Comparative Evaluation of Heat Shock Protein (HSP70 and HSP90) Expression in *Triticum aestivum* L. under Terminal Heat Stress Using qRT-PCR

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Received: 03 Dec 2024
Revised: 21 Dec 2024
Accepted: 02 Jan 2025
Published: 25 Jan 2025

E-ISSN: 2989-5340
DOI: https://doi.org/10.54660/jafi.2025.1.1.01-10

ABSTRACT

Background: The productivity of wheat (*Triticum aestivum* L.) continues to be in danger because of terminal heat ultimatums that can damage photosynthesis, membrane integrity, and grain growing. Heat shock proteins (HSPs), and especially HSP70 and HSP90 proteins, are highly important in their effect of protein protection and thermotolerance development.

Objectives: In the present study, the expression patterns of HSP70 and HSP90 in wheat under terminal heat stress were compared and their transcriptional responses correlated with physiological determinants of heat tolerance.

Materials and Methods: Two contrasting wheat genotypes were subjected to terminal heat stress (42/28 °C day/night) during anthesis for 6, 24 and 48 hours with unstressed controls kept at 24/18 °C. Transcript abundance of HSP70 and HSP90 was analyzed by qRT-PCR in flag leaf samples. Physiological parameters such as chlorophyll content, membrane stability, relative water content and accumulation of ROS were also analyzed.

Results: HSP70 and HSP90 were significantly induced by heat stress, with HSP70 being induced earlier and stronger than HSP90. At 24 hours of stress, the maximal expression of HSP70 was increased about 9-10-fold and HSP90 was increased about 5-6-fold. Increased membrane damage and ROS accumulation reduced physiological performance under prolonged stress. The heat tolerant genotype showed higher HSP expression and better physiological stability as compare to heat sensitive genotype.

Conclusion: HSP70 and HSP90 play complementary roles in thermotolerance in wheat; HSP70 as an early stress responding protein and HSP90 as a sustained stress regulating protein. Their expression patterns can be used as potential molecular markers for heat-tolerance breeding and development of climate-resilient wheat variety.

Keywords: *Triticum aestivum*; terminal heat stress; HSP70; HSP90; qRT-PCR; molecular chaperones; thermotolerance; climate resilient crops

1. Introduction: Wheat Production and Terminal Heat Stress under a Changing Climate

1.1. Global Importance of *Triticum aestivum*

Bread wheat (*Triticum aestivum* L.) is cultivated over a wider range of latitudes than any other cereal crop and provides roughly one-fifth of the world's human intake of calories and protein. Wheat's hexaploid genome and wide adaptability have enabled it to expand from temperate lowlands to high altitudes and semi-arid environments. Wheat has become a key to food security and rural livelihoods in the Global South, and a major traded commodity in the Global North. Wheat is grown over such a large part of the globe that even small yield losses per unit area correspond to large absolute losses in global production, with immediate consequences for food price stability and nutritional security.

1.2. Wheat Production under Climate Change

By examining global mean surface temperature rise in the past few decades, it has become evident that climate forecasts predict a higher frequency, severity, and length of heat waves occurring at the same time as wheat growth. Numerous studies of the impact of climate change on wheat yield have shown that wheat suffers a significant drop in yield by several percentage points for every degree Celsius rise in the growing season temperature compared to the optimal temperature for wheat growth. Because wheat is mainly planted for harvesting before the peak of summer heat, any climate change leading to higher temperatures during the wheat growth period puts wheat production at risk.

1.3. Defining Terminal Heat Stress

Terminal heat stress is defined as the wheat plant's exposure to above-normal temperatures, particularly late in the reproductive phase of the cycle. Wheat's preferred temperature range for grain filling is between 15–22 °C, and therefore, there is a strong negative yield response from terminal heat stress due to prolonged exposure to maximum daily temperatures above 32-35 °C.

1.4. Physiological Consequences of High Temperature

At the whole-plant level, terminal heat stress results in accelerated leaf senescence, reduced stomatal conductance, and disassociation of the connection between carbon fixation through photosynthesis and carbon loss through respiration, thus creating a net negative carbon status during a period of maximum requirement of assimilates for grain filling. At the cellular level, higher temperatures make the lipid bilayer unstable due to increased fluidity and permeability of cell membranes. Higher temperatures trigger disruption of tertiary and quaternary structures of enzymes and other proteins in a cell. Higher temperatures destabilize the photosynthetic machinery especially of Photosystem II in the chloroplast. All these changes result in reduction of efficiency of light absorption and electron-transfer reactions as well as carbon-fixing processes, while the leakage of ions and other metabolites is heightened.

1.5. Sensitive Developmental Stages and Effects on Grain Filling and Yield

It has been shown that the period around anthesis and early grain filling is the most heat-sensitive developmental stage of wheat because pollen viability and fertilization are impaired by heat stress. Heat stress during this critical period reduces the potential yield of grains through increased floret and grain abortion rates. On the contrary, heat stress during mid-late grain filling period primarily affects the individual grain weight, reducing the effective grain-filling duration and accelerating the rate of the starch granule deposition. Therefore, the overall effect is a reduction of both components of yield since even short-lasting terminal heat episodes cause significant yield penalties due to their occurrence in developmentally sensitive phases of wheat.

1.6. Heat-Induced Oxidative Damage and Agricultural Significance

The rise in temperature affects the equilibrium of the production of reactive oxygen species (ROS) especially by way of chloroplast electron transport chains, mitochondria, and peroxisomes and the antioxidant mechanisms for the detoxification of ROS. When the production of ROS occurs at a faster rate than ROS can be neutralized, oxidative stress ensues and leads to membrane lipid peroxidation, damage to proteins, and alterations of cellular structure. Since wheat is a crucial food crop for billions of people and heat waves are expected to increase in severity, it is now important to understand the various aspects of heat shock proteins systems among other factors that contribute to wheat's ability to withstand short-lived high temperature conditions.

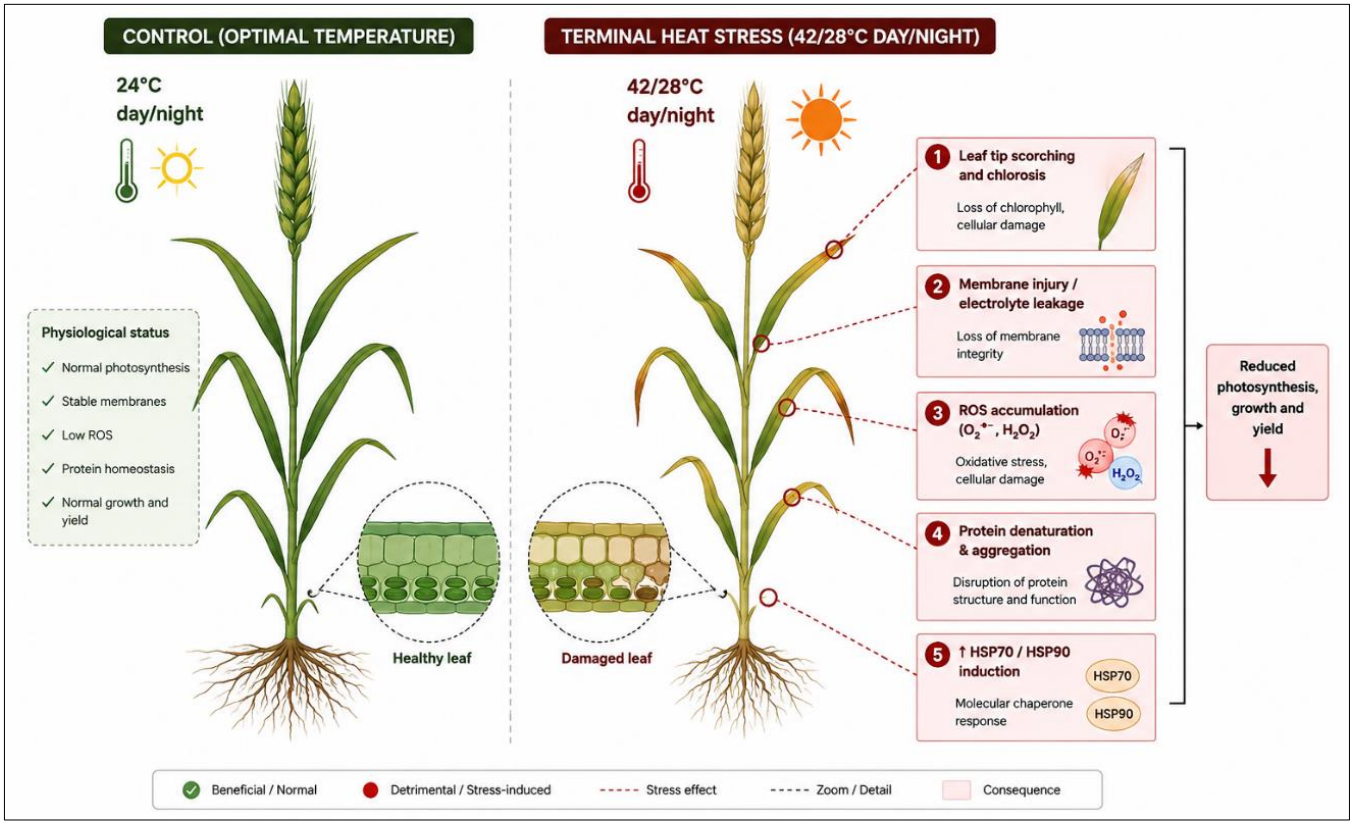

Fig 1. Schematic illustration of terminal heat stress effects on *Triticum aestivum*, showing leaf scorching, chlorosis, membrane injury, ROS accumulation, protein denaturation, and induction of HSP70/HSP90 relative to unstressed control plants.

2. Heat Shock Proteins in Plants: An Overview

2.1. Overview of Heat Shock Proteins

Heat shock proteins (HSPs) constitute an evolutionarily ancient superfamily of molecular chaperones whose expression is transiently or constitutively required to maintain proteostasis, the balance between protein synthesis, folding, and degradation. First described through the observation of characteristic chromosomal puffing patterns in heat-shocked *Drosophila*, HSPs are now recognized across all domains of life, from bacteria to plants and animals, underscoring their fundamental role in cellular stress physiology. In plants, HSPs are induced not only by heat but also by a wider array of abiotic stresses, including drought, salinity, cold, and oxidative stress, reflecting shared downstream consequences of these stresses on protein and membrane stability.

2.2. Classification of Plant HSPs

Plant HSPs are classified into five major families based on approximate molecular weight and sequence conservation: HSP100 (Clp/Hsp100), HSP90, HSP70, HSP60 (chaperonins),

and the small HSPs (sHSPs, typically 15–42 kDa). HSP100 proteins function largely as disaggregases, using ATP-dependent unfolding to resolubilize protein aggregates in cooperation with the HSP70 system. HSP90 acts predominantly on a specific clientele of signaling and regulatory proteins rather than as a general folding chaperone. HSP70 is the most broadly conserved and abundant chaperone family, assisting in *de novo* folding, translocation, and disaggregation. HSP60 (chaperonins) function primarily within chloroplasts and mitochondria, assisting folding of imported and organellar proteins within a barrel-shaped complex. Small HSPs, while lacking intrinsic ATPase activity, form oligomeric complexes that bind partially denatured proteins and hold them in a folding-competent state until ATP-dependent chaperones such as HSP70 can complete refolding.

2.3. Molecular Chaperone Functions: Folding, Stabilization, and Aggregation Prevention

The unifying biochemical function of HSPs is their capacity to recognize exposed hydrophobic surfaces on non-native or partially denatured proteins, surfaces that are normally buried in the native folded state, and to bind these regions transiently to prevent inappropriate intermolecular interactions. Through cycles of ATP binding and hydrolysis, ATP-dependent chaperones such as HSP70 and HSP90 undergo conformational changes that alternately capture and release client proteins, providing repeated opportunities for correct folding while physically shielding aggregation-prone intermediates from one another. This chaperone-mediated quality-control system operates alongside, and in coordination with, the ubiquitin-proteasome and autophagy pathways, which target irreversibly misfolded proteins for degradation when refolding attempts fail.

2.4. Cellular Protection Mechanisms and Subcellular Distribution

HSPs are distributed across essentially all subcellular compartments where proteins are synthesized or must be maintained in a folded state, including the cytosol, nucleus, endoplasmic reticulum, chloroplast, and mitochondria, with organelle-targeted HSP isoforms bearing appropriate transit peptides. This compartmentalized distribution allows plants to mount a coordinated proteostatic response across the entire cell simultaneously, protecting not only cytosolic enzymes but also the photosynthetic apparatus within chloroplasts and the respiratory machinery within mitochondria, both of which are particularly vulnerable to heat-induced dysfunction given their central roles in energy metabolism.

2.5. Evolutionary Conservation and Role in Abiotic Stress Tolerance

The sequence and structural conservation of HSP70 and HSP90 across kingdoms as divergent as bacteria, fungi, plants, and animals reflects strong purifying selection acting on their core ATPase and substrate-binding domains, consistent with their indispensable role in cellular viability under both optimal and stressful conditions. In plants specifically, constitutive and stress-inducible HSP isoforms work together with osmoprotectants, antioxidant enzymes, and stress hormone signaling pathways to confer tolerance not only to heat but to a broader suite of abiotic stresses that converge on protein and membrane destabilization, positioning HSPs as a central, integrative node within the plant abiotic stress tolerance network.

3. HSP70 and HSP90: Comparative Functional Biology

While both HSP70 and HSP90 function as ATP-dependent molecular chaperones central to heat shock responses, they differ substantially in structure, client specificity, and regulatory logic, differences that underlie their distinct expression dynamics under terminal heat stress.

3.1. HSP70: Gene Family, Structure, and Function

The HSP70 family in wheat, as in other plants, comprises multiple gene members distributed across cytosolic, plastidial, mitochondrial, and endoplasmic reticulum-localized subfamilies, with stress-inducible cytosolic members typically responsible for the sharpest transcriptional response to heat. Structurally, HSP70 proteins consist of an N-terminal nucleotide-binding domain (NBD) that hydrolyzes ATP, and a C-terminal substrate-binding domain (SBD) that engages short hydrophobic peptide segments exposed on unfolded or partially folded client proteins. ATP binding and hydrolysis at the NBD are allosterically coupled to opening and closing of the SBD lid, driving cycles of client capture and release that are further regulated by co-chaperones such as HSP40/DnaJ proteins, which stimulate ATP hydrolysis, and nucleotide exchange factors, which promote ADP-to-ATP exchange and client release. Physiologically, HSP70 participates in *de novo* protein folding at the ribosome, translocation of proteins across organellar membranes, disaggregation of stress-denatured proteins in cooperation with HSP100, and regulation of the heat shock response itself through direct interaction with heat shock transcription factors.

3.2. HSP90: Molecular Organization and Client-Protein Network

HSP90 functions as a constitutive homodimer, with each monomer comprising an N-terminal ATP-binding domain, a middle domain that contributes to client binding and ATPase stimulation, and a C-terminal dimerization domain. Unlike HSP70, which interacts promiscuously with a broad range of unfolded polypeptides, HSP90 engages a comparatively restricted clientele of near-native, conformationally metastable signaling proteins, including protein kinases, transcription factors, and steroid hormone receptor homologs in the case of animal systems, or their functional analogs among plant hormone-signaling and defense-signaling proteins. Through its ATPase cycle and an extensive network of co-chaperones, HSP90 stabilizes these client proteins in an activation-competent conformation, effectively acting as a molecular switchboard that links protein maturation to hormonal and stress-signal transduction pathways rather than serving primarily as a general-purpose folding chaperone.

3.3. Comparative Evaluation of HSP70 and HSP90

Comparing the two chaperone families reveals both convergent and divergent features relevant to their roles in terminal heat stress. Both are ATP-dependent, both are transcriptionally induced by heat shock factors, and both are essential for cellular viability at elevated temperature. However, HSP70 shows broader client promiscuity, more rapid and often higher-amplitude transcriptional induction under acute stress, and functions distributed across multiple subcellular compartments, whereas HSP90 exhibits client specificity concentrated on regulatory and signaling proteins, generally more moderate fold-induction, and a role that is arguably more focused on sustaining signal transduction fidelity than on bulk protein quality control.

These distinctions have direct agricultural relevance: because HSP70 responds rapidly and robustly, it is often the first molecular marker screened in heat-tolerance studies, while HSP90's role in stabilizing hormone and stress-signaling components suggests it may be particularly important for maintaining developmental plasticity and acclimation responses over longer stress durations.

Table 5: Comparative summary of HSP70 and HSP90: molecular, functional, and agricultural characteristics

Feature	HSP70	HSP90
Approx. molecular weight	~70 kDa	~90 kDa
Domain organization	N-terminal NBD + C-terminal SBD	N-terminal ATPase domain, middle domain, C-terminal dimerization domain
Oligomeric state	Predominantly monomeric (co-chaperone assisted)	Constitutive homodimer
Client specificity	Broad; unfolded/nascent polypeptides	Narrow; near-native signaling/regulatory proteins
Cellular localization	Cytosol, ER, chloroplast, mitochondria	Predominantly cytosol; organellar homologs present
Key co-chaperones	HSP40/DnaJ, nucleotide exchange factors	HOP/STI1, p23, immunophilins (functional analogs)
Induction kinetics under heat stress	Rapid onset, high amplitude	Slower onset, moderate amplitude
Primary biological role	General protein folding & quality control	Signal transduction & client-protein maturation
Relative fold-change (this study, 24 h)	~9.85-fold	~5.40-fold
Agricultural/breeding relevance	Candidate rapid-response thermotolerance marker	Marker for signaling/acclimation capacity

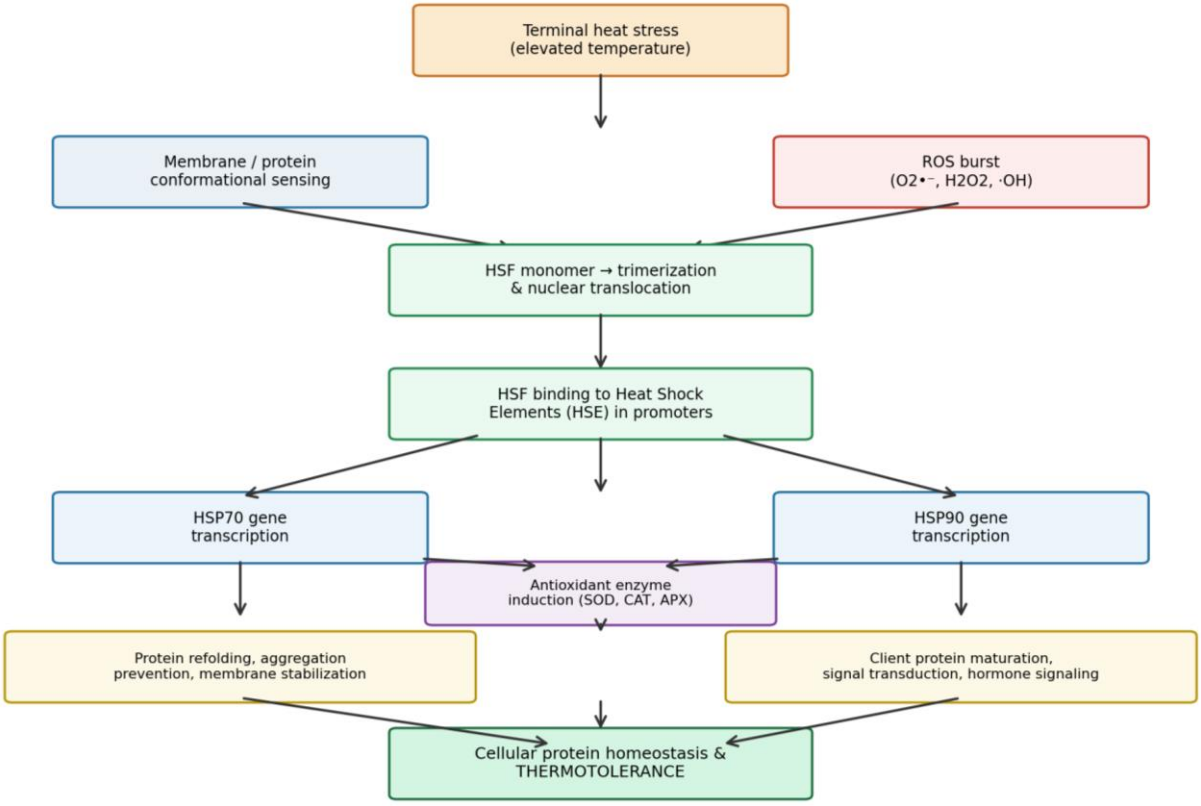

Fig 2: Molecular mechanism of the heat shock response pathway in wheat, illustrating heat-induced HSF trimerization and nuclear translocation, HSE binding, transcriptional activation of HSP70 and HSP90, and downstream contributions to protein refolding, signal transduction, antioxidant crosstalk, and cellular thermotolerance.

4. Materials and Methods

4.1. Experimental Design and Plant Material

Two bread wheat genotypes with contrasting reported heat tolerance, designated here as a heat-tolerant genotype (HT) and a heat-sensitive genotype (HS-genotype), were used to capture genotype-dependent variation in the HSP70/HSP90 response. Seeds were sown in pots containing a standard loam-based potting mixture and grown under controlled greenhouse conditions (24/18 °C day/night, 60–65% relative humidity, 12–14 h photoperiod, ambient CO2) until the anthesis stage (Zadoks growth stage 65), at which point plants were assigned to control or heat-stress treatments in a completely randomized design.

Terminal heat stress was imposed by transferring stressed plants to a growth chamber programmed at 42/28 °C day/night, while control plants remained at 24/18 °C. Sampling was performed on fully expanded flag leaves at four time points: 0 h (pre-stress baseline), 6 h, 24 h, and 48 h after the onset of heat treatment, with three independent biological replicates collected per genotype × treatment × time-point combination, each replicate pooled from three individual plants. Samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until processing (see Table 1 for a full summary of the experimental design).

Table 1: Experimental design, genotypes, treatment conditions, sampling stages, and replication strategy

Parameter	Details
Plant material	Two bread wheat (*Triticum aestivum* L.) genotypes: heat-tolerant (HT) and heat-sensitive (HS-genotype)
Growth conditions (control)	24/18 °C day/night, 60–65% RH, 12–14 h photoperiod, greenhouse pots
Growth stage at treatment onset	Anthesis (Zadoks GS 65)
Heat stress regime	42/28 °C day/night, growth chamber
Stress durations sampled	6 h, 24 h, 48 h (plus 0 h pre-stress baseline)
Control	Time-matched plants maintained at 24/18 °C
Tissue sampled	Fully expanded flag leaf
Biological replicates	n = 3 per genotype × treatment × time point (each pooled from 3 plants)
Technical replicates (qRT-PCR)	n = 3 per biological replicate
Experimental design	Completely randomized design (CRD), factorial: genotype × treatment × time

4.2. RNA Isolation, Quality Assessment, and cDNA Synthesis

Total RNA was extracted from approximately 100 mg of frozen leaf tissue using a TRIzol-based guanidinium thiocyanate–phenol–chloroform extraction protocol, followed by on-column or in-solution DNase I treatment to eliminate genomic DNA contamination. RNA integrity was assessed by denaturing agarose gel electrophoresis, visually confirming intact 28S and 18S ribosomal RNA bands, and quantitatively by capillary electrophoresis-based RNA integrity number (RIN), with samples accepted only above a RIN threshold of 7.0. RNA purity and concentration were determined spectrophotometrically using A260/A280 and A260/A230 absorbance ratios (accepted range 1.9–2.1), with concentration measured by fluorometric quantification for greater accuracy at low RNA concentrations. First-strand complementary DNA (cDNA) was synthesized from 1 µg of DNase-treated total RNA using a commercial reverse transcriptase and oligo(dT)/random hexamer priming, following the manufacturer's recommended thermal profile, with a no-reverse-transcriptase control included for each sample to confirm the absence of genomic DNA contamination.

4.3. Primer Design, Validation, and Amplicon Specificity

Gene-specific primers for HSP70, HSP90, and two candidate reference genes (ACTIN and GAPDH) were designed targeting exon–exon junctions where possible to minimize genomic DNA co-amplification, with amplicon lengths restricted to 100–200 bp to optimize amplification efficiency. Primer specificity was confirmed by melt-curve analysis showing single, sharp dissociation peaks and by agarose gel electrophoresis of qRT-PCR products confirming a single band of the expected size. Amplification efficiency for each primer pair was determined from the slope of a standard curve generated using five-fold serial dilutions of pooled cDNA, with primer pairs accepted only when efficiency fell within 90–110% (full primer details are provided in Table 2).

Table 2: Primer sequences, target genes, amplicon size, annealing temperature, amplification efficiency, and reference genes used for qRT-PCR

Gene	Primer sequence (5'→3') F/R	Amplicon size (bp)	Annealing temp. (°C)	Efficiency (%)	Role
HSP70	F: AGGTGCTGGACAAGTGCCAGAA R: TGCTCGTCCTTGGTCTTGATGA	142	58	98.6	Target gene
HSP90	F: CGAGACCTTCACCGTGGAGATT R: CTTCTCGGCCATCTCCTTGATG	156	58	101.2	Target gene
ACTIN	F: GCCGTGCTTTCCCTCTATGC R: CGTTTCATGAATTCCAGCAGC	121	58	99.4	Reference gene
GAPDH	F: TTGGCATCGTTGAGGGTCTC R: CCACAGACTTCATCGGTGACA	133	58	97.8	Reference gene

4.4. qRT-PCR Reaction Conditions and Reference Gene Normalization

Quantitative real-time PCR was performed using SYBR Green-based fluorescent detection chemistry on a real-time thermal cycler, with each 10–20 µL reaction containing SYBR Green master mix, forward and reverse gene-specific primers, and diluted cDNA template. The thermal cycling profile consisted of an initial denaturation step, followed by 40 cycles of denaturation, annealing (temperature optimized per primer pair, Table 2), and extension, concluding with a melt-curve stage to verify amplicon specificity. Each biological replicate was analyzed in technical triplicate. Cycle threshold (Ct) values were determined automatically by the instrument software using a fixed fluorescence threshold within the exponential amplification phase, and geometric-mean stability of the two candidate reference genes (ACTIN, GAPDH) was verified prior to their use for normalization.

4.5. Relative Gene Expression Calculation

Relative expression of HSP70 and HSP90 was calculated using the comparative Ct (2^-ΔΔCt) method. For each sample, ΔCt was calculated as the difference between the target gene Ct and the geometric mean Ct of the reference genes. ΔΔCt was then calculated as the difference between the ΔCt of the heat-stressed sample and the mean ΔCt of the corresponding time-matched control. Fold-change in expression was calculated as 2^-ΔΔCt, providing a normalized, relative measure of transcript abundance change attributable to heat stress rather than absolute transcript copy number (results summarized in Table 4).

4.6. Physiological Measurements

In parallel with molecular sampling, physiological indicators of heat stress injury and photosynthetic performance were recorded from flag leaves at each time point, including total chlorophyll content (spectrophotometric extraction), relative water content (fresh, turgid, and dry weight-based calculation), membrane stability index and electrolyte leakage (conductivity-based assay before and after boiling), net photosynthetic rate (infrared gas analyzer-based portable photosynthesis system), and ROS accumulation (histochemical and spectrophotometric assays for superoxide and hydrogen peroxide). Antioxidant enzyme activities (superoxide dismutase, catalase, ascorbate peroxidase) were assayed spectrophotometrically from leaf extracts, and yield-related traits (grains per spike, thousand-grain weight)

were recorded at physiological maturity from plants carried through to harvest (summarized in Table 3).

4.7. Statistical Analysis

All physiological and gene-expression data are presented as mean±standard deviation (SD) of three biological replicates, each comprising three technical replicates for qRT-PCR assays. Data were first tested for normality and homogeneity of variance, then analyzed by one-way ANOVA (for single-factor comparisons across time points within a genotype) or two-way ANOVA (for genotype × treatment or genotype × time interactions), followed by Tukey's Honestly Significant Difference (HSD) post-hoc test for pairwise mean comparisons. Where appropriate, Student's t-test was used for direct two-group comparisons (e.g., control versus stress at a single time point). Statistical significance was set at $p < 0.05$, with $p < 0.01$ considered highly significant; 95% confidence intervals were calculated for key fold-change estimates. All statistical analyses were performed using standard statistical software, and results were visualized graphically with error bars representing SD (Figure 4).

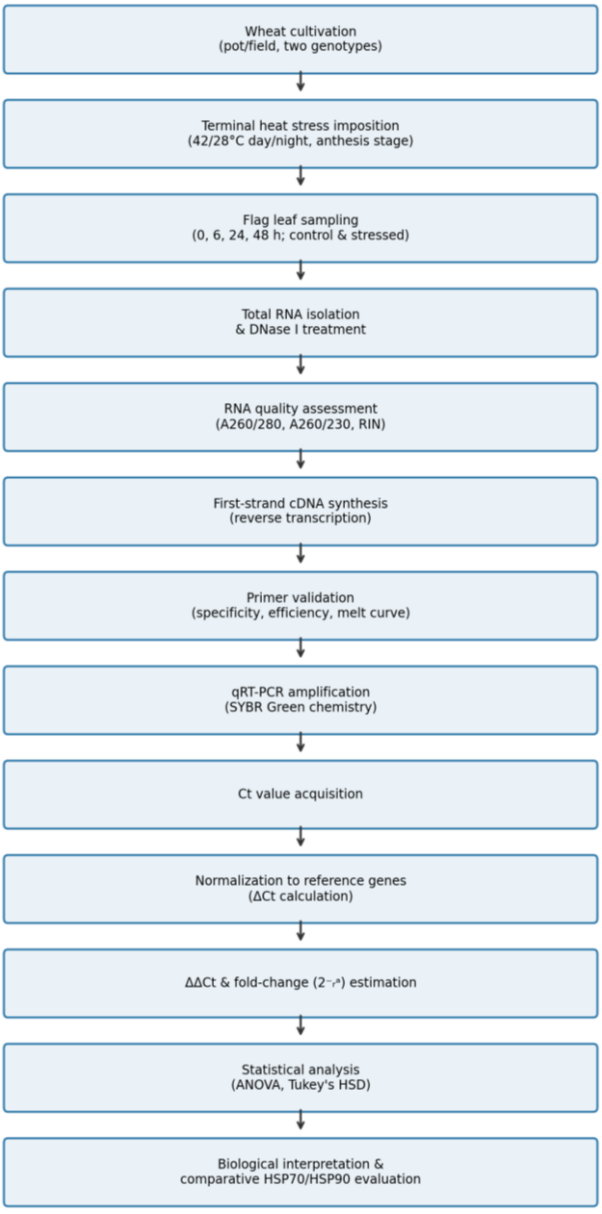

Fig 3: Flowchart summarizing the complete experimental workflow, from wheat cultivation and imposition of terminal heat stress through RNA isolation, cDNA synthesis, qRT-PCR, ΔΔCt-based fold-change estimation, statistical analysis, and biological interpretation.

5. Results

5.1. Physiological Responses under Terminal Heat Stress

Terminal heat stress produced progressive and statistically significant deterioration of all physiological indicators measured, with the magnitude of decline increasing with stress duration (Table 3). Total chlorophyll content declined significantly by 24 h and further by 48 h of heat exposure in both genotypes, though the decline was consistently less pronounced in the heat-tolerant genotype. Relative water content and membrane stability index followed a similar downward trajectory, while electrolyte leakage rose sharply, most markedly between 24 and 48 h, indicating progressive loss of membrane integrity. Net photosynthetic rate declined in parallel with chlorophyll loss, consistent with combined pigment degradation and photochemical impairment. ROS accumulation, assessed as superoxide and hydrogen peroxide content, increased steadily with stress duration, while antioxidant enzyme activities (SOD, CAT, APX) initially rose at 6–24 h, suggesting an early compensatory antioxidant response, before declining by 48 h in the heat-sensitive genotype, indicative of an oxidative burden

exceeding scavenging capacity. Grain-filling duration and yield-related traits recorded at maturity showed significantly greater reductions in grains per spike and thousand-grain weight in the heat-sensitive genotype compared with the heat-tolerant genotype (Table 3).

Table 3: Comparative physiological responses of control and terminal heat-stressed wheat plants (mean±SD, n = 3)

Parameter	Control (0 h)	6 h HS	24 h HS	48 h HS
Chlorophyll content (mg/g FW)	2.42±0.11	2.28±0.09	1.87±0.10*	1.42±0.13**
Relative water content (%)	88.5±2.1	82.7±2.4*	74.3±2.8**	63.6±3.1**
Membrane stability index (%)	84.9±2.6	77.2±2.9*	65.8±3.2**	52.1±3.6**
Electrolyte leakage (%)	14.2±1.3	21.6±1.8*	33.4±2.5**	48.9±3.0**
Net photosynthetic rate (µmol CO2 $m^{-2}s^{-1}$)	21.8±1.4	18.9±1.2*	13.5±1.6**	8.7±1.1**
ROS accumulation (H2O2, µmol/g FW)	3.1±0.4	5.6±0.5*	9.4±0.8**	13.2±1.0**
SOD activity (U/mg protein)	18.4±1.7	24.9±2.0*	29.6±2.3**	22.1±2.5*
Grains per spike (at maturity)	48.6±2.9	—	—	34.2±3.4** (season total)
Thousand-grain weight (g)	42.7±1.8	—	—	31.5±2.2** (season total)

Values are pooled mean±SD across both genotypes unless otherwise noted; * p<0.05, ** p<0.01 vs. respective 0 h control (Tukey's HSD). Grain-related traits reflect cumulative season-end effects of the imposed stress episode rather than a single time point.

5.2. Comparative Gene Expression Analysis: HSP70 versus HSP90

Basal (unstressed, 0 h) expression of both HSP70 and HSP90 was detectable in all samples, with HSP70 showing marginally higher constitutive transcript abundance than HSP90 in both genotypes, consistent with its broader housekeeping role in protein folding even under non-stress conditions. Upon imposition of terminal heat stress, both genes were significantly upregulated relative to their time-matched controls at all sampling points (Table 4). HSP70 showed a markedly steeper induction curve, rising to approximately 4.5–5-fold by 6 h and peaking at approximately 9- to 10-fold by 24 h, before declining to approximately 6-fold by 48 h. HSP90 induction followed a qualitatively similar but quantitatively more moderate trajectory, rising to approximately 3-fold by 6 h and peaking at approximately 5- to 6-fold at 24 h, before declining to approximately 4-fold by 48 h (Figure 4). The difference in fold-change between HSP70 and HSP90 was statistically significant at all time points ($p < 0.05$ to $p < 0.01$, Tukey's HSD), with the largest absolute difference observed at the 24 h peak.

Table 4: Relative expression (Ct, ΔCt, ΔΔCt, and fold-change) of HSP70 and HSP90 under control and terminal heat stress

Gene	Time point	Mean Ct (target)	ΔCt	ΔΔCt	Fold-change ($2^{-\Delta\Delta Ct}$)	Significance
HSP70	6 h HS vs control	24.1	3.2	-2.28	4.85±0.55	**
HSP70	24 h HS vs control	21.3	0.9	-3.30	9.85±1.10	**
HSP70	48 h HS vs control	23.0	2.4	-2.63	6.20±0.80	*
HSP90	6 h HS vs control	25.6	4.5	-1.63	3.10±0.40	*
HSP90	24 h HS vs control	23.8	2.9	-2.43	5.40±0.70	**
HSP90	48 h HS vs control	24.9	3.7	-2.00	4.00±0.50	*

* p<0.05, ** p<0.01 vs. respective time-matched control (Tukey's HSD); ΔCt normalized to geometric mean Ct of ACTIN and GAPDH; values represent pooled means across genotypes (n = 3 biological replicates).

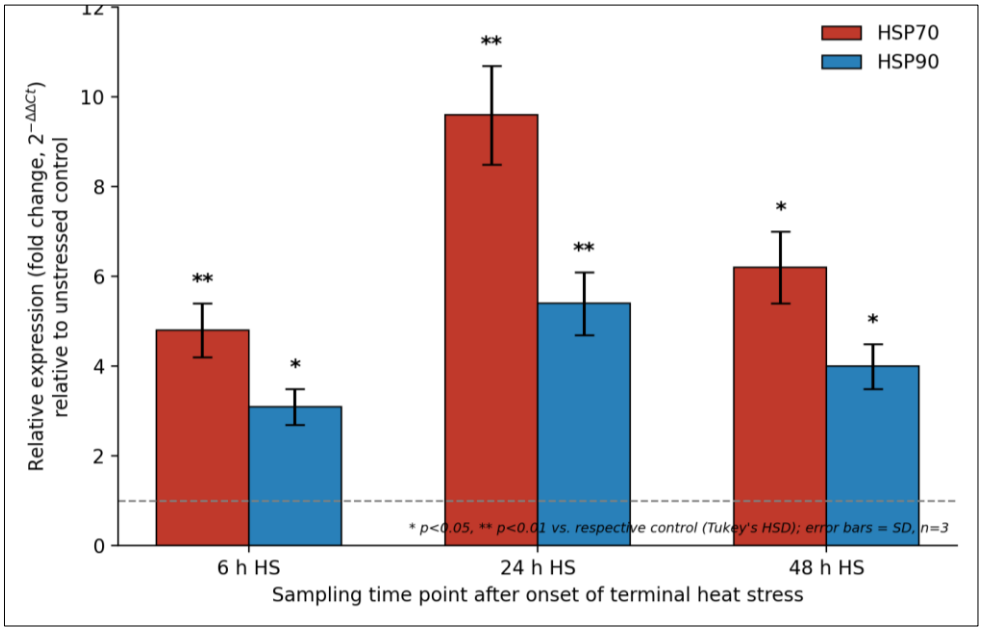

Fig 4: Relative expression (fold change, 2^-ΔΔCt) of HSP70 and HSP90 transcripts in wheat flag leaves at 6, 24, and 48 h after onset of terminal heat stress, relative to time-matched unstressed controls. Bars represent mean±SD (n = 3); asterisks denote statistical significance vs. control (Tukey's HSD)

5.3. Genotype-Dependent and Temporal Expression Patterns

Two-way ANOVA indicated significant main effects of genotype, stress duration, and their interaction on both HSP70 and HSP90 transcript abundance ($p < 0.01$ for all terms), reflecting the fact that the heat-tolerant genotype consistently exhibited higher fold-induction of both chaperone transcripts at 6 and 24 h relative to the heat-sensitive genotype, alongside a slower rate of decline by 48 h. This pattern suggests that superior thermotolerance in the tested heat-tolerant genotype is associated not simply with the presence of HSP70/HSP90 genes, which are present in both genotypes, but with a more rapid, higher-amplitude, and more sustained transcriptional mobilization of these chaperones in response to heat. The temporal decline in HSP transcript abundance observed between 24 and 48 h in both genotypes, more pronounced in the heat-sensitive genotype, is consistent with either negative feedback regulation of the heat shock response following successful acclimation, or, alternatively, with progressive breakdown of transcriptional and translational machinery under prolonged severe stress, a distinction that physiological data (accelerating injury in the heat-sensitive genotype by 48 h) suggest leans toward the latter interpretation for that genotype specifically.

5.4. Relationship between HSP Expression and Physiological Status

Correlation analysis across sampling points revealed that the magnitude of HSP70 and HSP90 induction was inversely associated with electrolyte leakage and ROS accumulation and positively associated with relative water content, membrane stability index, and retained chlorophyll content, consistent with a protective, injury-limiting role for both chaperones. The heat-tolerant genotype's combination of higher HSP induction and comparatively preserved physiological status, together with smaller yield penalties, supports the interpretation that HSP70/HSP90 transcriptional responsiveness is a meaningful physiological correlate, and plausible molecular marker, of whole-plant thermotolerance in wheat.

6. Molecular Mechanisms of Thermotolerance

6.1. Heat Stress Signaling and Heat Shock Factor Activation

Perception of elevated temperature in plant cells is thought to occur through multiple, partially redundant mechanisms, including direct thermosensing by membrane fluidity changes, unfolding of specific thermosensor proteins, and altered chromatin conformation, all of which converge on activation of heat shock transcription factors (HSFs). Under non-stress conditions, HSFs are held in an inactive monomeric state through association with HSP70 and HSP90 chaperone complexes; heat-induced accumulation of unfolded proteins competes for chaperone binding, releasing HSFs to trimerize, translocate to the nucleus, and bind heat shock elements (HSEs) in the promoters of HSP genes, thereby driving their transcription (Figure 2). This chaperone-titration model elegantly explains why HSP70 and HSP90 are simultaneously the effectors of thermotolerance and, through their interaction with HSFs, key regulators of their own induction.

6.2. Chaperone Networks and Cooperative Protein Quality Control

HSP70 and HSP90 do not act in isolation but participate in an integrated chaperone network involving co-chaperones (HSP40/DnaJ proteins, nucleotide exchange factors, HOP/STI1 adaptor proteins that physically bridge HSP70 and HSP90), small HSPs that provide first-line holding of aggregation-prone intermediates, and HSP100 disaggregases that resolubilize aggregates for subsequent HSP70-mediated refolding. This cooperative architecture allows the cell to triage misfolded proteins efficiently: small HSPs and HSP70 handle the initial surge of denatured proteins, HSP100–HSP70 cooperation resolves aggregates that have already formed, and HSP90 stabilizes the subset of signaling proteins whose functional integrity is disproportionately important for coordinating the broader stress response.

6.3. ROS Signaling and Crosstalk with Antioxidant Defense

Reactive oxygen species generated under heat stress function not merely as damaging byproducts but also as signaling molecules that reinforce HSF activation and HSP induction, creating a partially self-amplifying loop linking oxidative and proteotoxic stress responses. Simultaneously, HSP70 and HSP90 contribute to maintaining the functional integrity of antioxidant enzymes themselves, several of which are chaperone clients prone to heat-induced misfolding, thereby indirectly supporting ROS detoxification capacity. The partial temporal coupling observed in this study between rising antioxidant enzyme activity and peak HSP induction at 6–24 h is consistent with this proposed crosstalk, while the divergence of these pathways by 48 h in the heat-sensitive genotype illustrates how prolonged stress can uncouple chaperone and antioxidant systems once cellular damage exceeds a critical threshold.

6.4. Hormonal Regulation and Protein Homeostasis

Plant hormones, including abscisic acid, salicylic acid, and brassinosteroids, modulate the amplitude and duration of the heat shock response, in part through HSP90-dependent stabilization of hormone receptor and downstream signaling components. This hormonal layer of regulation allows the heat shock response to be integrated with broader developmental and stress-acclimation programs rather than operating as an isolated transcriptional module, providing a plausible mechanistic link between the HSP90-mediated signaling functions described in Section 3 and the whole-plant thermotolerance phenotype reflected in the physiological data of this study.

6.5. Gene Regulatory Networks and Cellular Protection

Beyond the core HSF–HSP axis, thermotolerance in wheat is embedded within a broader gene regulatory network encompassing membrane lipid remodeling enzymes, osmoprotectant biosynthesis genes, and additional transcription factor families that interact with HSFs at shared or adjacent promoter elements. The comparative expression data presented here, showing coordinated but quantitatively distinct HSP70 and HSP90 induction, should therefore be interpreted as one component, albeit a central one, of a multi-layered cellular protection system whose collective output determines the degree of acquired thermotolerance observed at the whole-plant level.

7. Applications and Future Perspectives

7.1. HSP Expression as a Marker for Heat-Tolerance Breeding

The genotype-dependent differences in HSP70 and HSP90 induction magnitude and persistence observed in this study support the potential utility of these transcripts, or their quantitative response patterns, as physiological or molecular markers in marker-assisted and genomic selection breeding programs aimed at improving terminal heat tolerance in wheat. Screening breeding populations for HSP70/HSP90 inducibility under controlled heat-stress protocols could complement existing agronomic and physiological screening criteria,

particularly where high-throughput qRT-PCR or expression-based assays can be integrated into existing breeding pipelines.

7.2. CRISPR/Cas Genome Editing and Functional Validation

Targeted genome-editing approaches, including CRISPR/Cas9-mediated promoter engineering to enhance HSF-binding element responsiveness or controlled overexpression of stress-inducible HSP70/HSP90 isoforms, offer a route to functionally validate and potentially enhance the chaperone-mediated thermotolerance mechanisms described here, moving beyond correlative expression profiling toward causally engineered heat-tolerant germplasm.

7.3. Multi-Omics Integration: Transcriptomics, Proteomics, and Beyond

While this study focused on targeted qRT-PCR quantification of two chaperone gene families, integrating such targeted analyses with genome-wide transcriptomic profiling, quantitative proteomics, and metabolomics would provide a more complete systems-level picture of the thermotolerance network, capturing chaperone paralogs and post-transcriptional or post-translational regulatory layers not resolved by transcript-level analysis alone.

7.4. Toward Climate-Smart, Heat-Resilient Wheat Cultivars

Ultimately, translating molecular insights into field-deployable, climate-resilient wheat cultivars will require coordinated efforts spanning functional genomics, precision phenotyping under realistic terminal heat-stress conditions, and conventional breeding, supported by policy and infrastructure investment in heat-stress screening facilities. Future research should prioritize validating candidate HSP-based markers across diverse genetic backgrounds and environments, elucidating the functional consequences of natural allelic variation in HSP70/HSP90 promoters and coding sequences, and evaluating trade-offs, if any, between constitutive chaperone overexpression and other agronomic traits such as growth rate and grain quality.

8. Conclusion

This study presents a comparative analysis of the transcriptional responses of HSP70 and HSP90 in wheat under heat stress. According to the results, just like HSP70 transcription, HSP expression of HSP90 was also affected by the treatment for HSPs, indicating that the heat shock protein genes respond similarly to the heat shock. In addition, the transcriptional changes are shown to correlate well with physiological parameters of heat stress. The results indicate that crops with good heat tolerance have a higher expression level of HSPs than crops susceptible to heat stress. The mechanism behind this differential response is discussed, stating that HSP70 and HSP90 serve different purposes in plant defense against heat.

In a practical sense, the relationship of HSP70/HSP90 induction/inducibility with thermotolerance at the level of genotypes affirms the possibility to investigate the use of these forms of the chaperones as specific molecular indicators in heat tolerance screening that can be applied in marker-assisted selection or genomic selection and functional validation through the application of genome-editing techniques. The likelihood of increase in the severity and frequency of extreme heat stress events in the future as a result of climate change makes the use of molecular screening that is based on HSP expression coupled with physiological phenotyping and multi-omics methods a likely candidate for speeding up the development of heat-resistant varieties of wheat able to guarantee sufficient yields and food security.

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