Journal of Life Sciences & Biomedicine, vol. 6(79), No 2, p. 06-12 (2024)

Proline accumulation and WRKY14/WRKY36 gene expression dynamics during drought-induced flag leaf senescence

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Received: September 1, 2024; Received in revised form: October 17, 2024; Accepted: November 26, 2024

Wheat is one of the most important cereal crops globally; however, its productivity is often reduced due to abiotic stresses, particularly drought, which accelerates leaf senescence. This study focuses on investigating the roles of proline and WRKY transcription factors, specifically *WRKY14* and *WRKY36*, in regulating drought-induced premature senescence in wheat genotypes with varying stress tolerance. The experiments included genotypes of bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* Desf.), which were cultivated under controlled conditions with normal and drought regimes. After the booting stage, irrigation was discontinued for the drought-treated plants. Flag leaf samples were collected 7, 14, 21, 28, and 35 days after anthesis. Proline accumulation was significantly higher in drought-tolerant genotypes, peaking 21 days after anthesis, compared to sensitive genotypes. Similarly, the expression of *WRKY14* and *WRKY36* genes showed genotype-specific dynamics. The *WRKY36* gene exhibited higher expression levels in drought-tolerant genotypes, particularly under drought conditions, while the transcriptional activity of *WRKY14* was more associated with senescence processes. Understanding the molecular mechanisms governing proline metabolism and the role of WRKY transcription factors can offer valuable insights for developing wheat varieties with improved resistance to premature senescence.

Keywords: *Wheat, aging, water deficiency, transcription factors, RT-PCR*

INTRODUCTION

Wheat is one of the most important cereal crops, meeting the primary dietary needs of the global population and holding strategic significance worldwide (Saeed et al., 2024). Leaf senescence is a key agronomic trait that limits crop productivity (Guo et al., 2021; Gregersen et al., 2013). Senescence is a tightly regulated, complex process with significant implications for plants. Extending the duration of photosynthesis by delaying senescence in leaves during the grain-filling stage has been identified as an important strategy for enhancing both yield and grain quality (Li et al., 2024). This process involves the programmed redistribution of nutrients to developing tissues, replacing cellular functions with programmed cell death to ensure plant survival (Lim et al., 2007). During senescence, macromolecules such as proteins, lipids, and nucleic acids are broken down, and their components are transported to developing or storage tissues, playing a vital role in stress tolerance and yield under adverse environmental conditions (Doan et al., 2024). In agriculture, leaf senescence significantly impacts yield and quality, especially in staple crops like wheat. The senescence process is regulated by intrinsic factors (e.g., aging) and external signals, including environmental stresses. Among them,

https://doi.org/10.59849/2710-4915.2024.2.6
Available online 31 December 2024

senescence plays a crucial role under drought and other abiotic stress conditions (Zhang et al., 2020).

Climate change remains one of the greatest challenges of the 21st century, profoundly impacting agriculture by altering growing conditions and posing severe threats to crop production. Increasing temperatures, unpredictable weather patterns, altered precipitation regimes, and more frequent extreme events such as droughts and floods are direct consequences of climate change, adversely affecting crops (Verma et al., 2024). These stress factors lead to reduced yields, decreased crop quality.

Proline plays a multifaceted role in plant senescence, acting as a critical adaptive molecule under both natural and stress-induced conditions. While proline is widely recognized for its contribution to stress tolerance, its role during senescence involves several interconnected physiological and molecular processes. Proline serves as both a stress mitigator and a regulator of senescence-associated processes. Its accumulation under stress conditions helps delay the detrimental effects of premature senescence, while its controlled metabolism facilitates nutrient redistribution and energy generation essential for programmed senescence. These complex roles position proline as a key molecule in elucidating the mechanisms underlying plant senescence, particularly under environmental stress conditions. Research on proline's regulatory networks and its interaction with other senescence-related factors is crucial for advancing our understanding of how plants adapt to stress and optimize their growth and productivity.

At the molecular level, leaf senescence involves significant changes in gene expression, regulated by senescence-associated genes (SAGs) (Cao et al., 2023). Transcription factors, including the WRKY family, play a pivotal role in these regulatory networks. WRKY proteins specifically bind to W-box elements in gene promoters, orchestrating various physiological responses, including nutrient redistribution and stress adaptation (Tan et al., 2024; Rushton et al., 2010). WRKY transcription factors are key regulators in plants, participating in diverse processes. They contain at least one WRKY domain, which is highly conserved across members, with approximately 60 amino acids in the N-terminal, and possess a characteristic zinc-finger structure in the C-terminal (Rushton et al., 2010). Based on the number of WRKY domains or zinc-finger structures, WRKYs are categorized into three groups. Group I contains two WRKY domains, including the CX4-5CX22-23HXH (C2H2) zinc-finger motif. Group IIa-e contains a single WRKY domain with a C2H2 motif, while Group III has a single WRKY domain and a CX7CX23HXC (C2HC) zinc-finger motif. The WRKY domain can specifically bind to the W-box ([T][T]TGAC[C/T]) in target gene promoters to regulate transcription (Rushton et al., 2010).

Recent studies reveal that several WRKY transcription factors play crucial roles in the regulatory networks of leaf senescence, exhibiting high expression levels in senescing leaves. Research on WRKY genes regulating senescence has primarily focused on model plants such as Arabidopsis and rice. However, there is limited information on senescence-related WRKYs in wheat. Recent findings have identified 116 WRKY members in the wheat genome, with 13 members upregulated during senescence in flag leaves (Borrill et al., 2019). Furthermore, 13 genes from the TaWRKY family have been confirmed as senescence-associated genes (SAGs).

The main objective of the study was to investigate the role of proline and WRKY transcription factors in regulating processes associated with drought-induced premature senescence in wheat plants.

MATERIALS AND METHODS

Cultivation of plant material. Seeds from local bread wheat (*Triticum aestivum* L.) genotypes, including the drought-tolerant Gyrmyzy gul 1 and the sensitive Tale 38**,** as well as durum wheat (*Triticum durum* Desf.) genotypes, the tolerant Vugar and the sensitive Tartar, were sourced from the gene pool of Research Institute of Crop Husbandry (Baku, Azerbaijan). The plants were grown in controlled environment chambers under control and drought conditions using a completely randomized design. During the entire experimental period, ambient

temperatures were controlled within the 19-29°C range, and relative humidity was maintained between 50% and 65%. Plants were cultivated under controlled conditions with a photoperiod of 16 hours of light and 8 hours of darkness. Flag leaves were collected at 7, 14, 21, 28, and 35 days post-anthesis. The collected leaf samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

Determination of proline concentrations. Extraction and measurement of free proline were performed according to the acid ninhydrin method described by Bates et al (1973).

RNA extraction and cDNA synthesis. Total RNA was isolated from leaf tissues using the Monarch Total RNA Miniprep Kit (New England Biolabs, Inc.) as per the manufacturer's guidelines. Genomic DNA was eliminated by treating the RNA with RNase-free DNase I. The integrity and purity of the extracted RNA were evaluated via agarose gel electrophoresis. RNA concentrations were determined using a NanoDrop Thermo Scientific-2000C spectrophotometer (USA). Complementary DNA (cDNA) synthesis was carried out from the isolated RNA using the LunaScript RT SuperMix Kit (New England Biolabs, Inc.), following the instructions provided by the manufacturer, in a final reaction volume of 20 µl.

Quantitative real-time PCR. PCR was performed using a Mic Real-Time PCR system with a total reaction volume of 20 µl. Each reaction mixture consisted of 10 µl of Luna Universal qPCR Mix (New England Biolabs, Inc.), 1 µl of 1:5 diluted cDNA, 0.5 µl each of forward and reverse primers (10 µM), and 7 µl of nuclease-free water. The amplification protocol began with an initial denaturation at 94°C for 60 seconds, followed by 45 cycles at 95°C for 15 seconds and 60°C for 30 seconds. No-template controls (NTCs) were included for every primer pair. Each reaction was carried out in triplicate (technical replicates) for three biological replicates. Elongation factor 1 alpha (Elf1-α) served as the reference gene. The primer sequences utilized for expression analysis are provided in Table 1. Primer efficiency for each set was evaluated using the standard curve method with serial dilutions of cDNA, calculated using the equation: Efficiency (%) = (10^(-1/slope) - 1) × 100. Dissociation curves for each amplicon were examined to ensure the specificity of amplification. Relative gene expression levels (stressed versus control conditions) were determined using the 2^−ΔΔCt approach (Livak and Schmittgen, 2001).

Table 1. Sequences of primers used for qRT-PCR.

Gene	Direction	Sequences
WRKY14	F	GATGACATAGATGCTGGAGGTGG
	R	TGTGGCGTCGCTGTGGTT
WRKY36	F	GTCAGCAGCCAGCCTTCCCTTAGCC
	R	CGTCGCCACGAGTATGGTCTTGTCC
Elf1-α	F	CAGATTGGCAACGGCTACG
	R	CGGACAGCAAAACGACCAAG

Statistical analysis: The statistical analysis was carried out using SAS software ver9.2 (SAS Institute, 2008). Standard deviation (SD) values are from at least three biological replicates.

RESULTS AND DISCUSSION

Proline content was measured in flag leaves at five-time points (7, 14, 21, 28, and 35 days after anthesis, DAA) during natural (age-induced) and drought-induced senescence in wheat genotypes with contrasting stress tolerance. In all genotypes, proline accumulation increased under drought conditions, peaking at 21 DAA, except for the highly drought-tolerant durum wheat genotype Vugar, which exhibited the highest increase in proline levels at 28 DAA (~6 µmol/mg fresh weight) (Fig. 1). This stage is likely critical for proline biosynthesis and coincides with increased sensitivity of plants to drought during the grain-filling period. Under control conditions, proline levels remained relatively stable and low at all developmental stages in all genotypes, with no distinct peaks. Despite a decline in proline levels at later stages, it remained higher than control values until the end of ontogenesis in drought-tolerant genotypes. In contrast, drought-sensitive genotypes (Tartar and Tale 38) also showed a peak in proline accumulation at 21 DAA (~5 and ~4 µmol/mg fresh weight, respectively); however, the decline in proline levels after the peak was more pronounced, indicating a less robust response to drought stress.

Plants have developed numerous defense mechanisms to mitigate the detrimental effects of

adverse environmental conditions. Among these mechanisms, the synthesis of protective compounds, including specific amino acids (e.g., proline), peptides (e.g., glutathione and phytochelatins), and polyamines (e.g., putrescine), plays a critical role (Hayat et al., 2012; Alcázar et al., 2020). The accumulation of proline under stress conditions has been confirmed in numerous plant species and is associated with adaptation to drought, salinity, or osmotic stress simulated by polyethylene glycol (PEG) (Hayat et al., 2012). Beyond its role as a widely distributed osmolyte, proline contributes to stress adaptation by scavenging reactive oxygen species (ROS), stabilizing proteins and intracellular structures, regulating intracellular redox potential, and acting as a signal molecule or heavy metal chelator (Hayat et al., 2012; Zdunek-Zastocka et al., 2021).

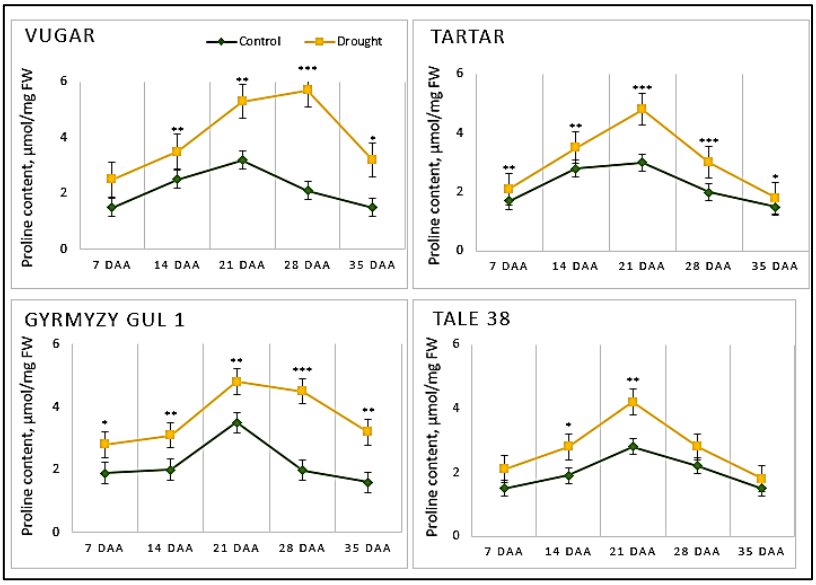

Fig. 1. Changes in proline content in wheat flag leaves during senescence. DAA – days after anthesis, FW – fresh weight. Data points represent the mean ± se (n = 5). Asterisks indicate the significance of differences (*P < 0.05, **P < 0.01, ***P < 0.001) using pairwise t-tests.

The expression of the *WRKY14* transcription factor gene exhibited varying dynamics depending on genotype and growth conditions (Fig. 2). In naturally senescing plants, the transcript levels of this gene sharply increased at the late stages of senescence. In drought-stressed plants, early senescence was observed in drought-tolerant genotypes, with the highly resilient durum wheat genotype Vugar showing an increase at 21 DAA, while in the tolerant bread wheat genotype Gyrmyzy gul 1, the rise occurred earlier, at 14 DAA. In drought-sensitive genotypes, a significant increase in *WRKY14* expression was observed at 35 DAA in the durum wheat genotype Tartar and at 28 DAA in the bread wheat genotype Tale 38. These findings indicate that the *WRKY14* transcription factor is more strongly induced by senescence than by the drought stress response itself and is more specific to the later stages of the senescence process.

The expression of the *WRKY36* transcription factor gene also varied depending on growth conditions, genotype, and plant developmental stage (Fig. 3). In plants grown under control conditions, the expression of this gene sharply increased at 35 DAA, during the most intensive phase of senescence. Under stress conditions, drought-tolerant genotypes exhibited earlier and higher *WRKY36* expression compared to sensitive genotypes. In the drought-tolerant durum wheat genotype, expression began increasing at 21 DAA, while in the drought-tolerant bread wheat genotype, the gene displayed significant transcription as early as 14 DAA. In contrast, sensitive genotypes showed a less pronounced increase in *WRKY36* expression, with a delayed peak at 35 DAA,

indicating less effective stress adaptation mechanisms. These findings underscore the critical role of *WRKY36* in enhancing plant resilience to abiotic stresses, particularly during the critical post-anthesis period.

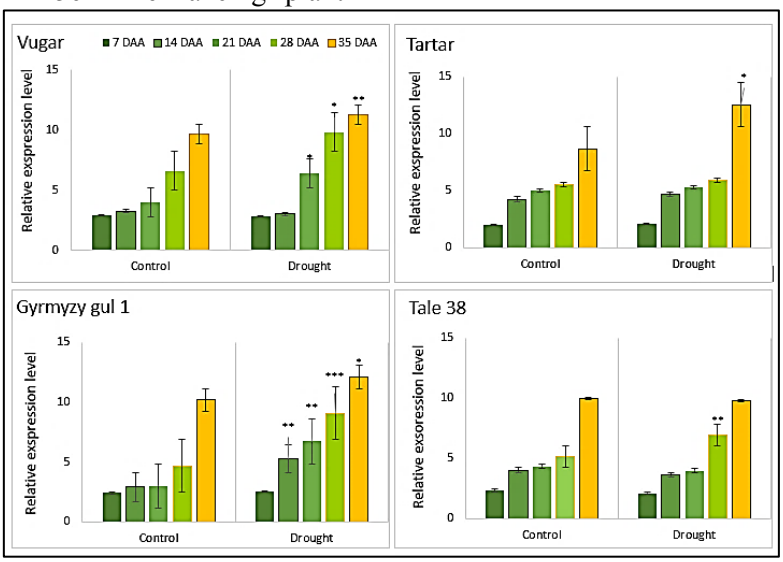

Fig. 2. Transcript level of WRKY14 transcription factor gene in flag leaves of wheat plants during natural and stress-induced senescence. DAA – days after anthesis. The fold change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean ± se (n=5). Asterisks indicate the significance of differences (*P < 0.05, **P < 0.01, ***P < 0.001) using pairwise t-tests.

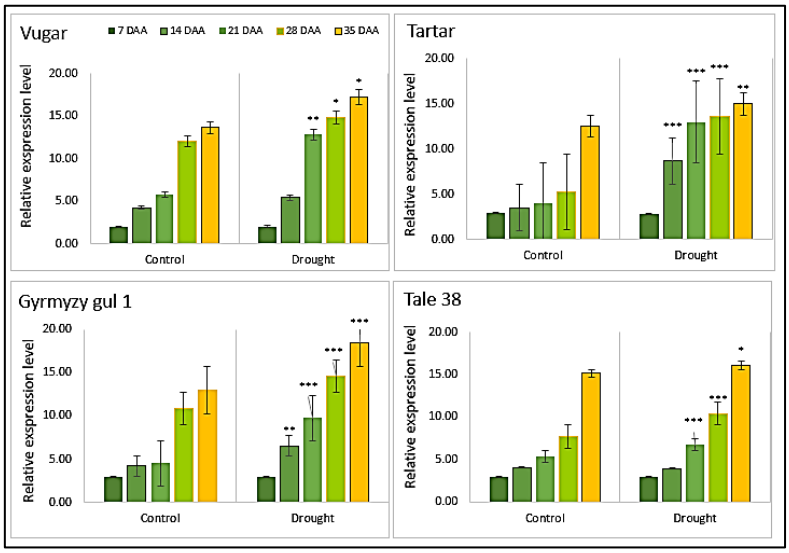

Fig. 3. Transcript level of WRKY36 transcription factor gene in flag leaves of wheat plants during natural and stress-induced senescence. DAA – days after anthesis. The fold change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean ± se (n = 5). Asterisks indicate the significance of differences (*P < 0.05, **P < 0.01, ***P < 0.001) using pairwise t-tests.

Its earlier activation in tolerant genotypes suggests that *WRKY36* contributes significantly to mitigating stress effects and delaying senescence under drought conditions.

Summarizing, it can be concluded that the *WRKY36* gene demonstrated higher expression

levels in drought-tolerant genotypes, especially under drought conditions, compared to *WRKY14*. This observation suggests a more active role for *WRKY36* in drought adaptation. The differences between the *WRKY14* and *WRKY36* genes are primarily attributed to their distinct signaling pathways and target genes. For instance, *WRKY14* targets include TaPR1 (pathogenesis-related protein), TaLOX2/3 (genes involved in jasmonate biosynthesis), and TaPAL (Baillo et al., 2019). In contrast, *WRKY36* regulates drought- and senescence-related genes such as *DREB2A* and *RD29A/B*. Despite these differences, both genes perform complementary functions, contributing to plant adaptation under stress conditions (Zhou et al., 2023). Increased expression of specific genes during senescence highlights the activation of pathways involved in the synthesis of proteins that either regulate or adapt to the aging process. For example, in rice, *AtWRKY6* directly regulates the transcription of senescence-induced receptor kinase (SIRK) genes via W-box promoter binding. Overexpression of *AtWRKY6* results in distinct phenotypes of premature and delayed senescence. Similarly, *AtWRKY53*, which regulates senescence-associated gene (SAG) expression, is induced by reactive oxygen species (ROS), emphasizing its role in oxidative stress signaling. Furthermore, *AtWRKY53* interacts with *AtWRKY30,* and its suppression by microRNAs prevents senescence phenotypes (Zeng et al., 2024). Other WRKY transcription factors such as *AtWRKY54, AtWRKY57*, and *AtWRKY70* also contribute significantly to regulating leaf senescence (Zhao et al., 2020). In wheat, *TaWRKY7* has been identified as a stress-responsive transcription factor, whose overexpression in Arabidopsis accelerates dark-induced leaf senescence. Its expression is upregulated under drought conditions and is associated with abscisic acid (ABA)-mediated stress responses. These findings highlight its dual role in senescence regulation and drought adaptation. Similarly, *TaWRKY42-B* has been identified as a positive regulator of leaf senescence in wheat. Its overexpression in Arabidopsis accelerates senescence, while its suppression in wheat delays both natural and dark-induced senescence. The interaction of *TaWRKY42-B* with jasmonate biosynthesis genes such as *TaLOX3* leads to jasmonate accumulation, triggering early senescence through JA-dependent signaling pathways.

Additionally, *TaWRKY14* has been shown to enhance chlorogenic acid biosynthesis in tobacco (*T. antungense*), illustrating its role in early stress adaptation. It also regulates the expression of pathogenesis-related genes and jasmonate biosynthesis genes, thereby enhancing plant defense against both biotic and abiotic stresses (Chen et al., 2023). Furthermore, its expression is upregulated under drought stress and linked to salicylic acid (SA)-dependent signaling pathways. These characteristics position *TaWRKY14* as a critical regulator of stress tolerance and senescence. Similarly, *TaWRKY36* has been associated with ABA-mediated signaling and the regulation of SAGs such as *SAG12* and *SAG29* during senescence. It also interacts with antioxidant defense genes, including catalase and superoxide dismutase, to mitigate ROS accumulation and maintain cellular stability under stress conditions (Vittozzi et al., 2024). These mechanisms highlight the functional importance of *WRKY36* in coordinating late-stage senescence and stress responses.

In conclusion, WRKY transcription factors play versatile roles in the regulation of senescence and stress responses. Their functional diversity and integration into hormonal and oxidative stress signaling pathways make them valuable targets for genetic improvement in crops. Further research on the precise mechanisms of WRKY-mediated regulation will provide critical insights for developing stress-resilient plant varieties.

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