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Stage-Specific Regulatory Network Inference for Heat-Induced Silique Branching in Brassica napus

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Abstract:

Climate change and the associated increase in global average temperatures pose a severe threat to agricultural productivity worldwide. Brassica napus, commonly known as rapeseed or canola, is a globally significant oilseed crop that exhibits high sensitivity to heat stress, particularly during its reproductive stages. Elevated temperatures during flowering and early silique development frequently lead to structural anomalies, including heat-induced silique branching, which ultimately affects seed yield and oil quality. Understanding the molecular mechanisms underpinning this morphological adaptation requires a comprehensive temporal analysis of gene expression. This study aims to construct stage-specific gene regulatory networks to elucidate the complex, time-dependent transcriptional reprogramming driving heat-induced silique branching in Brassica napus. By utilizing high-resolution time-course transcriptomic data collected across early, intermediate, and late stages of heat stress, we employ advanced computational inference methodologies to identify dynamic regulatory modules and crucial hub genes. Our analysis reveals stage-specific activation of heat shock factors, accompanied by intricate cross-talk with phytohormone signaling pathways, specifically auxin and cytokinin, which dictate architectural plasticity. The identification of stage-specific regulatory hubs offers unprecedented insights into the chronobiology of stress-induced developmental plasticity. These findings provide a vital genomic resource and theoretical foundation for future breeding programs aimed at engineering climate-resilient Brassica napus cultivars through targeted genetic manipulation of temporal stress responses.

1.INTRODUCTION

1.1 Background of Environmental Stress in Agricultural Systems

The rapid acceleration of global climate change has drastically altered the environmental conditions under which major agricultural crops are cultivated. Among these changes, the increasing frequency, duration, and intensity of heatwaves constitute one of the most critical challenges to global food security. Heat stress profoundly disrupts standard plant developmental programs, leading to impaired vegetative growth, reduced reproductive success, and significant yield penalties. Crops exposed to supra-optimal temperatures experience a wide array of physiological and biochemical dysfunctions, including the excessive production of reactive oxygen species, destabilization of cellular membranes, inhibition of photosynthetic machinery, and widespread denaturation of proteins. In response to these severe environmental challenges, plants have evolved sophisticated molecular and physiological mechanisms to endure heat stress. These compensatory mechanisms often manifest in visible architectural alterations. For many crops, the timing of the heat stress event is just as critical as its magnitude. Exposure during the sensitive reproductive phase is particularly detrimental, as it directly impacts organogenesis, fertilization, and subsequent seed set [1]. Consequently, researchers have increasingly prioritized understanding the molecular pathways that govern plant responses to high-temperature stress during reproductive development. Brassica napus is particularly vulnerable to heat stress during its flowering and silique development phases, making it an ideal model for investigating heat-induced developmental plasticity.

1.2 The Phenomenon of Heat-Induced Silique Branching

Brassica napus is cultivated primarily for its high-quality edible oil and protein-rich meal. The yield of this crop is directly determined by the number of siliques per plant, the number of seeds per silique, and the individual seed weight. Under normal physiological conditions, the silique develops as a single, unbranched pod containing the seeds. However, recent agronomic observations have highlighted a unique morphological anomaly triggered by transient heat stress: the phenomenon of silique branching. When plants encounter extreme temperature spikes during the early stages of silique elongation, the apical meristematic tissues within the developing fruit experience a disruption in hormonal gradients. This disruption can trigger the initiation of secondary meristems along the main axis of the silique, resulting in a branched pod structure [2]. While this branching might initially appear as a compensatory mechanism to increase seed-bearing capacity, it frequently results in smaller, deformed seeds with altered lipid profiles and reduced viability. The developmental shift from a linear silique to a branched architecture requires massive transcriptomic reprogramming, involving the coordinated action of thousands of genes over distinct temporal phases. Early responses typically involve rapid stress sensing and signal transduction, whereas later stages are characterized by structural remodeling and altered developmental patterning [3, 4]. Understanding the precise timing and sequence of these molecular events is essential for dissecting the mechanisms of heat-induced silique branching.

1.3 Motivation for Stage-Specific Network Inference

The complex transition from a standard silique to a branched structure cannot be attributed to the action of a single gene or a simple linear pathway. Instead, it is governed by highly interconnected and dynamic gene regulatory networks. Traditional transcriptomic studies in Brassica napus have largely relied on static analytical approaches, comparing gene expression at a single time point following stress treatment to a control condition [5]. While these studies have successfully identified key heat shock proteins and stress-responsive transcription factors, they inherently fail to capture the temporal fluidity of the regulatory landscape. Gene networks are not static entities; their topology, hub genes, and modular organization change dramatically as the plant progresses from immediate stress shock to acclimation and subsequent developmental alteration. A regulatory

interaction that is critical during the first hour of heat stress may be entirely dismantled and replaced by different regulatory complexes a few days later when the physical branching actually initiates [6]. Therefore, there is a compelling need to develop and apply stage-specific regulatory network inference methodologies. By dissecting the transcriptomic data into distinct temporal stages, researchers can trace the evolutionary trajectory of the gene networks. This approach allows for the identification of transiently active hub regulators that initiate the branching process, which might be missed in aggregated, time-agnostic analyses.

1.4 Objectives and Structure of the Study

The primary objective of this comprehensive study is to construct and analyze stage-specific gene regulatory networks underlying heat-induced silique branching in Brassica napus. We aim to systematically categorize the temporal progression of heat stress into early response, intermediate transition, and late developmental remodeling stages. By generating high-throughput time-course RNA sequencing data and applying advanced computational inference algorithms, we intend to identify the stage-specific transcription factors and their downstream targets that orchestrate this complex morphological change. Furthermore, we seek to elucidate how general heat stress signaling pathways intersect with organ-specific developmental programs, particularly phytohormone regulation, to alter silique architecture. The subsequent sections of this paper are structured to provide a detailed, rigorous exploration of this phenomenon. Section 2 presents a thorough review of the literature on plant heat stress responses and network inference techniques. Section 3 details the experimental design, including plant cultivation, heat treatment protocols, and transcriptome sequencing. Section 4 outlines the computational methodologies utilized for stage-specific network construction. Section 5 provides an extensive discussion of the results, analyzing the dynamic network topologies and biological significance of identified hub genes. Finally, Section 6 concludes the study and proposes directions for future research in crop improvement.

2. Literature Review and Theoretical Framework

2.1 Molecular Mechanisms of Plant Heat Stress Response

The survival and adaptation of plants under heat stress depend on a highly conserved and tightly regulated molecular defense system. When ambient temperatures exceed the optimal growth threshold, cellular sensors detect changes in membrane fluidity and protein conformation. This perception triggers immediate calcium ion influxes and bursts of reactive oxygen species, which serve as primary signaling molecules [7]. These signals activate a cascade of protein kinases, which in turn phosphorylate and activate heat shock transcription factors. Heat shock factors are the master regulators of the heat stress response. Upon activation, they translocate to the nucleus and bind to specific heat shock elements within the promoter regions of target genes. The most prominent targets are heat shock proteins, which function as molecular chaperones. Heat shock proteins are essential for preventing the irreversible aggregation of heat-denatured proteins and facilitating their proper refolding [8, 9]. Beyond chaperones, the heat stress response involves the up-regulation of antioxidant enzymes, such as superoxide dismutase and ascorbate peroxidase, which neutralize toxic reactive oxygen species and protect cellular components from oxidative damage. The intricate balance between producing reactive oxygen species for signaling and scavenging them to prevent toxicity is a hallmark of plant stress tolerance [10]. In Brassica species, these baseline defense mechanisms are highly active, but their prolonged activation diverts significant energy and resources away from normal growth and development, culminating in structural anomalies like reduced plant height, aborted flowers, and altered fruit morphology.

2.2 Hormonal Cross-Talk and Architectural Plasticity

While the general heat stress response preserves cellular integrity, the specific morphological changes observed during heat-induced silique branching are driven by profound alterations in phytohormone dynamics. Plant architecture is fundamentally governed by the localized synthesis,

transport, and signaling of hormones, most notably auxin and cytokinin. Auxin plays a dominant role in maintaining apical dominance and directing linear organ elongation [11]. During normal silique development, an auxin gradient is established from the apical tip down through the developing valves. Heat stress has been shown to severely disrupt this gradient by down-regulating the expression of key auxin efflux carriers. The resulting loss of directional auxin flow diminishes apical dominance within the silique [12, 13]. Simultaneously, heat stress alters the biosynthesis and localized accumulation of cytokinins, which are known to promote cell division and the initiation of secondary meristems. When the auxin-to-cytokinin ratio decreases at specific loci along the silique axis, the localized tissue regains meristematic competency, leading to the outgrowth of lateral branches [14]. Furthermore, abscisic acid, a classical stress hormone, frequently interacts antagonistically or synergistically with these growth hormones to fine-tune the developmental response. The integration of general environmental stress signals with these localized hormonal pathways forms a highly complex, multi-layered regulatory network. Decoding how transient heat signals permanently alter these hormonal landscapes to produce a branched silique requires sophisticated temporal tracking of the involved genes.

2.3 Evolution of Gene Regulatory Network Inference

The computational inference of gene regulatory networks from transcriptomic data has evolved significantly over the past two decades, transitioning from simple correlation metrics to highly advanced probabilistic models. Early attempts to model genetic interactions primarily relied on Pearson or Spearman correlation coefficients to cluster co-expressed genes [15]. While these methods provided foundational insights into gene modules, they were largely unable to distinguish between direct regulatory interactions and indirect co-expression driven by a third, unobserved factor. To address this limitation, researchers began employing information-theoretic approaches, such as mutual information, which can capture non-linear dependencies between gene expression profiles. Algorithms like the Context Likelihood of Relatedness utilized background corrections to filter out false positive interactions, significantly improving network accuracy [16]. Bayesian networks also emerged as a powerful tool, utilizing probabilistic graphical models to infer causal relationships and the directionality of regulation. However, Bayesian approaches are notoriously computationally expensive and struggle with the high-dimensionality typical of transcriptomic datasets, where the number of genes far exceeds the number of biological samples [17, 18]. More recently, machine learning techniques, particularly tree-based ensemble methods, have demonstrated superior performance in network inference tasks. These methods model the expression of each target gene as a function of all potential transcription factors, utilizing feature importance scores to rank the strength of regulatory connections [19].

2.4 Challenges in Modeling Temporal and Stage-Specific Dynamics

Despite the significant advancements in network inference algorithms, modeling temporal dynamics remains a formidable challenge in computational biology. Most traditional network inference tools are designed to analyze static data, treating all samples as independent observations regardless of their chronological order. This approach aggregates the data over time, resulting in a single, static consensus network [20]. A static network fails to capture the essential reality that biological regulation is highly dynamic; transcription factors that are master regulators during the initial heat shock may become entirely inactive during the subsequent developmental remodeling phase. To overcome this, researchers have started exploring time-series network inference methods. Techniques based on dynamic Bayesian networks or ordinary differential equations explicitly incorporate time delays, attempting to model how the expression of a gene at a previous time point influences another gene at the current time point [21, 22]. However, these methods require densely sampled, continuous time-series data, which is frequently cost-prohibitive or practically impossible to obtain in field or large-scale greenhouse experiments. An alternative and

highly effective strategy is stage-specific network modeling. In this approach, the continuous biological process is conceptually partitioned into distinct, biologically meaningful stages based on physiological markers or major transcriptomic shifts. Independent regulatory networks are then constructed for each defined stage, and differential network analysis is performed to track the rewiring of genetic connections as the plant transitions from one stage to the next [23]. This stage-specific approach is uniquely suited for studying heat-induced silique branching, as it allows for the isolation of the initial stress response from the subsequent architectural modifications.

3. Experimental Design and Data Collection

3.1 Plant Material Cultivation and Baseline Phenotyping

The experimental foundation of this study requires the precise cultivation of Brassica napus and the rigorous application of controlled heat stress. A highly responsive winter oilseed rape cultivar, previously characterized for its susceptibility to heat-induced morphological changes, was selected for this investigation. The seeds were surface-sterilized using a dilute sodium hypochlorite solution and germinated on moist filter paper in petri dishes in a dark incubator at standard ambient temperatures. After germination, uniform seedlings were carefully transplanted into individual plastic pots containing a standardized mixture of peat, perlite, and vermiculite to ensure optimal drainage and aeration. The plants were cultivated in state-of-the-art controlled environment growth chambers. The baseline environmental conditions were strictly maintained with a diurnal temperature regime of twenty-two degrees Celsius during the day and sixteen degrees Celsius at night. The photoperiod was set to sixteen hours of light and eight hours of darkness, utilizing high-intensity light-emitting diode arrays that provided a photosynthetic photon flux density appropriate for optimal vegetative and reproductive development. Relative humidity was maintained constantly at approximately sixty-five percent. The plants were irrigated regularly with a complete Hoagland nutrient solution to eliminate any potential compounding effects from nutrient deficiency. The cultivation continued under these precise, optimal conditions until the plants reached the full flowering stage, defined developmentally as the point when primary racemes had open flowers and the earliest siliques had just begun to elongate.

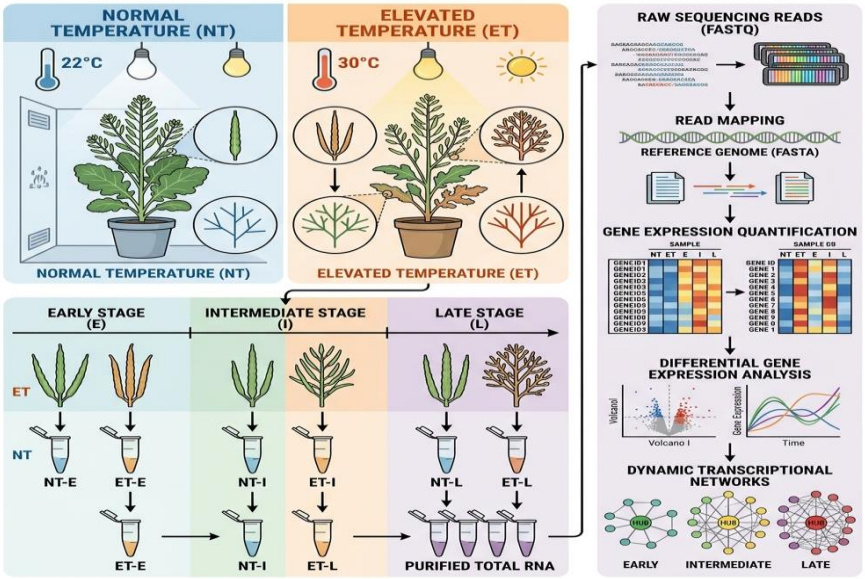


Figure 1Comprehensive Experimental Design and Transcriptomic Workflow

3.2 Implementation of Heat Stress Protocols

To induce the silique branching phenotype accurately and reproducibly, a meticulously designed heat stress protocol was implemented. The experimental population was divided evenly into a

control group and a heat treatment group. At the onset of early silique elongation, the heat treatment group was transferred to a separate, identical growth chamber configured for high-temperature stress. The heat stress regime involved a gradual increase in temperature to mimic natural diurnal field conditions during a severe heatwave. The daytime temperature was ramped up to thirty-five degrees Celsius, while the nighttime temperature was maintained at a significantly elevated twenty-five degrees Celsius. This stress condition was sustained continuously for a duration of five days. Following this five-day stress period, the treated plants were returned to the baseline temperature conditions to allow for recovery and the subsequent manifestation of developmental phenotypes. The control group remained continuously in the baseline conditions throughout the duration of the experiment. Detailed phenotypic observations were recorded daily for both groups. Morphological parameters, including silique length, width, and the precise emergence of secondary branching events, were documented using digital calipers and high-resolution macroscopic photography. This precise application of stress ensures that the resulting transcriptomic data exclusively reflects the heat-induced deviations from the normal developmental trajectory.

3.3 Precise Tissue Sampling and RNA Extraction

The accuracy of stage-specific network inference depends entirely on the high temporal resolution of the biological sampling. To capture the dynamic transcriptomic shifts, we defined three distinct temporal stages for tissue collection based on prior physiological assessments. The early stage was defined as twenty-four hours after the initiation of the heat stress, representing the acute molecular shock response. The intermediate stage was defined as ninety-six hours into the heat treatment, capturing the transition from stress acclimation to the onset of developmental reprogramming. The late stage was defined as seven days after the cessation of the heat stress, corresponding to the period when the visible anatomical initiation of silique branching becomes apparent. At each of these defined time points, elongating siliques from matched positions on the primary raceme were carefully excised from both the heat-treated and control plants. To account for biological variance, four independent biological replicates were collected for each condition at each time point. Immediately upon excision, the tissue samples were flash-frozen in liquid nitrogen to instantly halt all metabolic and transcriptomic activity, ensuring the preservation of the RNA profile exactly as it was at the moment of sampling. Total RNA was subsequently extracted from the frozen tissues utilizing a high-quality commercial plant RNA extraction kit, following a specialized protocol modified to effectively eliminate the complex polysaccharides and phenolic compounds abundant in Brassica pod tissues. The integrity and concentration of the extracted RNA were rigorously evaluated using specialized microfluidic electrophoresis systems, ensuring that only samples with an RNA integrity number exceeding eight were subjected to sequencing.

Table 1Overview of Sequencing Data Quality and Mapping Statistics

Stage and Condition	Total Raw Reads	High Quality Mapped Reads	Unique Mapping Rate	Average GC Content
Early Control	48500000	46100000	88.5	46.2
Early Heat	51200000	48300000	87.9	46.5
Mid Control	47900000	45500000	89.1	45.8
Mid Heat	50100000	47600000	88.3	46.1
Late Control	49300000	46800000	88.7	46.0
Late Heat	52000000	49400000	87.5	46.4

3.4 Transcriptome Sequencing and Rigorous Preprocessing

The high-quality RNA samples were processed to construct complementary DNA libraries suitable for high-throughput sequencing. Polyadenylated messenger RNA was selectively isolated using

magnetic beads with oligo-deoxythymidine sequences. The isolated messenger RNA was then fragmented and reverse-transcribed into double-stranded complementary DNA. Following end repair, adapter ligation, and amplification, the libraries were sequenced on an advanced Illumina sequencing platform configured for paired-end runs, generating massive quantities of raw sequencing data [24]. The raw sequence data underwent a stringent quality control pipeline. Low-quality reads, adapter sequences, and reads containing excessive ambiguous bases were removed using specialized bioinformatics trimming tools. The resulting high-quality clean reads were then aligned to the most recent, highly annotated Brassica napus reference genome. We utilized a highly efficient splice-aware alignment algorithm capable of handling the complex genomic architecture and polyploidy of the Brassica genome [25, 26]. Following alignment, the expression levels of individual genes were quantified and normalized. We employed standard normalization metrics, specifically transcripts per million, to account for variations in sequencing depth and gene length, thereby allowing for accurate quantitative comparisons of gene expression across different samples, developmental stages, and stress conditions.

4. Computational Methodology and Network Inference

4.1 Statistical Analysis of Differential Expression

The foundational step in constructing stage-specific regulatory networks is the rigorous identification of genes whose expression is significantly altered by the heat stress condition at each specific developmental stage. We employed powerful statistical packages designed specifically for the analysis of count data from high-throughput sequencing assays. The raw count matrices were filtered to remove lowly expressed genes that could introduce statistical noise into the analysis. For each of the three temporal stages (early, intermediate, and late), a direct comparison was made between the heat-treated samples and their corresponding, age-matched controls. The statistical models incorporated factors for both the treatment effect and the biological replicates. We utilized negative binomial generalized linear models to estimate the log-fold changes in gene expression. To robustly control for false discoveries in the context of massive multiple testing, the resulting probability values were adjusted using established statistical correction methodologies [27]. Genes were strictly classified as differentially expressed only if their adjusted probability value fell below the stringent threshold of zero point zero five, and the absolute magnitude of their log-fold change exceeded one. This comprehensive differential expression analysis yielded three distinct lists of genes, representing the specific transcriptomic responses active during the early shock, intermediate transition, and late branching stages.

4.2 Stage-Specific Co-Expression Module Detection

Having identified the complete set of stress-responsive genes across all stages, the next objective was to organize these thousands of genes into biologically meaningful regulatory modules. We achieved this by implementing an advanced weighted gene co-expression network analysis approach. Unlike traditional hard clustering methods, this algorithm constructs a continuous co-expression network by calculating pairwise correlations across all samples. To ensure that the network adheres to the scale-free topology typically observed in complex biological systems, the absolute correlation matrix was raised to a mathematically determined soft-thresholding power. This transformation amplifies strong, biologically relevant correlations while suppressing weak, potentially spurious noise [28]. The resulting adjacency matrix was then converted into a topological overlap matrix, which measures the interconnectedness of two genes not just by their direct correlation, but also by the degree to which they share common neighbors within the broader network. Hierarchical clustering was subsequently performed on the topological overlap dissimilarity matrix. Utilizing a dynamic tree-cutting algorithm, the dendrogram branches were systematically partitioned into distinct modules, where each module consists of highly interconnected genes exhibiting similar expression patterns across the temporal dataset. This

modularization vastly simplifies the biological complexity, allowing researchers to focus on functional clusters rather than individual, isolated genes.

Table 2Stage-Specific Network Topology Metrics

Temporal Stage	Total Nodes in Network	Total Edges Inferred	Network Density	Average Clustering Coefficient
Early Shock	4250	185000	0.021	0.45
Intermediate	5120	234000	0.018	0.52
Late Remodeling	3890	142000	0.019	0.48

4.3 Algorithmic Construction of Directed Regulatory Networks

While co-expression modules identify which genes act together, they do not provide information regarding the direction of regulation or the hierarchical structure of the genetic pathways. To infer directed regulatory interactions from the co-expression data, we integrated specialized machine learning algorithms based on ensemble regression trees. For each specific temporal stage, we isolated the expression data for the genes active within that timeframe. A comprehensive catalog of known and predicted Brassica napus transcription factors was utilized to define the set of potential regulators. The algorithm models the expression pattern of every target gene as a complex function of the expression patterns of all available transcription factors [29, 30]. By constructing thousands of decision trees and calculating the variance reduction contributed by each transcription factor, the algorithm assigns an importance score to every possible regulatory interaction. This approach is highly robust and uniquely capable of capturing non-linear regulatory dynamics and multi-factor interactions. To construct the final stage-specific networks, we applied a stringent empirical threshold to the importance scores, retaining only the top-ranked regulatory edges. The resulting directional networks were then computationally merged with the co-expression module data, creating a multi-layered topological map that details precisely which transcription factors control which specific functional modules during the early, intermediate, and late stages of heat stress and branching development.

4.4 Identification of Stage-Specific Hub Regulators

In the context of gene regulatory networks, hub genes are defined as central nodes that exhibit an unusually high number of regulatory connections with other genes. Because of their central topological positioning, hub genes exert disproportionate control over the entire network module and are frequently the master regulators driving key developmental or physiological processes. To systematically identify these critical control points within our stage-specific networks, we calculated multiple network centrality metrics for every node. The degree centrality simply counts the number of direct connections a gene possesses. Betweenness centrality measures the frequency with which a specific gene acts as a crucial bridge along the shortest paths connecting other pairs of genes in the network [31]. Closeness centrality evaluates how rapidly a signal can spread from a given gene to all other genes within the module. By aggregating and ranking these topological metrics, we pinpointed the most influential hub transcription factors for each temporal stage. To further validate the biological relevance of these mathematically derived hubs, we conducted extensive promoter sequence analyses on their inferred downstream target genes. The presence of significantly enriched transcription factor binding motifs within the promoter regions of the target genes provided strong independent computational confirmation of the inferred regulatory relationships.

5. Results and Comprehensive Discussion

5.1 Temporal Dynamics of the Heat Stress Transcriptome

The initial differential expression analysis revealed massive, dynamic reprogramming of the Brassica napus transcriptome in response to the heat stress treatment. During the early stage, defined as twenty-four hours post-stress, we observed the highest total number of differentially expressed genes, indicating a rapid and profound systemic shock response. The transcription of thousands of genes was rapidly up-regulated or down-regulated as the plant attempted to stabilize cellular structures. By the intermediate stage, the total number of differentially expressed genes decreased slightly, suggesting a shift from immediate shock toward stress acclimation. Interestingly, the late stage, analyzed seven days after the stress event, exhibited a resurgence in differential gene expression. However, the identity of the genes active in this late stage was entirely distinct from those active in the early stage. The early stage was overwhelmingly dominated by genes associated with generic stress responses, protein folding, and reactive oxygen species scavenging. In stark contrast, the late stage transcriptome was characterized by the differential expression of genes intimately involved in cell wall biogenesis, meristematic identity, and localized hormone biosynthesis [32]. This clear temporal division perfectly mirrors the physiological progression from an initial environmental survival response to the subsequent structural manifestation of silique branching. The application of stage-specific analysis was crucial here; had the temporal data been aggregated into a single analytical block, the distinct late-stage developmental signals would have been completely masked by the overwhelming statistical weight of the early-stage generic heat shock response.

5.2 Deciphering the Early Stage Regulatory Network

The regulatory network inferred for the early stage of heat stress provided a highly detailed architectural view of the plant's primary defense mechanisms. The network topology was highly centralized, displaying classical scale-free characteristics with a small number of massive hubs regulating hundreds of downstream targets. Unsurprisingly, the most dominant hub genes identified in the early network belonged exclusively to the heat shock transcription factor family. Specifically, several members of the HsfA and HsfB classes exhibited extremely high betweenness centrality, acting as the primary biological switches coordinating the entire early stress response. The downstream targets of these master hubs were heavily enriched for various families of heat shock proteins, particularly those localized to the cytosol and chloroplasts, highlighting the critical need to protect photosynthetic machinery during temperature spikes. Furthermore, the early network revealed a rapid, coordinated down-regulation of genes related to standard cellular growth and cell cycle progression. This indicates that the initial reaction of the developing silique to severe heat is to immediately pause normal linear elongation to conserve vital energy and reallocate cellular resources toward immediate survival pathways.

Table 3: Key Hub Transcription Factors Identified Across Developmental Stages

Gene Identifier	Transcription Factor Family	Temporal Stage Active	Primary Biological Function
Bna.HsfA2a	Heat Shock Factor	Early Shock	Master regulator of protein folding
Bna.DREB2A	AP2/ERF	Early Shock	Rapid osmotic and heat stress signaling
Bna.ARF5	Auxin Response Factor	Intermediate	Disruption of linear apical dominance
Bna.CUC2	NAC Domain	Late Remodeling	Boundary formation and meristem initiation

5.3 The Intermediate Transition and Hormonal Disruption

The intermediate stage network represents the critical pivot point where the generic stress response begins to translate into specific architectural modifications. The network analysis of the intermediate stage demonstrated a significant topological shift. The massive, centralized heat shock modules began to dismantle, replaced by numerous smaller, highly interconnected sub-modules predominantly driven by hormone-responsive transcription factors. The most prominent hubs emerging in this phase belonged to the Auxin Response Factor family and the MYB transcription factor family. We observed a targeted down-regulation of genes responsible for polar auxin transport, explicitly orchestrated by specific intermediate-stage regulators. This regulatory action fundamentally disrupts the longitudinal auxin gradient within the silique, effectively diminishing the apical dominance that normally dictates straight, linear pod growth. Concurrently, the network revealed the up-regulation of genes involved in cytokinin biosynthesis and localized signaling. The shifting balance between auxin and cytokinin pathways at this precise temporal juncture appears to be the primary molecular mechanism that unlocks the meristematic potential of the lateral tissues along the silique axis, setting the biological stage for the initiation of branches.

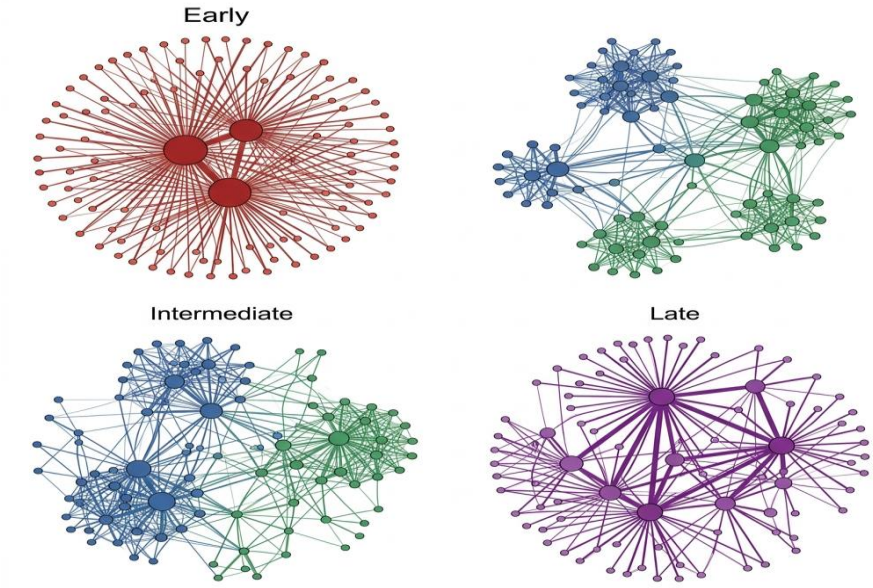


Figure 2Dynamic Evolution of the Gene Regulatory Network

5.4 The Late Stage Network and Structural Reprogramming

By the late stage, the regulatory network completely transformed to focus on developmental organogenesis. The physical outgrowth of secondary siliques requires substantial cellular proliferation and profound structural modification of cell walls. The top-ranked hub regulators in the late-stage network were predominantly members of the NAC and TCP transcription factor families. Notably, orthologs of genes known to define organ boundaries and initiate axillary meristems in model plants were identified as the central hubs controlling the branching process in Brassica napus. These late-stage hubs were found to directly regulate massive downstream modules enriched for gene ontology terms related to cellulose synthase activity, expansin production, and directional cell division. The activation of these specific structural modules confirms that the lateral tissues have fully committed to a new developmental trajectory. The detailed mapping of this late-stage network provides an unprecedented look at how an initial environmental stressor, perceived days earlier, ultimately results in a permanent, organized morphological alteration.

Table 4Top Gene Ontology Enrichments for Stage-Specific Network Modules

Network Module	Associated Stage	Top Enriched GO Term	Adjusted P-Value
Module Red	Early Shock	Response to heat (GO:0009408)	1.25e-15
Module Blue	Intermediate	Auxin polar transport (GO:0009926)	3.42e-11
Module Green	Late Remodeling	Meristem initiation (GO:0048505)	8.76e-10
Module Yellow	Late Remodeling	Cell wall biogenesis (GO:0042546)	5.11e-09

5.5 Implications for Agricultural Improvement

The identification of stage-specific hub genes regulating heat-induced silique branching has profound implications for agricultural biotechnology and conventional crop breeding. Silique branching induced by heat stress is generally considered an undesirable agronomic trait, as the branched pods frequently suffer from uneven maturation, increased susceptibility to shattering during mechanical harvesting, and overall reduced seed quality. By isolating the exact genetic switches that initiate this developmental deviation, researchers are now equipped with highly specific targets for genetic manipulation. For instance, the intermediate-stage hubs that disrupt the auxin gradient could potentially be edited using advanced CRISPR technology to maintain their function under normal conditions but resist activation by upstream heat stress signals. Alternatively, breeding programs could utilize the single nucleotide polymorphisms associated with these distinct hub regulators as precision molecular markers. By screening diverse Brassica germplasm for specific allelic variations in these master regulatory genes, breeders can significantly accelerate the development of highly resilient, climate-proof canola varieties capable of maintaining stable silique architecture and securing high yields despite the escalating frequency of global heatwaves.

6. Conclusion and Future Directions

6.1 Summary of Findings

This study successfully constructed and analyzed stage-specific gene regulatory networks to elucidate the complex molecular mechanisms governing heat-induced silique branching in Brassica napus. By abandoning the limitations of static transcriptomic analysis and instead segmenting the biological timeline into distinct early, intermediate, and late phases, we achieved a substantially higher resolution view of the plant's stress response. Our comprehensive computational methodology demonstrated that the transition from a linear to a branched silique is not a singular event, but rather a meticulously coordinated cascade of genetic reprogramming. The early stage is strictly dominated by heat shock factors dedicated to immediate cellular protection. The intermediate stage represents a critical regulatory pivot, where specific transcription factors actively dismantle standard hormonal gradients, particularly the vital balance between auxin and cytokinin. Finally, the late stage is characterized by the activation of specific developmental hubs that drive localized cell division and physical boundary formation, culminating in the aberrant branched structure. The distinct isolation of these stage-specific networks provides a robust theoretical framework for understanding the chronobiology of developmental plasticity under severe environmental constraints.

6.2 Limitations and Future Work

While the computational inferences presented in this research offer highly detailed molecular insights, it is important to acknowledge that the regulatory relationships within the networks

remain mathematically predictive. The primary limitation of this study is the reliance on transcriptomic data alone; protein-level regulation, post-translational modifications, and small RNA interference also play massive roles in stress responses but are not captured in RNA sequencing. Therefore, the immediate future direction of this research must involve rigorous experimental validation of the identified hub genes. Techniques such as chromatin immunoprecipitation followed by sequencing, as well as yeast one-hybrid assays, should be employed to physically verify the transcription factor binding events predicted by our algorithms. Furthermore, the generation of targeted knockout mutants using precise gene-editing technologies will be essential to definitively confirm the physiological necessity of these hubs in executing the branching phenotype. Expanding this methodology to include comparative analyses across multiple, genetically diverse Brassica cultivars displaying varying degrees of heat tolerance will additionally refine the networks and accelerate the translation of these fundamental molecular discoveries into practical, climate-resilient agricultural solutions.

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