International Information and Engineering Technology Association
Advancing the World of Information and Engineering

International Journal of Design & Nature and Ecodynamics
Vol. 20, No. 11, November, 2025, pp. 2581-2591
Journal homepage: http://iieta.org/journals/ijdne

Brassinolide and GABA Synergistically Upregulate Iron-Uptake Genes in Hydroponic Wheat Under Iron Deficiency

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https://doi.org/10.18280/ijdne.201109

Received: 28 September 2025
Revised: 14 November 2025
Accepted: 20 November 2025
Available online: 30 November 2025

Keywords:
iron deficiency, brassinolide, GABA, wheat, gene expression, iron uptake, hydroponic conditions

ABSTRACT

This research examined the synergistic interactions of iron (Fe), brassinolide (Br), and gamma-aminobutyric acid (GABA) on wheat (*Triticum aestivum* L.) gene expression under hydroponic conditions and specifically under iron-deficient conditions. Six wheat cultivars were treated with three concentrations of iron (1, 10, and 20 µmol L^{-1}) and Br, GABA, or a combination of both. Quantitative polymerase chain reaction (RT-qPCR) was employed to evaluate gene expression. The RT-qPCR analysis revealed that the expression of iron uptake genes was significantly upregulated, and *TaTOM* and *TaYSL* (4.41-fold and 2.99-fold, respectively) were significantly increased in iron-deficient conditions (1 µM). The Br + GABA treatment had a significant effect on increasing gene expression, where *TaTOM* was increased 2.26 times, *TaYSL* increased 1.97 times, and *TaNAAT* increased 1.27 times compared to control plants. Interestingly, the Super Max cultivar showed the greatest levels of expression, particularly when treated with low levels of iron and with the combination of Br + GABA. The findings indicate that the Br + GABA intervention facilitates the effective intake of iron through the up-regulation of significant genes, highlighting its potential for enhancing the efficiency of iron uptake in wheat and offering valuable insights for crop breeding initiatives that aim to find the most micronutrient-efficient and stress-resistant crops.

1. INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important strategic crops in the world. With the development of modern agricultural technologies, hydroponics has emerged as a promising solution to save water and soil and increase production efficiency [1]. However, the conditions in the hydroponic system may affect the availability of microelements and the physiological balance of the plant, which requires thoughtful interventions to stimulate its molecular performance [2]. In this context, the role of iron, the hormone brassinolide, and gamma-aminobutyric acid (GABA) comes as stimulating factors for gene expression and modifying the biological and physiological responses in wheat [3].

Iron (Fe) is a vital element in the life cycle of plants, playing a central role in key physiological processes, such as respiration and photosynthesis. Its significance lies particularly in its involvement in redox reactions, as it forms part of electron transfer proteins like cytochromes and ferredoxins [4]. Although abundant in soils, iron is often found in insoluble forms that limit its availability to plants. To cope with this challenge, plants activate specific genes such as *IRT1*, which facilitates iron uptake. Iron is a key micronutrient required for various plant functions, especially in energy production and chlorophyll formation [5]. Because iron is often poorly available in soils, plants use two main uptake strategies. Non-graminaceous plants reduce Fe^{3-} to Fe^{2-} at the root surface before absorption, while graminaceous plants, such as wheat, release natural chelators that bind iron and help transport it into root cells. These processes are influenced by internal signals and environmental conditions, ensuring plants maintain iron balance under different growth situations [6].

There is an urgent need to develop strategic crops with enhanced abilities to absorb essential micronutrients, most notably iron. It has been observed that iron plays a vital role in photosynthesis, respiration, and chlorophyll biosynthesis, which are all critical for plant development and productivity [7]. However, under iron-deficient conditions, the plant's physiological functions are compromised, leading to reduced growth, impaired chlorophyll synthesis, and ultimately lower yield [8]. Therefore, understanding the molecular and physiological mechanisms that enable wheat to adapt to iron deficiency is of paramount importance [9].

Recent studies have highlighted the crucial role of siderophores, small Fe^{3+} chelating molecules, in enhancing iron availability under deficiency conditions, as well as their regulation by specific biosynthetic genes in wheat. Key genes include *TaNAS,* which encodes nicotianamine (NA) synthase for nicotianamine production; *TaNAAT* and *TaDMAS*, which convert it to deoxymugineic acid (DMA), the primary phytosiderophore; *TaTOM*, a transporter that imports Fe^{3+}-DMA complexes into root cells; and *TYS*, a regulatory gene involved in modulating this pathway [10, 11].

Similarly, plant growth regulators like brassinolide (Br), a type of brassinosteroid, activate key molecular signalling pathways, including hormone transduction and calcium signalling. These pathways help plants adjust their metabolism, protect cells, and improve tolerance to drought stress [12], and GABA has gained attention for its roles in enhancing nutrient uptake and stress tolerance. Brassinolide promotes root development, modulates iron-uptake gene expression, and improves adaptation under micronutrient stress [13], while GABA contributes to oxidative homeostasis, secondary metabolite accumulation, and enhanced mineral acquisition, particularly iron, an amino acid that plays a central role in plant growth and stress adaptation [14]. It functions as an osmotic regulator that helps maintain cellular water balance and protects membrane stability under adverse conditions. In addition, GABA enhances the antioxidant defence system, thereby reducing oxidative damage caused by reactive oxygen species. Its accumulation is strongly associated with improved tolerance to drought, salinity, heat, and cold stresses. Beyond its protective effects, GABA interacts with key phytohormones, such as auxins and abscisic acid [15], which allows it to coordinate growth processes and activate stress-responsive genes. Thus, GABA represents a crucial signalling and metabolic molecule that strengthens plant resilience and contributes to sustaining productivity under challenging environments [16].

This study aimed to fill a gap in understanding how the interaction between iron, Br, and GABA influences the regulation of iron-uptake-related genes (*TaNAS*, *TaNAAT*, *TaDMAS*, *TaTOM*, and *TYS*) in wheat under hydroponic conditions. While iron is a critical micronutrient, its bioavailability in the soil is often limited, making the use of plant hormones such as Br and GABA an attractive approach to enhancing nutrient uptake and stress tolerance. Accordingly, we hypothesize that Br and GABA synergistically enhance the expression of key iron-uptake genes, including *TaNAS*, *TaNAAT*, and *TaTOM*, in wheat roots under iron-deficient conditions. We further propose that this synergistic effect differs among wheat genotypes. Using reverse transcription quantitative polymerase chain reaction (RT-qPCR) to monitor temporal gene expression, the study seeks to elucidate molecular responses underlying iron acquisition and identify promising genotypes for breeding programs that enhance micronutrient efficiency and support sustainable agriculture.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Source of plant materials

Six soft wheat cultivars were used as plant materials in this study. The local wheat cultivars used in this experiment (Bohoth, Ebaa, and Latifia) were provided by the Agricultural Research Center located in Abu Ghraib, Baghdad, which is responsible for maintaining and distributing certified native germplasm. The introduced cultivars (Dijla, Nizar, and Super Max) were obtained from certified agricultural suppliers authorized to distribute commercial seeds in Iraq.

2.2 Methodology

2.2.1 Hydroponic nutrient solution preparation

A hydroponic experiment was conducted using a nutrient solution based on Hoagland's formulation, providing a balanced supply of macro- and micronutrients essential for optimal wheat growth. The solution contained ammonium nitrate, calcium chloride dihydrate, potassium dihydrogen phosphate, potassium sulfate, magnesium chloride, magnesium sulfate, boric acid, manganese sulfate, zinc sulfate, copper sulfate, cobalt sulfate, and sodium molybdate. The nutrient solution contained iron, supplied as a combination of Fe-EDTA (1:1 ratio) and an organic iron compound, Fe(III)-citrate, to ensure balanced iron availability under controlled hydroponic conditions. Nutrient concentrations, pH, and electrical conductivity (EC) were carefully adjusted to meet the physiological requirements of wheat seedlings under controlled hydroponic conditions. This preparation followed recent recommendations for nutrient solution management in soilless culture systems [16].

2.2.2 Experimental setup and hydroponic design

Iron concentration in leaf and root tissues was determined using a flame atomic absorption spectrophotometer (novAA 350, Analytik Jena, Germany). Dried plant samples were digested with concentrated nitric acid until a clear solution was obtained. The digested extracts were filtered, diluted to a known volume with deionized water, and analyzed at the Fe-specific wavelength. Calibration was performed using a series of standard Fe solutions (1–9 mg L^{-1}), with the calibration curve showing excellent linearity ($R^2 = 0.99999$). The method detection limit (LOD) was 0.0105 mg L^{-1}. The measured Fe values provided a reliable physiological indicator of the plant's response to the applied treatments.

The experiment was carried out in the greenhouse of the Department of Biology, College of Education for Pure Sciences (Ibn Al-Haitham), University of Baghdad, during the winter season of 2023–2024. A hydroponic system was employed, following a factorial experiment based on a Completely Randomized Design (CRD) with three factors, each replicated three times. The experiment involved three factors. The first factor, cultivars, included six soft wheat (*Triticum aestivum* L.) varieties: three native (Bohooth, Ebaa, and Latifia) and three imported (Dijla, Nizar, and Super Max). The second factor, iron concentration, comprised three levels of FeNa-EDTA: 1, 10, and 20 μmol L^{-1}. The third factor, hormonal treatments, consisted of four applications: no spraying (control), Br (2 mg L^{-1}), GABA (50 mg L^{-1}), and Br + GABA (2 + 50 mg L^{-1}). The nutrient solution was renewed every five days, and the pH was maintained at 7. Samples for gene expression analysis were collected on 20/12/2024.

2.2.3 Hormone treatment

Br and GABA were applied as foliar sprays at a concentration of 2 mg L^{-1} and 50 mg L^{-1}, respectively, once every four days for a total of three applications, directly to the leaves of the wheat plants.

2.2.4 Sampling

Samples for gene expression analysis were collected 14 days after sowing (DAS), which coincided with the period of maximum treatment effect.

2.2.5 Primer validation

Primer efficiency was evaluated using a standard curve, with all primers showing an efficiency between 90% and 110%. Amplification specificity was confirmed by performing melt curve analysis, ensuring single-product amplification (Table 1).

Table 1. Nucleotide sequences of primers used in the study

Gene	D	Primer Sequence (5′–3′)	Tm (°C)	Product Size (bp)	Ref.
Actin	F	GCTGGAAGGTGCTGAGGGA	66.1	55	NCBI GenBank database
	R	GCATCGCCGACAGGATGAG	61.7		
TaNAS1	F	AGGCGCACTACTCCGACA	63.3	61	NCBI GenBank database
	R	GAAGATGCCGAGGTGGTC	61.7		
TaNAAT2-B	F	GACCATTTAGCCAAGGTTGC	58.7	50	NCBI GenBank database
	R	TACCTCGTCAGCAATCACCA	60.6		
TaDMAS1-A	F	CACCGTCAATCAGGTGGAG	61.7	58	NCBI GenBank database
	R	CCTCTGCAGAACTCCCTCA	62.8		
TaTOM	F	TGGAGAATGCAATGATAGGTTTT	55.9	35	NCBI GenBank database
	R	AGATGTTTTGCCTCGCTGTT	58.5		
TaYSL	F	TGCATGGAACCAAGATAAACAAG	57.4	39	NCBI GenBank database
	R	ACATATCAAAGCGGATGCAA	55.4		

D: Direction (F: Forward; R: Reverse)

Table 2. Components of the reverse transcription (RT) reaction for cDNA synthesis

Component	Vol (µl)
ES Reaction Mix	9
RNase-free Water	4
Random Primer (N9)	1
EasyScript RT/RI Enzyme Mix	1
Eluted RNA	5
Total Reaction Volume	**20**

2.2.6 Gene expression analysis via reverse transcription quantitative polymerase chain reaction (RT-qPCR)

To investigate the expression levels of iron-related genes (*TaNAS, TaNAAT, TaDMAS, TaTOM, TYS*), total RNA was extracted from root tissues of six wheat cultivars grown hydroponically using a commercial extraction kit. RNA integrity was assessed using the RNA integrity number (RIN), with values > 8 considered acceptable. RNA purity was evaluated by measuring the A_{260}/A_{280} and A_{260}/A_{230} ratios, which were found to be within the acceptable range (1.8–2.0). Then the isolated RNAs were reverse-transcribed into complementary DNA (cDNA) using EasyScript RT/RI Enzyme Mix and random primers, and the components and volumes of the reverse transcription reaction are shown in Table 2. The components and volumes used for the reverse transcription reaction are listed in Table 2. The TaActin gene was used as an internal reference to normalize gene expression levels. The qPCR reactions were performed in a final volume of 20 µL, consisting of 10 µL SYBR Green Master Mix (Thermo Fisher), 1 µL of 10 µM primer (forward and reverse), 2 µL of cDNA, and 7 µL of RNase-free water. Quantitative PCR (qPCR) was performed using SYBR Green chemistry on the Sacycler-96 system. Primers for the target and reference genes were synthesized by Macrogen (Korea), and their sequences, annealing temperatures, and product sizes are detailed in Table 1. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [17], according to the following equations:

Primer efficiency and amplification specificity were confirmed by standard curve and melt curve analysis.

$$\Delta \mathrm{CT} = CT\ of\ the\ target\ gene - CT\ of\ the\ reference\ gene$$

$$\Delta\Delta \mathrm{CT} = \Delta \mathrm{CT}\ \text{of each sample} - \text{mean}\ \Delta \mathrm{CT}\ \text{of the control group}$$

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

The data were statistically analyzed using Statistical Analysis System (SAS) software (version 9.4, SAS Institute Inc., Cary, NC, USA). A three-way analysis of variance (ANOVA) was applied to evaluate the effects of genotype, iron concentration, and hormonal treatments, as described by Morgan et al. [18].

3. RESULTS AND DISCUSSION

The current study demonstrated that the combined application of brassinolide and GABA induced a markedly stronger response in wheat compared to each treatment alone. This synergistic effect can be directly linked to the enhancement of iron uptake–related pathways, alongside improved physiological adjustments of roots under iron-deficient conditions. The recorded increases in gene expression, particularly those associated with Strategy II acquisition, support the notion that both Br and GABA act as metabolic modulators capable of reinforcing the plant's adaptive mechanisms. The data collectively indicate that the improvements observed in root morphology, organic acid secretion, and micronutrient homeostasis are not incidental but rather result from coordinated biochemical regulation triggered by the dual treatment.

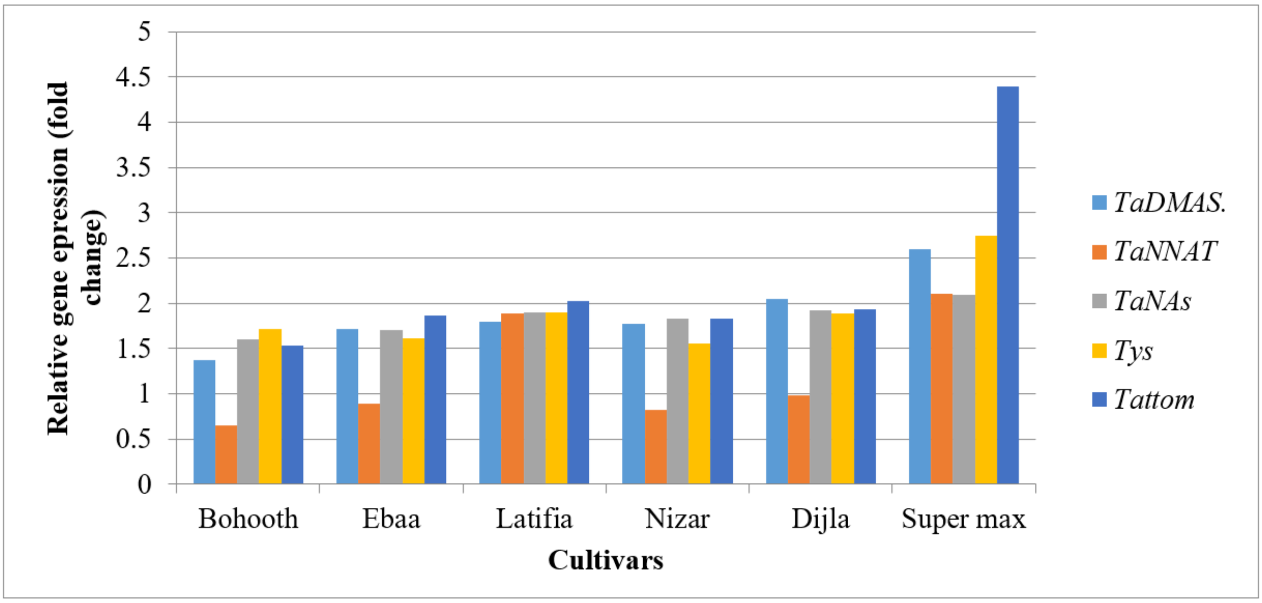
3.1 Effects of treatment and cultivar on gene expression in wheat

The relative gene expression analysis of *TaNAAT, TaDMAS, TaNAS, TaYSL*, and *TaTOM* using the relative gene expression method in wheat (*Triticum aestivum*) revealed that both treatment and cultivar significantly affect transcription, with strong evidence of a gene × cultivar × treatment interaction. All genes showed peak expression at the lowest treatment (~2.4–3.3-fold) before sharply declining at treatments 10 and 20, particularly for *TaDMAS, TaNAAT*, and *TYS*. The cultivar SuperMax exhibited the highest expression, notably of *TaTOM* (~4.5-fold) and *TaDMAS* (~2.5-fold), compared to other cultivars (1.4–2-fold). These results indicate that both treatment and cultivar exert strong independent effects, while *TaTOM* and *TaNAS* exhibit clear three-way interactions, with steep declines at higher treatments following high expression in Super Max under low treatment conditions (Figure 1(a)).

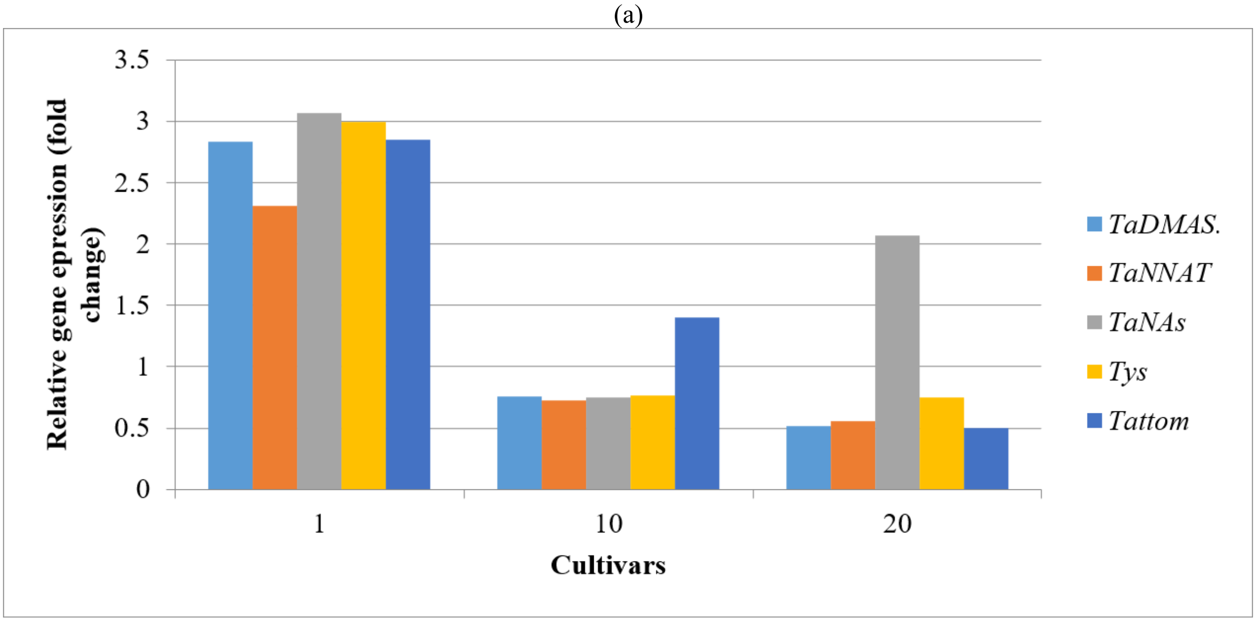
These findings align with recent transcriptomic studies in wheat, emphasizing that gene expression under multiple environmental or chemical stimuli is often non-linear and non-additive, relying on complex genotype-specific regulatory networks. For instance, non-additive transcriptional responses under combined drought and heat stress in wheat cultivars were reported to differ significantly between tolerant and sensitive lines [19]. This study focused on the role of key genes regulating iron uptake and transport in wheat. *TaNAS* and *TaNAAT* are central to phytosiderophore biosynthesis, *TaTOM* mediates their release from roots, and *TaYSL* facilitates long-distance transport of Fe-phytosiderophore complexes. Similar expression patterns were reported by Kenzhebayeva et al. [20], linking gene upregulation to enhanced iron accumulation in wheat grains.

Figure 1(b) shows that the expression of *TaDMAS*, *TaNAAT*, *TaNAS*, *TaYSL*, and *TaTOM* was markedly upregulated under iron deficiency (1 µM), while lower expression occurred at sufficient (10 µM) and excess (20 µM) iron levels, indicating that these genes are primarily induced as an adaptive response to limited iron availability. Regarding the spraying treatments, the individual effects of Br or GABA were moderate, while their combination resulted in a pronounced increase in gene expression, particularly in *TaDMAS* and *TaTOM*. This highlights a synergistic interaction where Br enhances growth and stress-related pathways, while GABA alleviates oxidative stress and improves metabolic balance, ultimately strengthening the efficiency of iron uptake and transport in wheat. In general, the studied genes exhibit strong primary effects of treatment, cultivar-dependent differences in expression magnitude, and probable three-way interactions that drive non-linear and genotype-dependent effects.

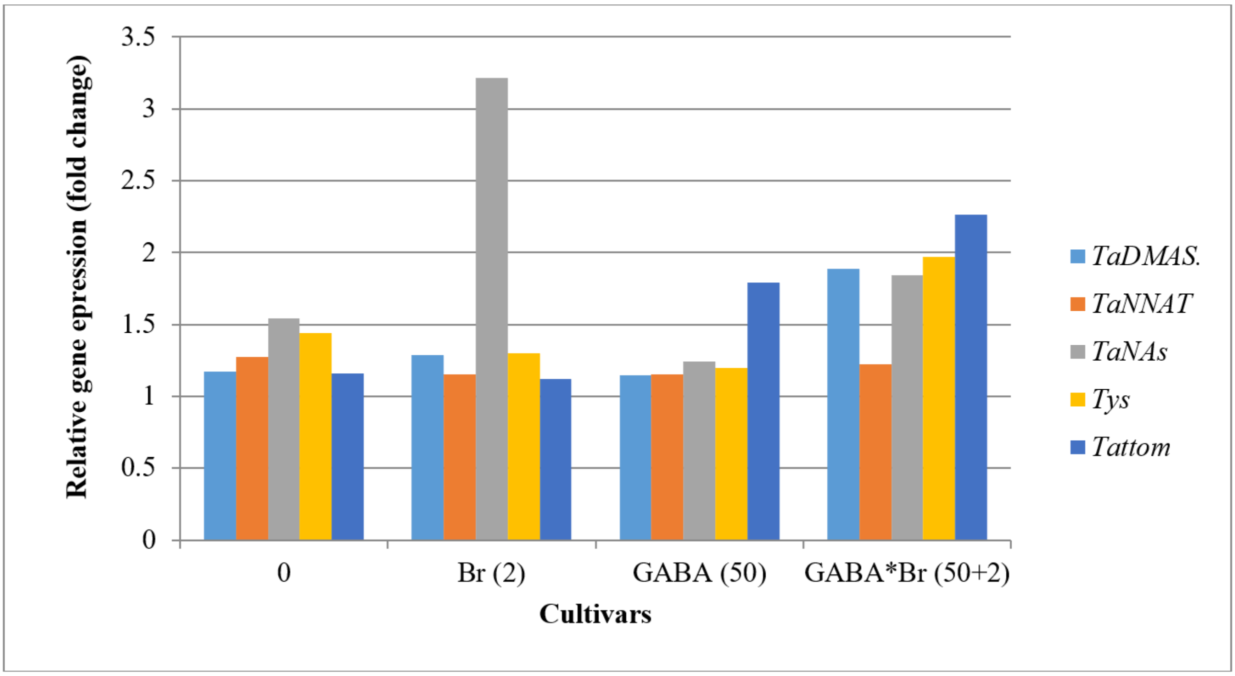
When wheat plants are exposed to iron deficiency, they activate a wide range of molecular responses to cope with the stress. Genes involved in the biosynthesis of iron chelators, such as nicotianamine (NA) and deoxymugineic acid (DMA), are strongly upregulated [21], enhancing the plant's ability to bind and transport iron [22]. In addition, regulatory genes encoding transcription factors, particularly those from the basic helix-loop-helix (bHLH) family, show a marked increase in expression in both roots and flag leaves [23]. These transcriptional adjustments reflect an adaptive strategy that improves iron acquisition and internal redistribution under nutrient-limited conditions.



(a)



(b)



(c)

Figure 1. (a) Gene expression in wheat cultivars under hydroponic conditions, (b) Effect of iron concentration on gene expression in wheat under hydroponic conditions, (c) Effect of Br and GABA on gene expression in wheat under hydroponic conditions

The results in Figure 1(c) clearly demonstrated that the combined spraying of Br + GABA (2 + 50 mgL^{-1}) significantly enhanced the expression levels of all studied genes compared to the non-sprayed plants. The highest expression value was recorded for *TaTOM* (2.26434) with a reduction rate of 82.31% relative to the control. Similarly, *TaYSL* showed an expression value of 1.9690, while *TaNAAT* reached 1.27144 with an increase of 3.49% over the control. For *TaDMAS*, the combined treatment achieved the highest expression value of 1.880, whereas *TaNAS* also exhibited a notable increase with a value of 1.8429. These findings highlight the consistent stimulatory effect of the combined Br + GABA treatment in upregulating genes involved in the mugineic acid pathway and iron transport in wheat.

The marked increase in gene expression related to iron uptake under the combined spraying of Br + GABA can be explained by their synergistic interaction. Brassinolide stimulates growth pathways and activates stress-responsive programs, thereby enhancing root capacity for nutrient uptake [24]. At the same time, GABA functions as a metabolic regulator that alleviates oxidative stress and improves cellular energy efficiency [25]. This synergy creates an optimal cellular environment that promotes the activation of *TaNAS–TaNAAT–TaDMAS* genes for iron chelator biosynthesis, along with upregulation of *TOM* and *YSL* genes for long-distance iron transport. As a result, plants exhibit improved iron acquisition and internal distribution, explaining the superior performance of the combined treatment compared to single or no spraying. The synergistic effect of Br and GABA can be attributed to their complementary roles in enhancing root development and mitigating oxidative stress, respectively. Brassinolide promotes growth and enhances nutrient uptake pathways, while GABA regulates stress responses and antioxidant systems, thus facilitating more efficient iron transport under deficient conditions.

3.2 *TaNAS* as a molecular indicator of iron uptake efficiency in wheat cultivars

As for the triple interaction of the study factors, the cultivar Latifia excelled in gene expression under the treatment of iron deficiency and the combined spraying treatment, Br + GABA (2 + 50 mg L^{-1}), with the highest value of gene expression reaching 4.5947 compared to the local cultivars, Ebaa and Bohooth (Figure 2). The Nizar cultivar exhibited superior performance under iron deficiency when treated with Br + GABA (2 + 50 mg L^{-1}), demonstrating synergism between Br, which activates transcription factors that stimulate stress-related genes, and GABA, which enhances stress tolerance mechanisms [26]. Gamma-aminobutyric acid acts as a rapid response regulator to environmental stress and contributes to enhancing root plasticity and ionic channel activity under iron deficiency [27]. Iron deficiency stimulates gene expression as a compensatory mechanism, activated through cell perception pathways and root response [28]. The Nizar variety exhibited the highest values under iron-deficient conditions when treated with Br alone, outperforming the other varieties and showing the greatest levels of gene expression.

This superiority reflects the ability of this cultivar to activate more efficient physiological and molecular responses under iron deficiency conditions, indicating the potential use of the *TaNAS* gene as a molecular indicator in breeding programs to improve iron uptake efficiency [29]. These results indicate that the *NAS* gene is one of the key pillars in wheat's response to iron deficiency, and it shows a high capacity for hormonal regulation under the influence of spraying with Br and GABA. This gene can also be considered an important functional marker for identifying superior cultivars in micronutrient uptake efficiency and stress tolerance.

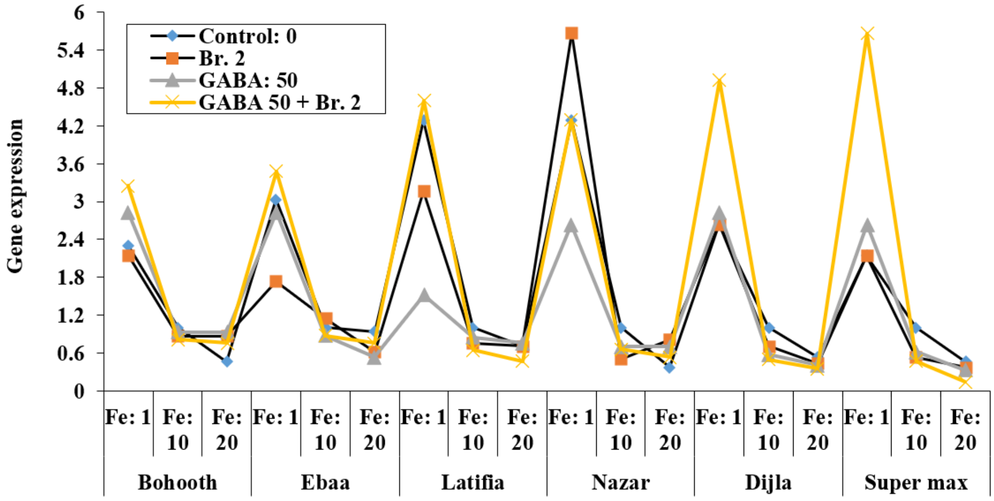


Figure 2. Effect of interactions among wheat cultivars, iron levels, and hormones (Br and GABA) on the expression of the *TaNAS* genes
Least Significant Difference (LSD) = 0.6149

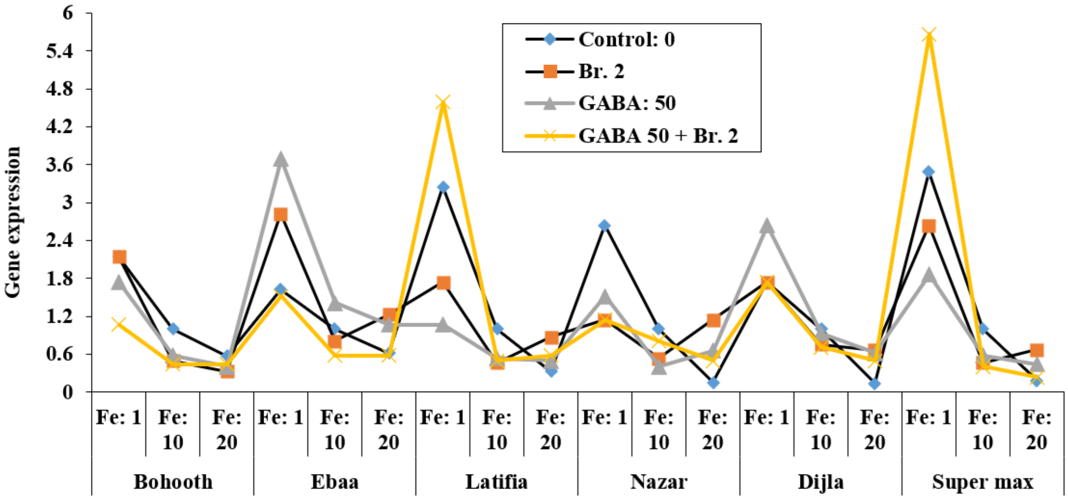


Figure 3. Effect of interactions among wheat cultivars, iron levels, and hormones (Br and GABA) on the expression of the *TANNAT* gene
LSD = 1.6708

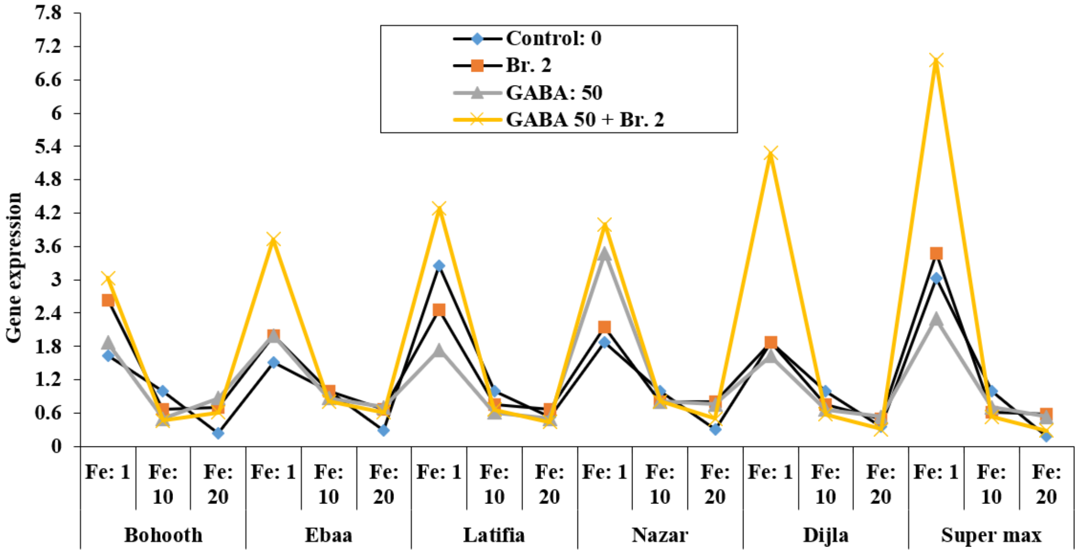


Figure 4. Effect of interactions among wheat cultivars, iron levels, and hormones (Br and GABA) on the expression of the *TaDMAS* gene
LSD = 1.6311

3.3 Influence of genotype and hormonal spraying on *TaNAAT*-mediated iron uptake

The results of the three-way interaction among the study factors, cultivar, iron concentration, and spraying treatment (Figure 3) revealed significant differences among the cultivars. The local cultivar Latifia and the imported cultivar Super Max exhibited the highest *TaNAAT* expression levels, recording 4.5947 and 5.6669, respectively, under the iron-deficient treatment (1 µmol L^{-1}) combined with Br + GABA spraying (2 + 50 mg L^{-1}), compared with the other local and imported cultivars. This indicates integration between the genotypic response and hormone-regulated spraying in improving iron uptake under stress conditions, supporting findings from previous studies, such as study [30]. The results indicate that the *TaNAAT* gene exhibits a differential response among the cultivars under the influence of iron and spraying, participating in the regulation of iron stress-sensing pathways and signal transduction. Moreover, there are promising cultivars, such as Latifia and Super Max, that can regulate this gene efficiently, making them candidates for cultivation in iron-deficient environments. These results underscore the importance of using growth regulators to improve the efficiency of iron uptake by affecting the associated gene expression. Activation of uptake genes and hormonal regulation [31].

3.4 *TaDMAS* as a genetic marker for iron deficiency tolerance in wheat

When examining the triple interaction among iron concentration, spraying treatment, and cultivar (Figure 4), the imported Super Max cultivar exhibited the highest expression value (6.96) under iron-deficient conditions (1 µmol L^{-1}) with the Br + GABA treatment (2 + 50 mg L^{-1}). This finding indicates the cultivar's strong ability to interact with hormonal treatments to compensate for iron deficiency. It also suggests the presence of a genetic structure that facilitates effective mobilization of biochemical adaptation mechanisms under stress conditions. These results imply that the *TaDMAS* gene may serve as an effective genetic marker for studying iron deficiency tolerance in wheat. Furthermore, the observed interaction between iron and hormones appears to play a pivotal role in enhancing the genetic response, consistent with the findings of Zhang et al. [32], who emphasized the importance of gene expression indicators in evaluating the effectiveness of biotic agricultural treatments.

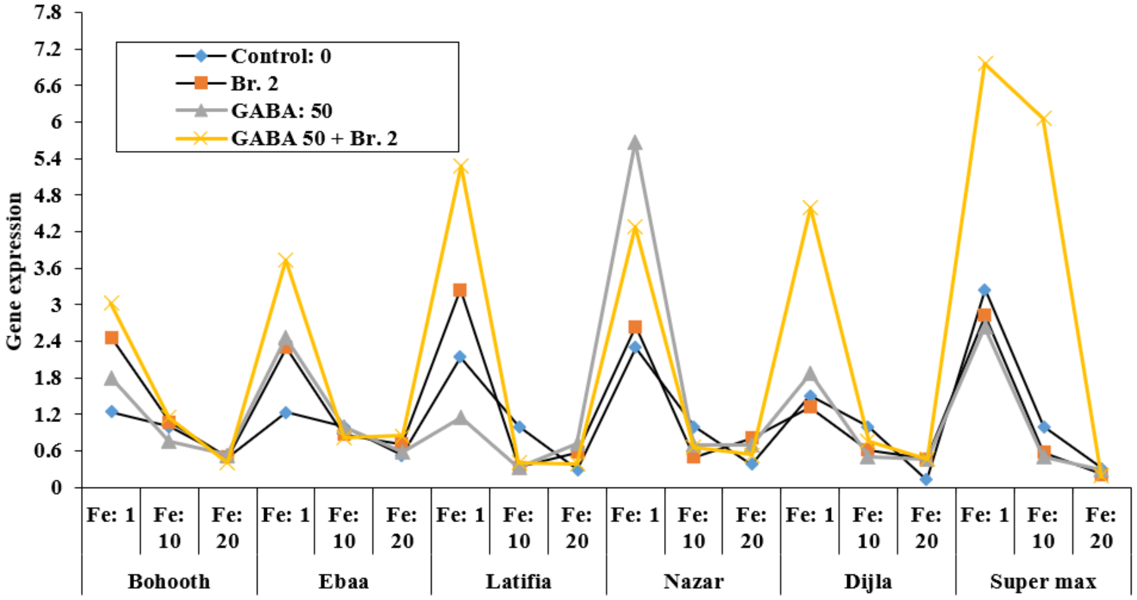


Figure 5. Effect of the interactions among wheat cultivars, iron levels, and hormonal treatment (Br and GABA) on the expression of the *TaTOM* gene

LSD =1.0889

3.5 Role of brassinolide and GABA in enhancing *TaTOM*-mediated iron transport in wheat

The triple interaction among the study factors revealed a significant effect. The local cultivar Latifia, when subjected to iron deficiency (1 µmol L^{-1}) and treated with the combined Br + GABA spray (2 + 50 mg L^{-1}), exhibited the highest expression value of 5.2780. Similarly, the imported cultivar Super Max recorded the highest average gene expression value of 6.9441 compared to the untreated plants (Figure 5). These findings indicate a strong synergistic effect between Br and GABA, as the combined hormonal treatment enhances the effectiveness of genetic pathways involved in iron transport and mitigates stress responses [33]. This confirms that the Latifia cultivar is genetically more efficient under iron deficiency, while Super Max excels under hormone interaction conditions. The hormone Br is considered a strong promoter of *TaTOM* expression, followed by the Br + GABA interaction, which showed the highest effect. The triple interaction (Br × GABA × Fe) is the most effective in activating gene expression, highlighting the importance of using chemical interactions to enhance plant resistance to nutrient deficiencies. The Super Max cultivar can be adopted in breeding programs related to improved nutrition conditions, while Latifia is suitable for harsh conditions.

3.6 Synergistic regulation of *TaYSL* in wheat by hormones and iron availability

Figure 6 shows that the triple interaction among the study factors had a significant effect on gene expression. The local Latifia cultivar treated with an iron concentration of 1 µmol L^{-1} and the combined Br + GABA spray (2 + 50 mg L^{-1}) exhibited the highest gene expression value (4.9226). Meanwhile, the Super Max cultivar recorded the highest average *TaYSL* expression value (7.4643) under iron-deficient conditions (1 µmol L^{-1}) combined with the Br + GABA spray (2 + 50 mg L^{-1}).

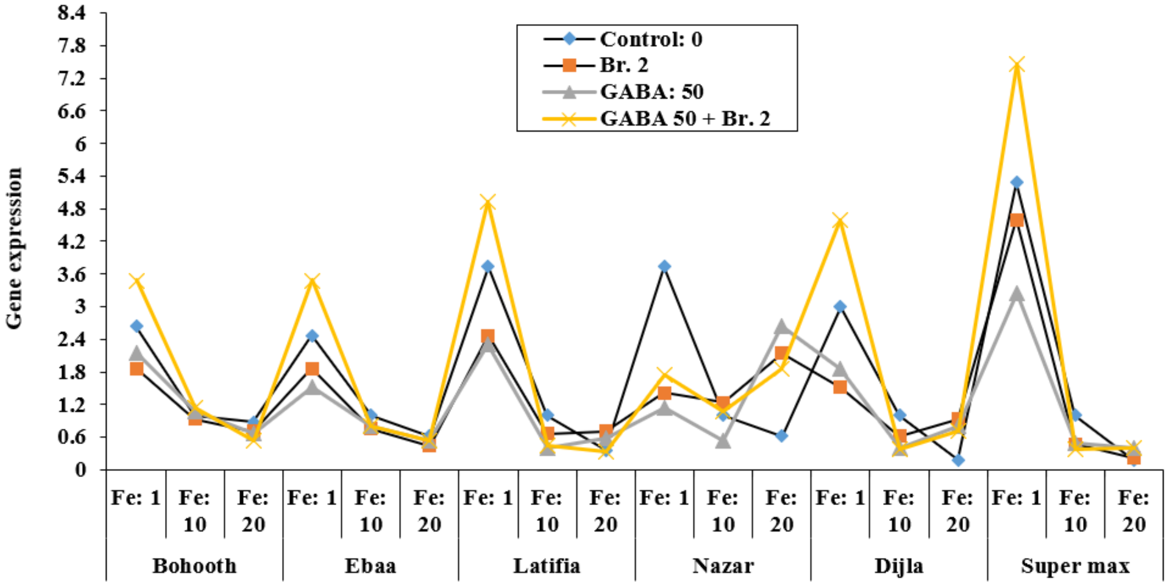


Figure 6. Effect of interactions among wheat cultivars, iron levels, and hormones (Br and GABA) on the expression of the *TaYSL* gene
LSD = 0.751

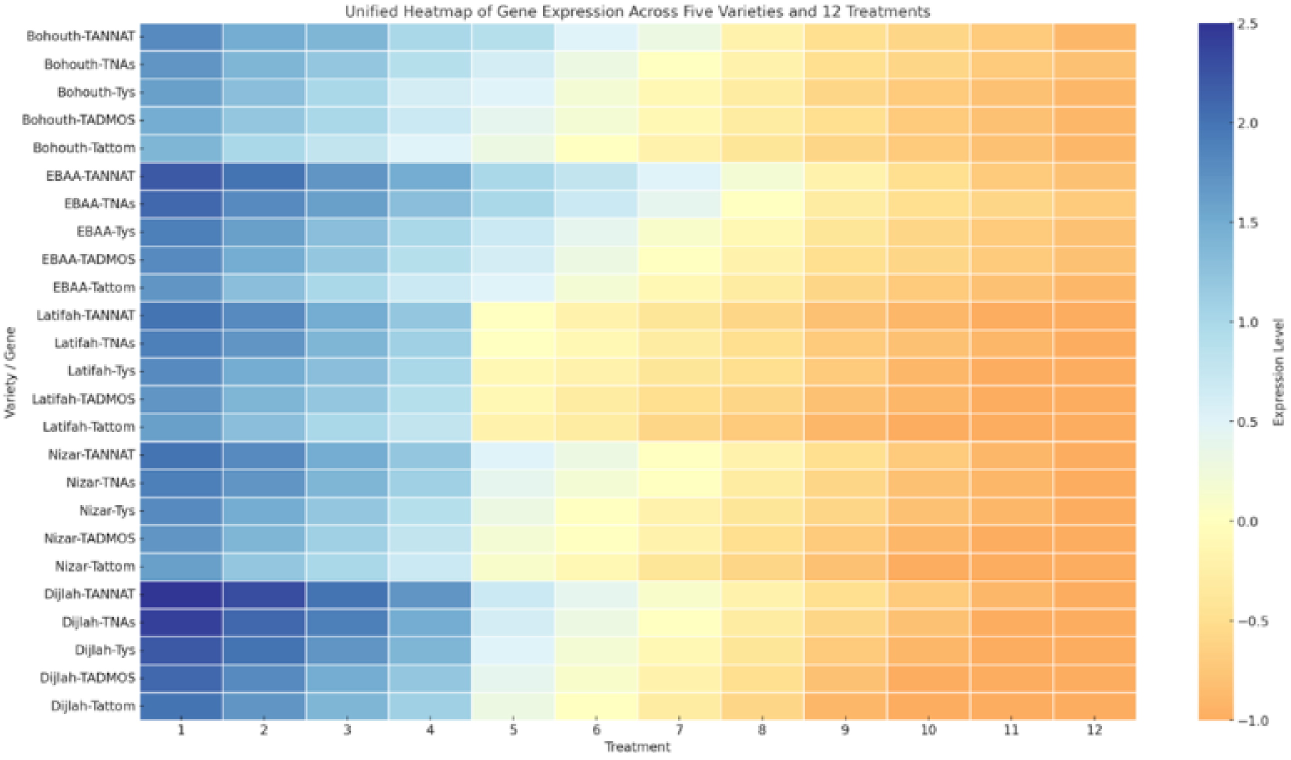


Figure 7. Unified heatmap showing the expression levels of five genes (*TaNAAT, TaNAS, TaYSL, TaDMAS*, and *TaTOM*) across five wheat varieties (Bohouth, Ebaa, Latifia, Nizar, and Dijlah) under twelve foliar iron treatments
The color gradient ranges from dark orange (strong downregulation) through yellow (neutral expression) to dark blue (strong upregulation), highlighting the impact of varying iron concentrations and the addition of GABA and/or Br on gene activity.

This result reinforces the hypothesis that the interaction between growth regulators and iron availability contributes to the reprogramming of genes associated with mineral transport [33, 34]. The *TaYSL* gene is believed to encode a protein associated with the synthesis of phytosiderophores, or factors that assist in iron transport. Its increased expression under stress conditions or when treated with Br + GABA reflects a physiological defence response at the transcriptional level, supporting the plant's ability to absorb micronutrients from the soil.

3.7 Genotype- and treatment-dependent expression patterns of wheat iron transport genes

The heatmap analysis (Figure 7) of *TaDMAS, TaNAAT, TaNAS*, *TaYST*, and *TaTOM* expression across the six wheat cultivars under twelve treatments revealed a distinct genotype- and treatment-dependent transcriptional response. Overall, low iron concentration (Fe = 1 µM) combined with biostimulant spraying, especially the dual Br + GABA treatment, consistently induced the highest gene expression levels across all cultivars. In single-factor responses, the highest expression levels were observed for *TaTOM* (~4.5-fold) and *TaDMAS* (~4.0-fold) under iron deficiency (Fe = 1 µM) with either Br or GABA applied individually. This indicates that early transcriptional activation is stimulated by low mineral availability in conjunction with a single exogenous treatment. In the three-way interaction, the most pronounced activation was observed in the Super Max and Dijlah cultivars, where *TaYSL*, *TaTOM*, and *TaDMAS* exhibited the highest expression under Fe = 1 µM combined with Br + GABA. Similarly, *TaNAS* maintained relatively high expression across multiple cultivars under low iron conditions with biostimulant sprays, highlighting its potential as a molecular biomarker for iron uptake and transport pathways. In contrast, high iron concentrations (Fe = 20 µM) without spraying resulted in a marked reduction of gene expression, reflecting transcriptional repression under mineral stress, which aligns with the concept of negative regulation of nutrient transport genes under excess mineral load.

These patterns align with recent studies emphasizing the synergistic effects of low iron and exogenous stimulants on wheat gene expression and stress adaptation [35, 36]. The pronounced peaks of *TaYSL*, *TaTOM*, and *TaDMAS* under low-iron, biostimulant-enriched treatments highlight their critical roles in regulating defense, transport, and metabolic pathways under suboptimal mineral conditions [37]. Overall, the heatmap approach clearly illustrates that dual bio-spraying under low iron maximizes transcriptional activation, supporting its potential for targeted agronomic strategies to enhance wheat tolerance to micronutrient stress. The three-way interaction between cultivar, iron concentration, and treatment significantly influenced gene expression. Notably, the combination of low iron concentration (1 µM) with Br + GABA treatment resulted in the highest upregulation of key genes across all cultivars, emphasizing the complex interplay between genotype and nutrient availability.

4. CONCLUSION

This study demonstrates that the combination of Br and GABA significantly upregulates iron uptake genes in wheat under iron deficiency, offering insights into potential strategies for improving micronutrient efficiency in crops. However, the study was limited by its focus on gene expression alone, and further research should integrate physiological data, such as protein levels and iron content, to validate the molecular findings.

Limitation

Although the present study provides valuable insights into the Br + GABA synergistic interaction, certain limitations should be acknowledged. Gene-expression analysis was performed at a single time point, which may not fully reflect the temporal dynamics of the underlying signaling pathways. The study quantified iron content in both leaves and roots, providing important physiological support for the transcriptional data; however, other physiological indicators such as total protein levels and antioxidant activity were not assessed. Including these measurements would have offered a more comprehensive view of the functional consequences of the molecular responses. Future studies are therefore recommended to include time-course sampling and broader physiological analyses.

ACKNOWLEDGEMENTS

The authors express their sincere thanks and gratitude to the Department of Biology, College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad, Baghdad, Iraq, for their support and assistance in carrying out this research.

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