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Regulation of the Thioredoxin System by Quercetin and Kaempferol in Arsenic Stressed Wheat (*Triticum aestivum* L.)

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Abstract

Thioredoxins (TRXs) are small proteins that function as redox regulators in various metabolic processes. However, due to the complexity of the thioredoxin system, research on its role in stress tolerance remains limited. This study aimed to elucidate the mechanisms by which quercetin (Q) and kaempferol (K) influence the transcription levels of NTRA, TRX15, TRX4, and TRXh1 genes, which are associated with the chloroplastic and cytoplasmic thioredoxin systems in wheat seedlings under arsenic (As) stress. The expression of TRX system-related genes was found to be reduced in wheat leaves subjected to As stress. While the expression of NTRA and TRX15 genes showed a slight increase in the As+Q group, Q did not exhibit a dominant effect on the regulation of the thioredoxin system. Similarly, As+K treatment led to an increase in TRXh1 gene expression. Although the regulatory effect of K was only noticeable with the combined application of Stress+Q+K, this effect was not strong enough to suggest that the thioredoxin system plays an active role in the stress response.

Keywords: Arsenic, Kaempferol, Oxidative stress, Quercetin, Redox regulation, Thioredoxin, Wheat

1. Introduction

Plants possess an evolutionarily conserved regulatory mechanism to protect light reactions in chloroplasts, regulate biogenesis, and maintain redox homeostasis under oxidative stress. This mechanism, known as the thioredoxin system, consists of small, multifunctional acidic proteins called thioredoxins (TRXs) (12-14 kDa molecular weight) and thioredoxin reductase (TRX reductase) enzymes, which reduce oxidized TRXs [1]. TRXs function as redox regulators in various metabolic and developmental processes across nearly all organisms. These proteins contain highly conserved cysteine residues in their redox-active sites, allowing them to modulate the activity of target enzymes through reversible thiol-disulfide exchanges [2].

In chloroplasts, two distinct TRX systems operate. Ferredoxin (Fd)-dependent TRX reductase (FTR) primarily reduces oxidized TRXs, playing a key role in photosynthetic electron transfer and enzyme activation [3]. Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent TRX reductase (NTR) and TRX-like proteins also contribute to redox homeostasis, particularly in chlorophyll metabolism, nitrogen assimilation, and stress responses [4].
Recent studies indicate that the thioredoxin system is activated in response to abiotic stresses, with increased expression of TRX-related genes localized in different organelles under stress conditions [5]. Moreover, research in *Arabidopsis* mutants has shown that NTR activity contributes to redox balance under stress conditions, supporting chloroplast function during abiotic stress [6]. However, due to the complex interplay between thioredoxins, redox-sensitive enzymes, and stress signaling pathways, further studies are required to fully elucidate the precise mechanisms underlying TRX-mediated stress responses. In particular, understanding how different abiotic stress conditions modulate TRX-dependent defense mechanisms in chloroplasts is essential for advancing stress-resistant crop development and improving agricultural productivity.

In recent years, significant progress has been made in identifying and characterizing TRX genes in wheat. Bhurta, et al. [7] conducted a genome-wide screening of TRX proteins in wheat plants, identifying 42 TRX genes localized on different chromosomes. Of these, TRX4 was found to localize in the chloroplasts, while TRX15 was localized to chloroplast membranes. Additionally, it has been shown that TRXh isoforms accumulate in the nucleus of cells under oxidative stress, suggesting their potential role in nuclear regulation of the stress response [8]. Among the TRXh proteins, the TRXh1 isoform has been identified as the most physiologically influential in wheat [9].

Arsenic (As) is a widespread metalloid in nature, yet it poses significant toxicity risks to living organisms, even in trace amounts, as it serves no biological purpose. In soils and waters, arsenic predominantly occurs as arsenate (AsV) and arsenite (AsIII), which differ in their toxic effects. AsV, resembling phosphate in structure, enters plant roots through phosphate transport systems, disrupting energy production and other critical phosphate-dependent processes [10]. Exposure to arsenic in plants hinders root function, leading to poor water and nutrient absorption. Photosynthetic efficiency declines sharply due to tissue damage, reduced chlorophyll content, and chloroplast impairment. This stress ultimately causes severe growth inhibition and biomass loss in crops like maize and wheat, as evidenced by various studies [11, 12].

Plants rely on a complex system of antioxidants to manage the harmful effects of reactive oxygen species (ROS), with both enzymatic and non-enzymatic mechanisms playing critical roles [13]. Phenolic compounds, a major group of non-enzymatic antioxidants, are secondary metabolites that support various physiological processes across a plant's life span. These compounds are synthesized under normal and stressful conditions, contributing to cell division, hormonal balance, photosynthesis, and reproduction [14]. During abiotic stress, plants increase the production of polyphenols like phenolic acids and flavonoids, along with the enzymes involved in their biosynthesis, to mitigate environmental stressors [15].

Kaempferol (K) is a natural flavonoid found in various plants and plant-based foods. Its role in apoptotic regulation and inhibition of cellular proliferation has been demonstrated in several cancer types, including breast cancer, ovarian cancer, and glioma [16, 17]. Quercetin (Q), another flavonol with a chemical composition similar to that of K, has been shown to act as a free radical scavenger in healthy cells [18]. In a study by Yao, et al. [19] on porcine zygotes, oxidative stress induced by H_2O_2 was observed to be neutralized by the application of 0.1 mM K. Kaempferol prevented the loss of membrane potential and the formation of ROS caused by H_2O_2. The application of K to stressed zygotes increased blastocyst formation and quality while reducing the level of DNA damage [19]. Similarly, a study on human granulosa cells demonstrated that Q application reduced H_2O_2-induced ROS production and apoptosis. It was also shown that Q protected cells from oxidative stress by enhancing the expression and activity of genes related to the TRX system [20]. Unfortunately, studies on the external application of K and Q to organisms have primarily focused on animal cells, as illustrated by the examples above [21]. One of the few studies in the literature involving plants was conducted by Parvin, et al. [22], who applied Q externally to tomato seedlings under salt stress. Their findings indicated that Q conferred tolerance to stressed plants by triggering antioxidant defense and glyoxalase systems. Although these studies highlight the potential of flavonoid applications in enhancing stress tolerance in plants, they do not address whether K and Q interact with the TRX system in plants. Therefore, the aim of the present study is to elucidate the mechanism by which exogenous Quercetin (Q) and Kaempferol (K) affect the transcription levels of genes associated with the thioredoxin system in wheat seedlings under arsenic stress.

2. Materials and Methods

2.1. Plant Material and Experimental Setup

Wheat seeds (*Triticum aestivum* L. cv. Ekiz) were sterilized with 5% NaOCl for 10 minutes, then rinsed five times with distilled water. The seeds were subsequently placed in moistened filter paper soaked with distilled water for germination. After germination, the wheat seedlings were grown under controlled conditions in a climate chamber using a water culture medium containing ½ Hoagland solution. Healthy 21-day-old wheat seedlings were used for the experiments. A preliminary trial was conducted to determine the appropriate application doses of Kaempferol (K) and Quercetin (Q). Based on the results, a concentration of 25 µM was selected as the optimal dose for phenolic applications under both control and stress conditions [22]. The doses of arsenic to be applied were determined by referencing comprehensive studies in the literature. Sil and Biswas [23] investigated arsenic content and antioxidant system elements in wheat plants by applying 25–50–100 µM sodium arsenate ($Na_2HAsO_4{\cdot}7H_2O$) and observed an increase in arsenic content and antioxidant enzyme activities after 21 days of stress. Consequently, As stress was applied at a concentration of 100 µM.

The experimental groups were arranged as follows:

Control Group: Plants grown without any additional treatments
Kaempferol Treatment (K): Plants treated with 25 µM Kaempferol
Quercetin Treatment (Q): Plants treated with 25 µM Quercetin
Arsenic Stress (As): Plants treated with 100 µM sodium arsenate
Combined Treatment (K + As and Q + As): Plants treated with 25 µM Kaempferol or Quercetin and 100 µM sodium arsenate

Each treatment group consisted of five biological replicates, and all plants were harvested 72 hours after treatment.

2.2. Molecular Analysis of Genes Related to the Thioredoxin System

2.2.1. RNA Isolation from Leaves and cDNA Library Construction

During harvest, 0.1 g leaf samples were collected under sterile conditions for RNA isolation and stored at -80°C until analysis. The total RNA was isolated from the samples using the Qiagen RNeasy kit. Remaining genomic DNA contamination was removed using DNase I (NEB). After determining the quantity and quality of the isolated total RNA, cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo K1622).

2.2.2. Gene Expression Analysis via qRT-PCR

For quantitative Real-Time PCR (qRT-PCR), ABM qPCR Master Mix was used. The mRNA content in each sample was normalized against the wheat Actin gene. The primers used in this study are listed in Table 1. The experiment was conducted with three independent replicates per reaction and run on the StepOnePlus™ Real-Time PCR System (Applied Biosystems™), with data analyzed using StepOne™ Software version 2.2.2. The qRT-PCR conditions were as follows: Initial denaturation at 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C for 15 seconds. Amplicon specificity was determined through melt curve analysis (65 to 97°C). The cDNA library of control *Triticum aestivum* plants was used as a reference, and the results were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. Primers used in gene expression analyses.

Gene	Forward and Reverse Primers
Actin	F- 5’ AGCGGTCGAACAACTGGTA 3’
	R- 5’ AAACGAAGGATAGCATGAGGAAGC 3’
TRX4	F- 5’ GATCGAATTCAGTTGTTCGGC 3’
	R- 5’ GTATAGGCTTGAGATGACATGGA 3’
TRX15	F- 5’ GTCTGAGAAGGACCACGACG 3’
	R- 5’ AATCGCACGCGCTAATTCA 3’
NTRA	F- 5’ TCTTCCGGAACAAGCCCATC 3’
	R- 5’ GTCCCACACGACCTGGATTT 3’
TRXh1	F- 5’ ACATGGCTAATGGCAAGGAGA 3’
	R- 5’ CGTTGTATGCTTCAGCGACGT 3’

2.3. Statistical Analyses

Each experiment was repeated at least three times, with each finding, consisting of two replicates (n=6). The obtained data were analyzed using one-way analysis of variance (ANOVA), and differences between means were compared using the Least Significant Difference (LSD) test. Values with $p < 0.05$ were considered statistically significant. Statistical analyses were conducted using the SPSS software (standard version 13.0).

3. Results and Discussion

In control conditions, Q and K treatments caused a 32% decrease in NTRA gene expression (Figure 1). Q+K treatment resulted in a 63% decreased NTRA expression compared to the control group. As exposure decreased NTRA expression in wheat leaves by 0.3-fold. As+Q increased NTRA expression by 41% and As+Q+K by 50% compared to the stress group.

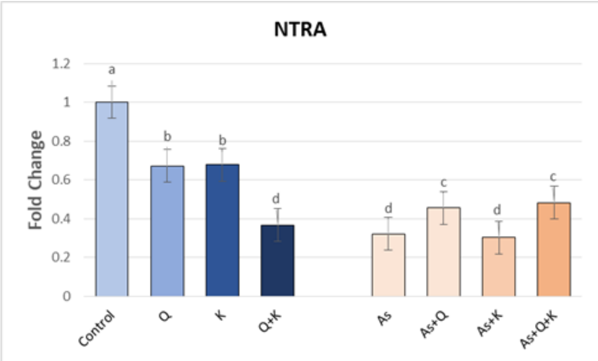

Figure 1. Effects of quercetin (Q) and kaempferol (K) applications on the gene expressions of NADPH-dependent thioredoxin reductase A (NTRA) in wheat leaves under arsenic (As, 100 µM) stress.

TRX15 gene expression increased 1.5-fold with Q treatment and decreased approximately 20% in K and Q+K treatments (Figure 2). TRX15 expression decreased by half in wheat shoots exposed to As stress. While a 20% increase in TRX15 transcript level was determined in the As+Q group compared to As stress, this rate increased to 50% in the As+Q+K group.

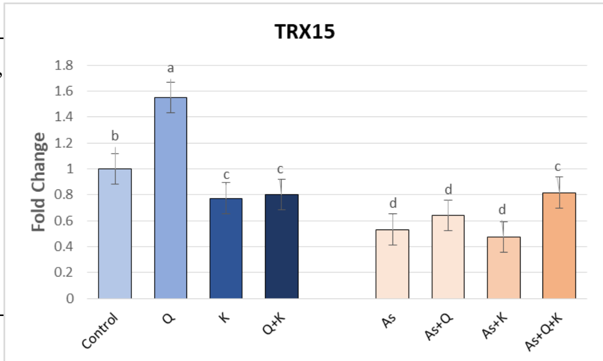

Figure 2. Effects of quercetin (Q) and kaempferol (K) applications on the gene expressions of thioredoxin 15 (TRX15) in wheat leaves under arsenic (As, 100 µM) stress.

All treatment groups caused decreases in TRX4 gene expression (Figure 3). TRX4 expression decreased by approximately 80% under As stress and was further reduced by Q and/or K treatments. Similarly, TRXh1 gene expression decreased with phenolic treatments under control conditions (Figure 4). TRXh1 expression decreased 0.4-fold in wheat leaves exposed to As stress. Under stress, an increase of 1.4-fold was determined in TRXh1 expression only in the As+Q+K combined treatment group.

Expression analyses of genes related to the thioredoxin system have demonstrated that all gene expressions are suppressed under arsenic (As) stress. Similar results were obtained in a study conducted by Yang, et al. [24] on rice seedlings under SCN^- tress, where the expressions of the thioredoxin system genes OsTrxF and OsTrxM5 were shown to decrease in parallel with the stress.

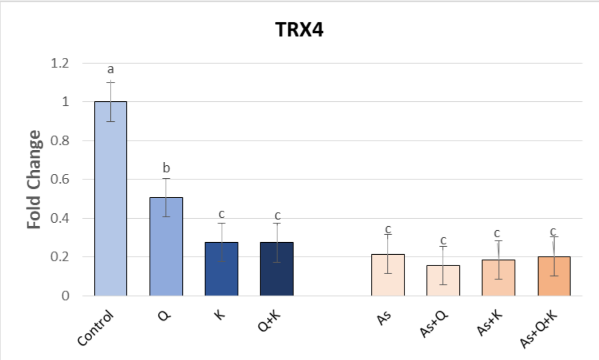

Figure 3. Effects of quercetin (Q) and kaempferol (K) applications on the gene expressions of thioredoxin 4 (TRX4) in wheat leaves under arsenic (As, 100 µM) stress.

Cazalis, et al. [9] reported a significant increase in Trxh1 gene expression in wheat seeds subjected to stress. However, in this study, Trxh1 gene expression decreased in wheat seedlings under stress. This discrepancy may be attributed to the involvement of the gene in the stress response during different developmental periods. In wheat seeds, h-type thioredoxin (Trxh) isoforms have been identified as playing significant roles during both germination and development, particularly by regulating the activity of enzymes responsible for the breakdown of storage proteins [9].

Following the discovery that Trxhs accumulate in the nuclei of cells under oxidative stress, it has been suggested that these proteins may also be involved in nuclear regulation related to the stress response [8]. Cazalis, et al. [9] also demonstrated that among the Trxh proteins in wheat, the Trxh1 isoform is the most influential in physiological changes. Similarly, Kamoun, et al. [25] demonstrated that the TdTrxh2 isoform of durum wheat plays a crucial role in abiotic stress tolerance by regulating redox homeostasis and enhancing stress tolerance in transgenic *Arabidopsis* through increased proline accumulation, antioxidant activity, and reduced reactive oxygen species levels. Another study highlighted the critical role of *Arabidopsis* TRXh5 protein in modulating the plant's defense response to biotic stress [26].

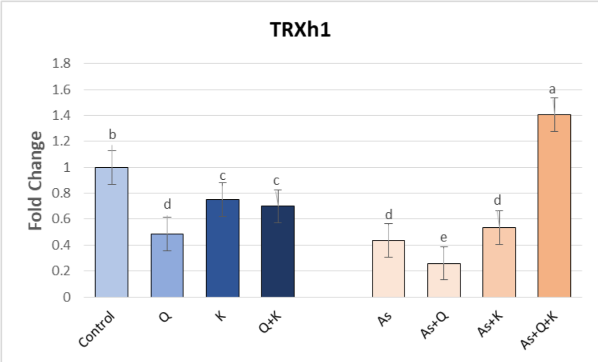

Figure 4. Effects of quercetin (Q) and kaempferol (K) applications on the gene expressions of thioredoxin h1 (TRXh1) in wheat leaves under arsenic (As, 100 µM) stress.

Trx proteins acquire their reducing power from Ferredoxin thioredoxin reductase (FTR) in plastids, and from NADPH thioredoxin reductase (NTR) in the cytosol and mitochondria [27]. Pulido, et al. [28] noted that the NTR/Trx system plays a role in preventing oxidative damage in wheat seeds. Additionally, Gan, et al. [29] demonstrated high oxidative stress levels in NTR activity inhibited tomato seedlings.

Quercetin (Q) applications induced the expressions of the NTRA and TRX15 genes in wheat leaves under As stress. Another study indicated that proline applications with antioxidant properties could enhance the expressions of genes associated with the thioredoxin system [24]. While K application led to a slight increase in the expression of only the TRXh1 gene, no effects were observed on the other genes. Conversely, the combined phenolic application (Q+K) significantly increased the expressions of the NTRA, TRX15, and TRXh1 genes, demonstrating its role in antioxidant protection. Nikkanen and Rintamaki [2] reported the impact of the thioredoxin system in regulating photosynthetic reactions in chloroplasts. Our results also consistent with the study of Tong, et al. [30] which showed several Trx genes were upregulated in exogenous nitric oxide treated *Liriodendron chinense.*

4. Conclusion

Although more detailed studies are needed to fully elucidate the role of the thioredoxin system in antioxidant defense, the findings so far support the potential of TRXs to confer tolerance to the chloroplastic antioxidant system under abiotic stress conditions. However, the results of this study indicate that under arsenic (As) stress, the selected thioredoxin system genes, including the NTRA enzyme gene, as well as the TRX15, TRX4, and TRXh1 protein genes, were not effective in generating a stress response or in antioxidant defense.

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Author's Contributions

Busra Arikan-Abdulveli: Drafted and wrote the manuscript, performed the experiment and result analysis.
Evren Yildiztugay: Supervised the experiment's progress, result interpretation and helped in manuscript preparation.

Ethics

There are no ethical issues after the publication of this manuscript.

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