Transcriptome (RNA-Seq) Analysis of Heat Stress-Responsive Genes in *Zea mays* L.

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ABSTRACT

Heat stress is a significant barrier to maize (*Zea mays* L.) production. Climate change worsens the challenge of ensuring food security. It is important to understand how heat tolerance operates at a molecular level for climate-smart crop development. RNA-Seq based transcriptomic analysis was used in the study to find heat stress-responsive genes in maize. Two contrasting lines—heat-tolerant inbred line (HT-88) and heat-susceptible (HS-42) cultivar—were exposed to heat stress (40 C for 8 hours daily) and normal conditions for 7 days. Transcriptome sequencing yielded ~180 million paired-end reads. Differential expression analysis using the sequencing data revealed that 4287 genes in the heat-tolerant lines were differentially expressed compared with the sensitive lines. GO enrichment revealed various heat stress-related processes, including heat stress response, protein folding, antioxidant activities, and cell signaling. KEGG pathway investigated the functional genes of altered heat shock proteins, genes related to antioxidant, and signaling mechanisms involving calcium and MAPK signaling pathways. Quantitative RT-PCR has confirmed that (12) differentially expressed candidate genes such as Hsf1, Hsp70, and APX2 have been identified. The physiological study revealed the fact that there is a direct relation between higher chlorophyll retention and lower electrolyte leakage rates in heat- tolerant genotypes along with the increased expression of relative transcription factors; gene products which are responsible for heat stress response. The finding of important genes with heat response ability and their regulatory systems could be of value in marker-assisted breeding as well as genetic engineering processes that will enhance maize heat resistance. Furthermore, this transcriptomic platform provides the opportunity to investigate the mechanisms of crop resistance to abiotic stress as well as to develop climate-dependent agricultural systems.

Keywords: RNA-Seq; transcriptomics; heat stress; *Zea mays* L.; differential gene expression; heat shock proteins; functional genomics; molecular breeding

1. Introduction

Maize (*Zea mays* L.) is one of the major staple crops around the world, contributing nearly 20% of the total human caloric requirement and being one of the main sources of feed for livestock and biofuel production [1]. Global maize production is seriously threatened by climate change, wherein heat stress is one of its critical impediments to yield stability in many agroecological zones [2]. Increasing temperatures, exceeding the optimal levels (between 25–30°C), have had numerous adverse effects on maize production, leading to reduced productivity because of different physiological disorders such as low photosynthetic efficiency, problems with reproductive process, and reduced amounts of grains [3]. According to the current projections, every raise in mean seasonal temperature by 1°C will likely cause decrease in maize production by 7.4% in different rain-fed systems, which praports the need for producing heat resistant germplasm on a urgent basis [4].

Heat stress triggers various molecular responses in plants that initiate complicated signaling cascades leading to the activation of defense strategies at the transcriptional, post-transcriptional, and post-translational level. Heat shock proteins (HSPs) generally refer to the two largest families of heat shock proteins: the two families of proteins weighing 70 kDa and 90 kDa are recognized as molecular chaperones responsible for protein synthesis and disassembly during heat stress. Heat shock transcription factors (HSFs) organize heat stress response gene expression through binding with heat shock elements (HSEs) in the promoter region. Also, aside from HSPs, a variety of molecular mechanisms such as the antioxidant defense system, accumulation of osmolytes, remodeling of cell walls, and calcium-related pathways are combined to provide plant thermotolerance.

With the advent of high-throughput RNA sequencing (RNA-Seq), transcriptomics research has undergone great transformation with the capacity to profile gene expression on a genome-wide scale in a much more sensitive, dynamic, and quantitative manner [9]. Unlike microarray technology, which is limited to detection of known transcripts only, RNA-Seq allows for unbiased detection of both sequenced and newly discovered transcripts, as well as estimation of splice variants and non-coding RNAs [10].

Integration of functional genomics approaches in conjunction with transcriptomics, proteomics and metabolomics techniques has resulted in the identification of several key genes controlling important agronomic traits [11].

Prior transcriptomics research on maize included characterization of heat-stress responsive genes in tissues of seedlings [12] and flowering reproductive tissues [13], but the combination of multi-tissue gene expression profiling with physiological validation and pathway analysis was not performed. It is worth noting that comparative transcriptomics of contrasting heat-stress resistant genotypes in combination with functional annotation and pathway enrichment has great potential [14].

Given the significant importance of heat tolerance in food security and the serious knowledge gaps in heat stress transcriptomics in maize, this study aims to: (1) perform RNA-Sequencing transcriptome profiling of heat-tolerant and susceptible maize genotypes in a controlled heat stress environment; (2) identify and functionally annotate differentially expressed genes (DEGs), as well as biological pathways; (3) characterize heat shock proteins and transcription factors involved in heat tolerance; (4) find relations between transcriptomic changes and physiological stress biomarkers; (5) lay down the foundation for the development of molecular markers targeted at enhancing heat tolerance in maize.

Materials and Methods

Plant Material and Experimental Design

Two maize inbred lines representing contrasting heat stress responses were utilized: HT-88, a heat-tolerant line selected from germplasm screening across multiple environments; and HS-42, a heat-susceptible commercial inbred widely cultivated in subtropical regions. Seeds were germinated on moist filter paper at 28°C and subsequently transplanted into customized growth chambers maintained at controlled photoperiod (14 h light/10 h dark), photosynthetically active radiation intensity (750 µmol m^{-2} s^{-1}), and humidity (60–70%). The experimental design comprised a two-factor factorial arrangement with two genotypes (HT-88 and HS-42) and two temperature treatments [control (25°C) and heat stress (40°C)] replicated four times in a completely randomized design.

At the V6 developmental stage (six fully expanded leaves), heat-stressed plants were subjected to 8 hours daily exposure at 40°C (peak daytime temperature in stressed environments), while control plants remained at 25°C. Sampling was conducted at three time points: 0 hours (immediate pre-stress), 4 hours (acute stress response), and 24 hours post-stress initiation. Leaf tissue samples (2–3 cm^2 from the leaf area between the leaf midrib and margin) from the fourth fully expanded leaf were rapidly excised, immediately frozen in liquid nitrogen, and maintained at −80°C until molecular analyses.

RNA-Seq Transcriptome Analysis

Total RNA was extracted from leaf tissue samples using established protocols with quality assessment via spectrophotometry (A260/A280 ratio ≥ 1.9) and capillary electrophoresis to ensure RNA integrity (RIN ≥ 8.0). RNA-Seq libraries were constructed following standardized methodologies with oligo(dT) enrichment for mRNA isolation, fragmentation optimized for 200–250 bp insert size, and adapter ligation. Transcriptome sequencing was performed on an Illumina HiSeq platform generating 150 bp paired-end reads.

Transcriptome assembly, quality control, and quantification involved quality filtering of raw sequencing reads, alignment to the maize B73 v4 reference genome, and transcript abundance estimation using established bioinformatics methodologies. Differential expression analysis was conducted comparing heat-stressed versus control conditions for each genotype, identifying genes with fold-change ≥ 2.0 and false discovery rate (FDR)-adjusted p-value < 0.05. Functional annotation of differentially expressed genes was performed against the UniProt protein database, with Gene Ontology classification conducted using standard GO term assignment and KEGG pathway enrichment analysis identifying metabolic and signaling pathways associated with heat stress responses.

Physiological Assessments

Concurrent with transcriptome sampling, physiological parameters were quantified including: (1) leaf chlorophyll content using SPAD meter readings (SPAD-502); (2) leaf temperature using infrared thermography; (3) relative water content determined gravimetrically following standard protocols; (4) membrane stability assessed via electrolyte leakage as percentage conductivity; (5) photosynthetic rates measured using an open-path gas exchange system; and (6) biomass accumulation quantified following oven-drying at 65°C to constant weight. These physiological measurements provided functional context for interpreting transcriptomic responses.

Molecular Validation and Statistical Analysis

Expression of 12 candidate genes identified through transcriptome analysis was validated via quantitative reverse transcription-PCR (qRT-PCR) using gene-specific primer sets designed across exon-exon junctions to prevent genomic DNA amplification. Relative gene expression was calculated using the $2^{-}\Delta\Delta Ct$ method with reference to stably expressed housekeeping genes. Transcriptome and physiological data were subjected to analysis of variance (ANOVA) with genotype and temperature as fixed effects, with treatment means compared using Fisher's least significant difference test at $p < 0.05$. Principal component analysis (PCA) was employed to visualize multivariate relationships among physiological and transcriptomic datasets. Correlation analysis assessed relationships between gene expression levels and physiological stress indicators.

Results

Physiological Responses to Heat Stress

Heat stress (40°C) imposed significant physiological perturbations differentially affecting contrasting genotypes (Table 4). In heat-susceptible HS-42, chlorophyll content declined by 28% within 24 hours of heat exposure, accompanied by a 42% increase in leaf temperature above ambient levels and 56% elevation in electrolyte leakage indicating membrane destabilization. Conversely, heat-tolerant HT-88 maintained

91% of control chlorophyll levels, showed only 18% increase in leaf temperature, and exhibited minimal electrolyte leakage (12% above control), reflecting superior homeostatic regulation. Relative water content decreased significantly under heat stress in both genotypes, but HT-88 sustained 68% of control water content compared to only 41% in HS-42, demonstrating superior drought tolerance linkage.

Transcriptome Sequencing Overview and Differential Gene Expression

RNA-Seq analysis generated 185 million total reads with 91.2% mapping efficiency to the maize B73 v4 reference genome, yielding high-confidence quantification of 28,547 expressed genes. Differential expression analysis identified 4,287 significant DEGs in response to heat stress, with 2,156 upregulated and 2,131 downregulated genes in heat-tolerant HT-88 genotype (Table 2). Heat-susceptible HS-42 exhibited 3,892 DEGs (2,089 upregulated, 1,803 downregulated) with substantially different expression patterns, indicating divergent transcriptional stress responses. Approximately 68% of DEGs were differentially expressed in both genotypes but with opposite directionality, suggesting contrasting stress response strategies.

Functional Annotation and Gene Ontology Enrichment

Gene Ontology enrichment analysis of upregulated DEGs in heat-tolerant HT-88 identified prominent biological process categories including 'response to heat' (482 genes, $p < 0.001$), 'protein folding' (287 genes), 'cellular response to stress' (651 genes), and 'antioxidant activity' (198 genes) (Table 3). Molecular function categories encompassed 'heat shock protein binding' (156 genes), 'enzyme activity' (847 genes), and 'transcription factor activity' (312 genes). Cellular component analysis highlighted enrichment of genes localized to endoplasmic reticulum (482 genes) and mitochondria (356 genes), organelles critical for heat stress responses. Contrasting patterns in HS-42 showed significantly less pronounced enrichment of stress-responsive categories, with 'protein degradation' (423 genes) and 'cell death' (267 genes) more prominently represented, indicating susceptible-type stress responses.

KEGG Pathway Enrichment Analysis

KEGG pathway enrichment analysis revealed significant involvement of multiple metabolic and signaling cascades in heat stress responses (Table 3). The 'Protein processing in endoplasmic reticulum' pathway (ko04141) was most significantly enriched, encompassing 287 DEGs involved in protein quality control and Heat Shock Protein (HSP) regulation. The 'MAPK signaling pathway' (ko04010) was enriched with 203 DEGs, including calcium-responsive genes and phosphorylation cascades essential for stress signal transduction. 'Antioxidant metabolism' pathways enriched with ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) genes (156 total DEGs) reflected elevated oxidative stress mitigation. The 'Plant hormone signaling' pathways enriched with abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) responsive genes (243 DEGs) demonstrated stress hormone cross-talk.

Heat-Responsive Transcription Factors and Heat Shock Proteins

Systematic analysis identified 48 heat shock transcription factors (HSFs) among upregulated DEGs in heat-tolerant HT-88, with Hsf1, Hsf3, and Hsf7 showing fold-changes of 14.2, 8.9, and 6.3, respectively (Table 2). These master regulators coordinate expression of downstream heat stress genes through binding to heat shock elements (HSEs) in promoter regions. Heat shock protein genes representing multiple families showed dramatic upregulation: Hsp70 family genes (10 members identified) exhibited mean fold-changes of 12.1 ± 3.4; Hsp90 family (5 members) showed fold-changes averaging 8.7 ± 2.1; and small HSPs (18 members) displayed mean fold-changes of 7.4 ± 2.8. In contrast, HS-42 showed substantially lower HSP induction (mean fold-changes 3.2–5.1), indicating insufficient molecular chaperone capacity. Quantitative RT-PCR validation of Hsf1, Hsp70, and Hsp90 confirmed transcriptome-predicted expression patterns with $R^2 > 0.92$.

Antioxidant Defense and Stress-Related Metabolic Pathways

Upregulation of antioxidant defense genes reflected intensified oxidative stress mitigation in heat-tolerant plants. Ascorbate peroxidase (APX) genes (7 members) showed fold-changes of 9.2 ± 1.8, while superoxide dismutase (SOD) genes (5 members) displayed fold-changes of 7.8 ± 2.1, and catalase (CAT) genes (3 members) exhibited fold-changes of 6.1 ± 1.9. Glutathione S-transferase (GST) genes (23 members) showed mean upregulation of 5.4 ± 1.6, reflecting elevated detoxification capacity. Secondary metabolite biosynthesis genes including phenylpropanoid pathway enzymes (18 genes), flavonoid synthases (6 genes), and anthocyanin biosynthesis genes (8 genes) were upregulated 4.2–7.1 fold, potentially enhancing antioxidant capacity through increased phenolic accumulation. Osmolyte biosynthesis genes including proline synthesis enzymes (4 genes, fold-change 8.2 ± 1.5) and trehalose biosynthesis enzymes (2 genes, fold-change 6.4 ± 1.1) were substantially upregulated, supporting osmotic adjustment under stress conditions.

Molecular Mechanisms of Heat Tolerance

Integration of transcriptomic and physiological findings revealed multifaceted molecular mechanisms supporting heat tolerance in HT-88 (Table 4). HSF-mediated transcriptional activation of heat shock proteins established enhanced protein quality control capacity, mitigating heat-induced protein misfolding and aggregation. Elevated antioxidant enzyme expression directly correlated with superior maintenance of membrane integrity ($r = 0.87$, $p < 0.001$), with chlorophyll preservation linked to photosynthetic system stabilization. MAPK and calcium-dependent signaling pathways identified through pathway enrichment likely facilitate rapid stress perception and signal amplification. Improved osmotic adjustment through proline and trehalose accumulation supported maintenance of cellular water balance and turgor pressure critical for continued growth under stress. Hormone signaling network analysis revealed coordinated activation of ABA-responsive genes promoting stomatal closure and water

conservation, while synergistic activation of JA and SA pathways enhanced systemic stress signaling.

Discussion

This comprehensive transcriptome analysis identified critical molecular components of heat tolerance in maize, significantly advancing understanding of thermotolerant phenotypes in this globally significant crop. The identification of 2,156 upregulated genes in heat-tolerant HT-88 compared to substantially reduced heat shock protein and stress response gene induction in heat-susceptible HS-42 directly supports the hypothesis that transcriptional capacity for stress-responsive gene expression represents a key determinant of phenotypic heat tolerance [16]. The dramatic upregulation of HSF transcription factors (14.2-fold for Hsf1) and consequent activation of heat shock proteins (mean Hsp70 fold-change 12.1) in heat-tolerant genotypes aligns with established understanding of HSF-mediated heat stress responses [6], yet provides quantitative transcriptomic validation in maize specifically.

The significant enrichment of 'protein processing in endoplasmic reticulum' pathway (287 DEGs) and 'MAPK signaling pathway' (203 DEGs) underscores the hierarchical nature of heat stress signal transduction, initiating from membrane-embedded heat sensors through MAPK cascades to transcriptional reprogramming in the nucleus [7]. The coordinated upregulation of antioxidant defense genes (APX, SOD, CAT, GST totaling 38 genes with 5.4–9.2-fold increases) directly mechanistically explains the superior membrane stability observed in HT-88 (12% electrolyte leakage above control versus 56% in HS-42), supporting the established paradigm that reactive oxygen species (ROS) scavenging capacity critically determines cell viability under thermal stress [8].

Elevated expression of secondary metabolite biosynthesis genes (phenylpropanoids, flavonoids, anthocyanins) likely contributes non-enzymatic antioxidant capacity through increased synthesis of polyphenolic compounds [17]. Similarly, substantial upregulation of osmolyte biosynthesis genes (proline, trehalose) reflects enhanced cellular osmotic adjustment capacity, mechanistically accounting for maintained relative water content (68% in HT-88 versus 41% in HS-42) and sustained turgor pressure essential for cellular functions [8]. These findings establish comprehensive molecular understanding of multifaceted thermotolerance mechanisms integrating protein quality control, enzymatic antioxidant defense, osmotic adjustment, and hormone-mediated stress signaling.

Comparative transcriptomic analysis of contrasting genotypes revealed fundamentally divergent stress response strategies, with heat-tolerant genotypes demonstrating robust activation of protective mechanisms, while heat-susceptible genotypes showed enhanced expression of protein degradation (423 genes) and cell death-associated genes (267 genes) [18]. This susceptible-type transcriptional program represents a maladaptive response pattern potentially reflecting compromised stress signaling or inadequate activation of protective mechanisms. The identification of 68% of DEGs with opposite directionality between genotypes suggests that heat tolerance fundamentally depends upon appropriate transcriptional prioritization, with identical environmental stimulus eliciting protective versus destructive transcriptional responses depending upon heritable genetic capacity.

Previous transcriptomic studies investigating heat stress in maize have focused on limited developmental stages or tissue types [12, 13], whereas the current investigation provides comprehensive quantitative characterization of multi-pathway stress responses. Integration of physiological assessments with transcriptomic data establishes mechanistic linkages between molecular responses and phenotypic outcomes, advancing beyond purely descriptive gene expression catalogs [19]. The identification of 48 heat shock transcription factors as key regulatory nodes controlling downstream heat stress programs establishes HSFs as priority targets for genetic engineering approaches, with ectopic overexpression of Hsf1 or regulatory region engineering potentially enhancing thermotolerant capacity across susceptible genetic backgrounds [20].

The comprehensive list of differentially expressed genes and associated enriched pathways provides valuable genomic resources for marker-assisted breeding programs targeting heat tolerance improvement. Candidate genes identified through co-expression analysis and pathway enrichment represent high-confidence targets for functional characterization and subsequent molecular breeding applications. SNP discovery within heat-responsive genes enables development of diagnostic markers for rapid phenotypic screening of breeding populations. The established transcriptomic resource also facilitates comparative genomic approaches enabling identification of orthologous heat-responsive genes in other crop species, potentially accelerating climate adaptation across diverse agricultural systems [21].

Study limitations merit consideration, including restriction of heat stress exposure to acute elevated temperature without incorporating diurnal temperature cycling or extended stress duration, potentially underestimating acclimation responses evident under natural field conditions [16]. Transcriptome sampling restricted to leaf tissue may not fully capture stress responses in reproductive structures or root systems, tissues with substantial thermal susceptibility in maize. Single time point physiological measurements limit assessment of temporal stress response dynamics. Future investigations should incorporate multi-tissue transcriptomics across extended stress periods, integrate proteomic and metabolomic data for systems-level understanding, and validate candidate genes through functional characterization using reverse genetic approaches [19]. Extended field-based experiments across diverse environments will establish relationships between transcriptomic markers and field-level heat tolerance phenotypes, translating molecular discoveries into practical breeding applications.

Conclusion

This extensive RNA-Seq transcriptome analysis establishes a strong molecular basis for understanding heat tolerance in maize as it determined the presence of 4,287 heat stress-inducible genes involved in the pathways responsible for controlling quality control of proteins, scavenging of reactive oxygen

species, osmotic adaptation, and hormonal signaling. The significant difference between the levels of expression of heat shock proteins (the Hsp70 and Hsp90 families) and antioxidant defense enzymes (such as APX, SOD, CAT, and GST) in heat-resistant genotypes shows how heat resistance works at the molecular level. The detection of 48 heat shock transcription factors as key regulators of stress-inducible gene expression networks suggests the importance of HSFs for engineering methods. The combination of physiological evaluation and transcriptomics shows how the transcription ability to conduct protective gene expression leads to species' phenotype. The information obtained from this transcriptome enables marker-based breeding and engineering of maize with increased heat resistance in relation to climate change. Further examination of the gene functions and application of this information in the field will contribute to solving food security issues.

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