 Heat stress resulted in down-regulation of many genes involved in multiple functional and metabolic processes at 2 DAT

In this study, comparative transcriptome analysis between heat treatment and control identified 9933 significantly DE transcripts (log2FC ≥ |0.5|, FDR ≤ 0.05) (Figure S4) with a balanced proportion of up and down-regulated genes in contrast between HS0vsC0 (0 DAT), and more down-regulated genes than up-regulated ones in the rest of the contrasts were identified. This suggests the

impact of heat treatment on the plant's transcriptome after 24 hours of heat stress, resulted in down-regulation of many genes involved in multiple functional and metabolic processes such as Glycolysis, Pentose phosphate pathway (PPP), Citrate cycle and Oxidative phosphorylation. In contrast HS12vsC12 (7 DOR), although the proportion of down-regulated genes was higher than the up-regulated ones, the total number of DEG decreased dramatically as the plant started to restore its metabolic functions after 7 days of recovery.

3.4.2 DEGs showed enrichment of pathways related to respiratory metabolism

The analysis of KEGG enriched pathways revealed the main underlying biochemical pathways altered in *B. napus* in response to heat stress (Figure 3.17-Figure 3.18 & Figure S5-Figure S7). In accordance with other reports (Estravis-Barcala et al., 2021; Li et al., 2022), DEGs showed an enrichment of pathways related to respiratory metabolism namely Glycolysis, Citrate cycle, Oxidative phosphorylation and PPP. At 2 DAT, the impact of heat stress on Glycolysis was manifested by the down-regulation of genes encoding the key enzymes phosphoglucomutase, aldose 1-epimerase, hexokinase, glucose-6-phosphate isomerase, and 6-phosphofructokinase (Figure 3.17). Moreover, most of the Citrate cycle genes were also down-regulated at 2 DAT (Figure 3.18). In the Electron transport chain (ETC), gene expression analysis showed that most of the involved genes were down-regulated under heat treatment, except for succinate dehydrogenase (*SDH*) which was up-regulated (Figure S9). In mitochondrial metabolism, SDH also known as complex II, plays a central role in the Citrate cycle and ETC (Huang & Millar, 2013). Additionally, SDH is thought to impact different components of the plant stress response, including stomatal conductance and ROS scavenging (Huang et al., 2019).

Although respiratory pathways have been found to have potential roles in adaptive response to abiotic stress, mainly through reducing the production of ROS (Van Dongen et al., 2011), under extreme conditions such as severe or prolonged heat stress, respiratory enzymes are deactivated, and proteins are denatured leading to culminating of ROS and a total breakdown of mitochondrial

respiration (Scafaro et al., 2021). This negatively impacts oxygen and carbon fluxes and eventually leads to more severe yield penalty (Rasmusson et al., 2020). In line with this, results from heat stress experiment on seagrass showed that high midday temperature stress of 40°C was associated with significant decrease in biomass (George et al., 2018). Likewise, Imp et al. (2019) found that under high night temperature, wheat tolerant cultivar exhibited smaller reductions in biomass and lower rates of both net photosynthesis and respiration compared to other wheat cultivars (Imp et al., 2019).

In the PPP, the oxidative phase (OPPP) was mainly affected at 1 DAT, and this was indicated by the differential expression of glucose-6-phosphate dehydrogenase (*G6PDH*) and 6-phosphogluconate dehydrogenase (*6PGDH*) enzymes (Figure S10). G6PDH and 6PGDH are the key enzymes in the OPPP. They catalyse the first and third steps of the pathway, respectively (Long et al., 2016) and have been reported to be associated with the response to various abiotic stresses in plants (Gong et al., 2012; Tian et al., 2021). Esposito (2016) reported the involvement of the OPPP in the early response to abiotic stress factors, forming a true metabolic sensor to oxidative stress. At 2 DAT, most of the oxidative and non-oxidative phase genes were down-regulated, including transketolase (*TK*), transaldolase and phosphofructokinase enzymes (Figure S11). TK has a central role in primary metabolism, where the products of the involved reactions produce the precursors for nucleic acids biosynthesis, aromatic amino acids, and vitamins (Bi et al., 2013). Bi et al (2013) showed that the cucumber *TK* gene (*CsTK*) was sensitive to temperature and light (Bi et al., 2013; Bi et al., 2019). Likewise, the transaldolase gene was found to be involved in the regulation of expression of genes involved in the ABA signalling pathway and the enzymes responsive to ROS (Rong et al., 2021). The results of this study add further evidence to the involvement of the PPP enzymes in the heat stress response in *B. napus*.

3.4.3 Heat stress altered GSL metabolism during treatment

At the transcriptomic level, heat treatment differentially affected the expression of GSL synthesis and regulatory genes (Figure 3.19-Figure 3.21). Several genes

encoding different UDP glycosyltransferases and glutathione s-transferases appeared down-regulated, while others encoding GSL related transcription factors were up-regulated, suggesting that these genes might have participated at different stages of the stress response.

The influence of heat stress on individual GSL was further evaluated through metabolomic analysis of two aliphatic GSL (progoitrin (PRO) and gluconapin (GNA), and one indolic GSL (glucobrassicin (GBS). The role of these GSL in response to different abiotic stress factors has been previously reported (Jasper et al., 2020; Ljubej et al., 2021). In the current study, the leaf concentration of PRO and GNA significantly increased in response to heat stress before it started to decline at 5 DAT (Figure 3.22 & Figure 3.23). This decline could have resulted from membrane damage caused by prolonged exposure to heat, leading to GSL degradation. Removal of heat stress after 5 DAT could also be another factor for the decline in GSL concentration. In addition to its primary role in response to plant pathogen interaction (Hopkins et al., 2009; Touw et al., 2020), GSL accumulation in response to heat stress has also been previously reported (Valente Pereira et al., 2002; Martínez-Ballesta et al., 2013; Jasper et al., 2020). When plants are stressed, growth is reduced, and carbon utilization is predominantly diverted towards the production of secondary metabolites. As part of the plant defence mechanism, GSL play important role as osmoprotective compounds (Martínez-Ballesta et al., 2013), where their concentration increases in response to stress, providing protection against oxidative damage. At 30/15°C (day/night) temperature regime, *Brassica oleracea* seedlings had significantly higher GSL concentration than plants cultivated at lower temperatures (22/15°C and 18/12°C) (Valente Pereira et al., 2002). Similarly, an *A. thaliana* GSL mutant experienced a remarkable decline in growth and development and was found to be less heat tolerant than wild-type plants upon exposure to high temperature (Ludwig-Müller et al., 2000).

In contrast, indolic glucosinolate GBS showed a downward trend where concentration decreased gradually in response to heat (Figure 3.24). Similar results have been found where GBS concentration was reduced in response to

heat treatment (Bohinc & Trdan, 2012). Also, during short term high temperature stress, the transcript level of the indolic GSL synthetic genes were predominantly down-regulated (Rao et al., 2021). Several studies reported that indole GSL are much more sensitive to heat treatment (Bones & Rossiter, 2006; Bohinc & Trdan, 2012) and can demonstrate more thermal degradation than aliphatic GSL at lower temperatures (Orlemans et al., 2006).

The results of our study indicate that the concentration of GSL in the leaves of *B. napus* is influenced by high temperature stress, which was previously reported in other Brassica species (Rask et al., 2000; Bohinc & Trdan, 2012). It also shows that different groups of GSL (aliphatic versus indolic) differ in their response to heat stress. The different accumulation trends of these groups could correspond to different protective mechanisms driven by individual GSL with diverse chemical structures and bioactivities (Figure 3.30) (Seo & Kim, 2017; Ljubej et al., 2021). These mechanisms may prioritise energy towards aliphatic GSL production, which are known to play crucial roles in ROS mitigation (Martínez-Ballesta et al., 2013). Additionally, the reduced availability of GSL precursors, such as nitrogen and sulphur, under stressful conditions may influence the allocation of GSL across plant tissues. In response, plants might break down their GSL and utilise the released sulphur to support primary metabolic processes, such as protein synthesis, within specific organs (Brown et al., 2003; Martínez-Ballesta et al., 2013). This plasticity highlights GSL players in balancing stress resilience and adaptation. By selectively modifying GSL content, plants can fine tune their responses to stress, meeting immediate defence demands while ensuring long-term survival and reproductive success.

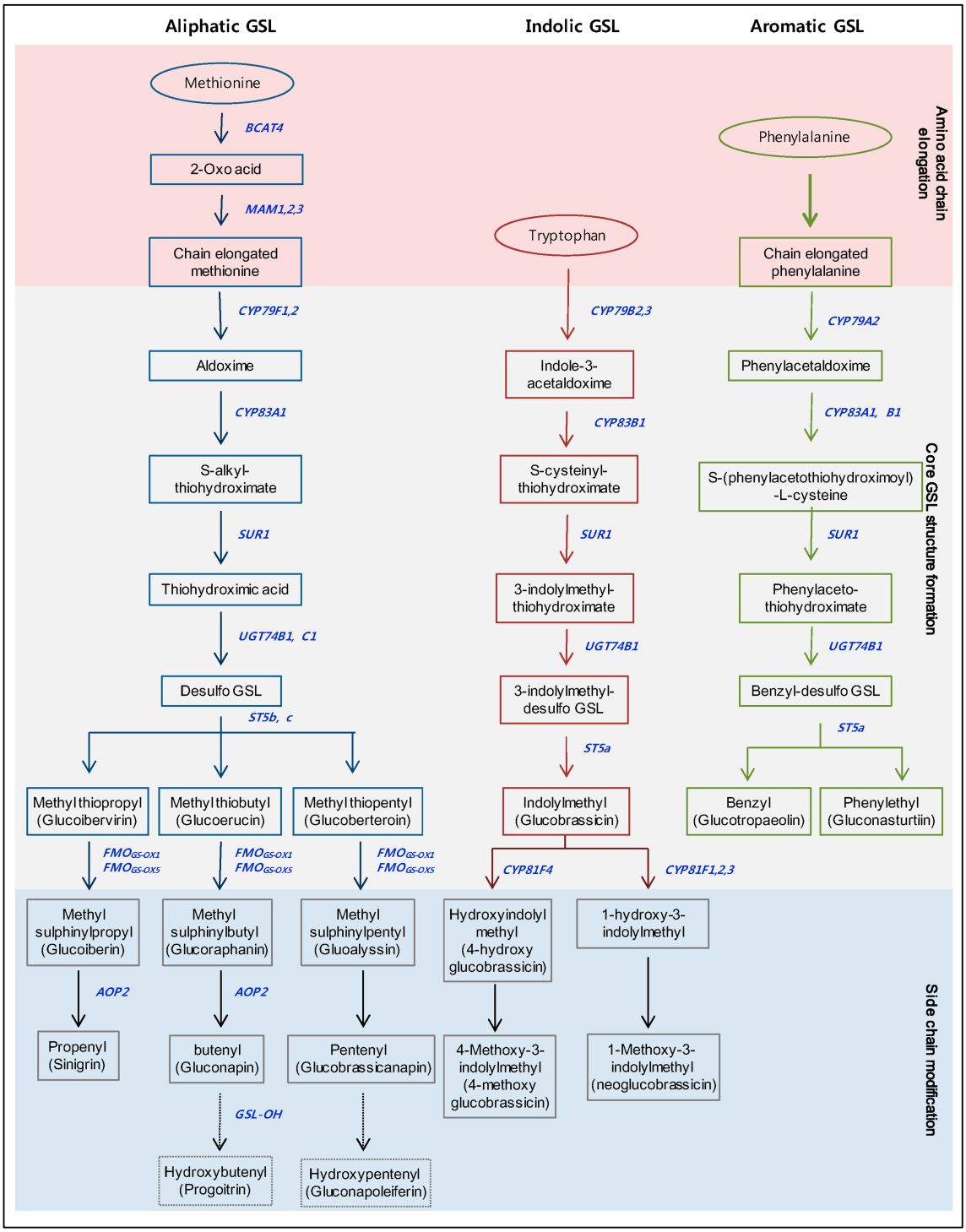

Figure 3.30 Three major biosynthesis pathways of the GSL in Brassicaceae. Outline of the pathways which can be divided into three parts; (1) amino acid chain elongation; (2) formation of the core structure of GSLs; (3) side chain modification. Blue bold indicates biosynthetic genes involved in each step. The names in parentheses denote common names (Seo & Kim, 2017).

3.4.4 Heat stress altered the expression of sulphur assimilation and transport genes

In the current study, the transcript levels of sulphur assimilation and transport genes were significantly down-regulated especially at 2 DAT (Figure 3.21). This included several adenylyl sulphate kinases and reductases, ATP sulphurylase (ATPS) and sulphate transporter genes. These genes are important for the activation, catalysis and transport of sulphur compounds (Capaldi et al., 2015). Moreover, enrichment pathway analysis showed that Cysteine and methionine metabolism pathway was among the top significantly enriched pathways at 1 & 2 DAT (Figure S5 & Figure 3.16). In this pathway, 11 transcripts mapping to s-adenosylmethionine synthetase and 11 transcripts mapping to homocysteine s-methyltransferases were down-regulated (Figure S8). These enzymes are among the key enzymes that control the methionine pool (Capaldi et al., 2015). Given that GSL compounds are rich in sulphur, assimilation of sulphur is closely associated with GSL biosynthesis. Cysteine, which is the terminal metabolite in sulphur assimilation, acts as a sulphur donor for methionine, a precursor for aliphatic GSL. This renders cysteine an important intersection point between sulphur assimilation and GSL biosynthesis (Rao et al., 2021). Such proximity between sulphur and these amino acids makes Cysteine and methionine metabolism pathway directly related to GSL biosynthesis.

3.4.5 Heat stress altered sugars metabolism during treatment and recovery periods

In the present study, heat treatment altered carbohydrate concentration as the amounts of glucose, fructose and sucrose increased with heat treatment (Figure 3.27-Figure 3.29). At the transcriptional level, the starch hydrolysing enzyme alpha-amylase was induced. Conversely, the starch synthesizing enzymes such as phosphoglucomutase (PGM), AGPase and hexokinase were down-regulated (Figure 3.17 & Figure S13). Likewise, heat induced the up-regulation of sucrose synthesizing enzymes and the down-regulation of sucrose hydrolysing enzymes such as cell wall invertase and sucrose synthase (Table S5 &

Table S6). Together with the inhibition of many sugars transporters genes such as *SWEET* and *ERD6-like* (Figure 3.25 & Figure 3.26) suggest that heat induced starch hydrolysis and promoted sucrose synthesis and accumulation in the leaves. Several reports showed that different environmental stress affect sugars metabolism and transport (Xalxo et al., 2020; Mathan et al., 2020). For example, drought and salinity stresses increased sucrose concentration in leaf and root tissues of rice (Mathan et al., 2020). Similarly, a significant accumulation of total sugars and proline was observed in different genotypes of heat stressed seedlings of moth bean (Harsh et al., 2016). Marias et al. (2017) also reported an accumulation of glucose and fructose under heat stress in the leaves of *Coffea arabica* (Marias et al., 2017). In maize ear leaf, the ^{13}C export rate was decreased by high temperature treatment, resulting in growth enhancement of vegetative plant parts and reduction in grain yield (Suwa et al., 2010). During heat stress, leaves are the first tissues to sense and experience damage by heat. Consequently, leaves use sugars to efficiently scavenge ROS and alleviate oxidative damage. In high respiration environment, it has been hypothesized that sugars acts as energy supply to ensure survival (Burke, 2007), as their role in osmotic readjustments helps to maintain cell-water balance and membrane integrity. Thereby, maintaining leaf sugars content through reducing source to sink export is considered a tolerance strategy (Kaushal et al., 2013). Additionally, the decrease in sink demand due to growth limitation, could also contribute to the accumulation of sugars in the source leaves under stressful conditions (Hummel et al., 2010; Lemoine et al., 2013).

3.5 Conclusion

Through a comprehensive transcriptomic and metabolomic analysis, this study brings evidence for the impact of high temperature stress during heatwaves on different functional and metabolic pathways, highlighting their roles in the plant stress defence system. More specifically, our results indicated that heat treatment:

1) Down-regulated important metabolic pathways, such as
   respiratory metabolism, Glycolysis, Pentose phosphate pathway, Citrate

cycle and Oxidative phosphorylation, especially after 48 hours of heat treatment.

2) Reduced yield and plant biomass and altered seed composition.
3) Led to changes in sugars and GSL levels with increased concentrations of glucose, fructose, sucrose and the aliphatic GSL progoitrin & gluconapin, while the indolic GSL glucobrassicin decreased.
4) Resulted in the differential expression of many genes involved in key biological processes such as heat shock, regulation of cellular processes, transcription factors, cell wall remodelling, sugars and secondary metabolites transport and metabolism.

Furthermore, the gradual increase in temperature utilised in the heat experiment helped to create an environment that mimics field conditions during heatwaves. Thus, this study represents an important step towards developing an understanding of the heat stress response and tolerance mechanisms, including knowledge that could be transferred to other plants.

Finally, the DEGs and pathways identified in response to heat stress provide a good genetic resource to further understand heat stress response in plants. As transcriptional regulation is regarded as one of the key mechanisms underpinning plant resistance in challenging environments (Chinnusamy et al., 2007), the next step in this study was to examine the regulatory mechanisms behind the transcription of these genes. Specifically, this involved exploring their interactions with TFs that play a crucial role in modulating various signal transduction pathways and regulating gene expression under both biotic and abiotic stress conditions. To facilitate this, a purpose-build Cytoscape plugin, NetViewer, has been developed as part of this work to reveal these interactions and enhance the understanding of molecular regulatory mechanisms involved in the heat stress response in *B. napus* and related species. Moreover, identifying TF-gene regulatory networks related to heat stress may expose new, targetable mechanisms for managing heat stress response. On this account, Chapter 4 describes NetViewer and provides a use case scenario in the context of plant heat stress response.

Author Contributions:

MK: Data curation, Formal analysis, Validation, Visualization, Writing – original draft. MA: Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. JH: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing. FM: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

4 NetViewer: A Cytoscape plugin for TF-gene network extension and enhancement

Exploring the interactions between genes and transcription factors (TFs) is an important step towards advancing the understanding of heat stress response in plants. Additionally, modelling and analysing these interaction networks is also essential for researchers. In recent years, several network graph visualisation platforms such as Cytoscape (Shannon et al. 2003) have been developed for this purpose. Additionally, with the use of plugins, these networks can be analysed by incorporating external data sources. However, plugins often require multiple preparatory steps, including data input and conversion into specific formats, which demand significant computational expertise. To simplify this process, I developed NetViewer plugin to automate these tasks and streamline network generation and data integration. This Chapter provides an overview of the NetViewer's design and architecture and presents a case study featuring DEGs identified in the heat treatment experiment described in Chapter 3. The source code for the plugin is available on GitHub at the following link: https://github.com/MariamKourani/NetViewer_NetBeansProject/tree/master/NetViewer

4.1 Introduction

TFs are proteins that bind to DNA and control essential cellular functions (Strader et al., 2022), making up about 6-10% of the plant genome coding sequences (Riechmann et al., 2000). In addition to their core functions in binding to their DNA target and recruiting the appropriate machinery for transcription, other TFs act as facilitators and play additional role in opening closed chromatin and making it available for other TFs to bind (Strader et al., 2022). In plants, TFs also known to have crucial roles in signal transduction, from the perception of stress signals to the expression of stress-responsive genes (Hrmova et al., 2021; Hussain et al., 2021).

In recent years, different TFs families have been identified in plants such as AP2/ERF, MYB, HSP, bZIP, NAC, DOF, MADS-box, etc. (Yamaguchi-Shinozaki and Shinozaki 2006; Ghorbani et al., 2020). While some members were found to

be involved in the regulation of developmental pathways in plants, others appear to have a role in the regulation of defence responses against environmental stresses (Hegedus et al, 2003).

In *A. thaliana*, more than a thousand stress responsive TFs were identified (Naika et al., 2013). More recently, a functional analysis of differentially expressed genes (DEGs) in *B. napus*, revealed that over 400 TFs were differentially expressed (DE) in response to cold stress. Interestingly, some of them were enriched in biological processes like photosynthesis, plant development, amino acid catabolism and stimulus response (Ke et al., 2020). Similarly, a total of 2,167 TFs belonging to five different families were identified with 7.6% identified as being novel and unique to *B. napus*. RNA-Seq analysis revealed that over 80% of TFs were inducible by abiotic stress, with 70% being responsive to heat after 12 hours of treatment and these include: 62 BnAP2/EREBPs, 32 BnbZIPs, 61 BnMYBs, 40 BnNACs, and 27 BnWRKYs (Wang et al., 2018). Moreover, a transcriptomic analysis of *B. napus* seedlings exposed to drought, salt, heat, cold and abscisic acid treatment showed that 99 BnWRKYs responded to at least one of these stress factors (Chen et al. 2021).

In view of the importance of these interactions, gene regulatory networks were developed to model physical and regulatory relationships between the genes and their regulators (Arda & Walhout, 2009). As a result, various network visualization tools were created to facilitate data integration and analysis. Cytoscape, is one of these tools that was originally designed for biological research. It is an open-source software that provides a basic set of features for data integration and two-dimensional network visualisation helping researchers intuitively observe gene interaction and obtain relevant information (Shannon et al. 2003). Moreover, Cytoscape's architecture is based on Apps, also called plug-ins. This architecture enables programmers to develop their own plugins, which significantly enhance the features of the basic environment, resulting in a more versatile network analysis tool. Some of these plugins belong to various categories such as network generation, graph analysis, literature mining, enrichment analysis, clustering and many more. CyTargetLinker is a plugin built to extend biological networks with regulatory interactions, such as miRNA-target, TF-target or drug-target

interactions. However, to create a gene network using CyTargetLinker, users need to upload a list of target genes and a database file that has been previously downloaded and converted into a format compatible with the plugin. This conversion process is done through a bash script on the command line, which requires considerable computational skills that may not be readily available, especially to biologists or non-technical users. To address this challenge, I developed NetViewer, a new Cytoscape plugin that builds upon CyTargetLinker's capabilities (Kutmon et al., 2018) and simplifies data integration and conversion, making it easier to generate biological networks and their associated regulatory interactions within Cytoscape GUI environment.

4.2 Methods

4.2.1 Input files

NetViewer's first input parameter is a gene list of interest in a tab-delimited format. To link these genes to regulatory information from the database, network nodes need to have an attribute containing a biological identifier, eg. Ensembl or NCBI Gene ID of *A. thaliana*.

To add additional layers of information to the displayed network, other data files such as differential gene expression, functional annotations, Gene Ontology (GO) terms or IDs can also be imported.

4.2.2 Network display workflow

Before running NetViewer, the corresponding *A. thaliana* IDs for the genes of interests must be obtained. These IDs will be used as attributes to match the genes of interest to the TFs in the database. Figure 4.1 shows in blue the steps needed for this task. The grey steps that follow represents the steps in NetViewer which is launched from Cytoscape App menu.

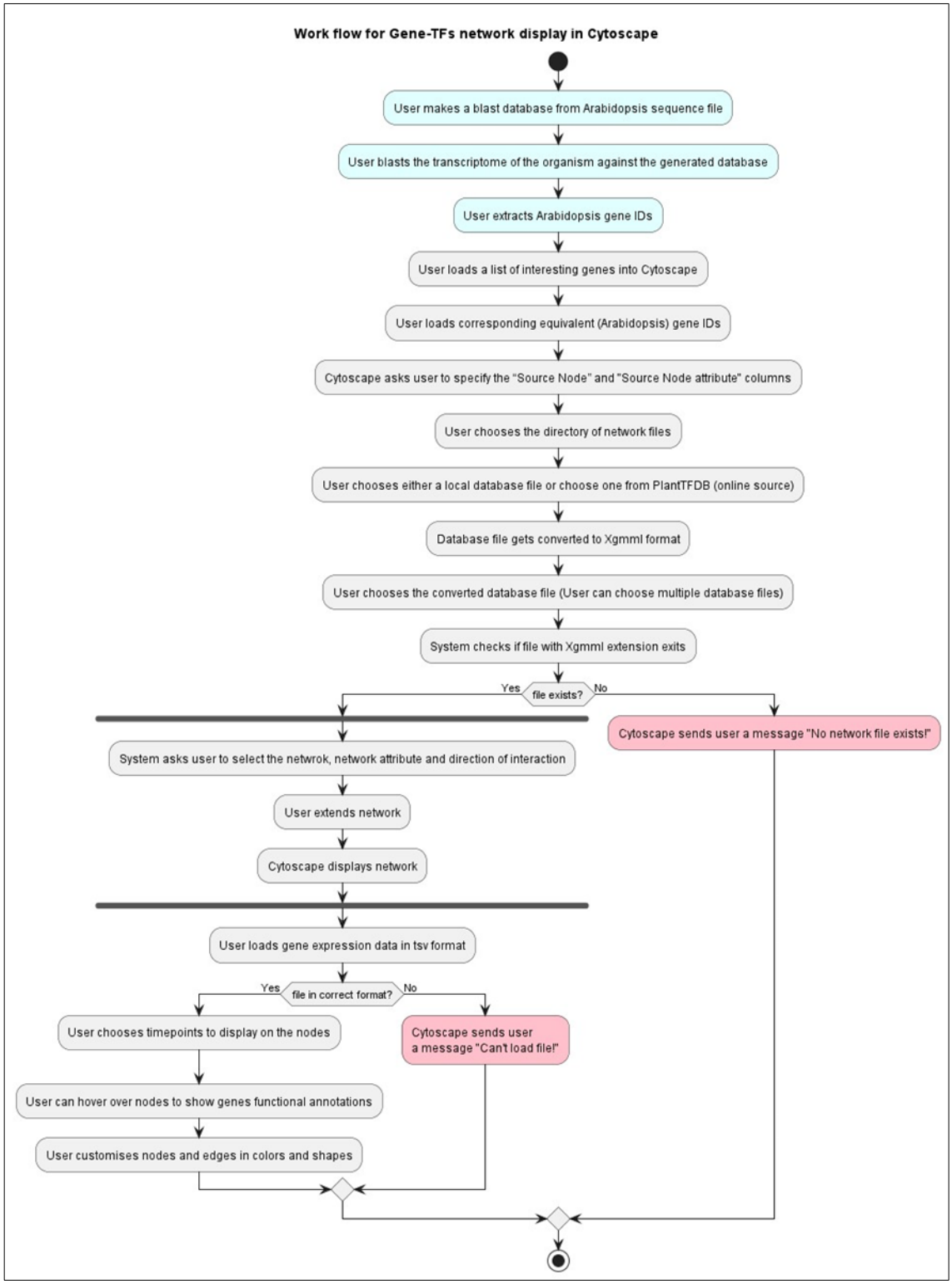

Figure 4.1 NetViewer functional workflow. Blue colour represents the steps that must be performed before launching Cytoscape. Grey colour represents the steps within NetViewer, and pink colour highlights information messages provided by NetViewer.

4.2.3 Regulatory interaction network file for the TFs

To create a regulatory interaction network file for the TFs (also called Linkset network file), NetViewer can either accept a local *A. thaliana* TFs database file or access one directly from the PlantTFDB website via the plugin menu. A Linkset is a network that holds regulatory information about the nodes within it. In this context, it represents gene-TF interactions. These networks are saved in xgmml (the eXtensible Graph Markup and Modeling Language) format, which is supported by Cytoscape. Each Linkset consists of interactions consisting of two nodes, a source and a target, connected by a single directed edge.

After selecting the TFs database file, NetViewer converts it into a Linkset network using a simple Java program that converts tab-delimited text files into xgmml Linksets, along with a configuration file that specifies details about each column of the TFs database. The configuration file is customizable, allowing adjustments based on the specific *A. thaliana* TFs database being used.

4.2.4 Network extension

As illustrated in Figure 4.2, following data input, NetViewer parses the provided Linksets and extracts relevant interactions for the selected network. Once the network is extended, it can be further enhanced through adding more information to the nodes based on the user's choice. The user can choose to display the gene functional annotations, GO terms or GO IDs. The resulting network can then be analysed and further explored using the large set of tools provided by the Cytoscape environment. For example, importing and displaying differential expression data such as Log2FC and Transcript per Million (TPM), customising the look and feel of the network and changing the visual style of the nodes and edges.

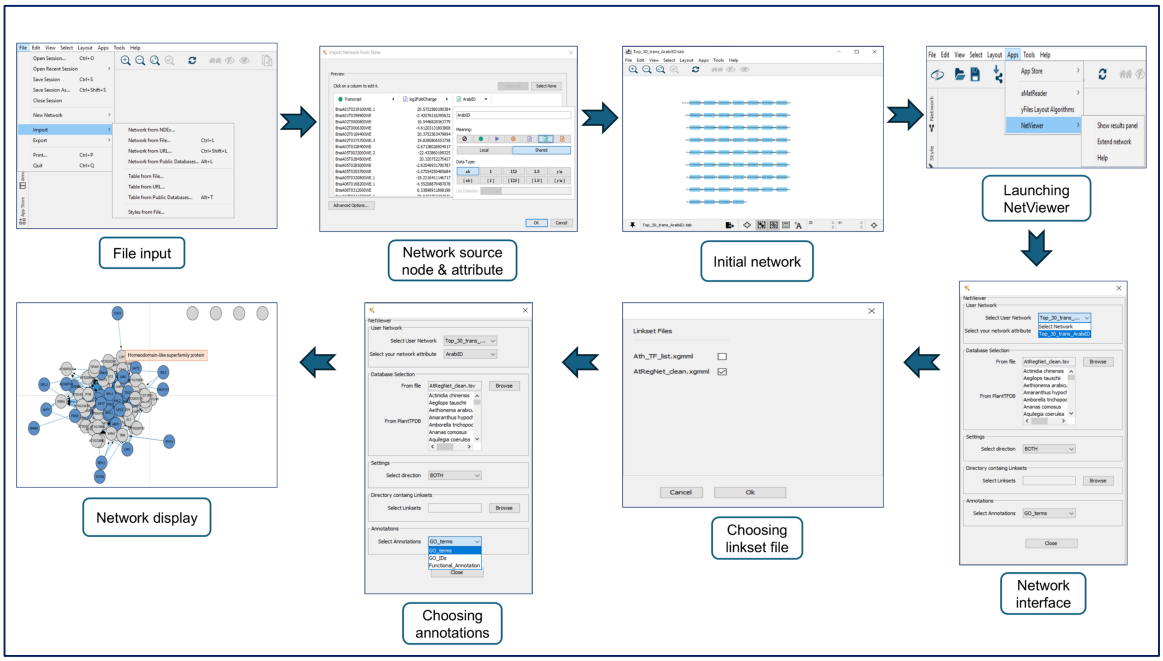

Figure 4.2 NetViewer workflow

4.2.5 Case study

To begin, a gene list of 60 top DEGs identified in the heat treatment experiment (described in Chapter 3) at 2 DAT (Days after Treatment) along with their corresponding *A. thaliana* gene IDs were imported in tab-delimited file into the Cytoscape environment. The rationale behind choosing the DEGs at 2 DAT is that comparative transcriptome analysis showed that heat stress resulted in the differential expression of many genes involved in crucial functional and metabolic processes at this timepoint which marks 48 hours of heat treatment. Investigating the TFs regulating these genes would be of paramount importance to understand the mechanisms involved in heat stress response in *B. napus.*

During the import process, Brassica gene IDs were designated as the network source nodes, and *A. thaliana* gene IDs were set as the network attribute. The network attribute is a column used to identify the nodes in the Linkset and must include one of the supported identifiers. Similarly, files for GO terms & IDs, functional annotations and expression data as TPM in tab-delimited format were also imported.

Next, the NetViewer interface was loaded from Cytoscape Apps menu. In the User Network section, the previously loaded gene set, and network attribute were selected. Then, a local TFs database file was chosen from the user's file system in the Database Selection section, which was then automatically converted into a Linkset. The database file is a tab-delimited file where columns hold information about the TFs and their target genes. This includes TF name, TF locus, TF family, target gene name, target gene locus and interaction type. Due to the lack of *B. napus* specific TFs databases, the TFs database used was exported from *A. thaliana* AtRegNet database. From the Settings, the direction of the interactions was set to "Both". Then, the generated Linkset file was selected through browsing the directory of Linkset files. Afterwards, the annotations to display upon hovering on nodes were chosen. Finally, the network was extended in the Cytoscape display pane, with nodes representing genes and TFs, and edges representing their interactions as in Figure 4.3 and Figure 4.4.

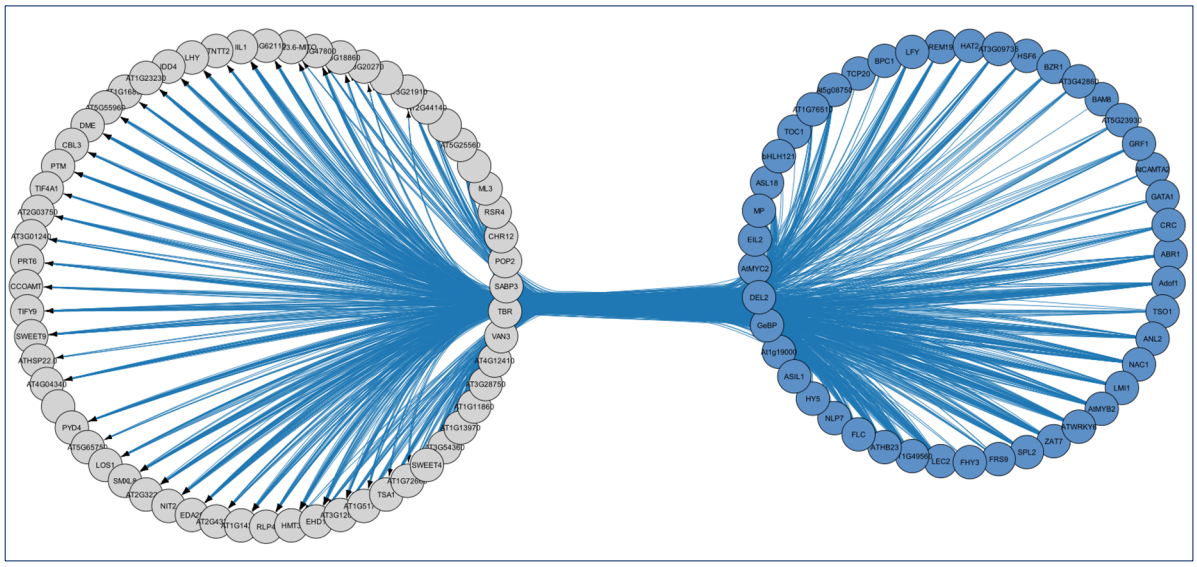

Figure 4.3 Extended network of interactions between top DEGs (grey nodes) and TFs (Blue nodes). The blue edges represent the interactions between the genes and TFs.

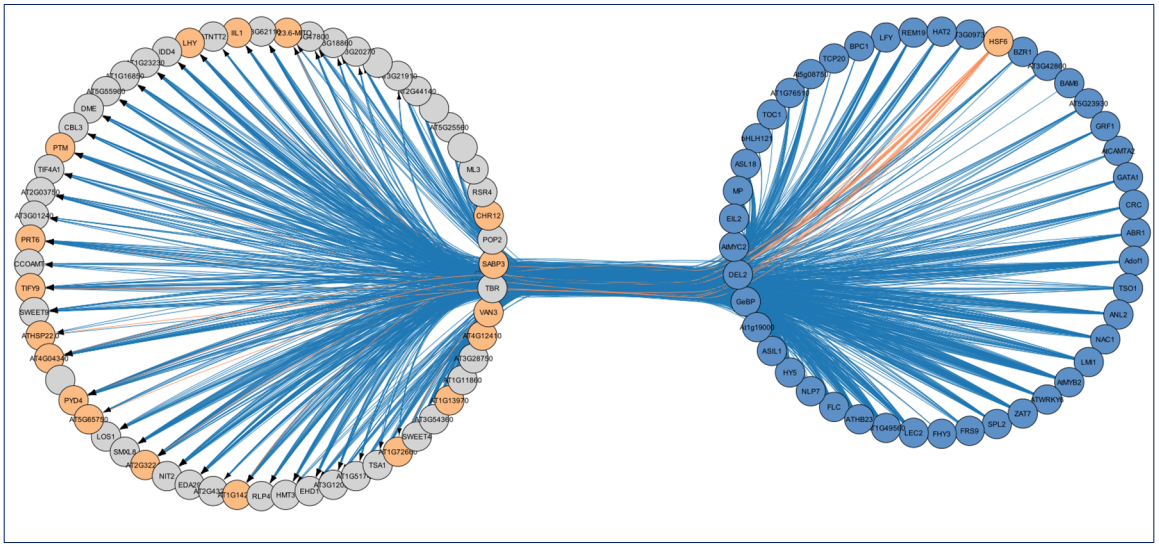

Figure 4.4 Extended network of interactions between top DEGs (grey nodes) and TFs (Blue nodes). As an example, the network also highlights in orange the interactions between one transcription factor (HSF6) and its interacting genes along with the edges connecting them.

4.3 Results and Discussion

NetViewer was used to model the relationships and display the interactions between a set of DE genes and their corresponding regulatory TFs. The gene set used corresponds to the top DEGs identified at 2 DAT of the heat experiment described in Chapter 3.

The displayed network showed that the genes are regulated with 45 TFs belonging to various TF families (Table 4.1). TFs belonged to MYB, NAC and WRKY families were among the top TFs that interacted with the highest number of genes.

In this case study, AtMYC2 was selected for further exploration because it interacts with a large number of genes and belongs to bHLH family, which is well recognised for its key roles in regulating stress-responsive genes (Hao et al., 2021; Song et al., 2022).

Table 4.1 Number of genes interacting with each TFs

TF name	TF family	No. of interacting genes
AtMYB2	MYB	52
At1g19000	MYB-related	51
NAC1	NAC	49
ATWRKY6	WRKY	46
AT1G49560	G2like	44
ZAT7	C2H2	44
Adof1	C2C2dof	44
ANL2	HB	40
AtMYC2	bHLH	37
ATHB23	ZFHD	37
LEC2	ABI3VP1	37
AT1G76510	ARID	36
HY5	bZIP	34
ABR1	AP2EREBP	34
ASIL1	Trihelix	30
LMI1	Homeobox	30
FLC	MADS	26
SPL2	SBP	26
LFY	ORPHAN	25
ASL18	LOBAS2	25
GeBP	GeBP	23
REM19	REM	22
MP	ARF	20
HSF6	HSF	18
AT3G09735	S1Falike	15
HAT2	Ecoli	15
CRC	C2C2YABBY	14
GATA1	GATA	13
TSO1	CPP	12
GRF1	GRF	11
AT5G23930	mTERF	10
BZR1	BZR	9
FRS9	ND	8
AT3G42860	zfGRF	7
AtCAMTA2	CAMTA	5
NLP7	RWPRK	4
BPC1	BBRBPC	3
EIL2	AP2EREBP	3
TCP20	C2C2dof	2
bHLH121	bHLH	2
DEL2	E2FDP	2
FHY3	FAR1	2
BAM8	BES1	1
TOC1	TOC1/APRR1	1

MYC transcription factors belongs to bHLH family of TFs and play a key role in regulating the Jasmonate (JA) signalling pathway, which is vital for plant growth, stress adaptation, and secondary metabolism (Song et al., 2022). When exposed to stress, plants trigger the JA signalling cascade, where regulatory elements bind to the promoters of target genes, altering their expression to help cope with stress (Song et al., 2022). Among these factors, MYC2, recognized as a master regulator of numerous biological processes (Lorenzo et al., 2004; Dombrecht et al., 2007), also plays a crucial role in stress response mechanisms, including the production of secondary metabolites (Luo et al., 2023). Unlike other MYC proteins, MYC2 has a greater activation capacity because tetramerised MYC2 facilitates the formation of DNA looping structures. This in turns, enhances the ability of MYC2 to bind DNA and activate gene expression (Kazan and Manners, 2013).

In this case study, the NetViewer generated network showed that AtMYC2 interacted with 37 DEGs after 48 hours of heat treatment (Table 4.2 & Figure 4.5). Functional annotations showed that several of these genes encode proteins that are involved in hormonal signalling such as jasmonate and auxin. In *A. thaliana*, the activation of JA signalling by MYC2 is precisely regulated by its repressor, the jasmonate-zim domain (JAZ) protein. Under non-stress conditions, JAZ binds to MYC2 and other TFs, keeping them inactive and preventing the transcription of downstream response genes (Thines et al., 2007; Song et al., 2022). Interestingly, the differential expression data from the heat treatment experiment (carried out in Chapter 3) revealed that 25 transcripts mapped to JAZ proteins were down-regulated (Table 4.3). Further, the results also showed that heat treatment led to the up-regulation of a *B. napus MYC2* transcript, which then gradually decreased over time but maintained a high level of expression throughout the treatment. After the removal of heat stress, *MYC2* expression began to decline, eventually returning to levels similar to the control by 7 days of recovery (DOR), suggesting that *MYC2* transcript was activated in response to heat stress (Figure 4.5).

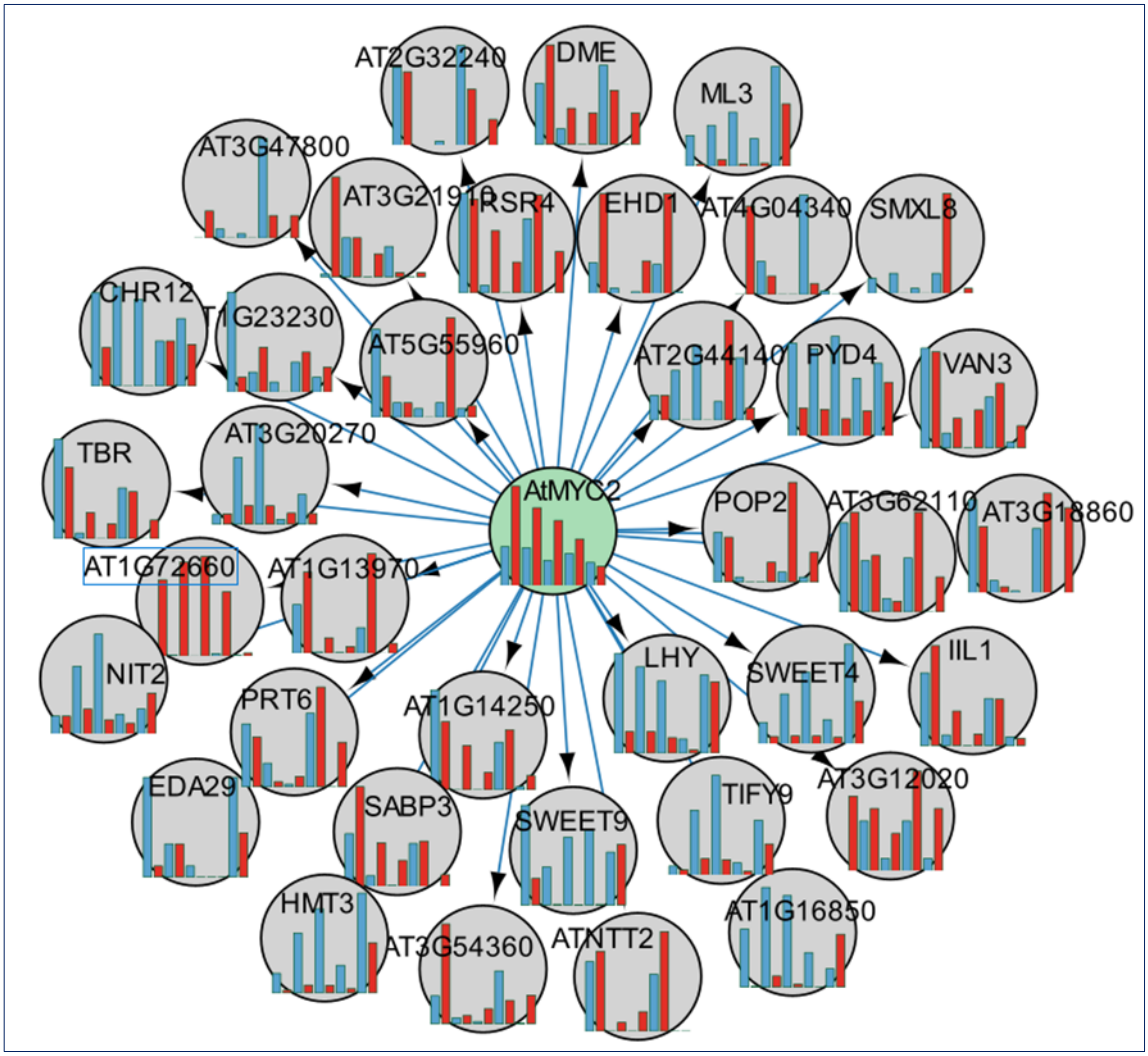

Figure 4.5 Transcription factor AtMYC2-genes interactions generated network. Green node (AtMYC2) and grey nodes (genes). Histogram bars represent expression levels in average transcript per million values (TPM) in response to heat treatment. Each timepoint is represented by two bars (blue: control condition, red: heat stress). The timepoints are as follows, from left to right: 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR.

Furthermore, among the genes interacting with AtMYC2 were those involved in sugars and energy transport *(SWEET4*, *SWEET9*, *ATNTT2*), carbohydrate metabolism (AT3G47800), protein binding and degradation (*EHD1*, *PRT6*, *AT2G44140*), DNA repair (*DME*), as well as several uncharacterized genes (*AT2G32240, AT5G55960, AT1G23230*) (Table 4.2). Gene Ontology terms showed that several of the AtMYC2 interacting genes were enriched with terms associated with response to different types of stresses such as oxidative stress, osmotic stress, heat stress and other types of abiotic stress, in addition to other terms related to regulation of transcription and gene expression. It has been

shown that MYC2 orchestrates a hierarchical transcriptional cascade through regulating multiple TFs, which in turn activate and amplify jasmonate-mediated response (Du et al., 2017; Luo et al., 2023). Interestingly, the network generated by NetViewer highlighted such interactions where AtMYC2 appears to engage with multiple genes coding for transcription factors, with some being up-regulated (e.g., *POP2*) and others down-regulated (e.g., *LHY*, *CHR12*, *EDA29*, *SMXL8*, and *AT4G04340*) (Figure 4.6). The dual function of MYC2 as both a transcriptional activator and repressor has been well-documented in the literature (Kazan & Manners, 2013). This was clearly demonstrated using NetViewer, where the generated networks distinctly showed this dual function. Under heat stress, *B. napus* MYC2 enhanced the expression of genes like *DME*, *RSR4*, *AT1G72660*, *IIL1*, *AT3G12020*, *VAN3*, *SABP3*, which exhibited higher TPM values under heat treatment (red bars) compared to control conditions (blue bars). MYC2's repressive effect was evident as well, with genes such as *SWEET4, SWEET9, HMT3, CHR12, PYD4, ML3, LHY, NIT2,* and *TIFY9* displaying higher TPM values under control conditions (blue bars).

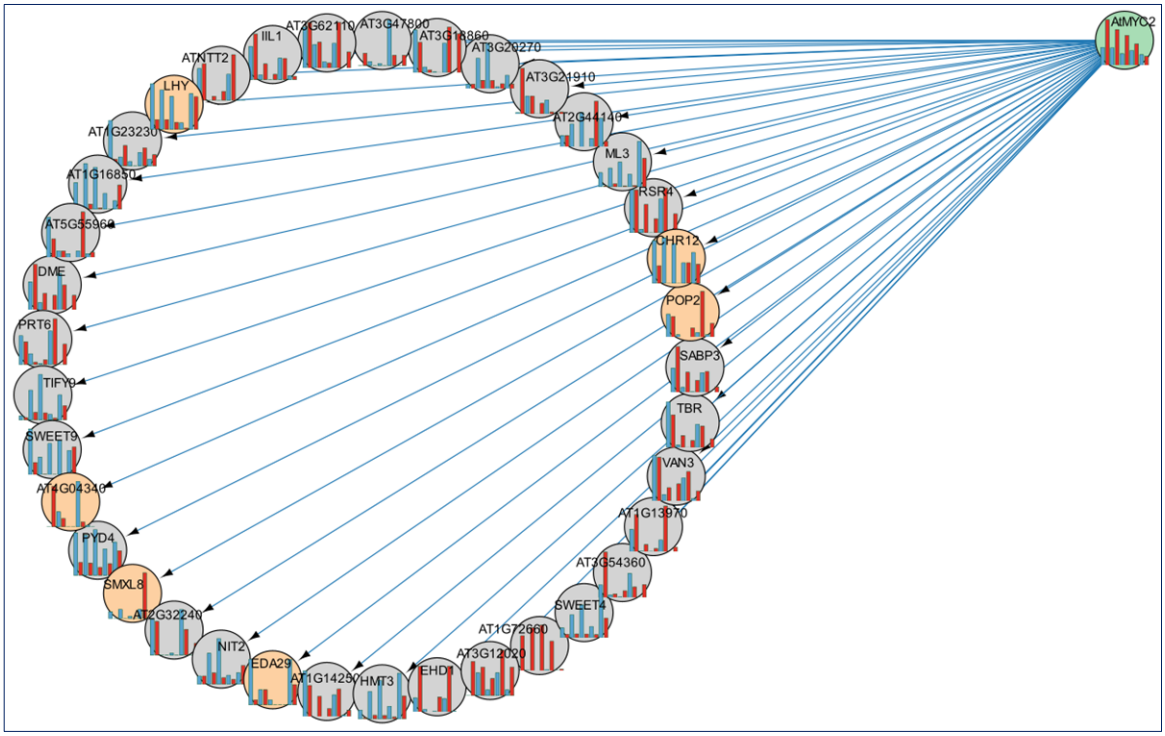

Figure 4.6 Transcription factor AtMYC2-genes interactions generated network. Green node (AtMYC2), orange nodes (genes encoding TFs) and grey nodes (other genes). Histogram bars represent expression levels in average TPM in response to heat treatment. Each timepoint is represented by two bars (blue: control condition, red: heat stress). The timepoints are as follows, from left to right: 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR.

Although AtMYC2 was found to interact with a large number of DEGs in response to heat stress, and its involvement in promoting thermotolerance through the Jasmonate signalling pathway has been established (Khan et al., 2023), it should be clearly noted that the identified genes also showed interactions with other TFs belonging to various families (Figure 4.7), and without clear annotations and differential expression data, the role of these TFs in regulating the obtained genes remains uncertain. Further, the DEGs used in this case study represents the top 60 DEGs in response to heat treatment. AtMYC2 could possibly be interacting with other heat stress responsive genes ranked below the top 60 DEGs. These challenges can be addressed by obtaining more comprehensive annotations for TFs and utilizing species-specific TF resources, which would offer more targeted information for the species in question, rather than relying solely on *A. thaliana*. Additionally, a deeper investigation into the impact of AtMYC2 on individual genes under specific conditions is needed. Reviewing literature on MYC2's role under various abiotic stresses would provide a clearer insight. This would help us

understand how different stresses affect the expression of this key TF and ultimately its downstream genes.

Finally, while experimental validation is required to confirm these predicted interactions, it is possible that MYC2's interactions with DEGs and other TFs in response to heat treatment could introduce additional functional diversity, enhancing its role as a multifaceted transcriptional regulator in various signalling pathways, particularly in heat stress response.

Taken together, this case study showcased NetViewer as a useful tool in visualising and exploring gene interaction networks. This is particularly important as genes-TFs interactions are essential in helping pinpoint new targets that may serve as valuable resources for crop improvement.

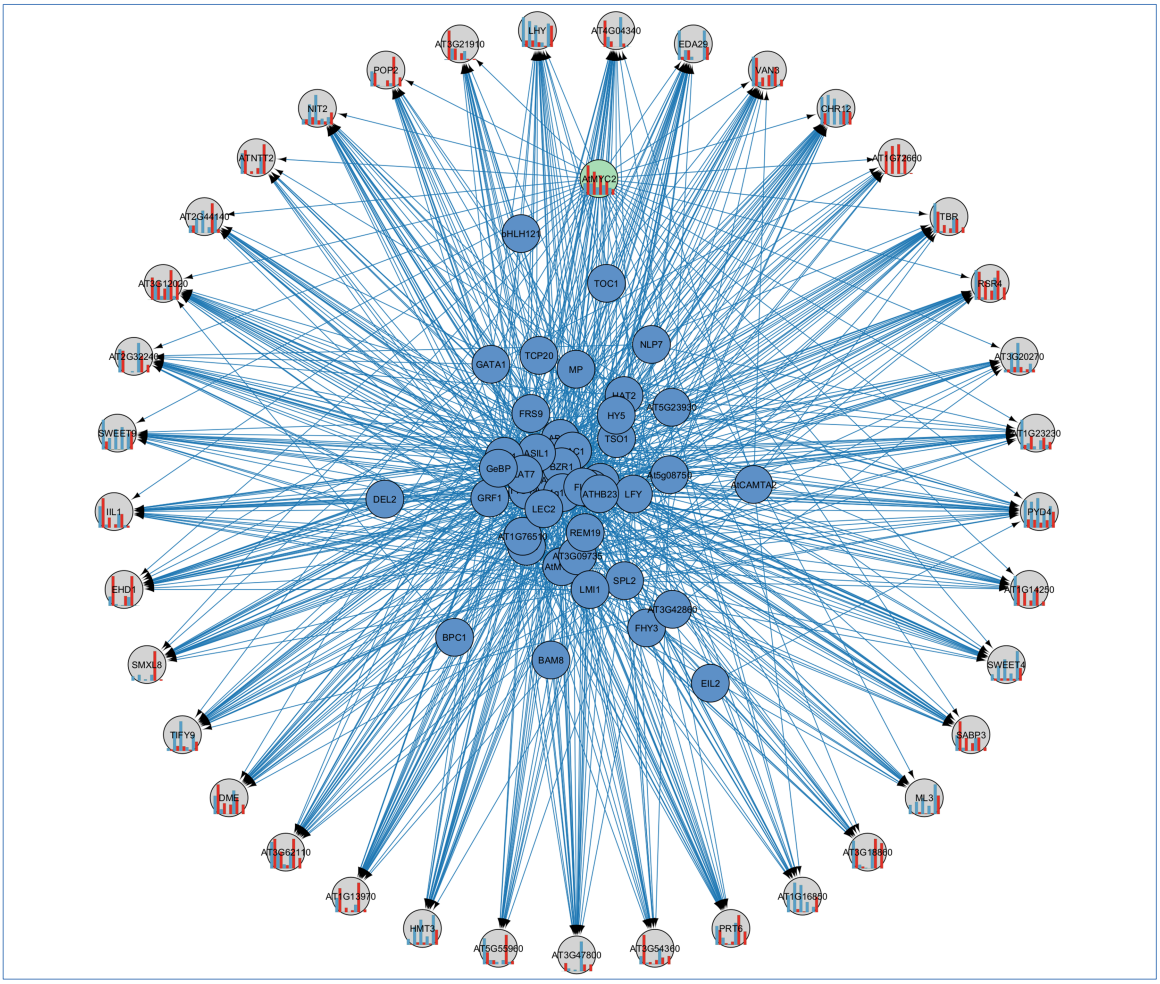

Figure 4.7 Multiple TFs regulate AtMYC2-regulated genes. Green node (AtMYC2), blue nodes (TFs) and grey nodes (genes). Histogram bars represent expression levels in average TPM in response to heat treatment. Each timepoint is represented by two bars (blue: control condition, red: heat stress). The timepoints are as follows, from left to right: 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR.

Table 4.2 AtMYC2 genes network table. C0, C1, C2, C6 & C12 represent TPM values at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively of control conditions. HS0, HS1, HS2, HS6 & HS12 represent TPM values at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively of heat stress conditions.

Gene ID	Arabi ID*	Gene Name	Functional Annotation	GO terms	C0	C1	C2	C6	C12	HS0	HS1	HS2	HS6	HS12
BnaC08T0442400WE	AT1G13970	*AT1G13970*	Protein of unknown function (DUF1336)	Molecular_function	19.6	0.6	0.3	10.3	0	32.7	6.3	2.8	40.1	4.1
BnaA10T0115800WE.1	AT5G55960	*AT5G55960*	NA	Integral component of membrane, molecular_function, biological_process	3	0.5	0.3	0.5	0.3	1.4	0.5	0	3.4	0.4
BnaC08T0293600WE.1	AT3G54360	*AT3G54360*	Zinc ion binding	Protein binding, nucleus, cytosol, positive regulation of catalase activity, zinc ion binding, nuclear heterochromatin, maintenance of DNA methylation, protein ubiquitination, ubiquitin protein ligase activity, regulation of methylation-dependent chromatin silencing	3.7	0.8	0.3	7	0.1	13.2	1.1	2	3.1	3.8
BnaA09T0588100WE	AT1G16850	*AT1G16850*	NA	Endoplasmic reticulum, response to salt stress, molecular_function	5.7	9.8	9	3.4	1.8	0.1	1.1	0.3	0	5.2
BnaA09T0425500WE	AT1G23230	*AT1G23230*	NA	Mediator complex, regulation of transcription, DNA-templated, molecular_function	2	0.4	0.2	0.6	0.3	0.3	0.9	0	0.8	0.5
BnaC07T0304700WE	AT3G28007	*SWEET4*	Nodulin MtN3 family protein	Protein binding, plasma membrane, protein homooligomerization, carbohydrate transmembrane transport, integral component of plasma membrane, sugars transmembrane transporter activity	17.3	40.1	57.5	19.6	79.9	6.1	6.6	6.5	5.4	34.4
BnaA02T0006300WE	AT5G23820	*ML3*	MD-2-related lipid recognition domain-containing protein	Plant-type vacuole, protein binding, defense response, ER body	5.9	7.8	10.3	5.3	19	0.4	1.3	0.4	0.6	11.9
BnaC03T0313100WE	AT3G08860	*PYD4*	PYRIMIDINE 4	Cellular response to nitrogen levels, photorespiration, mitochondrion, alanine-glyoxylate transaminase activity, pyridoxal phosphate binding, identical protein binding	6.9	6.5	7.4	4.3	5.4	2.1	2	1.3	1.9	4
AtMYC2	bHLH				4	3.9	2.6	3.3	2.4	10.2	8	6.7	4.8	2
BnaC06T0410600WE	AT1G72660	*AT1G72660*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	GTPase activity, GTP binding, GDP binding, cytoplasm	0.3	0.1	0	0.3	0.1	9.5	11.7	12.4	8	0.4
BnaA02T0000800WE	AT5G01410	*RSR4*	Aldolase-type TIM barrel family protein	Cytosol, plasma membrane, cellular amino acid metabolic process, response to lipid hydroperoxide, pyridoxine biosynthetic process, response to UV-B, response to non-ionic osmotic stress, endomembrane system, chlorophyll metabolic process, hyperosmotic salinity response, protein homodimerization activity, protein heterodimerization activity, pyridoxal phosphate	71.2	5.5	0	52.9	0	67.1	44.4	22	70	29.6

				biosynthetic process, pyridoxal 5'-phosphate synthase (glutamine hydrolysing) activity											
BnaA08T0301700WE	AT1G01060	*LHY*	Homeodomain-like superfamily protein	Transcription factor activity, sequence-specific DNA binding, response to cold, response to salt stress, response to ethylene, response to auxin, response to abscisic acid, response to gibberellin, response to salicylic acid, response to jasmonic acid, negative regulation of circadian rhythm, negative regulation of sequence-specific DNA binding transcription factor activity, transcription regulatory region DNA binding, response to cadmium ion, long-day photoperiodism, flowering, nucleus	14.8	12.8	10.7	2.1	11.6	3.2	3.2	2.3	0.5	10.6
BnaC03T0246700WE.1	AT4G04340	*AT4G04340*	ERD (Early-responsive to dehydration stress) family protein	Calcium activated cation channel activity, plasma membrane, cellular hyperosmotic response, regulation of calcium ion import, integral component of membrane, cation transmembrane transport, protein tetramerization	0	0.9	0	2.7	0.1	2.4	0.5	0	0.3	0
BnaA01T0299900WE	AT3G06010	*CHR12*	Homeotic gene regulator	Response to heat, response to water deprivation, response to salt stress, maintenance of root meristem identity, maintenance of shoot apical meristem identity, histone binding, helicase activity, nucleus, covalent chromatin modification, regulation of growth, DNA binding, ATP binding	2.9	3.1	2.7	1.4	2.1	1.2	0	0	1.4	1.3
BnaA08T0245200WE.1	AT1G15500	*ATNTT2*	TLC ATP/ADP transporter	Chloroplast envelope, ATP binding, integral component of membrane, ADP transport, ATP transport, chloroplast membrane, ATP:ADP antiporter activity	3.9	0	0	3.2	0	4.5	0.5	1.1	5.6	0
BnaA01T0219100WE.1	AT3G22200	*POP2*	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	Mitochondrion, sucrose metabolic process, inositol metabolic process, glutamate decarboxylation to succinate, glutamine metabolic process, response to oxidative stress, zinc ion binding, gamma-aminobutyric acid catabolic process, response to salt stress, pollen tube growth, pollen tube adhesion, response to organic substance, fruit development, pollen tube guidance, beta-alanine catabolic process, 4-aminobutyrate:pyruvate transaminase activity, root development, shoot system development, cobalt ion binding, pyridoxal phosphate binding	1	0.1	0	0.2	0.1	0.9	0	0.4	2	0.6
BnaC03T0161100WE	AT2G39060	*SWEET9*	Nodulin MtN3 family protein	Protein binding, plasma membrane, sucrose transmembrane transporter activity, vesicle membrane, trans-Golgi network membrane, nectar secretion, sucrose transport, cytoplasmic vesicle membrane, carbohydrate transmembrane transport, integral component of plasma membrane	25.8	9.9	17.6	19.3	13.7	6.9	0.2	0.1	0.2	15.7

BnaA08T0052500WE	AT4G13430	*IIL1*	Isopropyl malate isomerase large subunit 1	Chloroplast stroma, glucosinolate biosynthetic process, response to cadmium ion, intramolecular transferase activity, transferring hydroxy groups, 4 iron, 4 sulfur cluster binding, 3-isopropylmalate dehydratase activity, branched-chain amino acid biosynthetic process, leucine biosynthetic process, metal ion binding	25.5	3.7	0	16.5	3.1	35	12.1	4.1	16.3	2.6
BnaC05T0457400WE.1	AT3G12020	*AT3G12020*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	ATP binding, microtubule binding, microtubule, chloroplast, microtubule motor activity, kinesin complex, microtubule-based movement, ATPase activity	0	0.4	0.1	0.4	0.1	0.6	0.5	0.3	0.8	0.5
BnaC03T0004400WE	AT5G13220	*TIFY9*	Jasmonate-zim-domain protein 10	Nucleus, response to wounding, regulation of systemic acquired resistance, protein homodimerization activity, regulation of jasmonic acid mediated signaling pathway, regulation of transcription, DNA-templated, transcription corepressor activity, negative regulation of nucleic acid-templated transcription	2	14.1	21.6	2.7	11.9	1.2	3.6	3.4	0.9	6.8
BnaC05T0366800WE	AT3G20290	*EHD1*	EPS15 homology domain 1	Protein binding, endosome, endocytosis, plasmodesma, membrane, endocytic recycling, hyperosmotic salinity response, protein homooligomerization, GTP binding, plasma membrane, endosome membrane, hydrolase activity, calcium ion binding	1.7	0.3	0.1	1.6	0.1	5.5	0	1.8	5.5	0
BnaA07T0211500WE.1	AT3G62110	*AT3G62110*	Pectin lyase-like superfamily protein	Vacuole, extracellular region, lyase activity, cell wall organization, polygalacturonase activity, carbohydrate metabolic process	3.3	1.9	0.5	2	0	3.7	2.1	0.4	3.7	1.3
BnaC05T0336600WE	AT3G22740	*HMT3*	Homocysteine S-methyltransferase 3	Plasma membrane, methionine biosynthetic process, methylation, metal ion binding, S-adenosylmethionine-homocysteine S-methyltransferase activity	6.9	21.2	29.8	9.8	35.3	1	2.8	2.7	1.5	17.8
BnaA06T0168200WE.1	AT3G47800	*AT3G47800*	Galactose mutarotase-like superfamily protein	Carbohydrate binding, aldose 1-epimerase activity, galactose metabolic process	0	0.4	0.2	4.4	0	1.2	0	0	1	1
BnaC02T0000100WE	AT5G02310	*PRT6*	Proteolysis 6	ubiquitin-protein transferase activity, ubiquitin-dependent protein catabolic process, response to abscisic acid, regulation of seed germination, regulation of lipid catabolic process, zinc ion binding, protein ubiquitination, ligase activity, ubiquitin ligase complex, cytoplasm, ubiquitin protein ligase activity, ubiquitin-dependent protein catabolic process via the N-end rule pathway	2.4	0.9	0.1	2.8	0	1.9	0.2	0.4	3.8	1.7
Bnascaffold3139T0000400WE.6	AT3G01500	*SABP3*	Carbonic anhydrase 1	Carbonate dehydratase activity, plasma membrane, response to cold, chloroplast thylakoid membrane, chloroplast stroma, defense response to fungus, incompatible interaction, chloroplast	43.1	8.8	0.9	35.1	0	81.4	35.8	21.3	37.1	9.7

				envelope, response to carbon dioxide, regulation of stomatal movement, stromule, photosynthesis, defense response to bacterium, apoplast, negative regulation of stomatal complex development, zinc ion binding, carbon utilization											
BnaA05T0320800WE.1	AT3G18860	*AT3G18860*	Transducin family protein / WD-40 repeat family protein	Protein binding, nucleotide binding, biological_process	4.5	0.6	0.1	3.1	0	3.2	0.3	0	4.8	4.1
BnaC05T0109800WE.1	AT1G14250	*AT1G14250*	GDA1/CD39 nucleoside phosphatase family protein	Vacuole, ATP binding, integral component of membrane, hydrolase activity	6.3	0	0	3	0.2	4.3	2.8	1.1	3.8	0.9
BnaC09T0554000WE.1	AT5G06700	*TBR*	Plant protein of unknown function (DUF828)	Endosome, trans-Golgi network, plant-type cell wall modification, cellulose biosynthetic process, pectin biosynthetic process, integral component of membrane, O-acetyltransferase activity	5.3	0.3	0	2.7	0	3.8	1.4	0.8	2.5	1
BnaA05T0305700WE	AT3G20270	*AT3G20270*	Lipid-binding serum glycoprotein family protein	Lipopolysaccharide binding, regulation of gene expression, apoplast, reactive oxygen species biosynthetic process, protein binding, extracellular space	3	20.2	30.1	1.5	9	3.2	5.7	5.6	3.4	3.3
BnaC04T0530900WE.1	AT2G35940	*EDA29*	BEL1-like homeodomain 1	DNA binding, nucleus, cytoplasm, response to symbiotic fungus, response to abscisic acid, polar nucleus fusion, response to continuous far red light stimulus by the high-irradiance response system, protein homodimerization activity, protein heterodimerization activity, regulation of transcription, DNA-templated, transcription factor activity, sequence-specific DNA binding	0.9	0.3	0.1	0	0.9	0.1	0.3	0	0	0.4
BnaC09T0500200WE.1	AT5G13300	*VAN3*	ARF GTPase-activating protein	GTPase activator activity, protein binding, cytosol, plasma membrane, endocytosis, response to auxin, leaf morphogenesis, xylem and phloem pattern formation, phloem or xylem histogenesis, trans-Golgi network transport vesicle, phosphatidylinositol binding, positive regulation of GTPase activity, metal ion binding	10.8	1.6	0	5.5	0.6	10.4	3.2	4.1	7	2.4
BnaA05T0284500WE	AT3G21910	*AT3G21910*	Domain of unknown function (DUF26)	Extracellular region, molecular_function, biological_process	0.2	2.7	0	2.1	0	6.9	2.7	1.6	0.3	0.3
BnaC04T0492800WE	AT3G44300	*NIT2*	Nitrilase 2	cytosol, response to bacterium, response to cadmium ion, indole-3-acetonitrile nitrilase activity, indole-3-acetonitrile nitrile hydratase activity, nitrogen compound metabolic process, plasma membrane, indoleacetic acid biosynthetic process	1	3.8	5.6	1.1	1.4	1	1.4	0.8	0.6	2.3
BnaA05T0033000WE.2	AT2G44140	*AT2G44140*	Peptidase family C54 protein	Autophagosome assembly, mitophagy, cysteine-type endopeptidase activity, cytosol, C-terminal protein lipidation, protein targeting to membrane,	0.4	0.8	1.2	0.3	1	0.4	0	0	1.6	0.2

				protein processing, nucleophagy, protein delipidation										
BnaC04T0148200WE.1	AT2G32240	*AT2G32240*	NA	Plasma membrane, response to abscisic acid, response to cadmium ion, integral component of membrane, molecular_function	1.8	0	0.1	2.3	0	1.7	0	0	1.3	0.6
BnaA10T0276400WE.1	AT5G04560	*DME*	HhH-GPD base excision DNA repair family protein	DNA-(apurinic or apyrimidinic site) lyase activity, DNA methylation, regulation of gene expression by genetic imprinting, embryo development ending in seed dormancy, DNA N-glycosylase activity, polar nucleus, base-excision repair, 4 iron, 4 sulfur cluster binding, DNA binding, regulation of transcription, DNA-templated, metal ion binding	2.7	0.7	0	3.5	0	4.4	1.6	1.4	2.4	1.4
BnaC04T0067000WE.1	AT2G40130	*SMXL8*	Double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein	Response to strigolactone, ATP binding, protein metabolic process, nucleus, regulation of transcription, DNA-templated	0.3	0.4	0.1	0.4	0	0	0	0	2	0.1

**A. thaliana* gene ID

Table 4.3 Differential expression data of JAZ transcripts in log2FC. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively. (NA*: Not DE in this contrast)

Gene	HS0vsC0	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Sequence Description
BnaC03G0004400WE	NA*	-2.10111	-2.72948	-1.69371	NA	protein TIFY 9-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC02G0047400WE	NA	NA	-1.91786	-1.80735	NA	protein TIFY 9-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC08G0412700WE	NA	-1.33876	-1.6933	NA	NA	protein TIFY 10A-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA08G0230600WE	NA	-1.43237	-1.45255	NA	NA	protein TIFY 10A-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA01G0244800WE	-0.98225	-1.10666	-1.31969	-0.96064	-0.6695	protein TIFY 6B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC06G0296600WE	NA	-0.86871	-1.28004	NA	NA	protein TIFY 10B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC08G0207400WE	NA	-0.93257	-1.27907	-0.64677	-0.70899	protein TIFY 10A-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA05G0331400WE	-0.83664	-1.17669	-1.2069	-0.71593	NA	protein TIFY 6B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA02G0188100WE	NA	-0.93615	-1.1533	-0.94032	-0.83519	protein TIFY 7-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA07G0314600WE	NA	-0.77736	-0.9825	-0.64982	NA	protein TIFY 7; K13464 jasmonate ZIM domain-containing protein (A)
BnaC06G0429100WE	-0.69426	-0.71018	-0.94323	-0.52401	NA	protein TIFY 10B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC06G0408800WE	NA	NA	-0.89616	NA	NA	protein TIFY 11B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA09G0577900WE	NA	NA	-0.85238	NA	NA	protein TIFY 10A-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC05G0401200WE	-0.77013	-0.85134	-0.82705	-0.7499	NA	protein TIFY 6B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC01G0341600WE	NA	-0.85421	-0.81653	NA	NA	protein TIFY 6B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC02G0275300WE	NA	-0.68805	-0.73468	NA	NA	protein TIFY 10B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaC02G0242000WE	NA	-0.70767	-0.69984	-0.86821	NA	protein TIFY 7-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA02G0211500WE	NA	-0.6496	-0.65446	NA	NA	protein TIFY 10B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA07G0345500WE	-0.61338	NA	-0.64021	NA	NA	protein TIFY 10B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA10G0167300WE	NA	NA	-0.61823	NA	NA	protein TIFY 3B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA02G0047300WE	NA	NA	NA	-2.17619	NA	protein TIFY 9-like; K13464 jasmonate ZIM domain-containing protein (A)

BnaA03G0071000WE	NA	-1.78583	NA	NA	NA	protein TIFY 9-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA07G0245300WE	NA	-1.02175	NA	NA	NA	protein TIFY 10B-like; K13464 jasmonate ZIM domain-containing protein (A)
BnaA10G0219700WE	NA	NA	NA	NA	-1.32435	protein TIFY 9; K13464 jasmonate ZIM domain-containing protein (A)
BnaC06G0392900WE	NA	NA	NA	-0.71101	NA	protein TIFY 7; K13464 jasmonate ZIM domain-containing protein (A)
BnaC09G0500900WE	NA	NA	NA	NA	-1.53281	protein TIFY 9; K13464 jasmonate ZIM domain-containing protein (A)

5 General Discussion

Over the coming decades, climate change will continue to influence all aspects of crops. This has been made evident by the dramatic temperature fluctuations and the changes in precipitation regimes (Mittler et al, 2012; Parmesan & Hanley, 2015; Lamaoui et al., 2018). Due to their sessile nature, plants respond to these changes by modifying their physiology and metabolism and engaging in an intricate network of cellular and molecular interactions to acclimatise and ensure survival (Ding & Yang, 2022). However, when stress conditions surpass their adaptive capacity, plants struggle to maintain a steady growth, resulting in a profound reduction in yield and food supply.

Oilseed rape is an essential oilseed crop, accounting for approximately 12% of the world's vegetable oil production (Zheng & Liu, 2022). Its ability to grow in temperate climate has made it one of the predominant oil crops in Europe (Namazkar et al., 2016; Sun et al., 2017). While studies on various Brassica species have identified negative impacts of heat stress on seed yield and quality, the underlying mechanisms remain poorly understood and need further investigation (Yu et al., 2014). Therefore, to improve heat tolerance in *B. napus* and to produce climate resilient cultivars, it is imperative that heat stress response and adaptation mechanisms are well studied and understood. The first chapter of this thesis provides a brief introduction. It outlines the aims and objectives of the research project and explains the overall structure of the thesis. The second chapter presents a comprehensive review of the impact of heat stress on the physiology and genetics of *B. napus.* It summarizes the latest studies on this aspect with a focus on the reproductive stages. The chapter also outlines the different mechanisms that are involved in mediating heat stress response with a focus on phytohormones, transcription factors (TFs) and glucosinolates (GSL) (Figure 2.3). Additionally, it examines the role of polyploidy, alternative splicing, and epigenetic mechanisms like DNA methylation, histone modifications, and non-coding RNAs in heat stress responses. The chapter further explores the exploitation of systems biology approaches to study the genetic and physiological impacts of heat stress on *B. napus*.

Chapter three presents a heat treatment experiment that simulates a heatwave episode where the plants were exposed to a gradual increase in temperature reaching 30 °C in the day and 24 °C at night for a period of 6 days during the flowering stage of *B. napus* cv. Westar. Global transcriptome profiling using RNA-Seq showed a reduction in respiratory metabolism namely Glycolysis, Pentose phosphate pathway, Citrate cycle and Oxidative phosphorylation in response to heat treatment. This was manifested through the down-regulation of many genes involved in these pathways especially after 48 hrs of heat stress (Figure 3.17, Figure 3.18, Figure S9, Figure S11 & Figure S12). Differential expression analysis showed that some of the genes encoding sugars transporters, nitrogen transport and storage, cell wall modification and methylation were down-regulated (

Table 3.2 &

Table 3.4). In contrast, several genes mapping to small heat shock proteins (sHSP), other heat shock transcription factors, methylation and redox balance-related genes were up-regulated (Table 3.1 & Table 3.3). At the metabolomic level, HPLC and LC-MS were used to measure carbohydrate and GSL content respectively. The analysis showed that heat stress led to an increase in the concentration of glucose, fructose and sucrose in *B. napus* leaves (Figure 3.27-Figure 3.29). It also caused a rise in the concentration of the aliphatic GSL gluconapin and progoitrin (Figure 3.22 & Figure 3.23) while reducing the concentrations of the indolic GSL glucobrassicin (Figure 3.24). Additionally, a

reduction in yield (Figure 3.4) and significant changes in fatty acids compositions were observed in the seeds through NIRS analysis (Figure 3.5).

Studies showed that heat stress differentially modulates the concentrations of carbohydrates, lipids and proteins and destabilises membrane fluidity, enzyme efficiency and RNA stability, resulting in a state of metabolic imbalance and over-accumulation of ROS (Suzuki et al., 2011, Ahmad et al., 2021). To overcome this stress, plants undergo “multi-omic” reprogramming and produce a transcriptomic and a metabolic change to restore a steady state of balance necessary for the functioning and survival under high temperature stress. Once environmental conditions shift back to normal, a reverse process of deprogramming will take place (Mittler et al., 2012). This complex process was evident in the current heat experiment at both the transcriptomic and metabolic levels. During the recovery period, the total number of DEGs dropped dramatically (Figure S3). Notably, the number of DE sugars transporters genes (*SWEET* & *ERD6-like*) decreased significantly following the removal of heat stress, with only one gene, *SWEET4*, remaining down-regulated after one week of recovery (Figure 3.25 & Figure 3.26). A similar decline was seen in DEGs involved in GSL metabolism, including various UDP glycosyltransferases, glutathione s-transferases and GSL related transcription factors (Figure 3.19-Figure 3.21). Concurrently, plant leaves exhibited a remarkable decline in sugars and GSL, following their substantial increase in response to heat stress (Figure 3.27-Figure 3.29 & Figure 3.22-Figure 3.24).

Another aspect of the reprogramming process is the activation of heat shock HSFs, which regulate the expression of HSPs. These proteins play a vital role in binding to denatured proteins and inhibiting their aggregation, thus maintaining cellular homeostasis and contributing to thermotolerance (Su and Li, 2008; Scharf et al., 2012; Danilova et al., 2018). In the current study, several transcripts that mapped to HSPs and other HSFs were among the top DEGs. Notably, members of the sHSP family, also known as the HSP20 family proteins, such as HSP17.6, HSP22, and HSP23.6, showed high expression levels throughout the heat treatment, with expression reaching a log2FC of 11 at 2 DAT (Table 3.1 &

Table 3.3). This protein family is the most abundantly produced in plants in response to heat stress, and their role in plant responses to various abiotic stresses has been established (Sun et al., 2020). Other studies also reported the involvement of HSFs in different biotic and abiotic stress (Mishra et al., 2002; Mishra et al., 2019; Chowdhary et al., 2023). In *A. thaliana*, thermoprotection tests demonstrated the role of HSFA6b in thermotolerance acquisition (Huang YC et al., 2016). Moreover, Giorno et al. (2009) showed that HsfA2 and Hsp17-CII are induced under high temperature in tomato which are responsible for activating protection mechanisms during heat stress.

Other types of TF families such as bZIPs, NACs, WRKYs, AP2/ERFs, bHLHs and MYBs also contribute to stress tolerance in plants and their interaction with their target genes have been reported in response to heat stress (Guo et al., 2016; Wang et al., 2018; Meraj et al., 2020; Zhao et al., 2020; Li et al., 2022). Chapter four of this thesis demonstrates a Cytoscape application called NetViewer to visualise such interactions in a user-friendly environment. These interactions are presented in a network where genes and TFs are displayed as nodes and the relationship between them is displayed as edges. Moreover, the application also allows the user to add functional annotations and expression data for the displayed genes, thus complementing the default data. In a case study, NetViewer was used to display the interactions between the top DEGs identified in the heat treatment experiment in Chapter 3 and TFs (Figure 4.4). Members of the MYB, NAC and WRKY families were among the top TFs that interacted with the highest number of genes (Table 4.1). AtMYB2 appeared as a master regulator where it interacted with the highest number of genes. The characterization of different MYB members is based on the presence of highly conserved MYB domains involved in DNA binding. Generally, these domains consist of multiple repeats (R), with R2R3-MYB being unique to plants and playing vital roles in responding to various types of stresses (Baillo et al., 2019). AtMYB2 has primarily been associated with responses to salt and drought stress (Baek et al., 2015) and has also been linked to the regulation of secondary metabolite production during growth (Guo et al., 2011). Notably, our expression data revealed multiple transcripts corresponding to MYB-related transcription factors, though none were

specifically annotated as MYB2. Interestingly, some of these genes showed up-regulation in response to heat treatment, while others were down-regulated. NetViewer showed that AtMYB2 interacted with 52 genes mapping to various functions including biosynthesis and accumulation of secondary metabolites and carbohydrate binding and transport, in addition to stress response genes, such as *sHSP* (Table S7). The functional diversity of these genes reflects the broad regulatory roles of MYB TFs, making them important targets for the development of functional marker systems, such as simple sequence repeat (SSR) markers, single nucleotide polymorphisms (SNPs) or derived cleaved amplified polymorphic sequences (dCAPS) markers that will eventually aid in the identification of advantageous TFs across successive generations, hence, facilitating marker-assisted crop improvement (Shahzad et al., 2021). The case study further explored the regulatory role of transcription factor AtMYC2 which interacted with 37 DEGs in response to heat stress (Figure 4.5). Remarkably, in the heat treatment experiment carried out in Chapter 3, a *B. napus MYC2* transcript expression increased sharply at the onset of heat treatment, with a TPM value of 10.2 compared to 4 TPM in the control. Then, it gradually declined, reaching a similar level to the control after 7 days of recovery (Table 4.2). Despite this decline, *MYC2* maintained high expression throughout the heat treatment, highlighting its potential role in regulating heat-responsive genes. Additionally, NetViewer highlighted the AtMYC2's dual function as both an enhancer and a repressor, with the generated networks showing some regulated genes being up-regulated while others were down-regulated. Interestingly, some of these genes also mapped to other TFs such as suppressor of MAX2-like 8 (SMXL8), late elongated hypocotyl (LHY) and an ERD family protein (Figure 4.6). Additionally, the network also showed that the AtMYC2-interacting genes were also regulated by multiple other TFs such as HSF6, bHLH121 and ABR1 (Figure 4.7). However, due to lack of specific annotations available for these TFs in the Brassica family, it was challenging to align the differential expression data with certainty, based solely on annotations from *A. thaliana*. Further in vitro experimental validation of these interactions is of crucial importance to confirm these predictions in planta. For instance, the Yeast Two-Hybrid system assay can be employed to detect

interactions between proteins encoded by the *MYC2* gene and those encoded by MYC2-interacting genes. Another approach involves developing transgenic lines with markers, such as Green Fluorescent Protein (GFP), attached to heat stress-related genes (e.g., *sHSP*), enabling monitoring the interactions and localization of their encoded proteins. Applying such technologies would complement the predicted computational work and enable solid understanding with evidence of not only gene-TF interactions but also TF-TF interactions and its synergistic impact on the expression of specific genes under heat stress.

Altogether, the work carried out during this thesis is an attempt to tackle the question of how plants face and adapt to heat stress and to provide insights into the impact of a future warmer climate on plants especially during reproductive stages. The current work allowed the exploration of the stress response at a molecular and metabolomic level in *B. napus* during reproduction. Moreover, it highlighted different components and metabolic pathways responding to heat under similar field conditions. At the transcriptomic level, the key DEGs identified in this study could serve as important biomarkers that can be utilised by breeding programs to select cultivars with stronger resistance to heat. In particular, the identified genes could be further characterised and used as genetic markers for marker assisted selection, where plant breeders could select plant performance based on the composition of these genetic biomarkers rather than through waiting for the plants' phenotypic performance, thus accelerating traditional crop breeding for stress tolerance traits. Also, the identified genes could form a target for genome editing techniques such as the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated protein 9 (Cas9), an approach that has already been explored in crop improvement programs especially for rapid development of abiotic stress-tolerant crops (Zafar et al., 2020). This can be applied by modifying sensitive or negatively regulating genes and activating positively regulated genes involved in stress response pathways to enhance the plant's ability to withstand stress (Kumar et al., 2023). In tomatoes, the knockout of *SlMAPK3* gene which has been identified as a negative regulator of the plant defence response to heat stress, improved ROS homeostasis and increased tolerance to heat stress (Yu et al., 2019). In the

context of the current study, given the critical role of sHSP in plant heat stress response, enhancing the expression of key genes such as *HSP17.6*, *HSP22*, and *HSP23*.6, using CRISPR-mediated gene activation (CRISPRa) could contribute to an improved thermotolerance.

Additionally, the generated gene expression data could be incorporated into genomic prediction, where advanced machine learning models are applied to predict candidate gene selection. This selection process relies on data from a training population, including both phenotypic and genotypic information. After thorough training, these models generate predictions of a genomic estimated breeding value (GEBV) for traits of a target population consisting only of genotypic data (Kaler et al., 2022). To evaluate the performance of these models, cross-validation is carried out to compare different sets of models with distinct scenarios, such as including multiple traits, genes or other omics data (Alemu et al., 2024). Moreover, to select the genes with the largest importance and to reduce the dimensionality of the data, a feature selection approach can be performed to select the genes that can better predict the desired trait (Yoosefzadeh Najafabadi et al., 2021). The importance calculation can either be model based such as using random forest or estimated using a more general approach such as the variance threshold which removes features with zero variance. Gabur et al. (2022) demonstrated that by applying various feature selection methods, extracting a low-density SNP matrix with fewer predictor variables outperformed predictions using entire datasets, resulting in more stable outcomes for oilseed rape and wheat. Additionally, Yoosefzadeh Najafabadi et al. (2021) successfully predicted high-yielding soybean genotypes at early growth stages by using feature selection to identify key reflectance variables. These findings suggest that reducing the number of variables in plant breeding predictions could enhance prediction accuracy, significantly reduce computation time, and improve the identification of important agronomic traits.

At the metabolomic level, a marked increase in the concentrations of sugars (glucose, sucrose and fructose) and aliphatic GSL (progoitrin and gluconapin) was detected in *B. napus* leaves in response to heat treatment relative to control

conditions (Figure 3.27-Figure 3.29 & Figure 3.22 & Figure 3.23). It is well documented that these compounds function not only as osmoprotectants, helping to regulate osmotic balance and maintain membrane stability, but also as antioxidants, protecting against oxidative damage (Nishizawa et al. 2008; Martínez-Ballesta et al., 2013; Xalxo et al., 2020). Besides, the accumulation of sugars during heat stress, which can function as signalling molecules, triggering the expression of various heat responsive genes (Granot et al. 2013; Xalxo et al., 2020). These metabolites could also form potential markers that can be used to predict crop adaptability and resilience while developing stress tolerant varieties. Research indicates that metabolic markers are increasingly being used alongside, and sometimes replacing, traditional molecular markers in this field (Fernandez et al., 2016). This shift is due to the fact that metabolic profiling provides an early functional insight into the metabolic pathways involved in tolerance acquisition, even without knowledge of genetic variations between cultivars (Chang et al., 2019).

5.1 Limitations

This PhD thesis faced several limitations. Firstly, the heat treatment experiment was conducted using one variety of *B. napus*, while testing multiple varieties could have provided more insight into how different genotypes respond to similar treatment conditions. Secondly, sampling during heat treatment and recovery was carried out at specific timepoints over two weeks. However, daily sampling without gaps would have yielded a more representative dataset. The challenge with more frequent sampling, particularly of tissues like buds and leaves, is the potential to introduce additional stress to the plants. This issue could be mitigated by starting with a larger plant pool, allowing more plants per each biological replicate, and reducing sample size per plant. This would enable more frequent collections with additional timepoints. Additionally, the transcriptomic and metabolomic analysis were carried out on different plant tissues, but with a larger plant pool, it would also be possible to conduct multiple omics experiments on similar tissues, producing more comparable results. Thirdly, baseline sampling before the start of heat treatment introduced noise in the dataset which has led

to its exclusion from the analysis. And finally, relying on genes functional annotations from *A. thaliana* only, made it challenging to accurately match TFs to their corresponding differential expression genes in response to heat treatment.

6 Concluding Remarks and Future Perspectives

6.1 Conclusion

Several environmental stress factors are arising as result of the rapidly changing climate. Among them, heat stress in the form of more frequent and longer heatwaves, which has already shown negative impact on crops productivity and in specific on oilseed rape. Heat stress causes various physiological, morphological and molecular variations that drastically impact growth, yield and oil quality. Through simulating field conditions, this PhD project highlighted some of the mechanisms involved in the heat stress response in oilseed rape, an important crop species in Europe and worldwide. This was evident through a noticeable reduction in plant metabolism and in particular, down-regulation of many respiratory pathways and alteration in the movement and levels of metabolites such as sugars and glucosinolates under heatwaves conditions. Additionally, the involvement of heat shock factors and proteins were also at the forefront of the differentially expressed genes in response to heat as compared to control. The study also employed multi-dimensional omics where heat stress response was investigated at both transcriptomic and metabolomic levels. This approach along with other omics studies will continue to provide valuable information and comprehensive datasets that are crucial to elucidate key cellular processes and pathways necessary for plants survival under stress. This along

with the continuous improvement in breeding technologies coupled with predictive modelling would help in the development of stress resilient varieties that can withstand the heat without compromising on yield and nutritional qualities.

6.2 Future Perspectives

Indeed, more profound knowledge is needed in several key areas to draw more robust conclusions and to improve the predictions of how heat stress will impact future yield and quality. Firstly, most research on plant responses to temperature stress and their regulatory components such as TFs, has been conducted in model plants such as *A. thaliana* (Huang et al., 2015; Andrew et al., 2024). However, with climate predictions indicating more frequent and intense heatwaves, it is increasingly important to explore heat stress responses in non-model plants (Mokany et al., 2020). Shifting research on abiotic stress tolerance towards cultivated crops such as wheat, maize and oilseed rape is of paramount importance (Shahzad et al., 2021).

Secondly, designing experiments that involve multiple types of plant tissues will reveal how different parts of the plant respond to heat at a specific timepoint. Also, recent findings showed that plants' responses to combined abiotic stresses are unique and cannot be simply inferred from studying each stressor in isolation (Zandalinas et al., 2018). Therefore, future research should also focus on the simultaneous effects of multiple stress factors on plant tissues to better simulate real field conditions.

Thirdly, gaining insight into plant stress memory under extreme conditions such as heat, is essential for creating stress tolerant crops. Plants employ different types of stress memories regulated by epigenetic changes. Some of these changes are stable and can be transmitted as stress memory, potentially enabling inheritance across generations (Ramakrishnan et al., 2022; Jin et al., 2024). While these mechanisms are well-documented in *A. thaliana*, they remain underexplored in crop plants (Ramakrishnan et al., 2022). Therefore, identifying agriculturally significant epigenetic markers related to heat stress memory for use

in marker-assisted selection and genomic prediction could enhance the accuracy of predicting plant resilience, reduce the need for field trials, and ultimately increase crop yields.

Given the extensive genetic variation in *Brassica napus*, including wild relatives and landraces, conducting comparative studies across different genotypes is also crucial for crop improvement, especially as some variable genes have been linked to key agronomic traits (Bayer et al., 2017). The identified heat-responsive genes such as *sHSPs* and TFs with broad gene interaction networks (such as members of the MYB, NAC or WRKY families) can be further investigated to explore the relationships between gene variants and the variation in heat susceptibility. For instance, variant calling can be used to identify SNPs in aligned sequences from different genotypes. These SNPs can then undergo GO and pathway enrichment analysis to determine whether they are enriched in certain biological processes linked to heat stress response. Additionally, combining transcriptomic data with proteomics using approaches such as proteomics informed by transcriptomics (PIT) enables the comparison of differential gene expression with corresponding protein abundance. This makes it possible to identify genes with strong or weak correlations between mRNA and protein levels. Such analysis can uncover crucial post-transcriptional mechanisms, including heat-induced changes in protein abundance, stability, and modifications. Moreover, this approach can also detect new isoforms or splice variants that are uniquely expressed under heat stress, offering further insights into the molecular adaptations of plants to heat stress.

Finally, data collected from such studies will be instrumental for crop mechanistic modelling, improving prediction accuracy and helping design more climate-resilient cultivars. These integrated approaches are expected to accelerate the development of next generation of crops, making them more productive and better adapted to the ever-changing climate.

REFERENCES

Ahmad, M., Waraich, E.A., Skalicky, M., Hussain, S., Zulfiqar, U., Anjum, M.Z., Habib ur Rahman, M., Brestic, M., Ratnasekera, D., Lamilla-Tamayo, L., Al-Ashkar, I. and EL Sabagh, A. (2021). Adaptation strategies to improve the resistance of oilseed crops to heat stress under a changing climate: An overview. *Frontiers of Plant Science*. 12:767150. doi: 10.3389/fpls.2021.767150

Ahmed, W., Xia, Y., Li, R., Bai, G., Siddique, K. H. M., & Guo, P. (2020). Non-coding RNAs: Functional roles in the regulation of stress response in Brassica crops. *Genomics* 112(2), 1419–1424. Doi.org/10.1016/j.ygeno.2019.08.011

Aksouh-Harradj, N., Campbell, L. & Mailer, R. (2006). Canola response to high and moderately high temperature stresses during seed maturation. *Canadian Journal of Plant Science,* 86, 967–980

Alam, I., Liu, C.C., Ge, H.L., Batool, K., Yang, Y.Q., & Lu, Y.H. (2019). Genome wide survey, evolution and expression analysis of PHD finger genes reveal their diverse roles during the development and abiotic stress responses in *Brassica rapa* L. *BMC Genomics,* 20(1), 1–20. Doi.org/10.1186/s12864-019-6080-8

Alemu, A., Åstrand, J., Montesinos-López, O.A., Isidro Y Sánchez, J., Fernández-Gónzalez, J., Tadesse, W., Vetukuri, R.R., Carlsson, A.S., Ceplitis, A., Crossa, J., Ortiz, R., & Chawade, A. (2024). Genomic selection in plant breeding: Key factors shaping two decades of progress. *Molecular Plant*, 17(4), 552–578. https://doi.org/10.1016/j.molp.2024.03.007

Allis, C. D., & Jenuwein, T. (2016). The molecular hallmarks of epigenetic control. *Nature Reviews Genetics* 17(8), 487–500. Doi.org/10.1038/nrg.2016.59

Ambastha, V., & Leshem, Y. (2020). Differential cell persistence is observed in the Arabidopsis female gametophyte during heat stress. *Plant Reproduction*, 33(2), 111–116. Doi.org/10.1007/s00497-020-00390-0

Amjadi, Z., Hamzehzarghani, H., Rodriguez, V.M., Huang, Y.J, Farahbakhsh F. (2024). Studying temperature's impact on *Brassica napus* resistance to identify

key regulatory mechanisms using comparative metabolomics. *Scientific Reports.* 14(1):19865. Doi: 10.1038/s41598-024-68345-3

Andrew, S.C., Simonsen, A.K., Coppin, C.W., Arnold, P.A., Briceño, V.F., McLay, T.G.B., Jackson, C.J., Gallagher, R. V., & Mokany, K. (2024). Expression–environment associations in transcriptomic heat stress responses for a global plant lineage. *Molecular Ecology*, 33(16). https://doi.org/10.1111/mec.17473

Andrews, S. (2012). FastQC: A quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Angadi, S.V., Cutforth, H.W., Miller, P.R., Mcconkey, B.G., Entz, M.H., Brandt, S.A., & Volkmar, K.M. (2000). Response of three Brassica species to high temperature stress during reproductive growth. *Canadian Journal of Plant Science*, 80, 693–701

Antoun, M., and Ouellet, F. (2013). Growth temperature affects inflorescence architecture in *Arabidopsis thaliana*. *Botany,* 91, 642–651. doi: 10.1139/CJB2013-0011

Appel, R.D., Sanchez, J.C., Bairoch, A., Golaz, O., Miu, M., Vargas, J.R., & Hochstrasser, D.F. (1993). SWISS-2DPAGE: A database of two-dimensional gel electrophoresis images. *Electrophoresis*, 14(1), 1232–1238. Doi.org/10.1002/elps.11501401185

Arda, H.E., & Walhout, A.J. M. (2009). Gene-centered regulatory networks. *Briefings in Functional Genomics* 9(1), 4–12. https://doi.org/10.1093/bfgp/elp049

Ayaz, F.A., Glew, R.H., Millson, M., Huang, H.S., Chuang, L.T., Sanz, C., & Hayırlıoglu-Ayaz, S. (2006). Nutrient contents of kale (*Brassica oleraceae* L. var. acephala DC.). *Food Chemistry* 96(4), 572–579. https://doi.org/10.1016/j.foodchem.2005.03.011

Bac-Molenaar, J.A., Fradin, E.F., Becker, F.F. M., Rienstra, J.A., van der Schoot, J., Vreugdenhil, D., & Keurentjes, J.J.B. (2015). Genome-wide association mapping of fertility reduction upon heat stress reveals developmental stage-specific QTLs in *Arabidopsis thaliana*. *Plant Cell*, 27(7), 1857–1874. Doi.org/10.1105/tpc.15.00248

Baek, D., Chun, H.J., Kang, S., Shin, G., Park, S.J., Hong, H., Kim, C., Kim, D.H., Lee, S.Y., Kim, M.C., et al. (2016). A role for Arabidopsis miR399f in salt, drought, and ABA signalling. *Molecules and Cells*, 39:111–118. doi: 10.14348/molcells.2016.2188

Baillo, E.H., Kimotho, R.N., Zhang, Z., & Xu, P. (2019). Transcription factors associated with abiotic and biotic stress tolerance and their potential for crops improvement. *Genes*, 10(10): 771. https://doi.org/10.3390/genes10100771

Baron, K.N., Schroeder, D.F., & Stasolla, C. (2012). Transcriptional response of abscisic acid (ABA) metabolism and transport to cold and heat stress applied at the reproductive stage of development in *Arabidopsis thaliana*. *Plant Science*, 188–189, 48–59. Doi.org/10.1016/j.plantsci.2012.03.001

Bartel, D.P. (2009). MicroRNAs: Target recognition and regulatory functions. *Cell,* 136(2), 215–233. Doi.org/10.1016/j.cell.2009.01.002

Battisti, David. S., & Naylor, R. L. (2009). Historical warnings of future food insecurity with unprecedented seasonal heat. *Science*, 323(5911), 240-244. Doi.org/10.1126/science.1164363

Baux, A., Colbach, N., Allirand, J.M., Jullien, A., Ney, B., & Pellet, D. (2013). Insights into temperature effects on the fatty acid composition of oilseed rape varieties. *European Journal of Agronomy*, 49, 12–19. Doi.org/10.1016/j.eja.2013.03.001

Bayer, P.E., Hurgobin, B., Golicz, A.A., Chan, C.K., Yuan, Y., Lee, H., Renton, M., Meng, J., Li, R., Long, Y., Zou, J., Bancroft, I., Chalhoub, B., King, G.J., Batley, J., Edwards, D. (2017). Assembly and comparison of two closely related *Brassica napus* genomes. *Plant Biotechnology Journal*. 15(12):1602-1610. doi: 10.1111/pbi.12742

Begum, Y. (2022). Regulatory role of microRNAs (miRNAs) in the recent development of abiotic stress tolerance of plants. *Gene*, 821, 146283.

Bell, L., Oruna-Concha, M.J. & Wagstaff, C. (2015). Identification and quantification of glucosinolate and flavonol compounds in rocket salad (*Eruca sativa*, *Eruca vesicaria* and *Diplotaxis tenuifolia)* by LC–MS: Highlighting the potential for improving nutritional value of rocket crops. *Food Chemistry*, 172, 852–861. https://doi.org/10.1016/j.foodchem.2014.09.116

Benešová, M., Holá, D., Fischer, L., Jedelský, P. L., Hnilička, F., Wilhelmová, N., Rothová, O., Kočová, M., Procházková, D., Honnerová, J., Fridrichová, L., & Hniličková, H. (2012). The physiology and proteomics of drought tolerance in Maize: Early stomatal closure as a cause of lower tolerance to short-term dehydration? *PLoS ONE*, 7(6), e3801. Doi.org/10.1371/journal.pone.0038017

Bheemanahalli, R., Sathishraj, R., Manoharan, M., Sumanth, H. N., Raveendran, M., Ishimaru, T., & Krishna, J.S.V. (2017). Is early morning flowering an effective trait to minimize heat stress damage during flowering in rice? *Field Crops Research*, 203, 238–242. Doi.org/10.1016/j.fcr.2016.11.011

Bheemanahalli, R., Sunoj, V.S.J., Saripalli, G., Prasad, P.V.V., Balyan, H.S., Gupta, P.K., Grant, N., Gill, K.S., & Jagadish, S.V.K. (2019). Quantifying the impact of heat stress on pollen germination, seed set, and grain filling in spring wheat. *Crop Science*, 59(2), 684–696. Doi.org/10.2135/cropsci2018.05.0292

Bhogireddy, S., Kudapa, H., Bajaj, P., Garg, V., Chitikineni, A., Nayak, S., & Varshney, R.K. (2023). Transcriptome analysis of chickpea during heat stress unveils the signatures of long intergenic non-coding RNAs (lincRNAs) and mRNAs in the heat-QTL region. *Crop Design*, 2(1), 100026. https://doi.org/10.1016/j.cropd.2023.100026

Bi, H., Li, F., Wang, H. & Ai, X. (2019). Overexpression of transketolase gene promotes chilling tolerance by increasing the activities of photosynthetic enzymes, alleviating oxidative damage and stabilizing cell structure in *Cucumis*

sativus L. *Physiologia Plantarum*, 167, 502–515. https://doi.org/10.1111/ppl.12903

Bi, H., Wang, M., Dong, X. & Ai, X. (2013). Cloning and expression analysis of transketolase gene in *Cucumis sativus* L. *Plant Physiology and Biochemistry*, 70, 512–521. https://doi.org/10.1016/j.plaphy.2013.06.017.

Blümel, M., Dally, N., & Jung, C. (2015). Flowering time regulation in crops-what did we learn from Arabidopsis? *Current Opinion in Biotechnology* 32, 121 129. Doi.org/10.1016/j.copbio.2014.11.023

Bohinc, T. & Trdan S. (2012). Environmental factors affecting the glucosinolate content in Brassicaceae. *Journal of Food, Agriculture & Environment,* 10(2), 357-360

Bones, A.M. & Rossiter, J.T. (2006). The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry,* 67, 1053-1067.

Brown, P.D., Tokuhisa, J.G., Reichelt, M., Gershenzon, J. (2003). Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry*, 62, 471–481

Brunel-Muguet, S., D'Hooghe, P., Bataillé, M. P., Larré, C., Kim, T.H., Trouverie, J., Avice, J. C., Etienne, P., & Dürr, C. (2015). Heat stress during seed filling interferes with sulfur restriction on grain composition and seed germination in oilseed rape (*Brassica napus* L.). *Frontiers in Plant Science*, 6:213. Doi.org/10.3389/fpls.2015.00213

Burke, J.J. (2007). Evaluation of source leaf responses to water-deficit stresses in cotton using a novel stress bioassay. *Plant Physiology,* 143, 108121. https://doi.org/10.1104/pp.106.087783.

Canales, J., Verdejo, J.F., Calderini, D.F. (2023). Transcriptome and Physiological Analysis of Rapeseed Tolerance to Post-Flowering Temperature Increase. *International Journal of Molecular Science*, 24(21):15593. doi: 10.3390/ijms242115593.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., Corréa, M., da Silva, C., Just, J., Falentin, C., Koh, C.S., le Clainche, I., Bernard, M., Bento, P., Noel, B. et al. (2014). Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science*, 345(6199), 950-3. Doi: 10.1126/science.1253435

Chandrasekaran, U., Xu, W., & Liu, A. (2014). Transcriptome profiling identifies ABA mediated regulatory changes towards storage filling in developing seeds of castor bean (*Ricinus communis* L.). *Cell and Bioscience*, 4(1), 33. Doi.org/10.1186/2045-3701-4-33

Chang, C.Y., Lin, W.D., & Tu, S.L. (2014). Genome-wide analysis of heat-sensitive alternative splicing in *Physcomitrella patens*. *Plant Physiology*, 165(2), 826-840. Doi.org/10.1104/pp.113.230540

Chang, J., Cheong, B. E., Natera, S., & Roessner, U. (2019). Morphological and metabolic responses to salt stress of rice (Oryza sativa L.) cultivars which differ in salinity tolerance. *Plant Physiology and Biochemistry*, 144,427–435. https://doi.org/10.1016/j.plaphy.2019.10.01

Chao, L. M., Liu, Y. Q., Chen, D. Y., Xue, X. Y., Mao, Y. B., & Chen, X. Y. (2017). Arabidopsis transcription factors SPL1 and SPL12 confer plant thermotolerance at reproductive stage. *Molecular Plant*, 10(5), 735–748. Doi.org/10.1016/j.molp.2017.03.010

Chauhan, H., Khurana, N., Agarwal, P., & Khurana, P. (2011). Heat shock factors in rice (*Oryza sativa* L.): Genome-wide expression analysis during reproductive development and abiotic stress. *Molecular Genetics and Genomics*, 286(2), 171–187. Doi.org/10.1007/s00438-011-0638-8

Chaves, M.M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. *Annals of Botany,* 103, (4), 551–560. Doi.org/10.1093/aob/mcn125

Chaves, M. M., Pereira, J.S., Maroco, J., Rodrigues, M.L., Ricardo, C.P.P., Osório, M.L., Carvalho, I., Faria, T., & Pinheiro, C. (2002). How plants cope with water stress in the field. Photosynthesis and growth. *Annals of Botany*, 89, 907–916. Doi.org/10.1093/aob/mcf105

Chekanova, J.A. (2015). Long non-coding RNAs and their functions in plants. *Current Opinion in Plant Biology,* 27, 207–216. Doi.org/10.1016/j.pbi.2015.08.003

Chen, H., Wang, Y., Liu, J., Zhao, T., Yang, C., Ding, Q., Zhang, Y., Mu, J., & Wang, D. (2021). Identification of WRKY transcription factors responding to abiotic stresses in *Brassica napus* L. *Planta*, 255(1). https://doi.org/10.1007/s00425-021-03733-x

Chinnusamy, V., Zhu, J., Zhu, JK. (2007). Cold stress regulation of gene expression in plants. *Trends in Plant Science*, 12(10):444–451. doi: 10.1016/j.tplants.2007.07.002

Chowdhary, A.A., Mishra, S., Mehrotra, S., Upadhyay, S.K., Bagal, D., & Srivastava, V. (2023). Plant transcription factors: An overview of their role in plant life. *Plant Transcription Factors*, 3–20. https://doi.org/10.1016/b978-0-323-90613-5.00003-0

Coleman, H. D., Ellis, D. D., Gilbert, M., & Mansfield, S. D. (2005). Up-regulation of sucrose synthase and UDP-glucose pyrophosphorylase impacts plant growth and metabolism. *Plant Biotechnology Journal*, 4(1), 87–101. Wiley. https://doi.org/10.1111/j.1467-7652.2005.00160.x

Cui, P., & Xiong, L. (2015). Environmental Stress and Pre-mRNA Splicing. *Molecular Plant* 8(9), 1302–1303. https://doi.org/10.1016/j.molp.2015.07.006

Danilova, M. N., Kudryakova, N. V., Andreeva, A. A., Doroshenko, A. S., Pojidaeva, E. S., & Kusnetsov, V. V. (2018). Differential impact of heat stress on

the expression of chloroplast-encoded genes. *Plant Physiology and Biochemistry*, 129, 90–100. https://doi.org/10.1016/j.plaphy.2018.05.023

Del Olmo, I., Poza-Viejo, L., Piñeiro, M., Jarillo, J. A., & Crevillén, P. (2019). High ambient temperature leads to reduced FT expression and delayed flowering in *Brassica rapa* via a mechanism associated with H2A.Z dynamics. *Plant Journal*, 100(2), 343 356. Doi.org/10.1111/tpj.14446

Dellero, Y., Berardocco, S., & Bouchereau, A. (2024). U-13C-glucose incorporation into source leaves of *Brassica napus* highlights light-dependent regulations of metabolic fluxes within central carbon metabolism. *Journal of Plant Physiology* 292, 154162. Elsevier BV. https://doi.org/10.1016/j.jplph.2023.154162

Deng, M., Wang, Y., Kuzma, M., Chalifoux, M., Tremblay, L., Yang, S., Ying, J., Sample, A., Wang, H. M., Griffiths, R., Uchacz, T., Tang, X., Tian, G., Joslin, K., Dennis, D., McCourt, P., Huang, Y., & Wan, J. (2020). Activation tagging identifies Arabidopsis transcription factor AtMYB68 for heat and drought tolerance at yield determining reproductive stages. *Plant Journal,* 104(6), 1535-1550. Doi.org/10.1111/tpj.15019

Deng, X., Qiu, Q., He, K., & Cao, X. (2018). The seekers: how epigenetic modifying enzymes find their hidden genomic targets in Arabidopsis. *Current Opinion in Plant Biology,* 45, 75–81. Doi.org/10.1016/j.pbi.2018.05.006

Dhar, M. K., Vishal, P., Sharma, R., & Kaul, S. (2014). Epigenetic dynamics: role of epimarks and underlying machinery in plants exposed to abiotic stress. *International Journal of Genomics*, 187146. Doi.org/10.1155/2014/187146

Di, F., Jian, H., Wang, T., Chen, X., Ding, Y., Du, H., Lu, K., Li, J., & Liu, L. (2018). Genome-wide analysis of the PYL gene family and identification of PYL genes that respond to abiotic stress in *Brassica napus*. *Genes*, 9(3), 156. Doi.org/10.3390/genes9030156

Dikšaitytė, A., Viršilė, A., Žaltauskaitė, J., Januškaitienė, I. & Juozapaitienė, G (2019). Growth and photosynthetic responses in *Brassica napus* differ during stress and recovery periods when exposed to combined heat, drought and elevated CO_2. *Plant Physiology and Biochemistry*, 142, 59–72. Https://doi.org/10.1016/j.plaphy.2019.06.026

Ding, Y., & Yang, S. (2022). Surviving and thriving: How plants perceive and respond to temperature stress. *Developmental Cell*, 57(8):947–958. https://doi.org/10.1016/j.devcel.2022.03.010

Djanaguiraman, M., Schapaugh, W., Fritschi, F., Nguyen, H., & Prasad, P. V. V. (2019). Reproductive success of soybean (*Glycine max* L. Merril) cultivars and exotic lines under high daytime temperature. *Plant Cell and Environment*, 42(1), 321–336. Doi.org/10.1111/pce.13421

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., et al. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in Arabidopsis. *Plant Cell* 19, 2225-2245. doi: 10.1105/tpc.106.048017

Draeger, T., & Moore, G. (2017). Short periods of high temperature during meiosis prevent normal meiotic progression and reduce grain number in hexaploid wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*, 130(9), 1785–1800. Doi.org/10.1007/s00122-017-2925-1

Du, M., Zhao, J., Tzeng, D.T.W., Liu, Y., Deng, L., Yang, T., Zhai, Q., Wu, F., Huang, Z., Zhou, M., Wang, Q., Chen, Q., Zhong, S., Li, CB., Li, C. (2017). MYC2 orchestrates a hierarchical transcriptional cascade that regulates jasmonate-mediated plant immunity in tomato. *Plant Cell* 29, 1883-1906. doi: 10.1105/tpc.16.00953

Duan, H., Wu, J., Huang, G., Zhou, Sh, Liu, W., Liao, Y., Yang, X., Xiao, Z. & Fan, H. (2017). Individual and interactive effects of drought and heat on leaf physiology of seedlings in an economically important crop. *Annal of Botany Plants,* 9(1), plw090. Https://doi.org/10.1093/aobpla/plw090

Echer, F. R., Oosterhuis, D. M., Loka, D. A., & Rosolem, C. A. (2014). High night temperatures during the floral bud stage increase the abscission of reproductive structures in cotton. *Journal of Agronomy and Crop Science*, 200(3), 191–198. Doi.org/10.1111/jac.12056

Elferjani, R., & 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. Doi.org/10.3389/fpls.2018.01224

Erickson, A. N., & Markhart, A. H. (2002). Flower developmental stage and organ sensitivity of bell pepper (*Capsicum annuum* L.) to elevated temperature. *Plant, Cell and Environment*, 25(1), 123–130. Doi.org/10.1046/j.0016-8025.2001.00807.x

Esposito, S. (2016). Nitrogen assimilation, abiotic stress and glucose 6-phosphate dehydrogenase: The full circle of reductants. *Plants (Basel, Switzerland)*, 5(2), 24. https://doi.org/10.3390/plants5020024.

Estravis-Barcala, M., Heer, K., Marchelli, P., Ziegenhagen, B., Arana, MV. & Bellora,N. (2021). Deciphering the transcriptomic regulation of heat stress responses in *Nothofagus pumilio*. *PloS ONE,* 16(3), e0246615. https://doi.org/10.1371/journal.pone.0246615

Fahey, J.W., Zalcmann, A.T., & Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry,* 56(1), 5–51. Doi.org/10.1016/s0031-9422(00)00316-2

Fan, S., Liu, H., Liu, J., Hua, W., Xu, S., & Li, J. (2020). Systematic analysis of the DNA methylase and demethylase gene families in rapeseed (*Brassica napus* l.) and their expression variations after salt and heat stresses. *International Journal of Molecular Sciences*, 21(3), 253. Doi.org/10.3390/ijms21030953

Farkas, I., Dombrádi, V., Miskei, M., Szabados, L., Koncz, C. (2007). Arabidopsis PPP family of serine/threonine phosphatases. *Trends in Plant Science*. 12(4):169-76. doi: 10.1016/j.tplants.2007.03.003

Feng, J., Long, Y., Shi, L., Shi, J., Barker, G., & Meng, J. (2012). Characterization of metabolite quantitative trait loci and metabolic networks that control glucosinolate concentration in the seeds and leaves of *Brassica napus*. *New Phytologist*, 193(1), 96–108. Doi.org/10.1111/j.1469-8137.2011.03890.x

Feng, S. J., Zhang, X. D., Liu, X. S., Tan, S. K., Chu, S. S., Meng, J. G., Zhao, K. X., Zheng, J. F., & Yang, Z. M. (2016). Characterization of long non-coding RNAs involved in cadmium toxic response in: *Brassica napus*. *RSC Advances*, 6(85), 82157–82173. Doi.org/10.1039/c6ra05459e

Fernandez, O., Urrutia, M., Bernillon, S., Giauffret, C., Tardieu, F., Le Gouis, J., Langlade, N., Charcosset, A., Moing, A., & Gibon, Y. (2016). Fortune telling: metabolic markers of plant performance. *Metabolomics*, 12(10). https://doi.org/10.1007/s11306-016-1099-1

Fieldsend, J., & Milford, G. F. J. (1994). Changes in glucosinolates during crop development in single- and double-low genotypes of winter oilseed rape (*Brassica napus*): Production and distribution in vegetative tissues and developing pods during development and potential role in the recycling of sulphur within the crop. *Annals of Applied Biology*, 124(3), 531-542. Doi.org/10.1111/j.1744-7348.1994.tb04157.x

Finkelstein, R.R. & Gibson, S.I. (2002). ABA and sugar interactions regulating development: Cross-talk or voices in a crowd? *Current Opinion in Plant Biology*, 5, 26–32

Frenck, G., van der Linden, L., Mikkelsen, T. N., Brix, H., & Jørgensen, R. B. (2011). Increased [CO_2] does not compensate for negative effects on yield caused by higher temperature and [O_3] in *Brassica napus* L. *European Journal of Agronomy*, 35(3), 127–134. Doi.org/10.1016/j.eja.2011.05.004

Füßl, M., Lassowskat, I., Née, G., Koskela, M. M., Brünje, A., Tilak, P., Giese, J., Leister, D., Mulo, P., Schwarzer, D., & Finkemeier, I. (2018). Beyond histones: New substrate proteins of lysine deacetylases in Arabidopsis nuclei. *Frontiers in Plant Science*, 9, 461. Doi.org/10.3389/fpls.2018.00461

Gabur, I., Simioniuc, D. P., Snowdon, R. J., & Cristea, D. (2022). Machine learning applied to the search for nonlinear features in breeding populations. *Frontiers in Artificial Intelligence,* 5:876578. https://doi.org/10.3389/frai.2022.876578

Gao, G., Li, J., Li, H., Li, F., Xu, K., Yan, G., Chen, B., Qiao, J., & Wu, X. (2014). Comparison of the heat stress induced variations in DNA methylation between heat-tolerant and heat-sensitive rapeseed seedlings. *Breeding Science*, 64(2), 125 133. Doi.org/10.1270/jsbbs.64.125

Gao, Y., Li, T., Liu, Y., Ren, C., Zhao, Y., & Wang, M. (2010). Isolation and characterization of gene encoding G protein α subunit protein responsive to plant hormones and abiotic stresses in *Brassica napus*. *Molecular Biology Reports*, *37(*8), 3957–3965. Doi.org/10.1007/s11033-010-0054-x

Gawrysiak-Witulska, M., Rudzińska, M., Wawrzyniak, J., & Siger, A. (2012). The effect of temperature and moisture content of stored rapeseed on the phytosterol degradation rate. *Journal of the American Oil Chemists' Society*. 89(9), 1673–1679. Doi.org/10.1007/s11746-012-2064-4

Gawrysiak-Witulska, M., Siger, A., Wawrzyniak, J., & Nogala-Kalucka, M. (2011). Changes in tocochromanol content in seeds of *Brassica napus* L. during adverse conditions of storage. *Journal of the American Oil Chemists' Society*. 88(9), 1379–1385. Doi.org/10.1007/s11746-011-1793-0

George, R., Gullström, M., Mangora, M. M., Mtolera, M. S. P. & Björk, M. (2018). High midday temperature stress has stronger effects on biomass than on photosynthesis: A mesocosm experiment on four tropical seagrass species. *Ecology and Evolution,* 8, 4508–4517. doi: 10.1002/ece3.3952

Ghorbani, R., Zakipour, Z., Alemzadeh, A., & Razi, H. (2020). Genome-wide analysis of AP2/ERF transcription factors family in *Brassica napus*. *Physiology and Molecular Biology of Plants*, 26(7), 1463–1476. Doi.org/10.1007/s12298-020-00832-z

Giorno, F., Wolters-Arts, M., Grillo, S., Scharf, K.-D., Vriezen, W. H., & Mariani, C. (2009). Developmental and heat stress-regulated expression of HsfA2 and small heat shock proteins in tomato anthers. *Journal of Experimental Botany* 61(2):453–462. https://doi.org/10.1093/jxb/erp316

Giorno, F., Wolters-Arts, M., Mariani, C., & Rieu, I. (2013). Ensuring reproduction at high temperatures: The heat stress response during anther and pollen development. *Plants,* 2(3), 489–506. Doi.org/10.3390/plants2030489

Glaubitz, U,. Li, X., Schaedel, S., Erban, A., Sulpice, R., Kopka, J., Hincha, DK. & Zuther, E. (2017). Integrated analysis of rice transcriptomic and metabolomic responses to elevated night temperatures identify sensitivity- and tolerance-related profiles. *Plant Cell and Environment,* 40, 121–37. Https://doi. Org/10.1111/pce.12850

Goel, K., Kundu, P., Sharma, P. *et al.* (2023). Thermosensitivity of pollen: a molecular perspective. *Plant Cell Reports,* 42, 843–857

Gong, H., Chen, G., Li, F., Wang, X., Hu, Y. & Bi, Y. (2012). Involvement of G6PDH in heat stress tolerance in the calli from *Przewalskia tangutica* and *Nicotiana tabacum. Biologia plantarum*, 56, 422–430. https://doi.org/10.1007/s10535-012-0072-8

Granot, D., David-Schwartz, R., and Kelly, G. (2013). Hexose kinases and their role in sugar sensing and plant development. *Frontiers in Plant Science* 4:44

Guan, Q., Yue, X., Zeng, H., and Zhu, J. (2014). The protein phosphatase RCF2 and its interacting partner NAC019 are critical for heat stress-responsive gene regulation and thermotolerance in Arabidopsis. *Plant Cell* 26, 438–453. doi: 10.1105/tpc.113.118927

Guo, M., Liu, J.H., Ma, X., Luo, D.X., Gong, Z.H., Lu, M.H. (2016). The plant heat stress transcription factors (HSFs): Structure, regulation, and function in

response to abiotic stresses. *Frontiers in Plant Science*. 7(114). Doi: 10.3389/fpls.2016.00114

Guo, Y., Gan, S. (2011). AtMYB2 regulates whole plant senescence by inhibiting cytokinin-mediated branching at late stages of development in Arabidopsis. *Plant Physiology*, 156:1612–1619. doi: 10.1104/pp.111.177022

Guo, W., Yu, K., Han, L., Li, X., Wang, H., Liu, Y., & Zhang, Y. (2020). Global profiling of alternative splicing landscape responsive to salt stress in wheat (*Triticum aestivum* L.). *Plant Growth Regulation*, 92(1), 107–116. Doi.org/10.1007/s10725-020-00623-2

Hajiebrahimi, A., Owji, H., Hemmati, S. (2017). Genome-wide identification, functional prediction and evolutionary analysis of R2R3-MYB superfamily in *Brassica napus*. *Genome*. 60:797-814

Hanson, A.D., Beaudoin, G.A., McCarty, D.R., Gregory, J.F. (2016). Does abiotic stress cause functional B vitamin deficiency in plants? *Plant Physiology*. 172(4):2082-2097. doi: 10.1104/pp.16.01371

Hao, Y., Zong, X., Ren, P., Qian, Y., Fu, A. (2021). Basic Helix-Loop-Helix (bHLH) transcription factors regulate a wide range of functions in Arabidopsis. *International Journal of Molecular Sciences*, 22(13), 7152

Hara, M., Harazaki, A., & Tabata, K. (2012). Administration of isothiocyanates enhances heat tolerance in *Arabidopsis thaliana*. *Plant Growth Regulation*, 69(1), 71–77. Doi.org/10.1007/s10725-012-9748-5

Harsh, A., Sharma, Y.K., Joshi, U., Rampuria, S., Singh, G., Kumar, S. & Sharma, R. (2016). Effect of short-term heat stress on total sugars, proline and some antioxidant enzymes in moth bean (*Vigna aconitifolia*), *Annals of Agricultural Sciences*, 61(1), 57-64

Hasanuzzaman, M., Nahar, K., Alam, M. M. & Fujita, M. (2014). Modulation of antioxidant machinery and the methylglyoxal detoxification system in selenium-supplemented *Brassica napus* seedlings confers tolerance to high temperature stress. *Biological Trace Element Research*, 161(3), 297–307. Https://doi.org/10.1007/s12011-014-0120-7

He, Y., Mao, S., Gao, Y., Zhu, L., Wu, D., Cui, Y., Li, J., & Qian, W. (2016). Genome-wide identification and expression analysis of WRKY transcription factors under multiple stresses in *Brassica napus*. *PLoS ONE*, 11(6), e0157558. Doi.org/10.1371/journal.pone.0157558

Hedhly, A. (2011). Sensitivity of flowering plant gametophytes to temperature fluctuations. *Environmental and Experimental Botany,* 74(1), 9–16. Doi.org/10.1016/j.envexpbot.2011.03.016

Hegedus, D., Yu, M., Baldwin, D., Margaret, G., Sharpe, A., Parkin, I., Whitwill, S., Lydiate, D. (2003). Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress. *Plant Molecular Biology* 53, 383–39

Heinrich, M., Hettenhausen, C., Lange, T., Wünsche, H., Fang, J., Baldwin, I. T., & Wu, J. (2012). High levels of Jasmonic acid antagonize the biosynthesis of gibberellins and inhibit the growth of *Nicotiana attenuatastems*. *The Plant Journal* 73(4), 591–606. Doi.org/10.1111/tpj.12058

Hinojosa, L., Matanguihan, J.B. & Murphy, K.M. (2019). Effect of high temperature on pollen morphology, plant growth and seed yield in quinoa (*Chenopodium quinoa* Willd.). *Journal of Agronomy and Crop Science,* 205, 33–45

Hong, B., Ma, C., Yang, Y., Wang, T., Yamaguchi-Shinozaki, K., & Gao, J. (2009). Over-expression of AtDREB1A in Chrysanthemum enhances tolerance to heat stress. *Plant Molecular Biology*, 70(3), 231–240. Doi.org/10.1007/s11103-009-9468-z

Hoogland, C., Mostaguir, K., Appel, R.D., & Lisacek, F. (2008). The World-2DPAGE Constellation to promote and publish gel-based proteomics data through the ExPASy server. *Journal of Proteomics*, 71(2), 245–248. Doi.org/10.1016/j.jprot.2008.02.005

Hopkins, RJ., Van Dam, N.M. & Van Loon, J.J. (2009). Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annual Review of Entomology,* 54, 57–83

Hossain, Z., Nouri, M. Z., & Komatsu, S. (2012). Plant cell organelle proteomics in response to abiotic stress. *Journal of Proteome Research,* 11(1), 37–48. Doi.org/10.1021/pr200863r

Hrmova, M., Hussain, S.S. Plant transcription factors involved in drought and associated stresses (2021). *International Journal of Molecular Science*. 22(11):5662. doi: 10.3390/ijms22115662

Huang, L., Dong, H., Zhou, D., Li, M., Liu, Y., Zhang, F., Feng, Y., Yu, D., Lin, S., & Cao, J. (2018). Systematic identification of long non-coding RNAs during pollen development and fertilization in *Brassica rapa*. *Plant Journal*, 96(1), 203–222. Doi.org/10.1111/tpj.14016

Huang, Q., Wang, Y., Li, B., Chang, J., Chen, M., Li, K., Yang, G., & He, G. (2015). TaNAC29, a NAC transcription factor from wheat, enhances salt and drought tolerance in transgenic Arabidopsis. *BMC Plant Biology*,15(1) https://doi.org/10.1186/s12870-015-0644-9

Huang, R., Liu, Z., Xing, M., Yang, Y., Wu, X. & Liu, H., et al. (2019). Heat stress suppresses *Brassica napus* seed oil accumulation by inhibition of photosynthesis and BnWRI1 pathway. *Plant Cell Physiology,* 60, 1457–1470. doi: 10.1093/pcp/pcz052

Huang, S. & Millar, A. H. (2013). Succinate dehydrogenase: The complex roles of a simple enzyme. *Current Opinion in Plant Biology*, 16(3), 344–349. https://doi.org/10.1016/j.pbi.2013.02.007

Huang, S.Q., Xiang, A.L., Che, L.L., Chen, S., Li, H., Song, J.B., & Yang, Z.M. (2010). A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress. *Plant Biotechnology Journal*, 8(8), 887–899. Doi.org/10.1111/j.1467-7652.2010.00517.x

Huang, W., Ma, H.Y., Huang, Y., Li, Y., Wang, G.L., Jiang, Q., Wang, F. & Xiong, A.S. (2017). Comparative proteomic analysis provides novel insights into chlorophyll biosynthesis in celery under temperature stress. *Physiologia Plantarum,* 161, 468–485

Huang, Y.C, Niu, C.Y, Yang, C.R., Jinn, T.L. (2016). The heat stress factor HSFA6b connects ABA signalling and ABA-mediated heat responses. *Plant Physiology*. 172(2):1182-1199. doi: 10.1104/pp.16.00860

Hummel, I., Pantin, F., Sulpice, R., Piques, M., Rolland, G. & Dauzat, M., et al. (2010). Arabidopsis plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. *Plant Physiology,* 154, 357–372 10.1104/pp.110.157008

Hussain, Q., Asim, M., Zhang, R., Khan, R., Farooq, S., & Wu, J. (2021). Transcription factors interact with ABA through gene expression and signalling pathways to mitigate drought and salinity stress. *Biomolecules*. 11, 8, 1159. https://doi.org/10.3390/biom11081159

Ibrahim, S., Mira, M.M, Hill, R.D, Stasolla, C. (2024). The *Brassica napus* phytoglobin 1 (BnPgb1) mitigates the decrease in plant fertility resulting from high temperature stress. *Journal of Plant Physiology*, 300:154302. doi: 10.1016/j.jplph.2024.154302

Ikram, M., Chen, J., Xia, Y., Li, R., Siddique, K. H. M. & Guo, P. (2022). Comprehensive transcriptome analysis reveals heat-responsive genes in flowering Chinese cabbage (*Brassica campestris* L. ssp. chinensis) using RNA sequencing. *Frontiers in Plant Science,* 13 https://doi.org/10.3389/fpls.2022.1077920

Impa, S. M., Sunoj, V. S. J., Krassovskaya, I., Bheemanahalli, R., Obata, T. & Jagadish, S. V. K. (2019). Carbon balance and source-sink metabolic changes in winter wheat exposed to high night-time temperature. *Plant Cell and Environment,* 42, 1233–1246. doi: 10.1111/pce.13488

Ismaili, A., Salavati, A., & Mohammadi, P. P. (2015). A comparative proteomic analysis of responses to high-temperature stress in hypocotyl of Canola (*Brassica napus* L.). *Protein & Peptide Letters,* 2(3), 285-99. Doi: 10.2174/0929866521666141124102755

Jagadish, S.K., Way, D.A. & Sharkey, T.D. (2021). Plant heat stress: Concepts directing future research. *Plant Cell and Environment,* 44, 1992–2005.

Jasper, J. Wagstaff, C. & Bell, L. (2020). Growth temperature influences postharvest glucosinolate concentrations and hydrolysis product formation in first

and second cuts of rocket salad. *Postharvest Biology and Technology,* 163, 111157. doi:10.1016/j.postharvbio.2020.111157

Jedličková, V., Hejret, V., Demko, M., Jedlička, P., Štefková, M., Robert, H.S. (2023). Transcriptome analysis of thermomorphogenesis in ovules and during early seed development in *Brassica napus*. *BMC Genomics,* 24(1):236. doi: 10.1186/s12864-023-09316-2. PMID: 37142980

Jha, U. C., Nayyar, H., Jha, R., Khurshid, M., Zhou, M., Mantri, N., & Siddique, K. H. M. (2020). Long non-coding RNAs: Emerging players regulating plant abiotic stress response and adaptation. *BMC Plant Biology,* 20:466. Doi.org/10.1186/s12870-020-02595-x

Jhingan, S., Harloff, HJ., Abbadi, A., Welsch, C., Blümel, M., Tasdemir, D., Jung, C. (2023). Reduced glucosinolate content in oilseed rape (*Brassica napus* L.) by random mutagenesis of *BnMYB28* and *BnCYP79F1* genes. *Sci Rep* 13, 2344. https://doi.org/10.1038/s41598-023-28661-6

Jian, H., Wang, J., Wang, T., Wei, L., Li, J., & Liu, L. (2016). Identification of rapeseed MicroRNAs involved in early-stage seed germination under salt and drought stresses. *Frontiers in Plant Science*, 7, 658. Doi.org/10.3389/fpls.2016.00658

Jin, B., Li, W., Jing, W., Jiang, K.Z., Yang, W., Jiang, X.X., Ni, C.Y., Wang, Y.L. & Teng, N.J. (2011). The effect of experimental warming on leaf functional traits, leaf structure and leaf biochemistry in *Arabidopsis thaliana*. *BMC Plant Biology,* 11, 35. Https://doi.org/10.1186/1471-2229-11-35

Jin, Q., Chachar, M., Ali, A., Chachar, Z., Zhang, P., Riaz, A., Ahmed, N., & Chachar, S. (2024). Epigenetic regulation for heat stress adaptation in plants: New horizons for crop improvement under climate hange. *Agronomy*, 14(9), 2105. https://doi.org/10.3390/agronomy14092105

Joshi, R. K., Megha, S., Basu, U., Rahman, M. H., & Kav, N. N. V. (2016). Genome wide identification and functional prediction of long non-coding RNAs responsive to *Sclerotinia sclerotiorum* infection in *Brassica napus*. *PLoS ONE*, 11(7), e0158784. Doi.org/10.1371/journal.pone.0158784

Justen, V. L., & Fritz, V. A. (2013). Temperature-induced glucosinolate accumulation is associated with expression of BrMYB transcription factors. *HortScience*, 48(1), 47-52. Doi: 10.21273/HORTSCI.48.1.47

Kagale, S., Divi, U. K., Krochko, J. E., Keller, W. A., & Krishna, P. (2007). Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta*, 225(2), 353–364 Doi.org/10.1007/s00425-006-0361-6

Kaler, A.S., Purcell, L.C., Beissinger, T. *et al.* (2022). Genomic prediction models for traits differing in heritability for soybean, rice, and maize. *BMC Plant Biol* 22, 87. https://doi.org/10.1186/s12870-022-03479-y

Kanehisa, M. & Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research,* 28, 27–30 https://doi.org/10.1093/nar/28.1.27

Kannan, S., Halter, G., Renner, T., & Waters, E. R. (2018). Patterns of alternative splicing vary between species during heat stress. *AoB Plants*, 10(2), ply013. Doi.org/10.1093/aobpla/ply013

Kashyap, A., Garg, P., Tanwar, K., Sharma, J., Gupta, N.C., Ha, P.T.T., Bhattacharya, R.C., Mason, A.S., Rao, M. (2022). Strategies for utilization of crop wild relatives in plant breeding programs. *Züchter Genetics and. Breeding. Research,* 135:4151–4167. doi: 10.1007/s00122-022-04220-x.

Kaushal, N., Awasthi, R., Gupta, K., Gaur, P., Siddique, K.H.M. & Nayyar, H. (2013). Heat-stress-induced reproductive failures in chickpea (*Cicer arietinum*) are associated with impaired sucrose metabolism in leaves and anthers. *Functional Plant Biology,* 40:1334–1349. doi: 10.1071/FP13082.

Kazan, K., & Lyons, R. (2016). The link between flowering time and stress tolerance. *Journal of Experimental Botany*, 67(1), 47–60. Doi.org/10.1093/jxb/erv441

Kazan, K., Manners, J. M. (2013). MYC2: The master in action. *Molecular Plant,* 6, 686-703. Doi: 10.1093/mp/sss128

Ke, L., Lei, W., Yang, W., Wang, J., Gao, J., Cheng, J., Sun, Y., Fan, Z., & Yu, D. (2020). Genome-wide identification of cold responsive transcription factors in *Brassica napus* L. *BMC Plant Biology*, 20(1). https://doi.org/10.1186/s12870-020-2253-5

Khan, F.S., Goher, F., Paulsmeyer, M.N., Hu, C.G., & Zhang, J.Z. (2023). Calcium (Ca^{2+}) sensors and MYC2 are crucial players during jasmonates-mediated abiotic stress tolerance in plants. *Plant Biology* 25(7), 1025-1034. doi: 10.1111/plb.13560

Kim, S.Y., Hong, C.B., & Lee, Ilha. (2001). Heat shock stress causes stage-specific male sterility in *Arabidopsis thaliana*. *Journal of Plant Research*, 114, 301–307. Doi: 10.1007/PL00013991

Klungland, A., & Robertson, A. B. (2017). Oxidized C5-methyl cytosine bases in DNA: 5-hydroxymethylcytosine; 5-formylcytosine; and 5-carboxycytosine. *Free Radical Biology and Medicine,* 107, 62–68. Doi.org/10.1016/j.freeradbiomed.2016.11.038

Ko, J. H., Mitina, I., Tamada, Y., Hyun, Y., Choi, Y., Amasino, R. M., Noh, B., & Noh, Y.S. (2010). Growth habit determination by the balance of histone methylation activities in Arabidopsis. *EMBO Journal*, 29(18), 3208–3215. Doi.org/10.1038/emboj.2010.198

Koscielny, C.B., Hazebroek, J. & Duncan, R.W. (2018). Phenotypic and metabolic variation among spring *Brassica napus* genotypes during heat stress. *Crop Pasture Science,* 69, 284–295. doi: 10.1071/CP17259

Kosová, K., Prášil, I. T., & Vítámvás, P. (2013). Protein contribution to plant salinity response and tolerance acquisition. *International Journal of Molecular Sciences,* 14(4), 6757–6789. Doi.org/10.3390/ijms14046757

Kosová, K., Vítámvás, P., Urban, M. O., Klíma, M., Roy, A., & Tom Prášil, I. (2015). Biological networks underlying abiotic stress tolerance in temperate crops-a proteomic perspective. *International Journal of Molecular Sciences,* 16(9), 20913–20942. Doi.org/10.3390/ijms160920913

Kourani, M., Mohareb, F., Rezwan, F. I., Anastasiadi, M. & Hammond, J. P. (2022). Genetic and physiological responses to heat stress in *Brassica napus*. *Frontiers in Plant Science,* 13. https://doi.org/10.3389/fpls.2022.832147.

Krasensky, J., & Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany,* 63(4), 1593–1608. Doi.org/10.1093/jxb/err460

Krasucki, W., Tys, J., Szafran, K., Rybacki, R., Orlicki, Ł. (2002). Influence of drying temperature on chemical composition of seeds of oilseed rape. *Oilseed Crop*, 23, 427–438

Kumar, M., Prusty, M. R., Pandey, M. K., Singh, P. K., Bohra, A., Guo, B., & Varshney, R. K. (2023). Application of CRISPR/Cas9-mediated gene editing for abiotic stress management in crop plants. *Frontiers in Plant Science* (14). https://doi.org/10.3389/fpls.2023.1157678

Kutmon, M., Ehrhart, F., Willighagen, E.L., Evelo, C.T., Coort, S.L. (2018). CyTargetLinker app update: A flexible solution for network extension in Cytoscape. F1000Res. 7, 743. doi: 10.12688/f1000research.14613.2

Kwon, C.S., Lee, D., Choi, G., & Chung, W.II. (2009). Histone occupancy-dependent and independent removal of H3K27 trimethylation at cold-responsive genes in Arabidopsis. *The Plant Journal*. 60(1), 112–121 Doi.org/10.1111/j.1365-313x.2009.03938.x

Lafos, M., Kroll, P., Hohenstatt, M.L., Thorpe, F.L., Clarenz, O., Schubert, D. (2011). Dynamic regulation of H3K27 trimethylation during *Arabidopsis* differentiation. *PLoS Genetics* 7(4): e1002040. Doi:10.1371/journal.pgen.1002040

Laird, P. (2010). Principles and challenges of genome-wide DNA methylation analysis. *Nature Reviews Genetics,* 11(3), 191–203. Doi.org/10.1038/nrg2732

Lam, K.C, Ibrahim, R.K, Behdad, B., Dayanandan, S. (2007) Structure, function, and evolution of plant O-methyltransferases. *Genome*. 50(11):1001-13. doi: 10.1139/g07-077

Lamaoui, M., Jemo, M., Datla, R. & Bekkaoui, F. (2018). Heat and drought stresses in crops and approaches for their mitigation. *Frontiers in Chemistry*, *6*. Https://doi.org/10.3389/fchem.2018.00026.

Lee, D. G., Ahsan, N., Lee, S. H., Kyu, Y. K., Jeong, D. B., Lee, I. J., & Lee, B. H. (2007). A proteomic approach in analyzing heat-responsive proteins in rice leaves. *Proteomics*, 7(18), 3369–3383. Doi.org/10.1002/pmic.200700266

Lee, J. S., & Adams, K. L. (2020). Global insights into duplicated gene expression and alternative splicing in polyploid *Brassica napus* under heat, cold, and drought stress. *Plant Genome*, 13(3), e20057. Doi.org/10.1002/tpg2.20057

Lemoine, R., La Camera, S., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., Bonnemain, JL., Laloi, M., Coutos-Thévenot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J. & Durand, M. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science,* 24, 4-272. doi: 10.3389/fpls.2013.00272

Lesk, C., Rowhani, P., & Ramankutty, N. (2016). Influence of extreme weather disasters on global crop production. *Nature*, 529(7584), 84–87. Doi.org/10.1038/nature16467

Li, W., Wei, Z., Qiao, Z., Wu, Z., Cheng, L., & Wang, Y. (2013). Proteomics analysis of alfalfa response to heat stress. *PLoS ONE*, 8(12), e82725. Doi.org/10.1371/journal.pone.0082725

Li, J., Huang, Q., Sun, M., Zhang, T., Li, H., Chen, B., Xu, K., Gao, G., Li, F., Yan, G., Qiao, J., Cai, Y., & Wu, X. (2016). Global DNA methylation variations after short-term heat shock treatment in cultured microspores of *Brassica napus* cv. Topas. *Scientific Reports*, 6, 38401. Doi.org/10.1038/srep38401

Li, H., Tiwari, M., Tang, Y., Wang, L., Yang, S., Long, H., Guo, J., Wang, Y., Wang, H., Yang, Q., Jagadish, S. V. K., & Shao, R. (2022). Metabolomic and transcriptomic analyses reveal that sucrose synthase regulates maize pollen viability under heat and drought stress. *Ecotoxicology and Environmental Safety,* 246, 114191. https://doi.org/10.1016/j.ecoenv.2022.114191

Li, M., Li, J., Zhang, R., Lin, Y., Xiong, A., Tan, G., Luo, Y., Zhang, Y., Chen, Q. & Wang, Y. et al. (2022). Combined analysis of the metabolome and transcriptome to explore heat stress responses and adaptation mechanisms in celery (*Apium graveolens* L.). *International Journal of Molecular Science,* 23, 3367. https://doi.org/10.3390/ijms23063367

Li, B., Cai, H., Liu, K. *et al.* (2023). DNA methylation alterations and their association with high temperature tolerance in rice anthesis. *Journal of Plant Growth Regulation* 42, 780–794. https://doi.org/10.1007/s00344-022-10586-5

Liang, Z., Li, M., Liu, Z., & Wang, J. (2019). Genome-wide identification and characterization of the Hsp70 gene family in allopolyploid rapeseed (*Brassica napus* L.) compared with its diploid progenitors. *PeerJ,* 7, e7511. Doi.org/10.7717/peerj.7511

Liepman, A.H., Vijayalakshmi, J., Peisach, D., Hulsebus, B., Olsen, L. J., & Saper, M. A. (2019). Crystal structure of photorespiratory alanine:glyoxylate 1 (AGT1) From *Arabidopsis thaliana*. *Frontiers in Plant Science.*10. https://doi.org/10.3389/fpls.2019.01229

Lin, J.S., Kuo, C.C., Yang, I.C., Tsai, W.A., Shen, Y.H., Lin, C.C., Liang, Y.C., Li, Y.C., Kuo, Y.W., King, Y.C., Lai, H.M., & Jeng, S.T. (2018). MicroRNA160 modulates plant development and heat shock protein gene expression to mediate heat tolerance in Arabidopsis. *Frontiers in Plant Science*, 9, 68. Doi.org/10.3389/fpls.2018.00068

Ling, Y., Serrano, N., Gao, G., Atia, M., Mokhtar, M., Woo, Y. H., Bazin, J., Veluchamy, A., Benhamed, M., Crespi, M., Gehring, C., Reddy, A. S. N., & Mahfouz, M. M. (2018). Thermopriming triggers splicing memory in Arabidopsis. *Journal of Experimental Botany*, 69(10), 2659–2675. Doi.org/10.1093/jxb/ery062

Liu, C., Lu, F., Cui, X., Cao, X. (2010). Histone methylation in higher plants. *Annual Review of Plant Biology,* 61: 395–420. Doi.org/10.1146/annurev.arplant.043008.091939

Liu, Zhi-Bin, Wang, J. M., Yang, F. X., Yang, L., Yue, Y. F., Xiang, J. B., Gao, M., Xiong, F. J., Lv, D., Wu, X. J., Liu, N., Zhang, X., Li, X. F., & Yang, Y. (2014). A novel membrane-bound E3 ubiquitin ligase enhances the thermal resistance in plants. *Plant Biotechnology Journal*, 12(1), 93–104. Doi.org/10.1111/pbi.12120

Liu, T., Li, Y., Duan, W., Huang, F., & Hou, X. (2017). Cold acclimation alters DNA methylation patterns and confers tolerance to heat and increases growth rate in *Brassica rapa*. *Journal of Experimental Botany*, 68(5), 1213–1224. Doi.org/10.1093/jxb/erw496

Liu, G., Xia, Y., Liu, T., Dai, S., & Hou, X. (2018a). The DNA methylome and association of differentially methylated regions with differential gene expression during heat stress in *Brassica rapa*. *International Journal of Molecular Sciences*, 19(5), 1414. Doi.org/10.3390/ijms19051414

Liu, Z., Qin, J., Tian, X., Xu, S., Wang, Y., Li, H., Wang, X., Peng, H., Yao, Y., Hu, Z., Ni, Z., Xin, M., & Sun, Q. (2018b). Global profiling of alternative splicing landscape responsive to drought, heat and their combination in wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal*, 16(3), 714–726. Doi.org/10.1111/pbi.12822

Liu, C., Cheng, J., Zhuang, Y., Ye, L., Li, Z., Wang, Y., Qi, M., Xu, L., & Zhang, Y. (2019a). Polycomb repressive complex 2 attenuates ABA-induced senescence in Arabidopsis. *Plant Journal*, 97(2), 368–377. Doi.org/10.1111/tpj.14125

Liu, G., Khan, N., Ma, X., & Hou, X. (2019b). Identification, evolution, and expression profiling of histone lysine methylation moderators in *Brassica rapa*. *Plants*, 8(12), 526. Doi.org/10.3390/plants8120526

Ljubej, V., Radojčić, Redovniković I., Salopek-Sondi, B., Smolko, A., Roje, S. & Šamec, D. (2021). Chilling and freezing temperature stress differently influence glucosinolates content in *Brassica oleracea* var. *acephala*. *Plants*, 27, 10(7), 1305. doi: 10.3390/plants10071305.

Lohani, N., Singh, M.B., 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. doi: 10.3389/fpls.2022.905674

Lohani, N., Jain, D., Singh, M.B., & Bhalla, P.L. (2020a). Engineering multiple abiotic stress tolerance in Canola, *Brassica napus*. *Frontiers in Plant Science,* 11, 3. Doi.org/10.3389/fpls.2020.00003

Lohani, N., Singh, M.B., & Bhalla, P.L. (2020b). High temperature susceptibility of sexual reproduction in crop plants. *Journal of Experimental Botany,* 71(2), 555–568. Doi.org/10.1093/jxb/erz426

Long, X., He, B., Fang, Y. & Tang, C. (2016). Identification and characterization of the glucose-6-phosphate dehydrogenase gene family in the para rubber tree, *Hevea brasiliensis. Frontiers in Plant Science,* 7. https://doi.org/10.3389/fpls.2016.00215

Lorenzo, O., Chico, J. M., Sánchez-Serrano, J. J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell*, 16, 1938–1950. doi: 10.1105/tpc.022319

Love, MI., Huber, W. & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15, 550. doi:10.1186/s13059-014-0550-8

Ludwig-Müller, J., & Krishna, P. (2000). A glucosinolate mutant of *Arabidopsis* is thermosensitive and defective in cytosolic Hsp90 expression after heat stress. *Plant Physiology*, 123, 949–958. Doi: 10.1104/pp.123.3.949

Luo, L., Wang, Y., Qiu, L., Han, X., Zhu, Y., Liu, L., Man, M., Li, F., Ren, M., & Xing, Y. (2023). MYC2: A master switch for plant physiological processes and specialized metabolite synthesis. *International Journal of Molecular Sciences* 24(4), 3511. https://doi.org/10.3390/ijms24043511

Magno Massuia de Almeida, L., Coquemont-Guyot, M., Elie, N., Morvan-Bertrand, A., Avice, J.C., Mollier, A., & Brunel-Muguet, S. (2023). Repeated heat stress events during the reproductive phase impact the dynamic development of seeds in *Brassica napus* L. *Plant Science*, 327, 111559. Elsevier BV. https://doi.org/10.1016/j.plantsci.2022.111559

Magno, L., Avice, J.C., Morvan-Bertrand, A., Wagner, M.H., Gonz´alez-Centeno, M.R., Teissedre, P. L. ., Bessoule, J.J., Le Gu´edard, M., Kim, T.H., Mollier, A., Brunel-Muguet, S. (2021). High temperature patterns at the onset of seed maturation determine seed yield and quality in oilseed rape (*Brassica napus* L.) in relation to sulphur nutrition. *Environmental and Experimental Botany,* 185, https://doi.org/10.1016/j.envexpbot.2021.104400.

Magno, L., Coulon, M., Avice, J.C., Morvan-Bertrand, A., Bessoule, J.J., Le Gu´edard, M., Kim, T. H., Niare, A., Mollier, A., Bertin, N., Brunel-Muguet, S. (2022). Effects of two-generational heat stress exposure at the onset of seed maturation on seed yield and quality in *Brassica napus* L. *Environmental and*

Experimental Botany 195, 104788, https://doi.org/10.1016/j.envexpbot.2022.104788

Marconi, G., Pace, R., Traini, A., Raggi, L., Lutts, S., Chiusano, M., Guiducci, M., Falcinelli, M., Benincasa, P., & Albertini, E. (2013). Use of MSAP markers to analyse the effects of salt stress on DNA methylation in rapeseed (*Brassica napus* var. oleifera). *PLoS ONE*, 8(9), e75597. Doi.org/10.1371/journal.pone.0075597

Marias, D., Meinzer, F.C., & Still, C. (2017). Impacts of leaf age and heat stress duration on photosynthetic gas exchange and foliar nonstructural carbohydrates in *Coffea arabica*. *Ecology and Evolution,* 7, 1297–1310

Martínez-Ballesta, M.delC., Moreno, D.A. & Carvajal, M. (2013). The physiological importance of glucosinolates on plant response to abiotic stress in Brassica. *International Journal of Molecular Sciences,* 14, (6), 11607–11625. https://doi.org/10.3390/ijms140611607

Mathan, J., Singh, A. & Ranjan, A. (2020). Sucrose transport in response to drought and salt stress involves ABA-mediated induction of OsSWEET13 and OsSWEET15 in rice. *Physiologia Plantarum,* 171(4), 620–637. https://doi.org/10.1111/ppl.13210

Mathur, S., & Jajoo, A. (2014). Effects of heat stress on growth and crop yield of wheat (*Triticum aestivum*). *Physiological Mechanisms and Adaptation Strategies in Plants Under Changing Environment*, 163–191 Doi.org/10.1007/978-1-4614-8591-9_8

Meraj, T. A., Fu, J., Raza, M. A., Zhu, C., Shen, Q., Xu, D., & Wang, Q. (2020). Transcriptional factors regulate plant stress responses through mediating secondary metabolism. *Genes*. 11(4), 346 https://doi.org/10.3390/genes11040346

Met Office (n.d.). *UK temperature, rainfall and sunshine time series*. https://www.metoffice.gov.uk/research/climate/maps-and-data/uk-temperature-rainfall-and-sunshine-time-series.

Mills, J,T. (1996) Storage of Canola. Agriculture and Agri-Food Canada. http://www1.agric.gov.ab.ca

Mishra, S. K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L., & Scharf, K.-D. (2002). In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. *Genes & Development,* 16(12),1555–1567. https://doi.org/10.1101/gad.228802

Mishra, S., Chowdhary, A. A., Mehrotra, S., & Srivastava, V. (2019). Function of plant heat shock transcription factors in abiotic stress. *Energy, Environment, and Sustainability,* 113–126. https://doi.org/10.1007/978-981-15-0690-1_6

Mittler, R., Finka, A., & Goloubinoff, P. (2012). How do plants feel the heat? *Trends in Biochemical Sciences* 37(3), 118–125. Doi.org/10.1016/j.tibs.2011.11.007

Mohammadi, P. P., Moieni, A., & Komatsu, S. (2012). Comparative proteome analysis of drought-sensitive and drought-tolerant rapeseed roots and their hybrid F1 line under drought stress. *Amino Acids*, 43(5), 2137–2152. Doi.org/10.1007/s00726-012-1299-6

Mohapatra, M.D., Poosapati, S., Sahoo, R.K., & Swain, D.M. (2023). Helicase: A genetic tool for providing stress tolerance in plants. *Plant Stress*. 9, 100171. https://doi.org/10.1016/j.stress.2023.100171

Mokany, K., Ferrier, S., Harwood, T. D., Ware, C., Di Marco, M., Grantham, H. S., Venter, O., Hoskins, A. J., & Watson, J. E. M. (2020). Reconciling global priorities for conserving biodiversity habitat. *Proceedings of the National Academy of Sciences of the United States of America*, 117(18), 9906–9911. https://doi.org/10.1073/pnas.1918373117

Morrison, M. J., & Stewart, D. W. (2002). Heat stress during flowering in summer Brassica. *Crop Science*, 42(3), 797–803. Doi.org/10.2135/cropsci2002.7970

Müller, JL., Krishna, P. & Foreiter, C. (2000). A glucosinolate mutant of *Arabidopsis* is thermosensitive and defective in cytosolic Hsp90 expression after heat stress. *Plant Physiology*, 123*,* 949–958

Naika, M., Shameer, K., Mathew, O. K., Gowda, R., & Sowdhamini, R. (2013). STIFDB2: An updated version of plant stress-responsive transcription factor database with additional stress signals, stress-responsive transcription factor binding sites and stress-responsive genes in Arabidopsis and rice. *Plant and Cell Physiology*, 54(2), e8–e8. https://doi.org/10.1093/pcp/pcs185

Namazkar, S., Stockmarr, A., Frenck, G., Egsgaard, H., Terkelsen, T., Mikkelsen, T., Ingvordsen, C. H., & Jørgensen, R. B. (2016). Concurrent elevation of CO_2, O_3 and temperature severely affects oil quality and quantity in rapeseed. *Journal of Experimental Botany*, 67(14), 4117–4125. Doi.org/10.1093/jxb/erw180

Narayanan, S., Tamura, PJ., Roth, MR., Prasad, PVV., & Welti, R. (2016). Wheat leaf lipids during heat stress: High day and night temperatures result in major lipid alterations. *Plant Cell and Environment,* 39, 787–803

Narayanan, S., Zoong-Lwe, ZS., Gandhi, N., Welti, R., Fallen, B. & Smith, JR et al. (2020). Comparative lipidomic analysis reveals heat stress responses of two soybean genotypes differing in temperature sensitivity. *Plants,* 9, 457

Neilson, K. A., Gammulla, C. G., Mirzaei, M., Imin, N., & Haynes, P. A. (2010). Proteomic analysis of temperature stress in plants. *Proteomics,*10(4), 828–845. Doi.org/10.1002/pmic.200900538

Nishizawa, A., Yabuta, Y., and Shigeoka, S. (2008). Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiology* 147: 1251–1263

Nour-Eldin, H. H., Andersen, T. G., Burow, M., Madsen, S. R., Jørgensen, M. E., Olsen, C. E., Dreyer, I., Hedrich, R., Geiger, D., & Halkier, B. A. (2012). NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature*, 488(7412), 531–534. Doi.org/10.1038/nature11285

Nover, L., Bharti, K., Dö, P., Mishra, S. K., Ganguli, A., & Scharf, K.D. (2001). Arabidopsis and the heat stress transcription factor world: How many heat stress transcription factors do we need? *Cell Stress & Chaperones*, 6(3), 177-189

Nunez-Vazquez, R.; Desvoyes, B.; Gutierrez, C. (2022). Histone variants and modifications during abiotic stress response. *Frontiers in. Plant Science*, 13, 984702

Olejnik, D., Gogolewski, M., & Nogala-Kałucka, M. (1997). Isolation and some properties of plastochromanol-8. *Food / Nahrung* 41(2), 101–104. Doi: 10.1002/food.19970410209

OmicsBox – Bioinformatics Made Easy, BioBam Bioinformatics, March 3, 2019, https://www.biobam.com/omicsbox

Orlemans, K., Barrett, D.M., Bosch Suades, C., Verkerk, R. & Dekker, M. (2006). Thermal degradation of glucosinolates in red cabbage. *Food Chemistry,* 95, 19-29

Owji, H., Hajiebrahimi, A., Seradj, H., & Hemmati, S. (2017). Identification and functional prediction of stress responsive AP2/ERF transcription factors in *Brassica napus* by genome-wide analysis. *Computational Biology and Chemistry*, 71, 32–56. Doi.org/10.1016/j.compbiolchem.2017.09.004

Pachauri, R. K., Allen, M. R., Barros, V. R., Broome, J., Cramer, W., & Christ, R. et al. (2014). IPCC, 2014: Contribution of Working Groups, I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change. *Climate Change 2014: Synthesis Report*

Pacheco-Sangerman, F., Gómez-Merino, F. C., PeraltaSánchez, Ma. G., Alcántar-González, G., & Trejo-Téllez, L. I. (2023). Glucosinolates: Structure, classification, biosynthesis and functions in higher plants. *Agro Productividad*. 107-114. https://doi. org/10.32854/agrop.v16i3.2567

Parmesan, C., & Hanley, M. E. (2015). Plants and climate change: Complexities and surprises. *Annals of Botany,* 116(6), 849–864. https://doi.org/10.1093/aob/mcv169

Pasini, F., Verardo, V., Caboni, M.F. & D'Antuono, L.F. (2012). Determination of glucosinolates and phenolic compounds in rocket salad by HPLC-DAD-MS: Evaluation of *Eruca sativa Mill*. And *Diplotaxis tenuifolia* L. genetic resources. *Food Chemistry*, 133(3), 1025–1033.

Poidevin, L., Forment, J., Unal, D., & Ferrando, A. (2020). Transcriptome and translatome changes in germinated pollen under heat stress uncover roles of transporter genes involved in pollen tube growth. *Plant Cell and Environment*, 44(7), 2167-2184. Doi.org/10.1111/pce.13972

Pokharel, M., Chiluwal, A., Stamm, M., Min, D., Rhodes, D., & Jagadish, S. V. K. (2020). High night-time temperature during flowering and pod filling affects flower opening, yield and seed fatty acid composition in canola. *Journal of Agronomy and Crop Science*, 206(5), 579–596. Doi.org/10.1111/jac.12408

Politano, G., Benso, A., Savino, A., Di Carlo, S. (2014). ReNE: A Cytoscape plugin for regulatory network enhancement. PLoS One. 9(12):e115585. doi: 10.1371/journal.pone.0115585. PMID: 25541727; PMCID: PMC4277354.

Prasad, P. V., Bheemanahalli, R. & Jagadish, S. K. (2017). Field crops and the fear of heat stress — opportunities, challenges and future directions. *Field Crops Research,* 200, 114–121. doi: 10.1016/j.fcr.2016.09.024

Pujol, C., Bailly, M., Kern, D., Maréchal-Drouard, L., Becker, H., & Duchêne, A.M. (2008). Dual-targeted tRNA-dependent amidotransferase ensures both mitochondrial and chloroplastic Gln-tRNA Gln synthesis in plants. *Proceedings of the National Academy of Sciences*. 105(17), 6481–6485. https://doi.org/10.1073/pnas.0712299105

Rahaman, M., Mamidi, S., & Rahman, M. (2018). Genome-wide association study of heat stress-tolerance traits in spring-type *Brassica napus* L. under controlled conditions. *Crop Journal*, 6(2), 115–125. Doi.org/10.1016/j.cj.2017.08.003

Ramakrishnan, M., Zhang, Z., Mullasseri, S., Kalendar, R., Ahmad, Z., Sharma, A., Liu, G., Zhou, M., Wei, Q. (2022). Epigenetic stress memory: A new approach to study cold and heat stress responses in plants. *Frontiers in Plant Science*.13:1075279. doi: 10.3389/fpls.2022.1075279

Rang, Z. W., Jagadish, S. V. K., Zhou, Q. M., Craufurd, P. Q., & Heuer, S. (2011). Effect of high temperature and water stress on pollen germination and spikelet fertility in rice. *Environmental and Experimental Botany*, 70(1), 58–65. Doi.org/10.1016/j.envexpbot.2010.08.009

Rao, S.Q., Chen, X.-Q., Wang, K.-H., Zhu, Z.J., Yang, J. & Zhu, B. (2021). Effect of short-term high temperature on the accumulation of glucosinolates in *Brassica rapa*. *Plant Physiology and Biochemistry*, 161, 222–233. https://doi.org/10.1016/j.plaphy.2021.02.013.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. & Meijer, J. (2000). Myrosinase: Gene family evolution and herbivore defense in Brassicaceae. *Plant Molecular Biology,* 42:93-113.

Rasmusson, L. M., Buapet, P., George, R., Gullström, M., Gunnarsson, P. C. B. & Björk, M. (2020). Effects of temperature and hypoxia on respiration, photorespiration, and photosynthesis of seagrass leaves from contrasting temperature regimes. J. Norkko (Ed.), ICES *Journal of Marine Science,* 77(6), 2056–2065. https://doi.org/10.1093/icesjms/fsaa093.

Ratnayake WMN, Daun JK. (2004). Chemical composition of canola and rapeseed oils. Gunstone FD (ed) Rapeseed and canola oil. Production, processing, properties and uses. *CRC Boca Raton*, 37–78

Raza, A. (2020). Metabolomics: A systems biology approach for enhancing heat stress tolerance in plants. *Plant Cell Reports*. Doi.org/10.1007/s00299-020-02635-8

Raza, A., Razzaq, A., Mehmood, S. S., Hussain, M. A., Wei, S., He, H., Zaman, Q. U., Xuekun, Z., & Hasanuzzaman, M. (2021). Omics: The way forward to

enhance abiotic stress tolerance in *Brassica napus* L. *GM Crops and Food*, 12(1), 251–281. Doi.org/10.1080/21645698.2020.1859898

Razzaq, A., Sadia, B., Raza, A., Hameed, M. K., & Saleem, F. (2019). Metabolomics: A way forward for crop improvement. *Metabolites,* 9(12), 303. Doi.org/10.3390/metabo9120303

Ren, Y., Zhu, J., Zhang, H., Lin, B., Hao, P., & Hua, S. (2022). Leaf Carbohydrate Metabolism Variation Caused by Late Planting in Rapeseed (*Brassica napus* L.) at Reproductive Stage. *Plants* 11(13), 1696. MDPI AG. https://doi.org/10.3390/plants11131696

Rhee, J.-H., Choi, S., Lee, J.E., Hur, O.-S., Ro, N.Y., Hwang, A.J., Ko, H.C., Chung, Y.J., Noh, J.J., & Assefa, A. D. (2020). Glucosinolate Content in Brassica Genetic Resources and Their Distribution Pattern within and between Inner, Middle, and Outer Leaves. *Plants* 9(11), 1421. MDPI AG. https://doi.org/10.3390/plants9111421

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C.Z., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R. R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., & Yu, G.L. (2000). Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes. *Science*. 290(5499), 2105–2110. https://doi.org/10.1126/science.290.5499.2105

Rocco, M., Arena, S., Renzone, G., Scippa, G. S., Lomaglio, T., Verrillo, F., Scaloni, A., & Marra, M. (2013). Proteomic analysis of temperature stress-responsive proteins in *Arabidopsis thaliana* rosette leaves. *Molecular BioSystems*, 9(6), 1257–1267. Doi.org/10.1039/c3mb70137a

Rong, Y., Li, T., Liu, X., Shi, S., Wang, X. & Tu, pengfei. (2021). A transaldolase from *Aquilaria Sinensis* involves in ABA-mediated seed germination and root growth. https://doi.org/10.21203/rs.3.rs-234062/v1.

Rudzińska, M., Przybylski, R., & Wąsowicz, E. (2009). Products formed during thermo-oxidative degradation of phytosterols. *Journal of the American Oil Chemists' Society* 86 (7), 651–662. Doi.org/10.1007/s11746-009-1397-0

Sage, T. L., Bagha, S., Lundsgaard-Nielsen, V., Branch, H. A., Sultmanis, S., & Sage, R. F. (2015). The effect of high temperature stress on male and female reproduction in plants. *Field Crops Research*, 182, 30–42. Doi.org/10.1016/j.fcr.2015.06.011

Sahni, S., Prasad, B. D., Liu, Q., Grbic, V., Sharpe, A., Singh, S. P., & Krishna, P. (2016). Overexpression of the brassinosteroid biosynthetic gene DWF4 in *Brassica napus* simultaneously increases seed yield and stress tolerance. *Scientific Reports*, 6:28298. Doi.org/10.1038/srep28298

Saminathan, T., Nimmakayala, P., Manohar, S., Malkaram, S., Almeida, A., Cantrell, R., Tomason, Y., Abburi, L., Rahman, M. A., Vajja, V. G., Khachane, A., Kumar, B., Rajasimha, H. K., Levi, A., Wehner, T., & Reddy, U. K. (2015). Differential gene expression and alternative splicing between diploid and

tetraploid watermelon. *Journal of Experimental Botany*, 66(5), 1369–1385. Doi.org/10.1093/jxb/eru486

Sanyal, R. P., Misra, H. S., & Saini, A. (2018). Heat-stress priming and alternative splicing-linked memory. *Journal of Experimental Botany,* 69(10), 2431–2434. Doi.org/10.1093/jxb/ery111

Saraswat, S., Yadav, A. K., Sirohi, P., & Singh, N. K. (2017). Role of epigenetics in crop improvement: Water and heat stress. *Journal of Plant Biology,* 60(3), 231–240. Doi.org/10.1007/s12374-017-0053-8

Scafaro, A. P., Fan, Y., Posch, B. C., Garcia, A., Coast, O. & Atkin, O. K. (2021). Responses of leaf respiration to heatwaves. *Plant, Cell & Environment,* 44(7), 2090–2101. https://doi.org/10.1111/pce.14018.

Scharf, K. D., Berberich, T., Ebersberger, I., & Nover, L. (2012). The plant heat stress transcription factor (Hsf) family: Structure, function and evolution. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 1819(2), 104–119. Doi.org/10.1016/j.bbagrm.2011.10.002

Schiessl, S. (2020). Regulation and subfunctionalization of flowering time genes in the allotetraploid oil crop *Brassica napus*. *Frontiers in Plant Science,* 11, 605155. Doi.org/10.3389/fpls.2020.605155

Schonhof, I., Kläring, H. P., Krumbein, A., Clauβen, W. & Screiner, M. (2007). Effect of temperature increase under low radiation conditions on phytochemicals and ascorbic acid in greenhouse grown broccoli. *Agriculture, Ecosystems and Environment,* 119, 103-111.

Seo, M.S., & Kim, J. (2017). Understanding of MYB Transcription Factors Involved in Glucosinolate Biosynthesis in Brassicaceae. Molecules, 22(9), 1549. https://doi.org/10.3390/molecules22091549.

Seneviratne, SI. et al (2012). Changes in climate extremes and their impacts on the natural physical environment. *Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation* [Field C. B. et al. (eds)]. A Special Report of Working Groups I and II of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge, UK, and New York, NY, USA, 109–230.

Seth, R., Maritim, TK., Parmar, R. & Sharma, RK. (2021). Underpinning the molecular programming attributing heat stress associated thermotolerance in tea (*Camellia sinensis* (L.) O. Kuntze). *Horticulture Research,* 8(1). Https://doi.org/10.1038/s41438-021-00532-z.

Shahzad, R., Jamil, S., Ahmad, S., Nisar, A., Amina, Z., Saleem, S., Zaffar Iqbal, M., Muhammad Atif, R., & Wang, X. (2021). Harnessing the potential of plant transcription factors in developing climate resilient crops to improve global food security: Current and future perspectives. *Saudi Journal of Biological Sciences*, 28(4), 2323–2341. https://doi.org/10.1016/j.sjbs.2021.01.028

Shamloo-Dashtpagerdi, R., Razi, H., Ebrahimie, E., & Niazi, A. (2018). Molecular characterization of *Brassica napus* stress related transcription factors, BnMYB44

and BnVIP1, selected based on comparative analysis of *Arabidopsis thaliana* and *Eutrema salsugineum* transcriptomes. *Molecular Biology Reports*, 45(5), 1111–1124. Doi.org/10.1007/s11033-018-4262-0

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

Shen, E., Zhu, X., Hua, S., Chen, H., Ye, C., Zhou, L., Liu, Q., Zhu, Q. H., Fan, L., & Chen, X. (2018). Genome-wide identification of oil biosynthesis-related long non-coding RNAs in allopolyploid *Brassica napus. BMC Genomics*, 19(1), 745. Doi.org/10.1186/s12864-018-5117-8

Shen, Y., Sun, S., Hua, S., Shen, E., Ye, C. Y., Cai, D., Timko, M. P., Zhu, Q. H., & Fan, L. (2017). Analysis of transcriptional and epigenetic changes in hybrid vigor of allopolyploid *Brassica napus* uncovers key roles for small RNAs. *Plant Journal*, 91(5), 874–893. Doi.org/10.1111/tpj.13605

Siger, A., Gawrysiak-Witulska, M., & Wawrzyniak, J. (2018). Changes in contents of phenolic compounds (sinapic acid derivatives) in seeds of *Brassica napus* L.under adverse storage conditions. *Acta Scientiarum Polonorum Technologia Alimentaria*.17(4), 367–375. Doi.org/10.17306/j.afs.2018.0596

Simpson, G. G. (2004). The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. *Current Opinion in Plant Biology*, 7(5), 570–574. Doi.org/10.1016/j.pbi.2004.07.002

Solís, M. T., Rodríguez-Serrano, M., Meijón, M., Cañal, M. J., Cifuentes, A., Risueño, M. C., & Testillano, P. S. (2012). DNA methylation dynamics and MET1a-like gene expression changes during stress-induced pollen reprogramming to embryogenesis. *Journal of Experimental Botany*, 63(18), 6431–6444. Doi.org/10.1093/jxb/ers298

Soltis, D. E., Visger, C. J., & Soltis, P. S. (2014). The polyploidy revolution then...and now: Stebbins revisited. *American Journal of Botany*, 101(7), 1057–1078. Doi.org/10.3732/ajb.1400178

Song, C., Cao, Y., Dai, J., Li, G., Manzoor, M. A., Chen, C., & Deng, H. (2022). The multifaceted roles of myc2 in plants: Toward transcriptional reprogramming and stress tolerance by jasmonate signaling. *Frontiers in Plant Science* (13). https://doi.org/10.3389/fpls.2022.868874

Song, JM., Guan, Z. & Hu, J. *et al* (2020). Eight high-quality genomes reveal pan-genome architecture and ecotype differentiation of *Brassica napus*. *Nature Plants,* 6, 34–45. https://doi.org/10.1038/s41477-019-0577-7

Song, X., Liu, G., Huang, Z., Duan, W., Tan, H., Li, Y., et al. (2016). Temperature expression patterns of genes and their coexpression with LncRNAs revealed by RNA-Seq in non-heading chinese cabbage. BMC Genomics 17:297. doi: 10.1186/ s12864-016-2625-2

Strader, L., Weijers, D., & Wagner, D. (2022). Plant transcription factors — being in the right place with the right company. *Current Opinion in Plant Biology*, 65, 102136. https://doi.org/10.1016/j.pbi.2021.102136

Su, PH., & Li, H. (2008). Arabidopsis stromal 70-kd heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiology*, 146(3), 1231–1241. Doi.org/10.1104/pp.107.114496

Sun, F., Fan, G., Hu, Q., Zhou, Y., Guan, M., Tong, C., et al. (2017). The high quality genome of *Brassica napus* cultivar 'ZS11' reveals the introgression history in semi-winter morphotype. *Plant Journal*, 92, 452–468. doi: 10.1111/tpj.13669

Sun, M., Yang, Z., Liu, L., & Duan, L. (2022). DNA Methylation in Plant Responses and Adaption to Abiotic Stresses. *International Journal of Molecular Sciences*, 23(13), 6910. https://doi.org/10.3390/ijms23136910

Sun, X., Zhu, J., Li, X. *et al.* (2020). AsHSP26.8a, a creeping bentgrass small heat shock protein integrates different signaling pathways to modulate plant abiotic stress response. *BMC Plant Biology,* 20, 184. https://doi.org/10.1186/s12870-020-02369-5

Suwa, R., Hakata, H., Hara, H., El-Shemy, HA., Adu-Gyamfi, JJ., Nguyen, NT., Kanai, S., Lightfoot, DA., Mohapatra, PK. & Fujita, K. (2010). High temperature effects on photosynthate partitioning and sugar metabolism during ear expansion in maize (*Zea mays* L.) genotypes. *Plant Physiology Biochemistry,* 48, 124-30. doi: 10.1016/j.plaphy.2009.12.010.

Suzuki, N. et al. (2011) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ. DOI: 10.1111/j.1365-3040.2011.02336.x

Talarico, E., Zambelli, A., Araniti, F., Greco, E., Chiappetta, A., Bruno, L. (2024). Unravelling the epigenetic code: DNA methylation in plants and its role in stress response. *Epigenomes*, 8*,* 30. https://doi.org/10.3390/epigenomes8030030

Tan, X., Li, S., Hu, L., & Zhang, C. (2020). Genome-wide analysis of long non-coding RNAs (lncRNAs) in two contrasting rapeseed (*Brassica napus* L.) genotypes subjected to drought stress and re-watering. *BMC Plant Biology*, 20(1), 81. Doi.org/10.1186/s12870-020-2286-9

Tang, X., Hao, Y.-J., Lu, J.-X., Lu, G., & Zhang, T. (2019). Transcriptomic analysis reveals the mechanism of thermosensitive genic male sterility (TGMS) of *Brassica napus* under the high temperature inducement. *BMC Genomics,* 20(1), 644. Doi.org/10.1186/s12864-019-6008-3

Tang, Y., Zhang, G., Jiang, X., Shen, S., Guan, M., Tang, Y., Sun, F., Hu, R., Chen, S., Zhao, H., et al. (2023). Genome-Wide Association Study of Glucosinolate Metabolites (mGWAS) in *Brassica napus* L. *Plants* 12, 639. https://doi.org/10.3390/ plants12030639

Teperino, R., Schoonjans, K., & Auwerx, J. (2010). Histone methyl transferases and demethylases; Can they link metabolism and transcription? *Cell Metabolism* 12(4), 321–327. Doi.org/10.1016/j.cmet.2010.09.004

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. *Nature* 448, 661–665. doi: 10.1038/nature05960

Touw, AJ., Verdecia Mogena, A., Maedicke, A., Sontowski, R., van Dam, N. M. & Tsunoda, T. (2020). Both biosynthesis and transport are involved in glucosinolate accumulation during root-herbivory in *Brassica rapa*. *Frontiers in Plant Science,* 10. https://doi.org/10.3389/fpls.2019.01653.

Ueda, M., & Seki, M. (2020). Histone modifications form epigenetic regulatory networks to regulate abiotic stress response1. *Plant Physiology*, 182(1), 15–26. Doi.org/10.1104/pp.19.00988

USDA. (2025). Oilseeds: World markets and trade. *United States Department of Agriculture, Foreign Agricultural Service.* https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf

Valente Pereira, F. M. V., Rosa, E., Fahey, J. W., Stephenson, K. K., Carvalho, R. & Aires, A. (2002). Influence of temperature and ontogeny on the Levels of glucosinolates in Broccoli (*Brassica oleracea* Var. italica) sprouts and their effect on the induction of mammalian phase 2 enzymes. *Journal of Agricultural and Food Chemistry*, 50(21), 6239–6244. https://doi.org/10.1021/jf020309x.

Van Dongen, J.T., Gupta, K.J., Ramírez-Aguilar, S.J., Araújo, W.L., Nunes-Nesi, A. & Fernie, A.R. (2011). Regulation of respiration in plants: A role for alternative metabolic pathways. *Journal of Plant Physiology,* 168(12), 1434–1443. https://doi.org/10.1016/j.jplph.2010.11.004.

Verma, V., Ravindran, P., & Kumar, P. P. (2016). Plant hormone-mediated regulation of stress responses. *BMC Plant Biology*, 16(1), 86. Doi.org/10.1186/s12870-016-0771-y

Vlahakis C, Hazebroek J (2000) Phytosterol accumulation in canola, sunflower, and soybean oils: Effects of genetics, planting location, and temperature. *J Am Oil Chem Soc* 77:49–53

Wahid, A., Gelani, S., Ashraf, M. & Foolad, MR. (2007). Heat tolerance in plants: An overview. *Environment and Experimental Botany,* 61:199–223. Https://doi.org/10.1016/j.envexpbot.2007.05.011.

Wan, H., Chen, L., Guo, J., Li, Q., Wen, J., Yi, B., Ma, C., Tu, J., Fu, T., & Shen, J. (2017). Genome-wide association study reveals the genetic architecture underlying salt tolerance-related traits in rapeseed (*Brassica napus* L.). *Frontiers in Plant Science,* 8, 593. Doi.org/10.3389/fpls.2017.00593

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: A revolutionary tool for transcriptomics. *Nature Reviews Genetics,* 10(1), 57–63. Doi.org/10.1038/nrg2484

Wang, J., Meng, X., Yuan, C., Harrison, A.P., Chen, M. (2016). The roles of crosstalk epigenetic patterns in *Arabidopsis thaliana*. *Brief Funct Genomics* 15(4),278–287

Wang, P., Yang, C., Chen, H., Luo, L., Leng, Q., Li, S., Han, Z., Li, X., Song, C., Zhang, X., & Wang, D. (2018). Exploring transcription factors reveals crucial members and regulatory networks involved in different abiotic stresses in *Brassica napus* L. *BMC Plant Biology*, 18, 202. Doi.org/10.1186/s12870-018-1417-z

Wang, R., Liu, H., Liu, Z., Zou, J., Meng, J., & Wang, J. (2019a). Genome-wide analysis of alternative splicing divergences between *Brassica* hexaploid and its parents. *Planta*, 250(2), 603–628. Doi.org/10.1007/s00425-019-03198-z

Wang, A., Hu, J., Gao, C., Chen, G., Wang, B., Lin, C., Song, L., Ding, Y., & Zhou, G. (2019b). Genome-wide analysis of long non-coding RNAs unveils the regulatory roles in the heat tolerance of Chinese cabbage (*Brassica rapa* ssp. chinensis). *Scientific Reports*, 9, 5002. Doi.org/10.1038/s41598-019-41428-2

Wang, J., Yu, H., Zhao, Z., Sheng, X., Shen, Y., & Gu, H. (2019c). Natural variation of glucosinolates and their breakdown products in Broccoli (*Brassica oleracea* var. italica) Seeds. *Journal of Agricultural and Food Chemistry*. 67(45), 12528–12537. https://doi.org/10.1021/acs.jafc.9b06533

Wang, L., Ma, K.B., Lu, Z.G., Ren, S.X., Jiang, H.R., Cui, J.W., Chen, G., Teng, N.J., Lam, H.M. & Jin, B. (2020). Differential physiological, transcriptomic and metabolomic responses of Arabidopsis leaves under prolonged warming and heat shock. *BMC Plant Biology,* 20(1). Https://doi.org/10.1186/s12870-020-2292-y.

Wang R, Zhao W, Yao W, Wang Y, Jiang T, Liu H (2023a). Genome-wide analysis of strictosidine synthase-like gene family revealed their response to biotic/abiotic stress in Poplar. *International Journal of Molecular Science*. 14;24(12):10117. doi: 10.3390/ijms241210117

Wang, L., Wang, L., Tan, M. *et al.* (2023b). The pattern of alternative splicing and DNA methylation alteration and their interaction in linseed (*Linum usitatissimum* L.) response to repeated drought stresses. *Biological Research* 56, 12. https://doi.org/10.1186/s40659-023-00424-7.

Wassan, G.M., Khanzada, H., Zhou, Q., Mason, A.S., Keerio, A.A., Khanzada, S., Solangi, A.M., Faheem, M., Fu, D., & He, H. (2021). Identification of genetic variation for salt tolerance in *Brassica napus* using genome-wide association mapping. *Molecular Genetics and Genomics* 296(2), 391–408. Doi.org/10.1007/s00438-020-01749-8

Way, DA. & Yamori, W. (2014). Thermal acclimation of photosynthesis: On the importance of adjusting our definitions and accounting for thermal acclimation of respiration. *Photosynthesis Research,* 119, 89–100. Https://doi.org/10.1007/s11120-013-9873-7.

WeatherOnline (n.d.). *Max temperature London - Observations 05.2017 interval: 02 Weeks | United Kingdom Weather History*. https://www.weatheronline.co.uk/weather/maps/city.

Werteker M, Lorenz A, Johannes H, Berghofer E, Findlay CS. 2010. Environmental and varietal influences on the fatty acid composition of rapeseed, soybeans, and sunflowers. *Journal of Agronomy and Crop Science* 196, 20–27. Doi.org/10.1111/j.1439-037X.2009.00393.x

Wu, F., Deng, L., Zhai, Q., Zhao, J., Chen, Q., and Li, C. (2020). Mediator subunit MED25 couples alternative splicing of JAZ genes with fine-tuning of jasmonate signalling. *Plant Cell* 32, 429–448. doi: 10.1105/tpc.19.00583

Wu, T.-Y., & Urano, D. (2018). Genetic and systematic approaches toward G protein-coupled abiotic stress signalling in plants. *Frontiers in Plant Science*. 9. https://doi.org/10.3389/fpls.2018.01378

Wu, X. L., Liu, Z. H., Hu, Z. H., & Huang, R. Z. (2014). BnWRI1 coordinates fatty acid biosynthesis and photosynthesis pathways during oil accumulation in rapeseed. *Journal of Integrative Plant Biology*, 56(6), 582–593. Doi.org/10.1111/jipb.12158

Xalxo, R., Yadu, B., Chandra, J., Chandrakar, V. & Keshavkant, S. (2020). Alteration in carbohydrate metabolism modulates thermotolerance of plant under heat stress. *Heat Stress Tolerance in Plants,* 77–115. https://doi.org/10.1002/9781119432401.ch5.

Xie, F. L., Huang, S. Q., Guo, K., Xiang, A. L., Zhu, Y. Y., Nie, L., & Yang, Z. M. (2007). Computational identification of novel microRNAs and targets in *Brassica napus*. *FEBS Letters*, 581(7), 1464–1474. Doi.org/10.1016/j.febslet.2007.02.074

Xin, M., Wang, Y., Yao, Y., Xie, C., Peng, H., Ni, Z., & Sun, Q. (2010). Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum* L.). *BMC Plant Biology,* 10, 123. Doi.org/10.1186/1471-2229-10-123

Xu, Y., Zhang, S., Lin, S., Guo, Y., Deng, W., Zhang, Y., & Xue, Y. (2017). WERAM: A database of writers, erasers and readers of histone acetylation and methylation in eukaryotes. *Nucleic Acids Research*, 45(D1), D264–D270. Doi.org/10.1093/nar/gkw1011

Xue, G. P., Sadat, S., Drenth, J., & McIntyre, C. L. (2014). The heat shock factor family from *Triticum aestivum* in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. *Journal of Experimental Botany*, 65(2), 539–557. Doi.org/10.1093/jxb/ert399

Yamaguchi-Shinozaki K., Shinozaki K. (2006).Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology*, 57,781–803. doi: 10.1146/annurev.arplant.57.032905.105444.

Yan, G., Lv, X., Gao, G., Li, F., Li, J., Qiao, J., Xu, K., Chen, B., Wang, L., Xiao, X., & Wu, X. (2016). Identification and characterization of a glyoxalase I gene in a rapeseed cultivar with seed thermotolerance. *Frontiers in Plant Science*, 7, 150. Doi.org/10.3389/fpls.2016.00150

Yoosefzadeh-Najafabadi, M., Earl, H. J., Tulpan, D., Sulik, J., & Eskandari, M. (2021). Application of machine learning algorithms in plant breeding: Predicting yield from hyperspectral reflectance in soybean. *Frontiers in Plant Science* (11). https://doi.org/10.3389/fpls.2020.624273

Yoshida, T., Sakuma, Y., Todaka, D., Maruyama, K., Qin, F., Mizoi, J., Kidokoro, S., Fujita, Y., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2008). Functional analysis of an Arabidopsis heat-shock transcription factor HsfA3 in the transcriptional cascade downstream of the DREB2A stress-regulatory system. *Biochemical and Biophysical Research Communications*, 368(3), 515–521. Doi.org/10.1016/j.bbrc.2008.01.134

You, Y., Sawikowska, A., Neumann, M., Posé, D., Capovilla, G., Langenecker, T., Neher, R. A., Krajewski, P., & Schmid, M. (2017). Temporal dynamics of gene expression and histone marks at the *Arabidopsis* shoot meristem during flowering. *Nature Communications* 8(1). Doi.org/10.1038/ncomms15120

Young, L.W., Wilen, R.W., & 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. Doi.org/10.1093/jxb/erh038

Yu, E., Fan, C., Yang, Q., Li, X., Wan, B. & Dong, Y. et al. (2014). Identification of heat responsive genes in *Brassica napus* siliques at the seed-filling stage through transcriptional profiling. *PLoS One* 9:e101914. doi: 10.1371/journal. pone.0101914.

Yu, E., Fan, C., Yang, Q., Li, X., Wan, B. & Dong, Y. et al. (2014). Identification of heat responsive genes in *Brassica napus* siliques at the seed-filling stage through transcriptional profiling. *PLoS One* 9:e101914. doi: 10.1371/journal. pone.0101914.

Yu, W., Wang, L., Zhao, R., Sheng, J., Zhang, S., Li, R., et al. (2019). Knockout of SlMAPK3 enhances tolerance to heat stress involving ROS homeostasis in tomato plants. BMC Plant Biol. 19, 1–13. doi: 10.1186/s12870-019-1939-z

Yu, X., Wang, H., Lu, Y., de Ruiter, M., Cariaso, M., Prins, M., van Tunen, A., & He, Y. (2012). Identification of conserved and novel microRNAs that are responsive to heat stress in *Brassica rapa*. *Journal of Experimental Botany*, 63(2), 1025–1038. Doi.org/10.1093/jxb/err337

Yuenyong, W., Sirikantaramas, S. & Qu, LJ. *et al.* (2019). Isocitrate lyase plays important roles in plant salt tolerance. *BMC Plant Biology,* 19, 472. https://doi.org/10.1186/s12870-019-2086-2.

Zafar, I., Hussain, A.I., Fatima, T., Abdullah Alnasser, S.M., & Ahmad, A. (2022). Inter-varietal variation in phenolic profile, sugar contents, antioxidant, anti-proliferative and antibacterial activities of selected brassica species. *Applied Sciences* 12(12), 5811. https://doi.org/10.3390/app12125811

Zafar, S.A., Zaidi, S.S.E.A., Gaba, Y., Singla-Pareek, S.L., Dhankher, O.P., Li, X., et al. (2020). Engineering abiotic stress tolerance via CRISPR/Cas-mediated

genome editing. *Journal of Experimental Botany*, 71, 470–9. doi: 10.1093/jxb/erz476

Zandalinas, SI., Rivero, RM., Martínez, V., Gómez-Cadenas, A. & Arbona, V. (2016). Tolerance of citrus plants to the combination of high temperatures and drought is associated to the increase in transpiration modulated by a reduction in abscisic acid levels. *BMC Plant Biology,* 16, 1-16

Zandalinas, S.I., Mittler, R., Balfagón, D., Arbona, V., & Gómez-Cadenas, A. (2018). Plant adaptations to the combination of drought and high temperatures. *Physiologia Plantarum,* 162(1), 2–12. Doi.org/10.1111/ppl.12540

Zandalinas, S.I., Fritschi, F.B., & Mittler, R. (2021). Global warming, climate change, and environmental pollution: Recipe for a multifactorial stress combination disaster. *Trends in Plant Science*, 26(6), 588–599. Doi.org/10.1016/j.tplants.2021.02.011

Zhang, P. G., Huang, S. Z., Pin, A. L., & Adams, K. L. (2010). Extensive divergence in alternative splicing patterns after gene and genome duplication during the evolutionary history of Arabidopsis. *Molecular Biology and Evolution*, 27(7), 1686–1697. Doi.org/10.1093/molbev/msq054

Zhang, T., Shen, L.Y., Wang, R.X., Zou, Y., Zhao, J.H., Li, R.C., Liang, J.L., Gong, H.M. (2012). Fertility alteration and utilization of male-sterile line 160S in *Brassica napus*. *Acta Botan Boreali-Occiden Sin*. 32(1), 35–41

Zhang, H., Yang, B., Liu, W.-Z., Li, H., Wang, L., Wang, B., Deng, M., Liang, W., Deyholos, M. K., & Jiang, Y.-Q. (2014a). Identification and characterization of CBL and CIPK gene families in canola (*Brassica napus* L.). *BMC Plant Biology*, 14, 8. Doi.org/10.1186/1471-2229-14-8

Zhang, Y.C., Liao, J.Y., Li, Z.Y., Yu, Y., Zhang, J.P., Li, Q.F., Qu, L.H., Shu, W.S., & Chen, Y.Q. (2014b). Genome-wide screening and functional analysis identify a large number of long noncoding RNAs involved in the sexual reproduction of rice. *Genome Biology*, 15(12), 512. Doi.org/10.1186/s13059-014-0512-1

Zhang, S.S., Yang, H., Ding, L., Song, Z.T., Ma, H., Chang, F. & Liu, J.X. (2017). Tissue-specific transcriptomics reveals an important role of the unfolded protein response in maintaining fertility upon heat stress in Arabidopsis. *Plant Cell*, 29(5), 1007-1023. https://doi.org/10.1105/tpc.16.00916

Zhang, H., Zhang, X., Xiao, J. (2023). Epigenetic regulation of nitrogen signalling and adaptation in plants. *Plants*, *12*, 2725.

Zhao, J., Lu, Z., Wang, L., & Jin, B. (2020). Plant responses to heat stress: physiology, transcription, noncoding RNAs, and epigenetics. *International Journal of Molecular Sciences.* 22(1), 117. https://doi.org/10.3390/ijms22010117

Zhao, Y., Chan, Z., Gao, J., Xing, L., Cao, M., Yu, C., Hu, Y., You, J., Shi, H., Zhu, Y., Gong, Y., Mu, Z., Wang, H., Deng, X., Wang, P., Bressan, R. A., & Zhu, J.-K. (2016). ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proceedings of the National Academy of Sciences*,113(7), 1949–1954. Doi.org/10.1073/pnas.1522840113

Zheng, G., Tian, B., Zhang, F., Tao, F. & Li, W. (2011). Plant adaptation to frequent alterations between high and low temperatures: Remodelling of membrane lipids and maintenance of unsaturation levels. *Plant Cell and Environment,* 34, 1431–42

Zheng, Q., & Liu, K. (2022). Worldwide rapeseed (*Brassica napus* L.) research: A bibliometric analysis during 2011–2021 (2022). *Oil Crop Science*, 7(4),157–165. https://doi.org/10.1016/j.ocsci.2022.11.004

Zhou, L., Yan, T., Chen, X., Li, Z., Wu, D., Hua, S. & Jiang, L. (2018). Effect of high night temperature on storage lipids and transcriptome changes in developing seeds of oilseed rape. *Journal of Experimental Botany*, 69(7), 1721-1733. https://doi.org/10.1093/jxb/ery004

Zhou, R., Moshgabadi, N., & Adams, K. L. (2011). Extensive changes to alternative splicing patterns following allopolyploidy in natural and resynthesized polyploids. *Proceedings of the National Academy of Sciences of the United States of America*, 108(38), 16122-16127. Doi.org/10.1073/pnas.1109551108

Zhu, X., Huang, C., Zhang, L., Liu, H., Yu, J., Hu, Z., & Hua, W. (2017). Systematic analysis of HsF family genes in the *Brassica napus* genome reveals novel responses to heat, drought and high CO2 stresses. *Frontiers in Plant Science,* 8, 1174. Doi.org/10.3389/fpls.2017.01174

Zhu, Y., Wang, Z., Jing, Y., Wang, L., Liu, X., Liu, Y., & Deng, X. (2009). Ectopic over-expression of BhHsf1, a heat shock factor from the resurrection plant *Boea hygrometrica*, leads to increased thermotolerance and retarded growth in transgenic Arabidopsis and tobacco. *Plant Molecular Biology*, 71(4–5), 451–467. Doi.org/10.1007/s11103-009-9538-2

Zoong Lwe, Z., Sah, S. & Persaud, L. *et al* (2021). Alterations in the leaf lipidome of *Brassica carinata* under high-temperature stress. *BMC Plant Biology,* 21, 404. https://doi.org/10.1186/s12870-021-03189-x

Zou, Y., Xiao, H., Bianchi, F. J. J. A., Jauker, F., Luo, S., and van der Werf, W. (2017). Wild pollinators enhance oilseed rape yield in small-holder farming systems in China. *BMC Ecology*, 17, 6. Doi.org/10.1186/s12898-017-116-111

Appendix A

Supplementary Figures and Tables

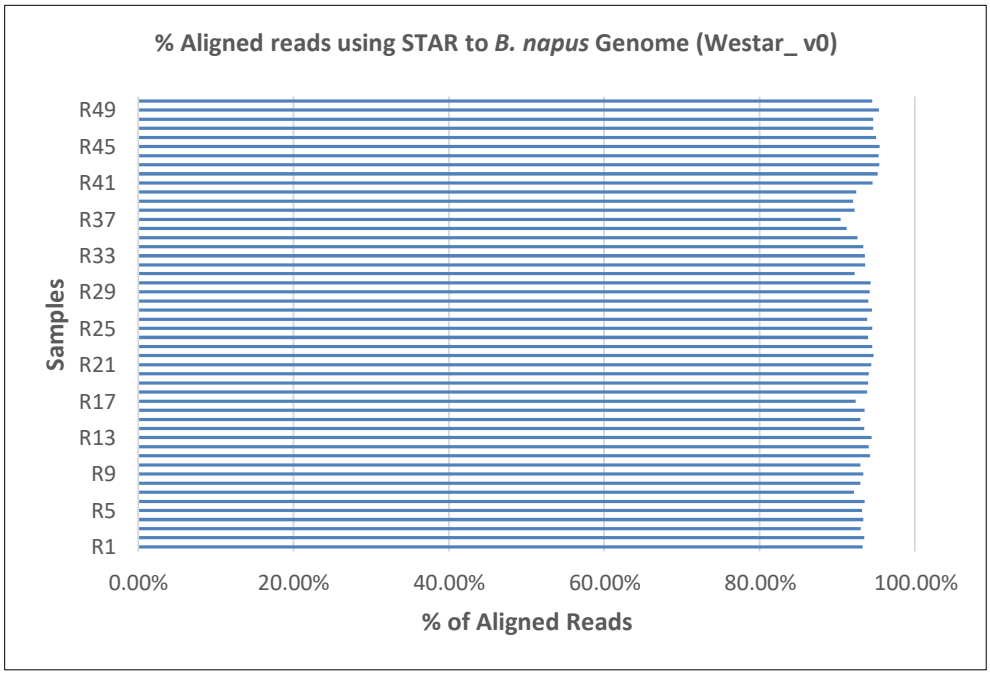

Figure S1 Percentage of aligned reads per sample. Around 90%- 95.49% total reads per sample were mapped to the *B. napus* cv. Westar v0 reference genome.

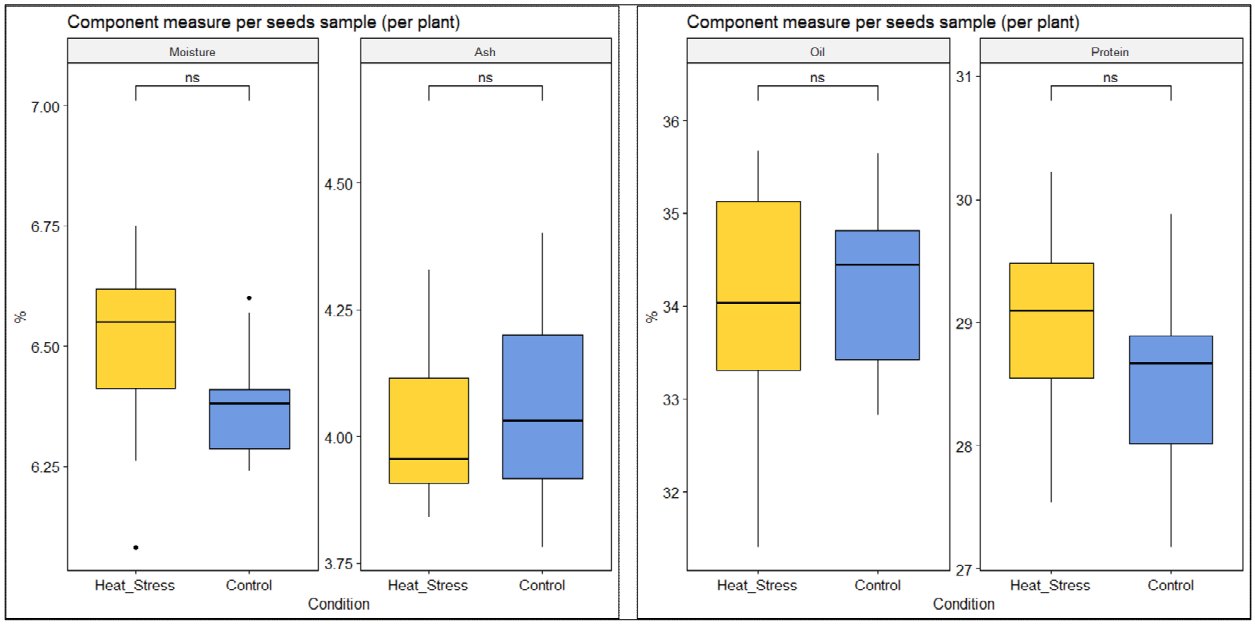

Figure S2 Moisture, ash, oil and Protein content in seeds per plant. “ns” denotes non-significance.

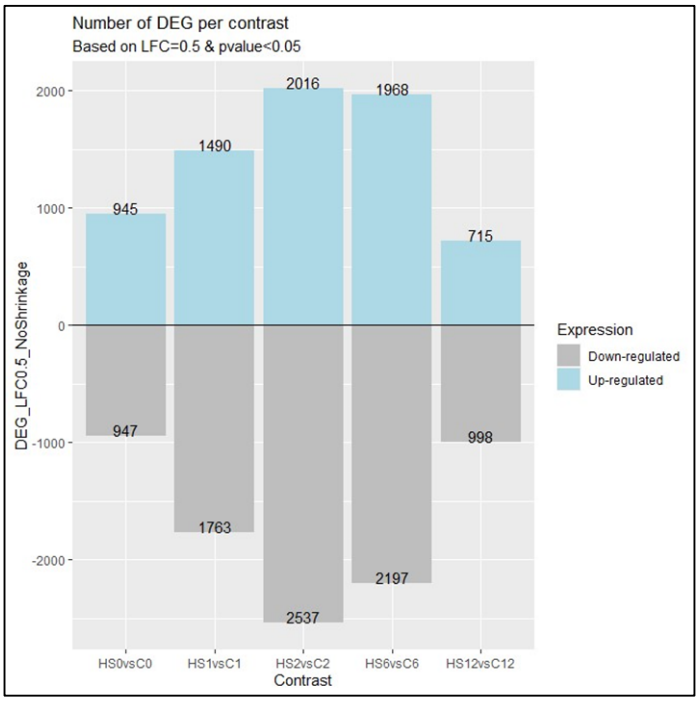

Figure S3 Pairwise comparison of the number of differentially expressed genes between heat treatment and control at each timepoint based on Log2FC =0.5 and p-value ≤0.05. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6, and HS12vsC12 contrasts correspond to pairwise comparisons between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

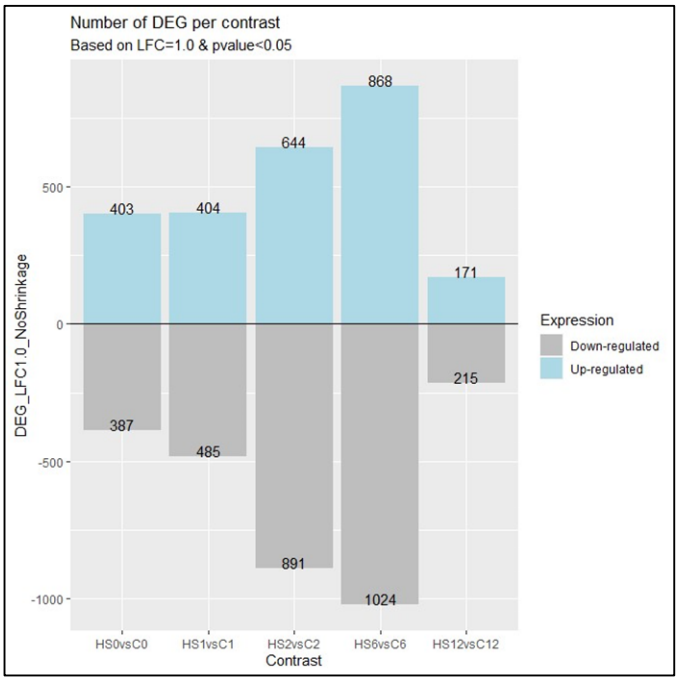

Figure S4 Pairwise comparison of the number of DEG between heat treatment and control at each timepoint based on Log2FC = 1.0 and p-value ≤0.05. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6, and HS12vsC12 contrasts correspond to pairwise comparisons between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

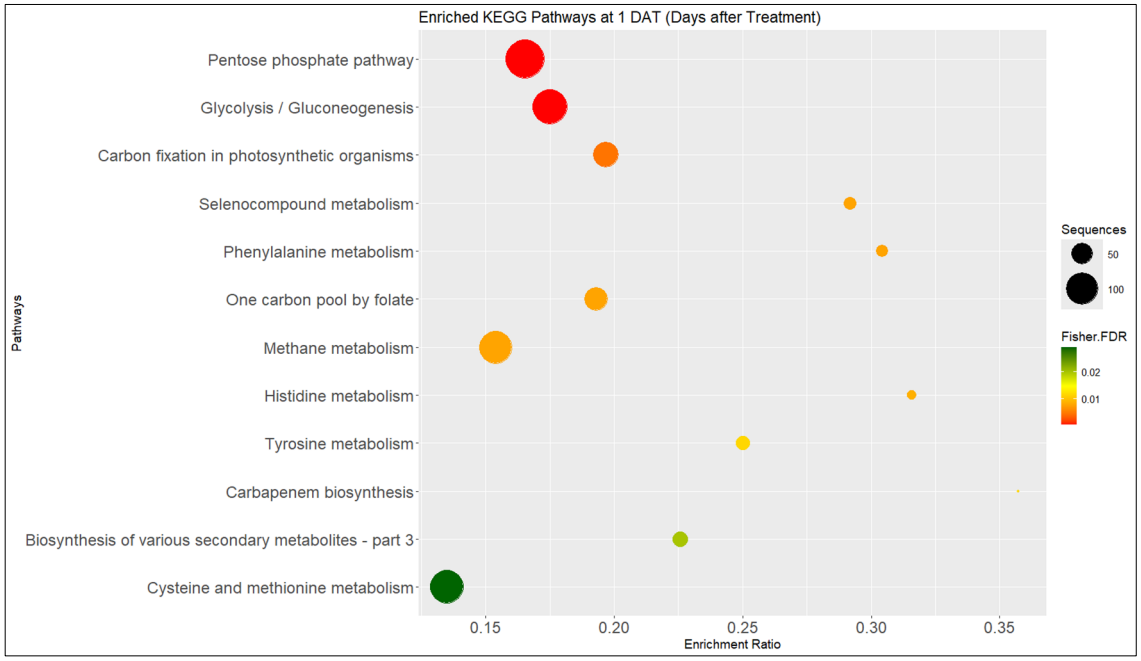

Figure S5 The top enriched KEGG pathways and their corresponding number of genes at 1 DAT at p-value ≤ 0.05. The x-axis represents the enrichment factor. The y-axis represents KEGG pathways. The circle represents the number of genes mapped to pathways and the red to green indicates the significance.

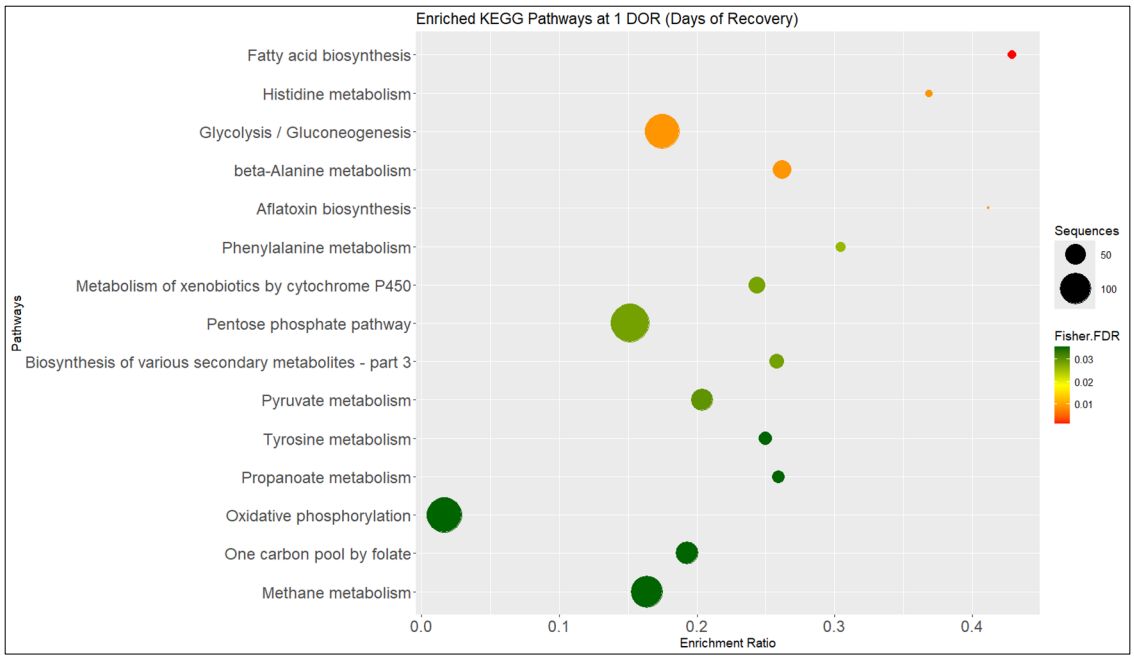

Figure S6 The top enriched KEGG pathways and their corresponding number of genes at 1 DOR at p-value ≤ 0.05. The x-axis represents the enrichment factor. The y-axis represents KEGG pathways. The circle represents the number of genes mapped to pathways and the red to green bar indicates the significance.

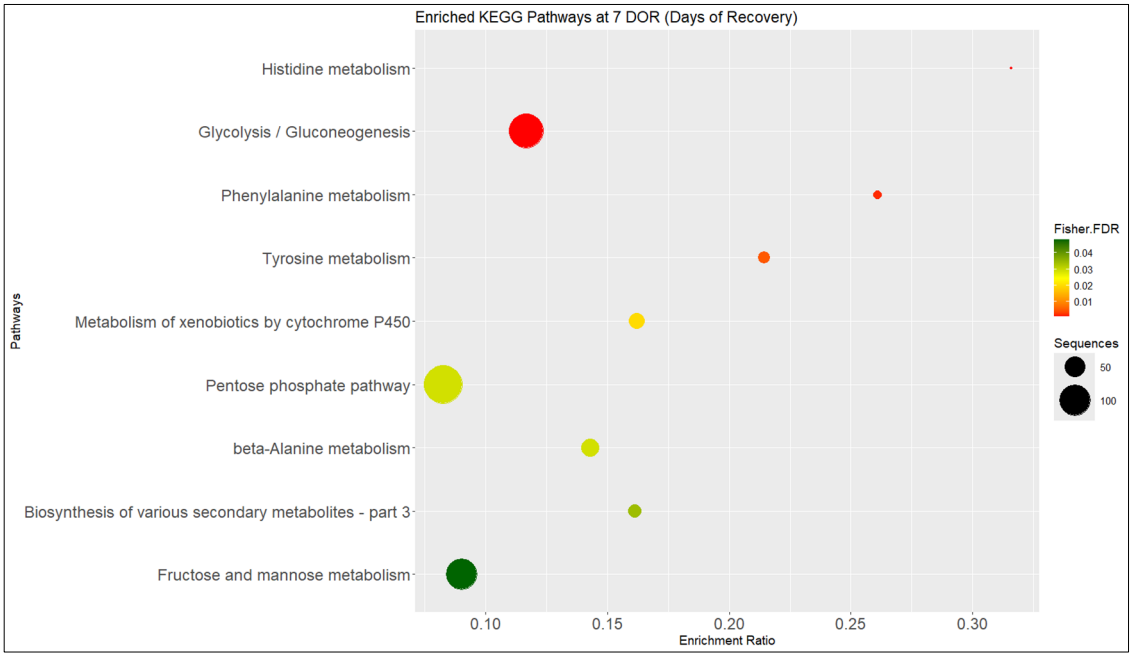

Figure S7 The top enriched KEGG pathways and their corresponding number of genes at 7 DOR at p-value ≤ 0.05. The x-axis represents the enrichment factor. The y-axis represents KEGG pathways. The circle represents the number of genes mapped to pathways and the red to green bar indicates the significance.

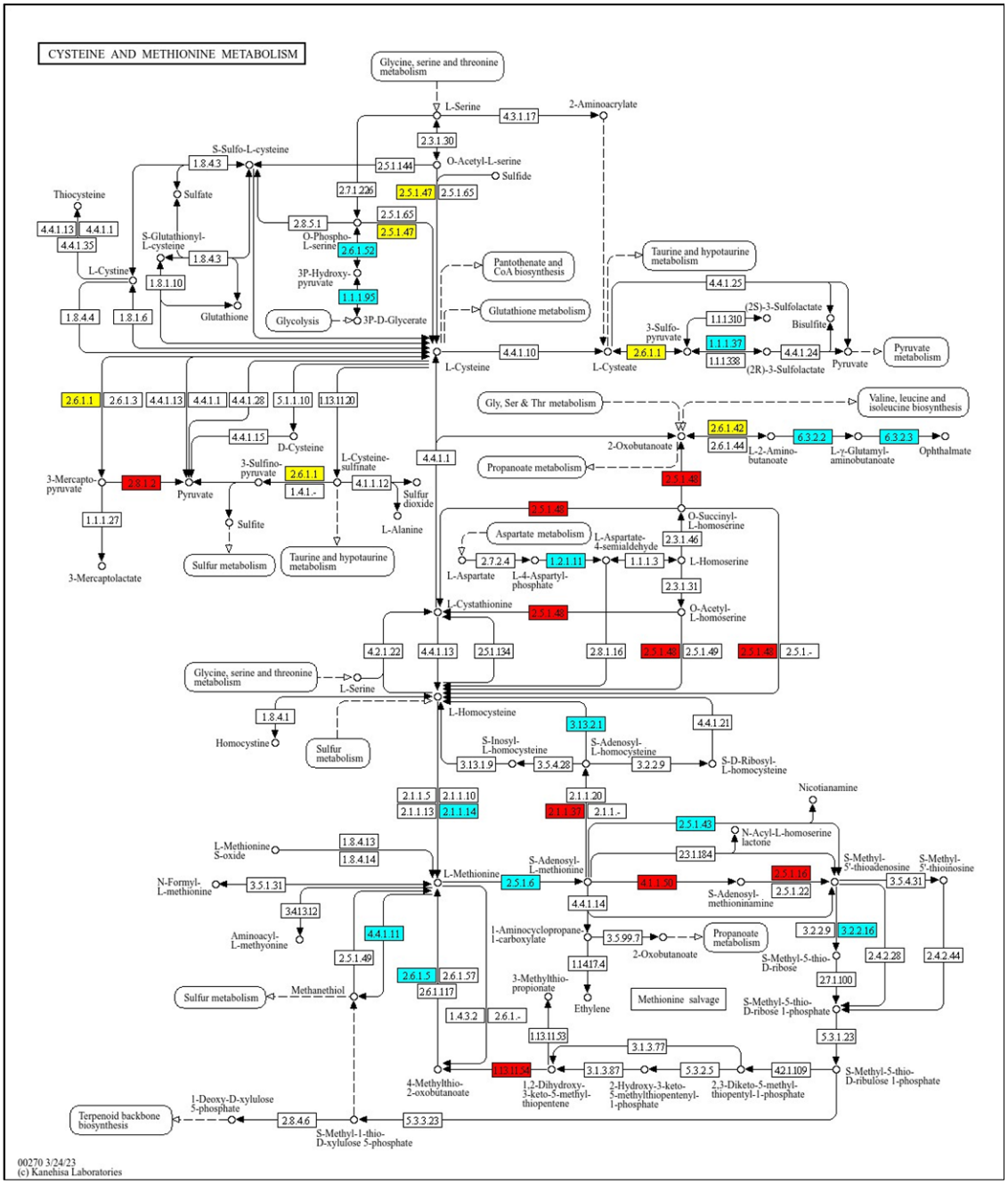

Figure S8 KEGG pathway for Cysteine and methionine metabolism at 2 DAT. *Blue denotes down-regulated genes, red denotes up-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

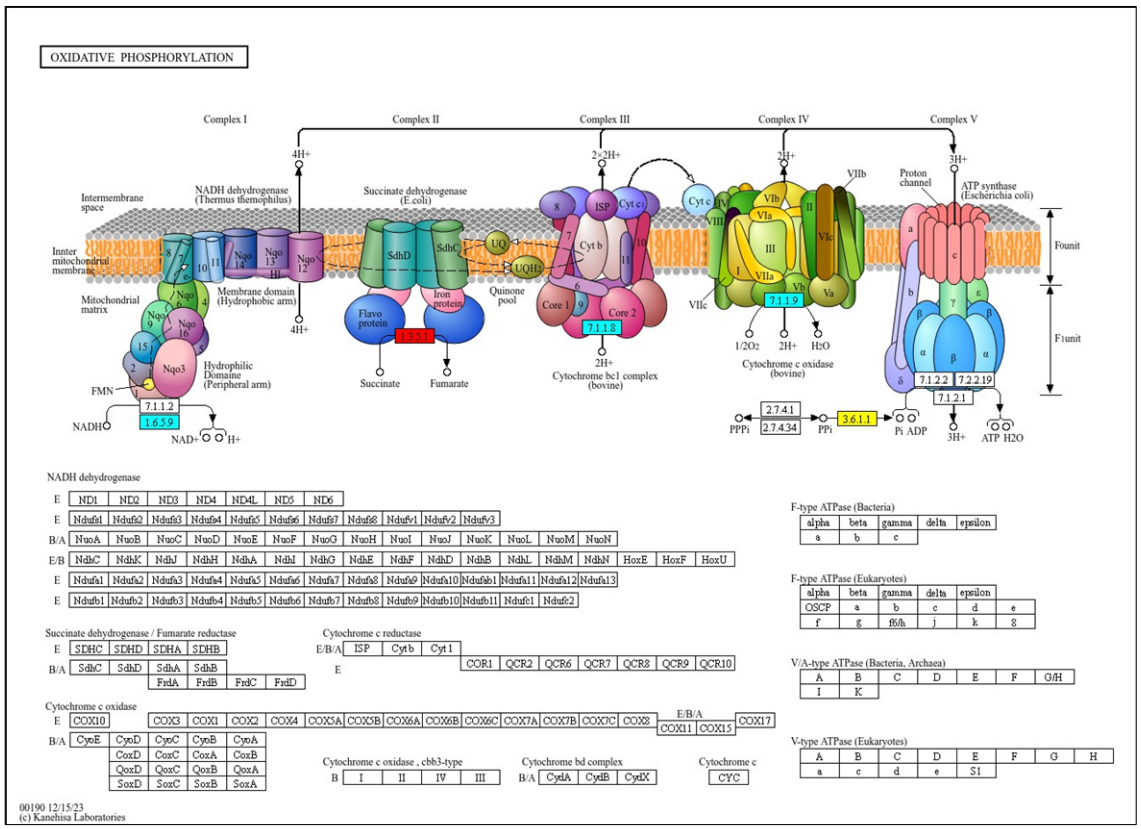

Figure S9 KEGG pathway for Oxidative phosphorylation at 2 DAT. *Blue denotes down-regulated genes, red denotes up-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

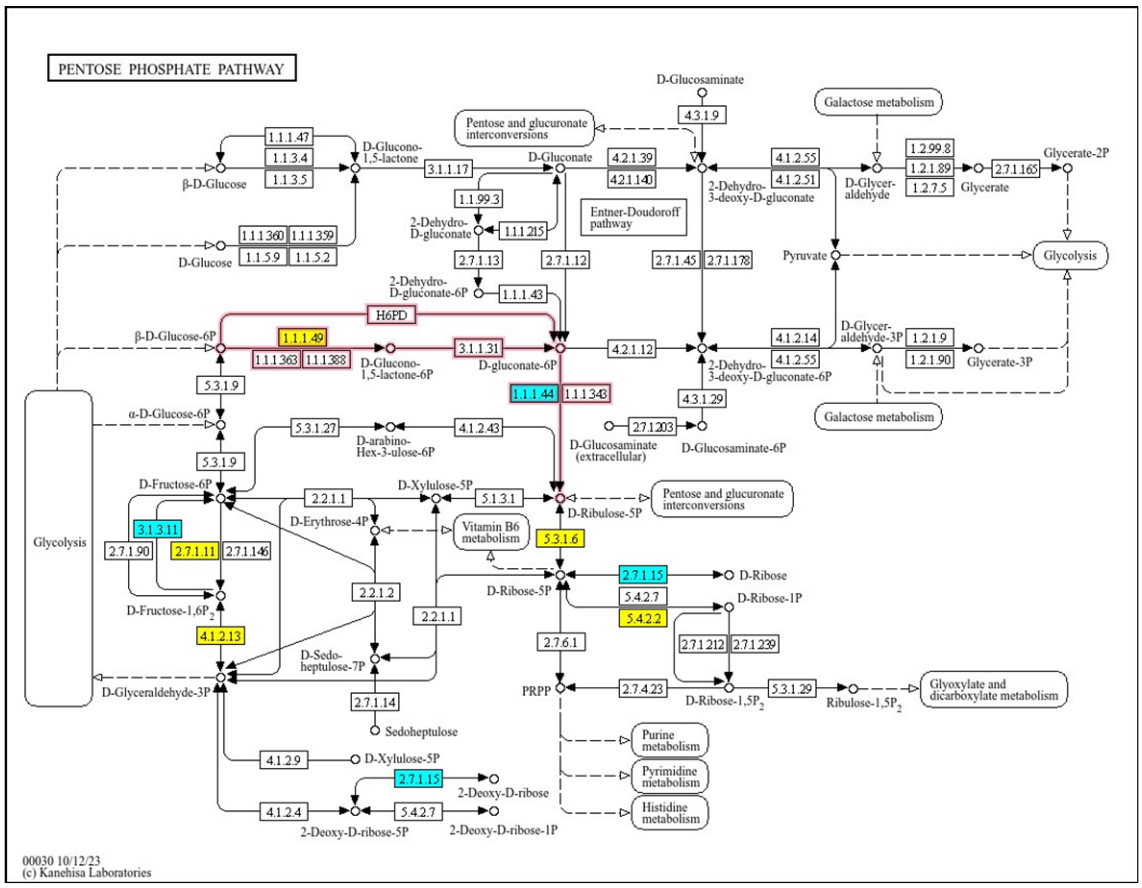

Figure S10 KEGG pathway for Pentose phosphate pathway (Oxidative branch is denoted by the red arrows) at 1 DAT. *Blue denotes down-regulated genes, and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

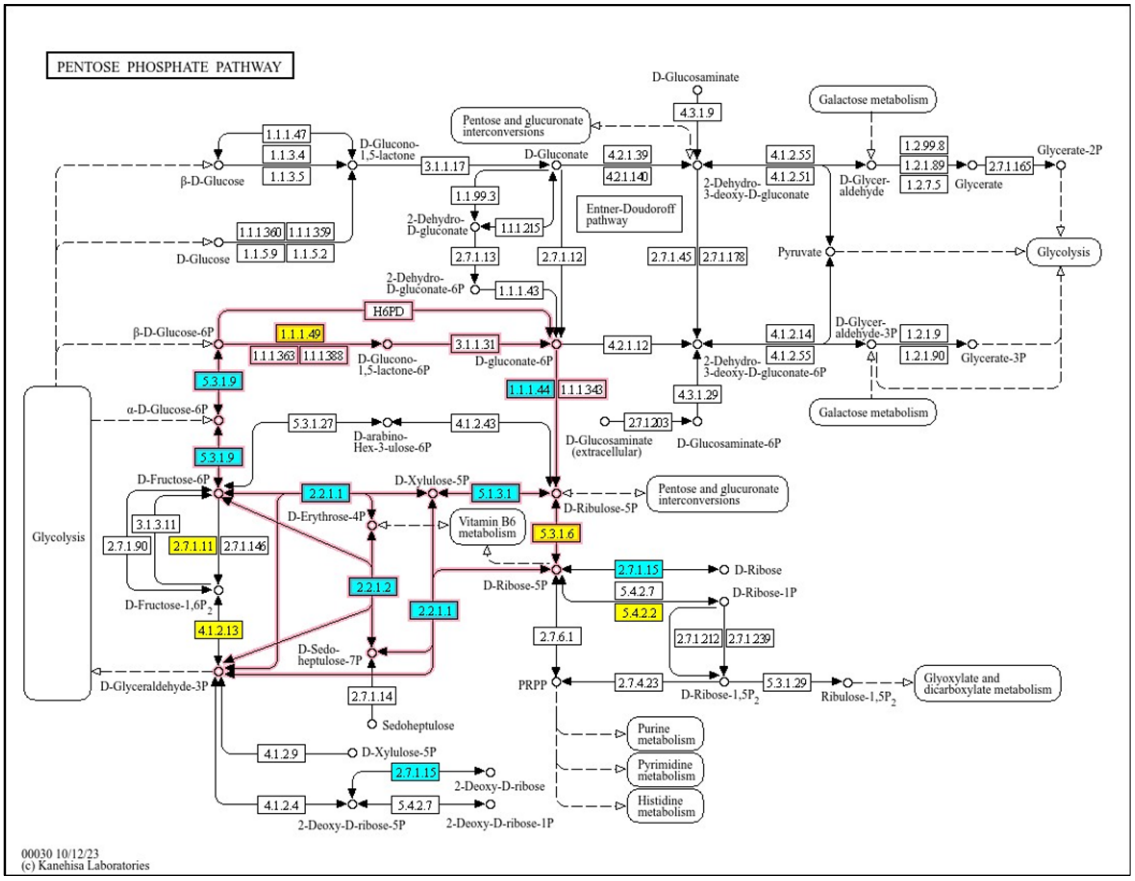

Figure S11 KEGG pathway for Pentose phosphate pathway (Oxidative and Non-Oxidative branches are denoted by red arrows) at 2 DAT. *Blue denotes down-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

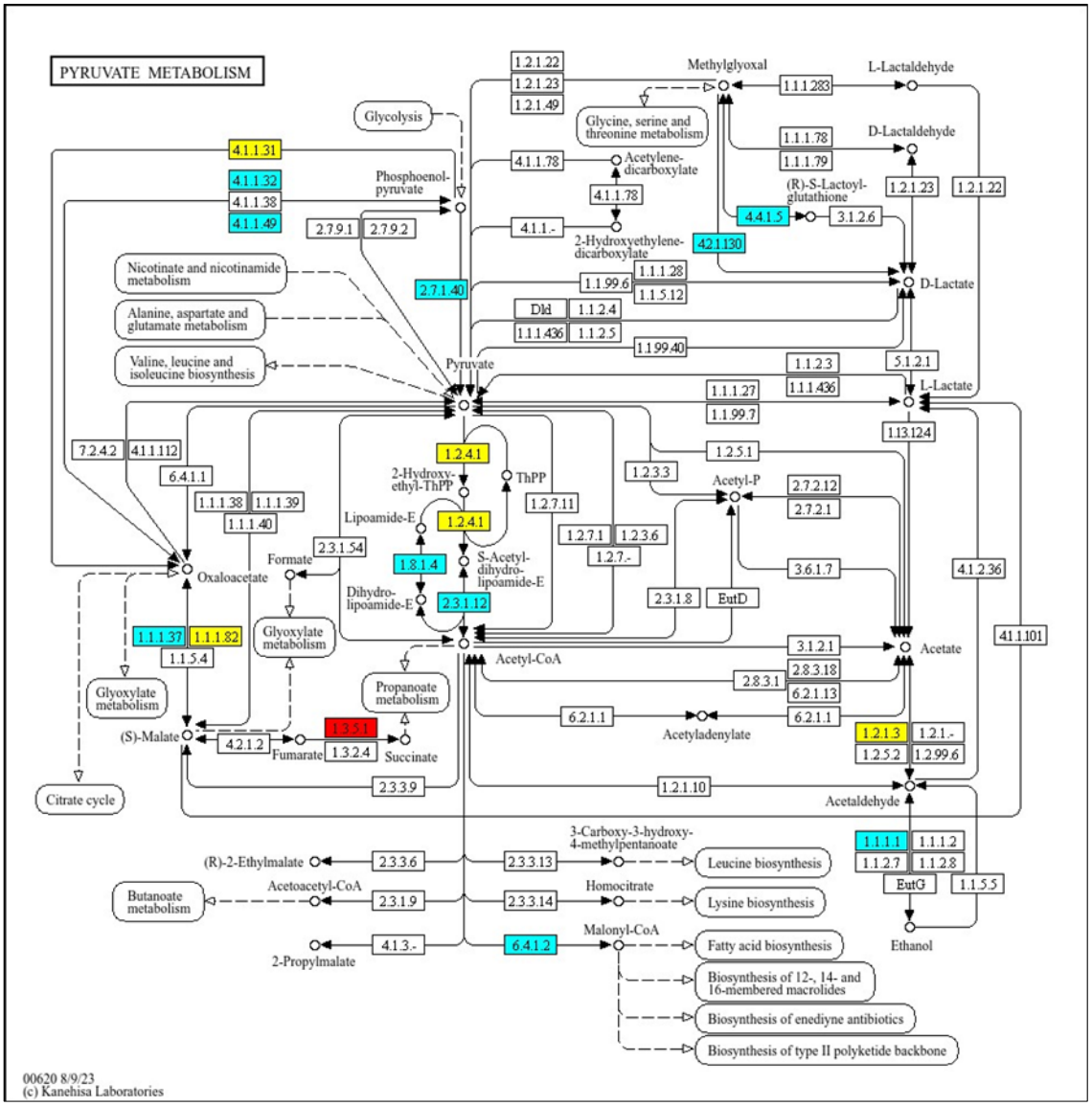

Figure S12 KEGG pathway for Pyruvate pathway at 2 DAT. *Blue denotes down-regulated genes, red denotes up-regulated genes and yellow denotes some isoforms were up-regulated and some isoforms were down-regulated.

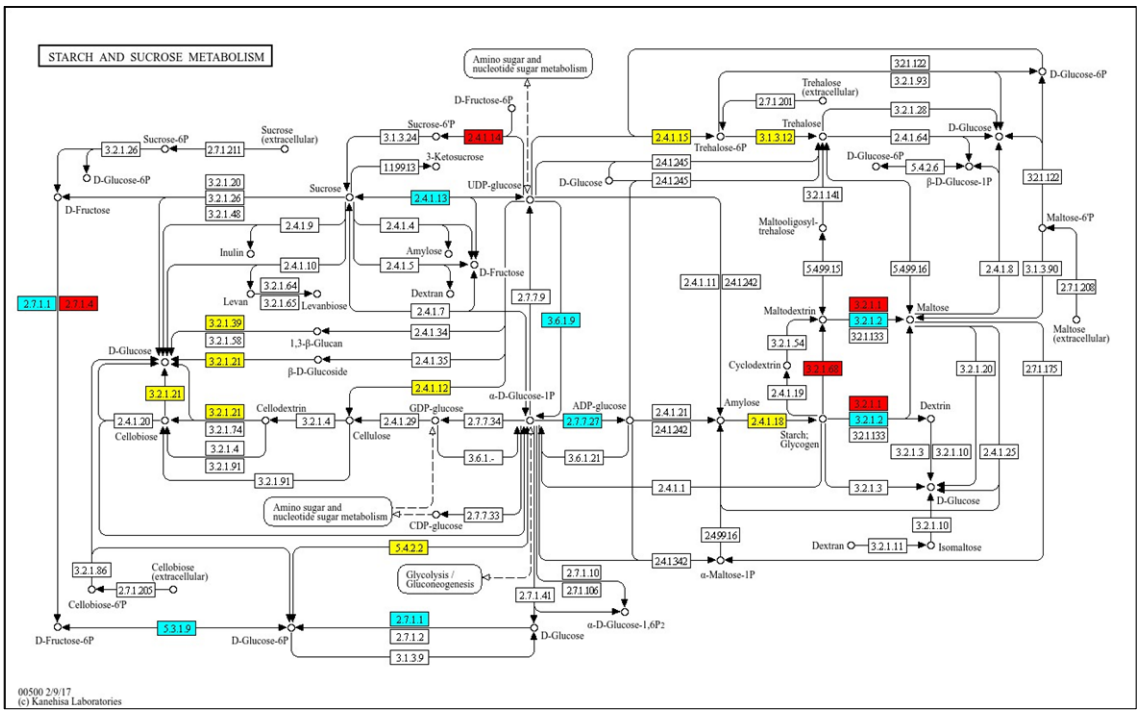

Figure S13 KEGG pathway for Starch and sucrose metabolism at 2 DAT. *Blue denotes down-regulated genes, and red denotes up-regulated genes.

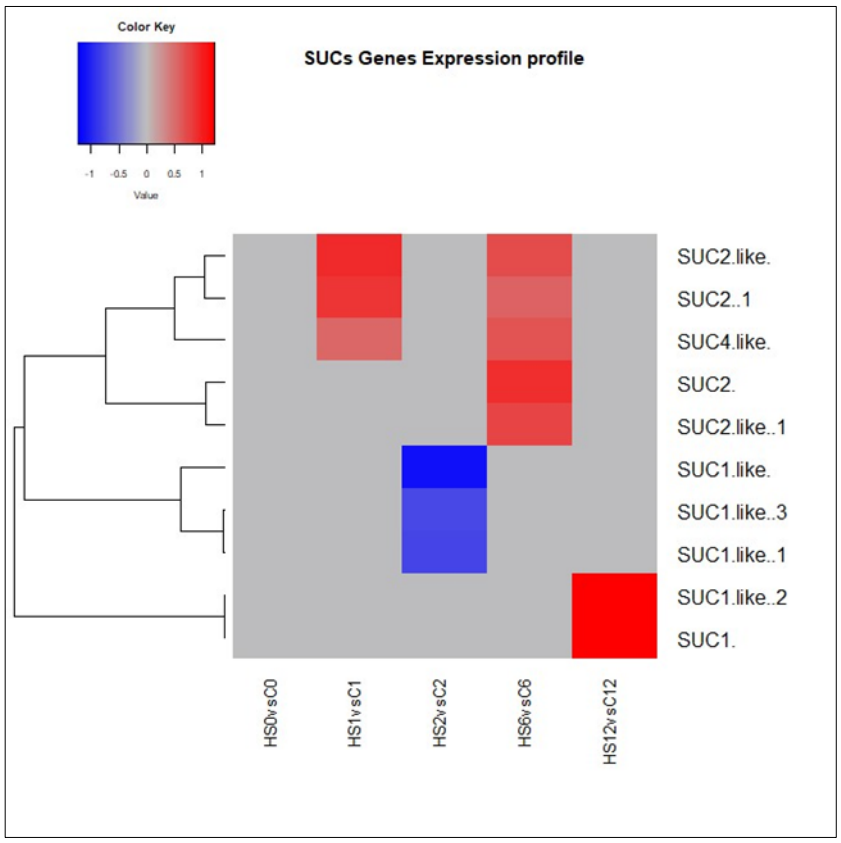

Figure S14 Heatmaps of DE sucrose transporters genes (*SUCs*) at each pairwise comparison. *B. napus* plants were subjected to heat treatment (30 °C/24 °C day/night) at 0-5 DAT or maintained under control temperatures (20 °C/14 °C day/night) at all timepoints. Total RNA was extracted and used to generate RNA seq data, with DEGs identified between heat and controls samples using |log2FC| ≥ 0.5 and adjusted P-value < 0.05 (using the Benjamini and Hochberg method) with n=5. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

Table S1 Data Quality Summary

Sample ID	Treatment Day	Condition	Tissue	Library ID	Raw reads	Raw data	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
D0C1	0	Control	Flower Buds	R11	42546892	6.4	98.24	0.03	96.74	91.33	43.59
D0C2	0	Control	Flower Buds	R12	43146158	6.5	98.22	0.03	96.35	90.41	43.11
D0C3	0	Control	Flower Buds	R13	48200214	7.2	98.23	0.03	96.63	91.05	43.06
D0C4	0	Control	Flower Buds	R14	47204032	7.1	98.31	0.03	95.99	89.72	43.62
D0C5	0	Control	Flower Buds	R15	46625810	7	98.33	0.03	96.13	89.91	43.29
D0HS1	0	Heat stress	Flower Buds	R16	48823460	7.3	98.42	0.03	96.11	89.91	43.61
D0HS2	0	Heat stress	Flower Buds	R17	42361588	6.4	98.21	0.03	95.72	89.14	43.52
D0HS3	0	Heat stress	Flower Buds	R18	47472104	7.1	98.3	0.03	96.54	90.88	43.36
D0HS4	0	Heat stress	Flower Buds	R19	40668522	6.1	98.3	0.03	96.35	90.47	43.35
D0HS5	0	Heat stress	Flower Buds	R20	44058576	6.6	98.2	0.03	96.1	89.98	43.34
D1C1	1	Control	Flower Buds	R21	44407298	6.7	98.53	0.03	96.46	90.65	44.78
D1C2	1	Control	Flower Buds	R22	42572118	6.4	98.55	0.03	96.4	90.57	45.12
D1C3	1	Control	Flower Buds	R23	45707162	6.9	98.54	0.03	96.65	91.14	44.99
D1C4	1	Control	Flower Buds	R24	39779066	6	98.55	0.03	96.25	90.28	44.6
D1C5	1	Control	Flower Buds	R25	48614394	7.3	98.21	0.03	96.3	90.47	44.96
D1HS1	1	Heat stress	Flower Buds	R26	45844218	6.9	98.37	0.03	96.43	90.59	43.9
D1HS2	1	Heat stress	Flower Buds	R27	47542486	7.1	98.21	0.03	96.07	89.82	44.54
D1HS3	1	Heat stress	Flower Buds	R28	47371258	7.1	98.46	0.03	96.52	90.88	44.61
D1HS4	1	Heat stress	Flower Buds	R29	46407612	7	98.58	0.03	96.58	91.05	44.59
D1HS5	1	Heat stress	Flower Buds	R30	42705194	6.4	98.7	0.03	95.71	89.03	44.59
D2C1	2	Control	Flower Buds	R31	48709190	7.3	98.45	0.03	96.39	90.54	45.57
D2C2	2	Control	Flower Buds	R32	50232808	7.5	98.68	0.03	96.24	90.25	45.61
D2C3	2	Control	Flower Buds	R33	42394356	6.4	98.72	0.03	96.86	91.66	45.25
D2C5	2	Control	Flower Buds	R35	49019624	7.4	98.26	0.03	96.62	90.98	45.2
D2HS1	2	Heat stress	Flower Buds	R36	43939042	6.6	98.46	0.03	96.65	91.08	45.12
D2HS2	2	Heat stress	Flower Buds	R37	45232502	6.8	98.6	0.03	96.83	91.45	44.77

D2HS3	2	Heat stress	Flower Buds	R38	41291490	6.2	98.43	0.03	96.6	90.94	44.93
D2HS4	2	Heat stress	Flower Buds	R39	44141240	6.6	98.52	0.03	96.57	90.87	44.94
D2HS5	2	Heat stress	Flower Buds	R40	40421822	6.1	98.54	0.03	96.48	90.68	44.36
D6C1	6	Control	Flower Buds	R41	45618730	6.8	98.3	0.03	96.09	89.94	43.28
D6C2	6	Control	Flower Buds	R42	47279800	7.1	98.36	0.03	96.88	91.63	43.5
D6C3	6	Control	Flower Buds	R43	49797014	7.5	98.42	0.03	96.2	90.13	43.04
D6C4	6	Control	Flower Buds	R44	48275232	7.2	98.45	0.03	95.79	89.21	43.72
D6C5	6	Control	Flower Buds	R45	41275426	6.2	98.51	0.03	95.91	89.47	43.53
D6HS1	6	Heat stress	Flower Buds	R46	45090682	6.8	98.26	0.03	96.04	89.81	43.4
D6HS2	6	Heat stress	Flower Buds	R47	46214376	6.9	97.73	0.03	96.51	90.89	43.35
D6HS3	6	Heat stress	Flower Buds	R48	44791070	6.7	98.28	0.03	96.38	90.48	43.85
D6HS4	6	Heat stress	Flower Buds	R49	43154676	6.5	98.09	0.03	95.9	89.59	43.06
D6HS5	6	Heat stress	Flower Buds	R50	48451812	7.3	98.31	0.03	96.35	90.49	43.31
D12C1	12	Control	Flower Buds	R51	42706732	6.4	98.61	0.03	96.19	90.2	45.62
D12C2	12	Control	Flower Buds	R52	45100386	6.8	98.52	0.03	97.7	93.57	45.32
D12C3	12	Control	Flower Buds	R53	49761388	7.5	98.61	0.03	97.43	92.94	45.52
D12C4	12	Control	Flower Buds	R54	55353976	8.3	98.67	0.03	97.45	92.98	45.8
D12C5	12	Control	Flower Buds	R55	43229896	6.5	98.7	0.03	97.49	93	45.51
D12HS1	12	Heat stress	Flower Buds	R56	39276294	5.9	98.68	0.03	97.39	92.84	44.61
D12HS1	12	Heat stress	Flower Buds	R57	43958400	6.6	98.8	0.03	97.61	93.31	44.4
D12HS2	12	Heat stress	Flower Buds	R58	49089438	7.4	98.67	0.03	97.43	92.92	44.69
D12HS3	12	Heat stress	Flower Buds	R59	43623028	6.5	98.71	0.03	97.54	93.2	44.56
D12HS4	12	Heat stress	Flower Buds	R60	42641344	6.4	98.62	0.03	97.47	93.04	44.27

Table S2 PCR timepoints tested per gene

Timepoints / Genes	0 (baseline)	1 DAT (24 hrs of HS*)	2 DAT (48 hrs of HS)	5 DAT (5 days of HS)	1 DOR (1 day of recovery)	7 DOR (7 days of recovery)
ACTIN	✓	✓	✓	✓	✓	✓
SS5	✓	X	✓	✓	✓	✓
HSP20	✓	X	✓	✓	✓	✓

***** HS: Heat Stress

Table S3 List of primer sequences of 3 selected genes and reference gene used for the qt-PCR analysis

Primer name	Forward primer sequence ((5' to 3')	Reverse primer sequence ((5' to 3')
BnaSS5	GGAGAAAACGCAGGGAAAG	TCAGGCAAACCAAGAACATC
BnaHSP20	CAAGGAGTATCAGCCAGGTG	TAGAGGGCATCGTCTTTCTC
BnaACTIN	TGGGTTTGCTGGTGACGAT	TGCCTAGGACGACCAACAATACT

Table S4 Correlation analysis of the results obtained from RNA-seq and qt-PCR for *SS5*, *HSP20* genes at different timepoints. HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

Log2 qt-PCR & RNA-seq values				
	SS5		***HSP20***	
Timepoints	**Qt-PCR**	**RNA-seq**	**Qt-PCR**	**RNA-seq**
HS0vsC0	0.141107	NA*	6.981295	7.6
HS1vsC1		NA		7.3
HS2vsC2	-0.48492	-1.1	8.550429	6.1
HS5vsC5	0.163089		5.673896	
HS6vsC6	0.814462	NA	3.146389	6.2
HS12vsC12	0.397126	NA	2.671107	NA
R	0.860104		0.631955	
R2	0.73978		0.399368	

NA*: Not DE at this contrast

Table S5 Differential expression data of sucrose synthesizing enzyme (sucrose phosphate synthase). HS0vsC0, HS1vsC1, HS2vsC2, HS6vsC6 & HS12vsC12 correspond to pairwise comparison of expression between heat stress and control at 0 DAT, 1 DAT, 2 DAT, 1 DOR & 7 DOR respectively.

Gene	HS0vsC0	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Seq. Description	Enz. Code
BnaA06G0367000WE	NA*	0.69	NA	0.9	0.7	Probable sucrose-phosphate synthase 2	EC:2.4.1.14
BnaC02G0093600WE	0.5	0.76	0.77	NA	NA	Sucrose-phosphate synthase 1-like	EC:2.4.1.14
BnaC07G0352200WE	0.8	1.1	1.0	1.4	0.94	Probable sucrose-phosphate synthase 2	EC:2.4.1.14
BnaA02G0084400WE	NA	-0.5	NA	NA	NA	sucrose-phosphate synthase 1-like	EC:2.4.1.14
BnaA10G0171700WE	-0.52	NA	NA	NA	NA	sucrose-phosphate synthase 1	EC:2.4.1.14

NA*: Not DE in this contrast

Table S6 DE of sucrose hydrolysing enzymes (cell wall invertase and sucrose synthase).

Gene	HS0vsC0	HS1vsC1	HS2vsC2	HS6vsC6	HS12vsC12	Seq. Description	Enz. Code
BnaA03G0187500WE	-1.1	NA*	-1.2	NA	NA	CWINV4-1; beta-fructofuranosidase	EC:3.2.1.26
BnaC01G0375500WE	NA	NA	NA	1.7	NA	CWINV1; beta-fructofuranosidase	EC:3.2.1.26
BnaC03G0139900WE	NA	NA	-1.7	NA	NA	CWINV4-1; beta-fructofuranosidase	EC:3.2.1.26
BnaC05G0441400WE	0.9	NA	NA	1.5	NA	CWINV5-2; beta-fructofuranosidase	EC:3.2.1.26
BnaA05G0141800WE	NA	NA	-1.19	NA	NA	Sucrose synthase 5	EC:2.4.1.13
BnaA07G0333600WE	NA	NA	-1.45	NA	NA	Sucrose synthase 6	EC:2.4.1.13
BnaA09G0016500WE	NA	NA	NA	-1.04	NA	Sucrose synthase 3	EC:2.4.1.13
BnaC06G0143400WE	NA	NA	-1.16	NA	NA	Sucrose synthase 5	EC:2.4.1.13
BnaA10G0167800WE	NA	NA	NA	NA	-0.8	Sucrose synthase -1 like	EC:2.4.1.13

NA*: Not DE in this contrast

Table S7 AtMYB2 interacting genes

Gene ID	Gene Name	Functional Annotation	GO terms
AT3G21910	*AT3G21910*	Domain of unknown function (DUF26)	extracellular region, molecular_function, biological_process
AT3G01500	*SABP3*	Carbonic anhydrase 1 (response to stress)	carbonate dehydratase activity, plasma membrane, response to cold, chloroplast thylakoid membrane, chloroplast stroma, defense response to fungus, incompatible interaction, chloroplast envelope, response to carbon dioxide, regulation of stomatal movement, stromule, photosynthesis, defense response to bacterium, apoplast, negative regulation of stomatal complex development, zinc ion binding, carbon utilization
AT4G04340	*AT4G04340*	ERD (early-responsive to dehydration stress) family protein	calcium activated cation channel activity, plasma membrane, cellular hyperosmotic response, regulation of calcium ion import, integral component of membrane, cation transmembrane transport, protein tetramerization
AT2G44140	*AT2G44140*	Peptidase family C54 protein	autophagosome assembly, mitophagy, cysteine-type endopeptidase activity, cytosol, C-terminal protein lipidation, protein targeting to membrane, protein processing, nucleophagy, protein delipidation
AT5G06700	*TBR*	Plant protein of unknown function (DUF828)	endosome, trans-Golgi network, plant-type cell wall modification, cellulose biosynthetic process, pectin biosynthetic process, integral component of membrane, O-acetyltransferase activity
AT5G13300	*VAN3*	ARF GTPase-activating protein	GTPase activator activity, protein binding, cytosol, plasma membrane, endocytosis, response to auxin, leaf morphogenesis, xylem and phloem pattern formation, phloem or xylem histogenesis, trans-Golgi network transport vesicle, phosphatidylinositol binding, positive regulation of GTPase activity, metal ion binding
AT2G39060	*SWEET9*	Nodulin MtN3 family protein	protein binding, plasma membrane, sucrose transmembrane transporter activity, vesicle membrane, trans-Golgi network membrane, nectar secretion, sucrose transport, cytoplasmic vesicle membrane, carbohydrate transmembrane transport, integral component of plasma membrane
AT5G25560	*AT5G25560*	CHY-type/CTCHY-type/RING-type Zinc finger protein	protein binding, zinc ion binding
AT4G12410	*AT4G12410*	SAUR-like auxin-responsive protein family	molecular_function, response to auxin
AT5G13220	*TIFY9*	jasmonate-zim-domain protein 10	nucleus, response to wounding, regulation of systemic acquired resistance, protein homodimerization activity, regulation of jasmonic acid mediated signaling pathway, regulation of transcription, DNA-templated, transcription corepressor activity, negative regulation of nucleic acid-templated transcription
AT3G28750	*AT3G28750*	NA	
AT5G23820	*ML3*	MD-2-related lipid recognition domain-containing protein	plant-type vacuole, protein binding, defense response, ER body
AT1G11860	*AT1G11860*	Glycine cleavage T-protein family	mitochondrion, chloroplast thylakoid, chloroplast stroma, chloroplast envelope, membrane, cytosolic ribosome, response to cadmium ion, apoplast, protein binding,

			glycine catabolic process, transaminase activity, methylation, aminomethyltransferase activity
AT5G02310	*PRT6*	proteolysis 6	ubiquitin-protein transferase activity, ubiquitin-dependent protein catabolic process, response to abscisic acid, regulation of seed germination, regulation of lipid catabolic process, zinc ion binding, protein ubiquitination, ligase activity, ubiquitin ligase complex, cytoplasm, ubiquitin protein ligase activity, ubiquitin-dependent protein catabolic process via the N-end rule pathway
AT5G01410	*RSR4*	Aldolase-type TIM barrel family protein	cytosol, plasma membrane, cellular amino acid metabolic process, response to lipid hydroperoxide, pyridoxine biosynthetic process, response to UV-B, response to non-ionic osmotic stress, endomembrane system, chlorophyll metabolic process, hyperosmotic salinity response, protein homodimerization activity, protein heterodimerization activity, pyridoxal phosphate biosynthetic process, pyridoxal 5'-phosphate synthase (glutamine hydrolysing) activity
AT1G13970	*AT1G13970*	Protein of unknown function (DUF1336)	molecular_function
AT3G01240	*AT3G01240*	NA	molecular_function, biological_process
AT3G06010	*CHR12*	Homeotic gene regulator	response to heat, response to water deprivation, response to salt stress, maintenance of root meristem identity, maintenance of shoot apical meristem identity, histone binding, helicase activity, nucleus, covalent chromatin modification, regulation of growth, DNA binding, ATP binding
AT3G54360	*AT3G54360*	zinc ion binding	protein binding, nucleus, cytosol, positive regulation of catalase activity, zinc ion binding, nuclear heterochromatin, maintenance of DNA methylation, protein ubiquitination, ubiquitin protein ligase activity, regulation of methylation-dependent chromatin silencing
AT2G03750	*AT2G03750*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	cytoplasm, sulfotransferase activity
AT3G22200	*POP2*	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein (auxin biosynthesis)	mitochondrion, sucrose metabolic process, inositol metabolic process, glutamate decarboxylation to succinate, glutamine metabolic process, response to oxidative stress, zinc ion binding, gamma-aminobutyric acid catabolic process, response to salt stress, pollen tube growth, pollen tube adhesion, response to organic substance, fruit development, pollen tube guidance, beta-alanine catabolic process, 4-aminobutyrate:pyruvate transaminase activity, root development, shoot system development, cobalt ion binding, pyridoxal phosphate binding
AT3G28007	*SWEET4*	Nodulin MtN3 family protein (transport of sugars, hormone etc..)	protein binding, plasma membrane, protein homooligomerization, carbohydrate transmembrane transport, integral component of plasma membrane, sugars transmembrane transporter activity
AT1G72660	*AT1G72660*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	GTPase activity, GTP binding, GDP binding, cytoplasm
AT5G35210	*PTM*	metalloendopeptidases;zinc ion binding;DNA binding (remodelling of ECM)	RNA polymerase II core promoter sequence-specific DNA binding, nucleus, chloroplast outer membrane, chloroplast-nucleus signaling pathway, positive regulation of transcription, DNA-templated, protein

			binding, zinc ion binding, integral component of membrane
AT1G52410	*TSA1*	TSK-associating protein 1 (induced by methyl jasmonate)	tryptophan biosynthetic process, tryptophan synthase activity, calcium ion binding, protein binding, vacuole, peroxisome, chloroplast thylakoid membrane, gravitropism, photomorphogenesis, defense response to fungus, nuclear envelope, endoplasmic reticulum lumen, cellular amino acid biosynthetic process, auxin biosynthetic process, indole-3-glycerol-phosphate lyase activity
AT4G26570	*CBL3*	calcineurin B-like 3	protein binding, plasma membrane, plant-type vacuole membrane, potassium ion homeostasis, calcium ion binding, detection of calcium ion
AT1G51745	*AT1G51745*	Tudor/PWWP/MBT superfamily protein	molecular_function, biological_process
AT5G04560	*DME*	HhH-GPD base excision DNA repair family protein	DNA-(apurinic or apyrimidinic site) lyase activity, DNA methylation, regulation of gene expression by genetic imprinting, embryo development ending in seed dormancy, DNA N-glycosylase activity, polar nucleus, base-excision repair, 4 iron, 4 sulfur cluster binding, DNA binding, regulation of transcription, DNA-templated, metal ion binding
AT3G12020	*AT3G12020*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	ATP binding, microtubule binding, microtubule, chloroplast, microtubule motor activity, kinesin complex, microtubule-based movement, ATPase activity
AT3G20290	*EHD1*	EPS15 homology domain 1	protein binding, endosome, endocytosis, plasmodesma, membrane, endocytic recycling, hyperosmotic salinity response, protein homooligomerization, GTP binding, plasma membrane, endosome membrane, hydrolase activity, calcium ion binding
AT1G16850	*AT1G16850*	NA	endoplasmic reticulum, response to salt stress, molecular_function
AT3G22740	*HMT3*	homocysteine S-methyltransferase 3	plasma membrane, methionine biosynthetic process, methylation, metal ion binding, S-adenosylmethionine-homocysteine S-methyltransferase activity
AT1G23230	*AT1G23230*	NA	mediator complex, regulation of transcription, DNA-templated, molecular_function
AT1G28340	*RLP4*	receptor like protein 4	endosome, trans-Golgi network, protein binding, integral component of membrane, signal transduction
AT2G02080	*IDD4*	indeterminate(ID)-domain 4	metal ion binding, DNA binding, regulation of transcription, DNA-templated, chloroplast, transcription factor activity, sequence-specific DNA binding
AT1G14250	*AT1G14250*	GDA1/CD39 nucleoside phosphatase family protein	vacuole, ATP binding, integral component of membrane, hydrolase activity
AT1G01060	*LHY*	Homeodomain-like superfamily protein	transcription factor activity, sequence-specific DNA binding, response to cold, response to salt stress, response to ethylene, response to auxin, response to abscisic acid, response to gibberellin, response to salicylic acid, response to jasmonic acid, negative regulation of circadian rhythm, negative regulation of sequence-specific DNA binding transcription factor activity, transcription regulatory region DNA binding, response to cadmium ion, long-day photoperiodism, flowering, nucleus

AT2G43210	*AT2G43210*	Ubiquitin-like superfamily protein	protein binding, cellular_component, biological_process
AT1G15500	*ATNTT2*	TLC ATP/ADP transporter	chloroplast envelope, ATP binding, integral component of membrane, ADP transport, ATP transport, chloroplast membrane, ATP:ADP antiporter activity
AT2G35940	*EDA29*	BEL1-like homeodomain 1	DNA binding, nucleus, cytoplasm, response to symbiotic fungus, response to abscisic acid, polar nucleus fusion, response to continuous far red light stimulus by the high-irradiance response system, protein homodimerization activity, protein heterodimerization activity, regulation of transcription, DNA-templated, transcription factor activity, sequence-specific DNA binding
AT4G13430	*IIL1*	isopropyl malate isomerase large subunit 1 (2nd metabolism)	chloroplast stroma, glucosinolate biosynthetic process, response to cadmium ion, intramolecular transferase activity, transferring hydroxy groups, 4 iron, 4 sulfur cluster binding, 3-isopropylmalate dehydratase activity, branched-chain amino acid biosynthetic process, leucine biosynthetic process, metal ion binding
AT3G44300	*NIT2*	nitrilase 2 (auxin responsive)	cytosol, response to bacterium, response to cadmium ion, indole-3-acetonitrile nitrilase activity, indole-3-acetonitrile nitrile hydratase activity, nitrogen compound metabolic process, plasma membrane, indoleacetic acid biosynthetic process
AT3G62110	*AT3G62110*	Pectin lyase-like superfamily protein (cell wall biosynthesis)	vacuole, extracellular region, lyase activity, cell wall organization, polygalacturonase activity, carbohydrate metabolic process
AT2G32240	*AT2G32240*	NA	plasma membrane, response to abscisic acid, response to cadmium ion, integral component of membrane, molecular_function
AT4G25200	*HSP23.6-MITO*	mitochondrion-localized small heat shock protein 23.6	response to cadmium ion, mitochondrion, molecular_function, response to heat
AT2G40130	*SMXL8*	Double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein	response to strigolactone, ATP binding, protein metabolic process, nucleus, regulation of transcription, DNA-templated
AT3G47800	*AT3G47800*	Galactose mutarotase-like superfamily protein (carbohydrate and sugars binding)	carbohydrate binding, aldose 1-epimerase activity, galactose metabolic process
AT1G56070	*LOS1*	Ribosomal protein S5/Elongation factor G/III/V family protein	copper ion binding, nucleolus, vacuolar membrane, cytosol, plasma membrane, plasmodesma, chloroplast, cold acclimation, response to cytokinin, apoplast, GTP binding, translational elongation, translation elongation factor activity, GTPase activity
AT3G18860	*AT3G18860*	transducin family protein / WD-40 repeat family protein (flavonoid bio)	protein binding, nucleotide binding, biological_process
AT5G65750	*AT5G65750*	2-oxoglutarate dehydrogenase, E1 component (2nd metabolism-flavonoid syn etc..)	mitochondrion, zinc ion binding, response to cadmium ion, cobalt ion binding, thiamine pyrophosphate binding, oxoglutarate dehydrogenase (succinyl-transferring) activity, tricarboxylic acid cycle, oxoglutarate dehydrogenase complex

AT3G20270	*AT3G20270*	lipid-binding serum glycoprotein family protein	lipopolysaccharide binding, regulation of gene expression, apoplast, reactive oxygen species biosynthetic process, protein binding, extracellular space
AT3G08860	*PYD4*	PYRIMIDINE 4 (b-alanine aminotransferase)-lignin biosynthesis	cellular response to nitrogen levels, photorespiration, mitochondrion, alanine-glyoxylate transaminase activity, pyridoxal phosphate binding, identical protein binding

Table S8 Two-way mixed ANOVA table for fructose

Effect	DFn	DFd	F	p	p..05	ges
ow names nt	1	8	2.772	1.34e-01		0.074
Timepoint	4	32	19.793	2.75e-08	*	0.656
Treatment:Timepoint	4	32	3.774	1.30e-02	*	0.266

Table S9 Two-way mixed ANOVA table for glucose

Effect	DFn	DFd	F	p	p..05	ges
Treatment	1	8	6.005	4.00e-02	*	0.066
Timepoint	4	32	10.598	1.39e-05	*	0.545
Treatment:Timepoint	4	32	5.016	3.00e-03	*	0.362

Table S10 Two-way mixed ANOVA table for sucrose

Effect	DFn	DFd	F	p	p..05	ges
Treatment	1.00	8.00	0.235	0.641000		0.013
Timepoint	1.47	11.75	16.180	0.000813	*	0.535
Treatment:Timepoint	1.47	11.75	3.443	0.077000		0.197

Table S11 Two-way mixed ANOVA table for glucobrassicin (GBS)

Effect	DFn	DFd	F	p	p..05	ges
Treatment	1	8	1.675	0.23200		0.066
Timepoint	4	32	7.337	0.00026	*	0.378
Treatment:Timepoint	4	32	4.002	0.01000	*	0.249

Table S12 Two-way mixed ANOVA table for gluconapin (GNA)

Effect	DFn	DFd	F	p	p..05	ges
Treatment	1	8	19.392	2.00e-03	*	0.347
Timepoint	4	32	6.729	4.77e-04	*	0.396
Treatment:Timepoint	4	32	11.218	8.41e-06	*	0.523

Table S13 Two-way mixed ANOVA table for progoitrin (PRO)

Effect	DFn	DFd	F	p	p..05	ges
Treatment	1	8	5.717	4.40e-02	*	0.129
Timepoint	4	32	9.919	2.45e-05	*	0.495
Treatment:Timepoint	4	32	7.875	1.55e-04	*	0.438

Appendix B

NetViewer Workflow

This section provides a step-by-step explanation on how to extend a gene network with interactions from transcription factors (TFs) using NetViewer app in Cytoscape.

B.1 System Requirements

- An internet connection
- Cytoscape software installed with version 3.7 as a minimum version requirement. Cytoscape installation steps can be found here: https://cytoscape.org/download.html

B.2 Install NetViewer in Cytoscape

- To install the application, the user is required to add the application jar file into Cytoscape configuration at the following location: C:\Users\PathToCytoscapeConfigurationFolder\CytoscapeConfiguration\3\apps\installed
- The application jar file is available at the project target folder.
- The user is also required to add the jgoodies-forms-1.0.5.jar and collections-generic-4.0.jar to Cytoscape configuration available at: https://mvnrepository.com/artifact/jgoodies/forms/1.0.5 and https://mvnrepository.com/artifact/collections-generic/collections-generic/4.01 respectively.
- The application can be accessed from Cytoscape menu bar as follows: Apps » App Store » Install Apps from File
- Then, selecting the application jar file from the application project target folder.
- The config files required for database files conversion should be available to the user prior to running the pipeline.

B.3 Load the initial Network into Cytoscape

- After launching Cytoscape, the gene list of interest is imported in a tab-delimited file from Cytoscape file menu (Figure B1), forming the initial network, as follows:

  File » Import » Network from File

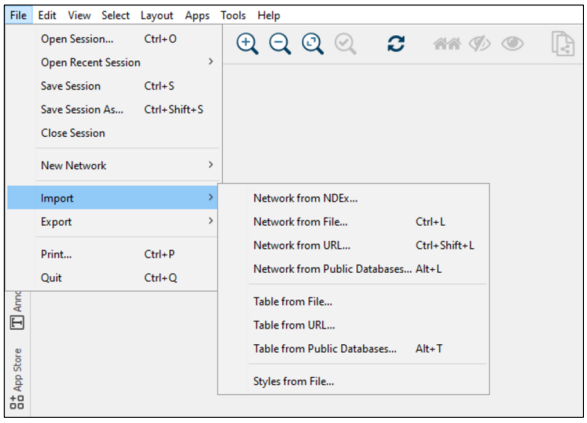

Figure B1 Importing input files

- The corresponding *A. thaliana* IDs can either be added as a column to the initial gene list file or imported as a separate tab-delimited file from Cytoscape file menu, as follows:

  File » Import » Table from File

- Additional data such as differential expression data, functional annotations, GO terms and IDs can also be imported in the same way, should the user wish to display this information on the extended network.
- Cytoscape will then ask the user to choose the source node and attribute columns. This can be done by clicking on the column name, then choosing the green circle icon for source node and the green page icon for the attribute as displayed in Figure B2. After importing the gene list and attributes, an initial network is displayed (Figure B3).

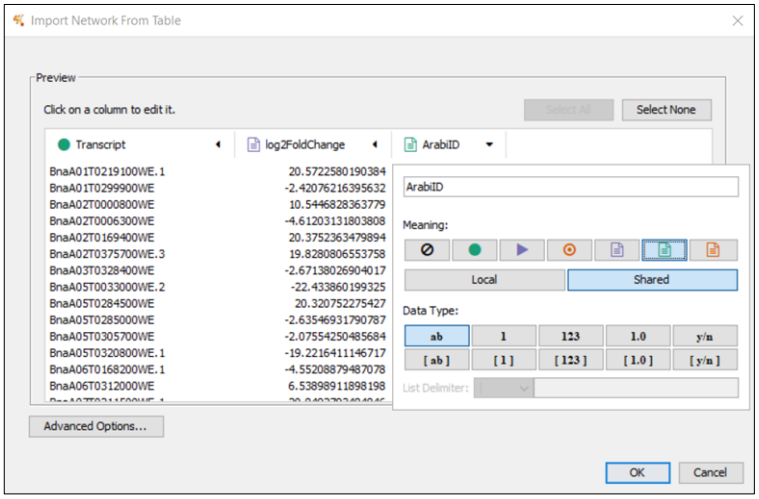

Figure B2 Selecting source nodes and attribute columns

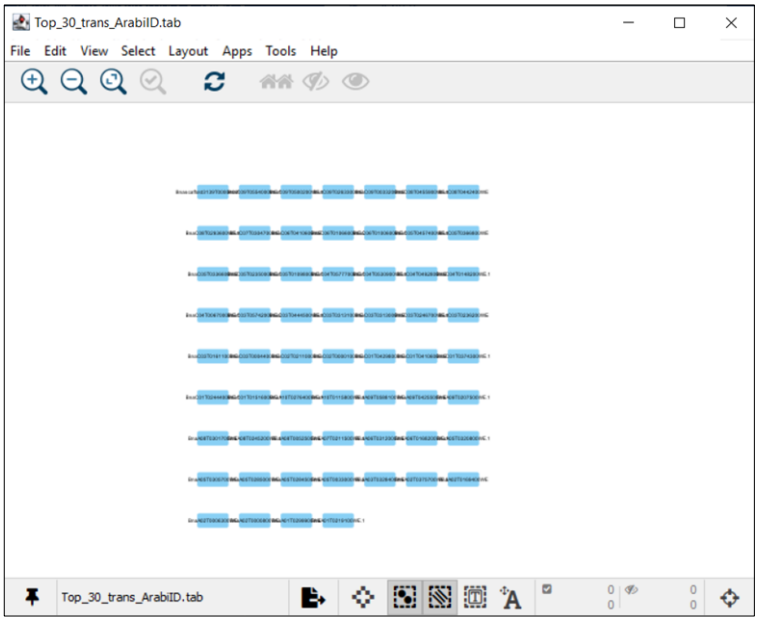

Figure B3 Initial network

B.4 Launching NetViewer

- NetViewer can then be launched from Cytoscape Apps menu, as shown in Figure B4.

  Apps » NetViewer » Extend network

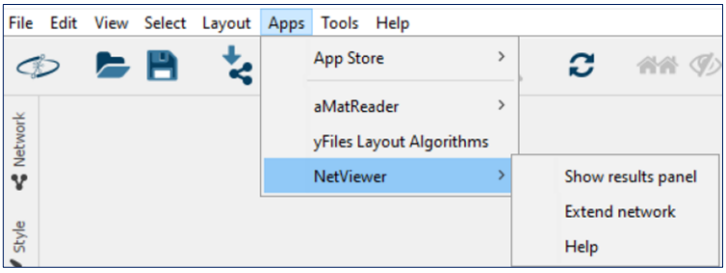

Figure B4 Launching NetViewer from Cytoscape Apps menu

B.5 Extend network with regulatory information

- As Figure B5 shows, the user selects the network that they initially uploaded in section B.3, through a drop-down list from the User Network section. The user also needs to select the network attribute column. This column serves as an identifier mapping to map the genes in the database and needs to contain one of the supported identifiers.

- Using Database selection feature, the user has the choice to upload a local database file through navigating the user's file system or access an online database from PlantTFDB. Once the database file is chosen, the file will then be converted into Linkset file in an XGMML format (the eXtensible Graph Markup and Modelling Language).

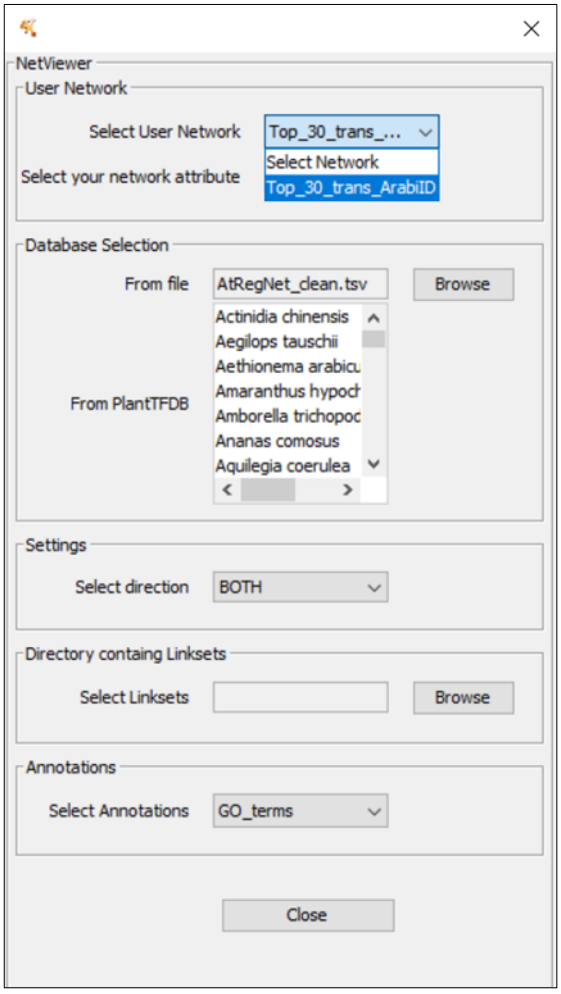

Figure B5 NetViewer interface

- Once the conversion of the database file is finished and the Linkset file is generated, a message informing the user with the completion of file conversion is displayed (Figure B6). Next, the user will choose the direction of interactions from the Settings. The available options are: Both, Sources and Targets.

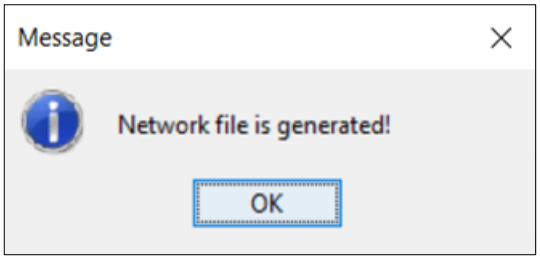

Figure B6 A message to inform the user that database file conversion is complete and the Linkset file is generated.

- From the Directory containing Linksets section, the user navigates through the user's file system to choose the generated Linkset file. A new window will open giving the user the choice to select one or more files (Figure B7).

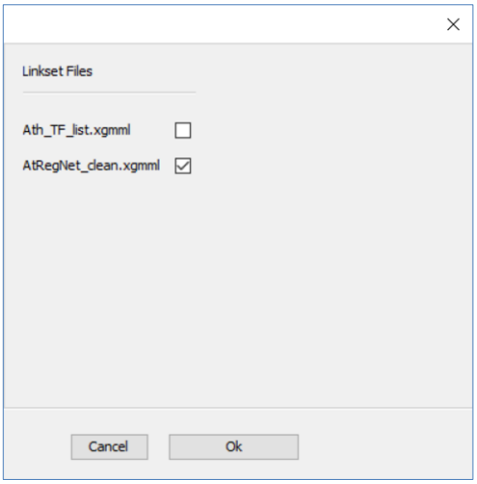

Figure B7 Linksets files selection

- This will be followed by choosing which annotations to display upon hovering on the network nodes from the Annotations section. The user has the option to display GO terms, GO IDs or a short description of the genes function.
- By clicking Ok, the network is extended with nodes representing genes and TFs and edges representing the interactions between them (Figure B8). Network display might take some time depending on the size of the original network and the Linkset file.

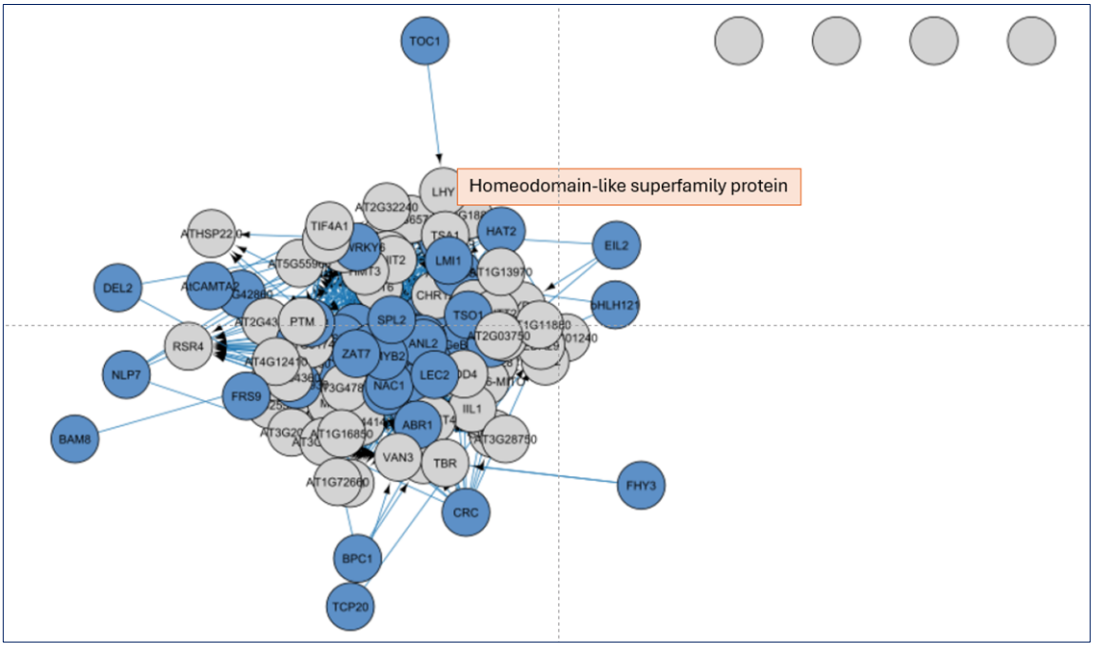

Figure B8 Extended network of interactions between genes (grey nodes) and TFs (Blue nodes). Grey nodes represent the genes and blue nodes represent TFs. The blue edges represent the interactions between the genes and TFs. Upon hovering on the nodes, the selected annotation will appear (orange box).

- After displaying the network, the user can use Cytoscape functionalities to customise the layout of the network. If more than one Linkset file were selected, the user can display the number of interactions per each Linkset file, as follows:

  Apps » NetViewer » Show result panel

- The right panel in Figure B9 shows results from two linksets files used to display interactions between a list of genes and TFs. Different colours for edges representing interactions generated from different linksets files.

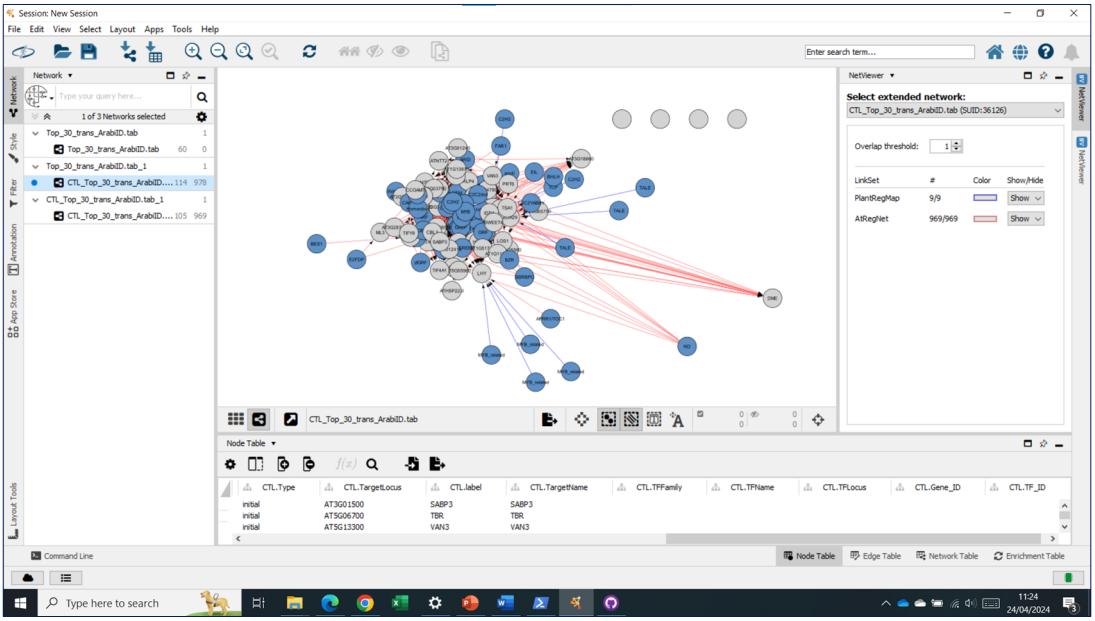

Figure B9 Main panel shows the extended network with interactions generated from two Linksets files. Right panel shows the number of interactions per Linkset file