JBS Vol. 01 No. 01 (December 2025)

Expression Analysis of *Trehalose* Biosynthesis Gene under Drought Stress in Wheat

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Abstract

Trehalose is a glucose dimer and has been detected in a number of organisms. During physiological stress conditions such as salinity and desiccation, it functions as a complete protectant. Although trehalose biosynthesis has been observed in higher plants, the details of its physiological functions and the regulation of its pathways remain unknown. In recent years, however, a growing consensus has emerged that trehalose plays a role in plant growth and metabolism, as well as in tolerance against abiotic and biotic stresses. Recent data have revealed that trehalose is not just a protective sugar, but a multifunctional molecule, as it acts as a signaling molecule and impacts many pathogenic microorganisms and the infectivity of several others. Trehalose is both biosynthesized and applied exogenously. It increases the tolerance of plants to abiotic stresses. Recent studies have identified trehalose biosynthesis genes in local germplasm of wheat, such as the variety "Millat 1" under drought stress for different durations (2h, 4h, 6h, and 8h), compared to control plants.

Keywords: *Trehalose, Abiotic stress, Drought tolerance*

Introduction

One of the 21st century's most urgent issues is ensuring food security worldwide. By 2050, there will likely be more than 9 billion people on the planet, which will increase demand for sustainable agricultural production, particularly in developing nations like Asia and Africa, where more than half of the world's population lives (Mc Carthy et al., 2018).

The need to feed this expanding population is accompanied by a number of challenges for agriculture, such as growing input costs, a shortage of arable land, and increasing environmental stressors, especially those brought on by climate change. Drought stress is still the main one of these, seriously hindering plant growth and lowering crop yields all over the world. Drought is a major concern in agricultural research and policy because it is estimated to affect almost 45% of the world's agricultural land (Stefanis, 2014).

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https://doi.org/10.58932/MULL0005

An agrarian nation like Pakistan is especially susceptible to these environmental stresses. Pakistan's economy is based primarily on agriculture, which employs more than 43% of the workforce and accounts for 18.98% of the country's GDP. One of the most significant staple crops in Pakistan is wheat (Triticum aestivum L.), which is grown widely in Punjab and Sindh. Proteins, carbohydrates, fats, dietary fiber, and vitamins B and E are among the vital nutrients it offers. 3.02 percent of the national GDP and 11.3% of the agricultural sector are derived from wheat (Dogar, 2023). However, the productivity of wheat is increasingly challenged by water scarcity, a key factor influencing physiological processes such as cell division, photosynthesis, protein synthesis, and hormonal balance in plants (Hena et al., 2019).

One of the most common abiotic stressors, drought causes a series of molecular, physiological, and biochemical alterations in plants. These include altered gene expression, impaired water absorption, reduced stomatal conductance, and disrupted cell proliferation. Furthermore, related stressors like salinity, high temperatures, and nutrient shortages exacerbate the negative impacts on plant development and yield (Bashir et al., 2021). Drought tolerance in crops varies depending on species, genotype, developmental stage, and the duration and intensity of stress. In wheat, water stress particularly affects tiller number, stem elongation, and grain filling, directly impacting yield (Wahab et al., 2022).

Several studies have demonstrated that plants adopt different adaptive mechanisms to mitigate drought stress, including drought avoidance and drought tolerance strategies. Drought avoidance involves minimizing water loss and maximizing water uptake, while drought tolerance refers to the plant's ability to maintain metabolic activity under low water potential (Haghpanah et al., 2024). These strategies are supported by physiological responses such as osmotic adjustment, antioxidant production, and membrane stabilization. Osmoprotectants like trehalose, proline, and glycine betaine play a pivotal role in conferring stress tolerance by maintaining cell turgor, protecting cellular structures, and scavenging reactive oxygen species (ROS) (Singh et al., 2015).

The half of the food needs of the world is fulfilled by wheat (*Triticum aestivum)* as compared to the other crops, wheat is a protein rich and carbohydrate with sufficient calories. Wheat is enriched with all kinds of micro and macro nutrient molecules like proteins, starch, lipids, fibers, vitamins B &E. Wheat contains multiple nutrients like proteins, fats, carbohydrate, iron, dietry fibers, and starch which serve as a main commercial product of wheat after gluten. In Pakistan, it is observed wheat is the main crop which is cultivated in almost all region of the country but in Punjab and Sindh it is cultivated more than any other region of the country (Arzani & Ashraf, 2017).

Different kind of abiotic stresses like drought, salinity, and high temperature are often interweaved and bring a similar set of plant responses by triggering the similar or interrelating trails. The all kind of stresses such as solute accumulation, proteins stress and antioxidant properties can be studied at different cellular level. Under abiotic stresses to increase the plant recital needs to meet a set of stresses due to an inadequate thoughtful of plant stress acceptance mechanism. The stress tolerances can be improved by readapting metabolism signaling and programming gene expression through studying different complex system. Quality food demand can be fulfilled by adopting viable plant production approach is environmentally sustainable (Iqbal et al., 2022).

However, in stress environment, to manage crop production the current breeding methods lack suitable methods. In contrast, stress tolerant plants production and development can be dependent on genetic engineering of plants. Recent breeding methods combine with transgenic approaches. The transgenic approaches aim to transfer several kinds of genes intricate in multiple pathways such as companionable solutes osmoprotectants and proteins for production of stress tolerance plants. Without sacrificing crop production yield, identification of different genes is through use of transgenic approach which is a bottleneck approach to transfer the genes in plants (Singh, 2023).

Trehalose, a non-reducing disaccharide composed of two glucose molecules, has emerged as a multifunctional molecule in plant stress physiology. Initially recognized for its role as a protective sugar in prokaryotes and lower eukaryotes, trehalose is now acknowledged for its involvement in signaling pathways, energy metabolism, and transcriptional regulation in plants. Although its concentration in most higher plants is very low, trehalose and its precursor, trehalose-6-phosphate (T6P), are known to modulate sugar signaling and developmental processes such as embryo formation, flowering, and stress responses (Eh et al., 2024; Paul, 2007).

Homologs of these genes have been identified in various crops, including rice and wheat. Transgenic studies have shown that overexpression of trehalose biosynthetic genes can enhance plant tolerance to drought, salinity, and oxidative stress, although excessive trehalose accumulation may cause developmental abnormalities such as dwarfism and altered leaf morphology (Liu et al., 2025).

Trehalose functions not only as an osmoprotectant but also as a signaling molecule influencing the SnRK1 protein kinase pathway, which is crucial for plant energy homeostasis. Synthetic T6P analogs, when delivered into plant cells, have been found to regulate stress responses and improve yield under water-limited conditions. This dual role underscores the potential of trehalose and its intermediates as key targets in crop improvement strategies (Tsai & Gazzarrini, 2014).

In wheat, the study of gene expression related to drought tolerance is vital for understanding and improving resilience under water-deficient conditions. Gene expression profiling, particularly using quantitative real-time PCR (qRT-PCR), allows researchers to quantify the transcriptional activity of stress-responsive genes under varying environmental conditions. This approach facilitates the identification of differentially expressed genes and enables the selection of candidate genes for genetic engineering or marker-assisted selection (Nguyễn et al., 2024; Zhao et al., 2015).

The wheat variety 'Millat-11' has demonstrated considerable potential for drought tolerance, making it an ideal candidate for studying stress-responsive gene expression during critical developmental stages. Monitoring the expression of trehalose biosynthesis genes during the embryonic stage can provide insights into their functional role in stress mitigation and developmental regulation. Understanding how these genes respond to drought stress at various time intervals can inform breeding programs aimed at developing high-yield, drought-resilient wheat cultivars (Singh et al., 2025).

The current study focuses on the expression profiling of key trehalose biosynthesis genes in Millat-11 wheat under controlled drought stress conditions. By analyzing gene expression patterns at different durations of water stress, this research aims to elucidate the molecular mechanisms through which trehalose contributes to drought tolerance. The findings will enhance our understanding of stress adaptation in wheat and support the development of molecular tools for improving crop performance in arid and semi-arid regions (Singh et al., 2025).

Materials and Methods

Study design

The wheat variety Millat 11 was used in this research. This wheat variety was obtained from Faisalabad's Ayub Agricultural Research Institute.

Selection of the Genes

Two drought stress-responsive genes (Trehalose (TPS) and OTS) and their sequences from Arabidopsis, rice, cotton, and maize were obtained from the GenBank. The primers were designed using gene sequences that were found to be comparable to wheat sequences. The program 'Primer 3' was used to design the primers (You et al., 2008).

Sowing of the Seeds

Wheat variety Millat 11 seeds were planted in 20 cm × 20 cm soil pots and housed in the FCCU's climate-controlled room. The room maintained conditions ideal

for plant growth (temperature: 28°C, humidity: 26.1%, light intensity: 69001lux) (Schillinger et al., 2017).

Stress Treatment

Plants were separated into control and stress groups. Control plants were maintained under optimal growth conditions, while stressed plants were subjected to water deprivation for durations of 2, 4, 6, and 8 hours. Root samples were collected, frozen in liquid nitrogen, and stored at -80°C to preserve mRNA integrity (Datta et al., 2011).

RNA Extraction

Total RNA was extracted from plant samples using the Invitrogen Plant Purification Reagent, following the manufacturer's instructions. To assist phase separation, 100 mg of finely powdered tissue was combined with NaCl and chloroform. Following centrifugation, the clear aqueous layer containing RNA was carefully removed and precipitated with isopropanol. The precipitated RNA was then washed with 70% ethanol to eliminate contaminants, air-dried quickly, and redissolved in RNase-free water. The RNA's integrity was validated using agarose gel electrophoresis, and its concentration and purity were determined using a NanoDrop spectrophotometer (Razak et al., 2022).

cDNA Synthesis

CDNA was synthesized using Thermo Fisher Maxima H minus First Strand cDNA Synthesis Kit. 1 µl RNA was used with oligo (dT) 18 primers, reaction buffer, RNase inhibitor, dNTP mix, and reverse transcriptase. The reaction was incubated at 42°C for 60 min and terminated at 70°C for 5 min (Kolenda et al., 2019).

Reverse Transcriptase PCR

Newly synthesized cDNA was amplified using actin primers. PCR conditions included initial denaturation (95°C, 5 min), denaturation (95°C, 1 min), annealing (58°C, 1 min), extension (72°C, 1 min), and final extension (72°C, 10 min) (Dudziak et al., 2020).

PCR Optimization

Gene-specific PCR master mixes were prepared for OTS, TPS2, and TPS3 genes. The PCR products were analyzed on 2%–2.5% agarose gels using standard protocols and ladders for band size verification (Silva et al., 2014).

Quantitative Real-time PCR

qRT-PCR was performed to evaluate the expression levels of selected genes using SYBR Green dye. Each reaction included cDNA, forward and reverse primers, and nuclease-free water. Samples were analyzed in triplicates, and standard curves were used to assess PCR efficiency (Dudziak et al., 2020).

Table 1: *Primers for Genes and their Annealing Temperatures*

Gene	Primer Name	Sequence (5′–3′)	Annealing Temperature (°C)
OTS	OTS-1F	ATGAGTCGTTTAGTCGTAGTA	58°C
OTS	OTS-2R	CCCACTATCCTTCGCAAGACC	58°C
TPS	TPS-1F	TCAACACAGAGATCACCTCACTGC	65°C
TPS	TPS-1R	CTAGGGATCCTACATCGAGCACAAG	65°C
TPS	TPS-2F	GTCGGGAATTCCACACGATGCTCTC	65°C
TPS	TPS-2R	AACCTGTCGATCCTGTAACTGCTG	65°C

Results and Discussion

Drought Stress Treatment

As plants were removed from their pots, they were imperiled to water stress by cutting off the water stream for 2 hours, 4 hours, 6 hours, and 8 hours, until plants exhibited signs of wilting and symptoms of wilting, as reported in wheat variety Millet 11. This was measured and reported using a thermometer as plants exposed at room temperature at 28ºC. All wheat Millet 11 plants displayed withering after 20-25 days of growth. Twenty-day plant stage and Twenty-five-day plant stage.

RNA Isolation

Total RNA was isolated from wheat plant Millet 11 that had been treated to four distinct hours of stress (control and stressed samples) at both development phases, which were 20-25 days old. Under UV light, RNA was run on a 1% agarose gel (Appendix-I) to assess the integrity bands.

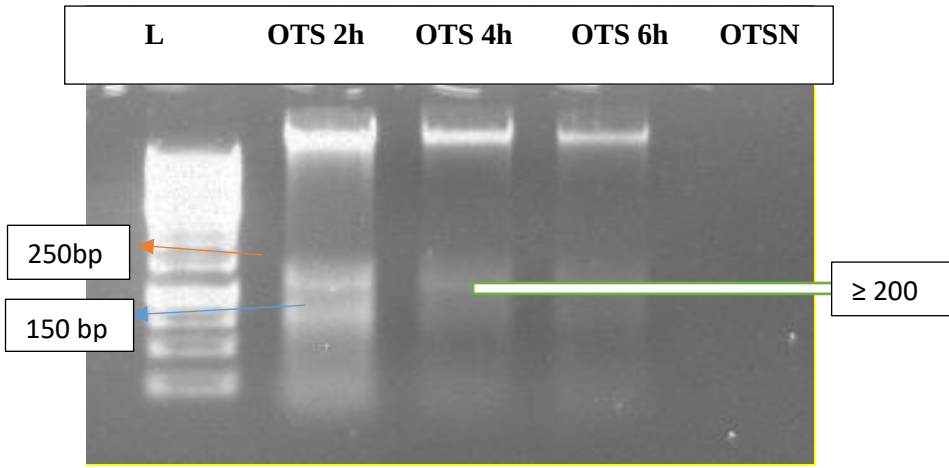
Reverse Transcriptase PCR

The RNA template was converted to cDNA. CDNA was used as a template for PCR, using wheat universal internal control actin primers and gene specific primers. There were also negative controls utilised. The actin amplicon size was about 365 base pairs, as determined by running the PCR product on a 1% agarose gel (Appendix-I) using a 1 Kb DNA ladder (Appendix-IV).

PCR Optimization of OTS Gene

The annealing range chosen for OTS primer optimization was 48-65 ºC. At the annealing temperature of 58ºC, the primer displayed significant brilliant bands of 200bp. Each negative reaction was conducted in tandem with each stress of varied hours. The PCR product was run on a 2 & 2.5% agarose gel and visualised under UV light with a 50 bp ladder (Figure 1).

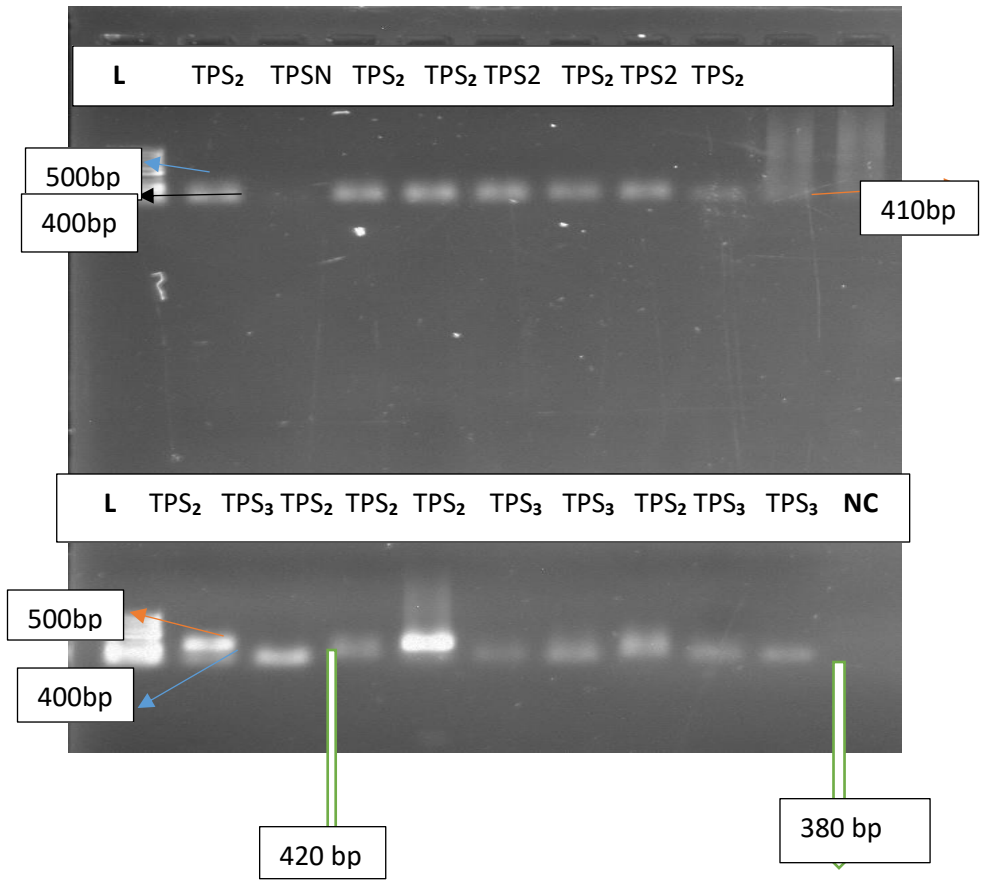
Figure 1: *PCR Optimization of OTS Gene at Different Hours Stress with Negative L= 50 bp DNA Ladder & Negative*



PCR optimization of TPS2 &TPS3

The annealing range chosen for OTS primer optimization was 48-65ºC. At the annealing temperature of 58ºC, the primer displayed significant brilliant bands of 200bp. Each negative reaction was conducted in tandem with each stress of varied hours. The PCR product was run on a 2 & 2.5% agarose gel and visualised under UV light with a 50 bp ladder (Figure 2)

Figure 2: *PCR Optimization Reaction of TPS2 &TPS 3 with Different Hours of Stress like 2h, 4h, 6h & 8h and Negative Control*



Using primers designed from known TPS/TPP sequences, we confirmed the presence of trehalose-biosynthetic genes in the drought-tolerant wheat cultivar 'Millet 11'. Quantitative RT-PCR (normalized to an actin reference) showed that these genes were expressed in leaves. Under progressive drought (simulated by withholding water), trehalose levels in Millet 11 leaves increased substantially compared to well-watered controls, consistent with activation of the pathway under stress. This parallels earlier observations in other wheat cultivars: for example, drought-resistant wheat accumulated significantly more trehalose in roots and leaves than sensitive cultivars (Grennan, 2007). Although the absolute trehalose concentration in wheat remains low (often µg/g fresh weight), such increases are considered physiologically meaningful,

since even small trehalose pools can stabilize enzymes and membranes during dehydration (Lunn et al., 2014).

Our findings suggest that Millet 11's drought tolerance may be partly linked to its trehalose metabolism. The induction of trehalose under stress is in line with its role as a compatible solute in many species (Lunn et al., 2014). Moreover, the essential nature of TPS1 in Arabidopsis embryo development implies that wheat TPS genes may also influence growth under stress. Consistent with this, transgenic approaches in other plants have shown improved drought resilience through trehalose-pathway engineering (Grennan, 2007). Thus, even though trehalose itself accumulates only in trace amounts, its biosynthetic pathway acts as a stress-response hub.

In conclusion, our preliminary results indicate that the trehalose biosynthesis gene is present and stress-responsive in Pakistan's wheat germplasm. Millet 11's ability to upregulate trehalose under drought correlates with its stress tolerance, supporting the hypothesis that the trehalose pathway contributes to drought adaptation (Grennan, 2007). Further studies are warranted to quantify TPS/TPP expression and enzymatic activity across genotypes and to determine how trehalose metabolism interacts with known drought-adaptation pathways in wheat.

Conclusion

The study concludes that trehalose biosynthesis genes play a significant role in wheat's response to drought stress. The expression profiling of these genes, particularly TPS and OTS, indicates their potential involvement in enhancing drought tolerance through osmoprotectant accumulation and signaling pathways. Understanding the regulation of these genes under water-deficit conditions can inform breeding strategies aimed at developing drought-resilient wheat cultivars, thereby contributing to sustainable agriculture and food security in arid and semi-arid regions.

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