

Article

Understanding the Role of Durum Wheat Thioredoxin *h*-Type TdTrxh2 in Biotic Stress Tolerance

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Abstract

The thioredoxin *h*-type (Trxh) proteins play a crucial role as convergence points within plants' responses to abiotic and biotic stresses. Previously, we demonstrated that the protein TdTrxh2 of durum wheat (*Triticum durum* Desf.) has a chaperone function and it promotes tolerance to abiotic stress. The aim of this study was to evaluate the antimicrobial effect of TdTrxh2 and its role in the response of durum wheat to *Fusarium graminearum* attack. First, we demonstrated the involvement of TdTrxh2 in the response of durum wheat to this fungus via the analysis of its expression profile under this fungus attack. In fact, the outcomes showed that the induction of TdTrxh2 expression is spatiotemporal in leaves and roots of durum wheat under *F. graminearum* infection. Interestingly, this induction was accompanied by H₂O₂ accumulation under short- and long-term stress in roots and leaves, respectively. Besides, the *cis* elements related to the two phytohormones ET and MeJA, and those implicated in defense and wound stress, were identified in the TdTrxh2 promoter's sequence. Second, the purified TdTrxh2 protein possessed antimicrobial effects against a diverse range of bacteria and fungi in vitro. Finally, the expression of TdTrxh2 in transgenic *Arabidopsis* plants enhanced their tolerance to *F. graminearum* attack through the activation of the two H₂O₂-scavenging enzymes, CAT and POD, and via the induction of a subset of SA- and ABA-related genes. Moreover, the exogenous SA and ABA applications improved the growth of the transgenic lines compared to the non-transformed plants. Taken together, the results highlighted that TdTrxh2 generates tolerance of durum wheat's response to *F. graminearum* attack, via the regulation of H₂O₂ homeostasis and the induction of hormone-associated genes. Thus, the TdTrxh2 gene could be considered as an interesting candidate gene to improve wheat tolerance to *F. graminearum* attack.



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Keywords: biotic stress; durum wheat; H₂O₂ homeostasis; plant antimicrobial protein; thioredoxin; tolerance mechanisms

1. Introduction

Biotic stresses, such as attacks by pests and pathogens, disrupt various physiological and molecular mechanisms in plants, therefore affecting plant growth and productivity. Indeed, regarding the yield loss of the major food crops, wheat is about one-fifth of the global annual loss, consequently presenting a threat to global food security [1,2]. To mitigate biotic stress, plants activate various strategies at apoplastic and symplastic levels, including

the activation of secondary metabolites, antioxidant enzymes, and defense proteins, like defensins, thionins, and lectins [3,4]. Under biotic stress, the recognition of pathogens via the nucleotide-binding leucine-rich repeat receptors (NLR) produces the hypersensitive response (HR), which is a local cell death. The distal parts perceive signals from the local site of stress perception and activate the systemic defense in the entire plant for subsequent attacks [5]. This rapid systemic signal is regulated in different cell compartments during stress through the reactive oxygen species (ROS), nitric oxide (NO), calcium, and hydraulic pressure [6,7]. The ROS wave is an essential signal in response not only to biotic and abiotic stresses but also in development and growth processes in plants [8]. The imbalance between ROS production and scavenging produces oxidative stress that causes widespread cellular damage. Therefore, plant cells regulate their redox environment through the activation of the ROS-scavenging enzymes, like catalase (CAT) and superoxide dismutase (SOD), the redox sensors proteins, such as peroxiredoxins (PRX), and the redox transmitter proteins, including thioredoxins (Trxs) and glutaredoxins (GRX) [9]. In the antioxidant defense mechanism, Trxs scavenge peroxides with PRX to produce water and oxygen and cause post-translational modifications, including S-nitrosylation (S-NO), S-sulfenation (S-OH), and S-thiolation (S-S), of several target proteins [10]. The regulation of several signaling proteins by S-nitrosothiols (RSNO) is a critical process in the plant immune system [11].

Trxs proteins are able to reduce disulfide bonds of target proteins through the involvement of the two cysteine residues in the WCGPC active site [11]. According to their active site, Trxs proteins are categorized as typical, featuring the canonical WCGPC motif, and atypical Trxs, possessing the WCXXC motif in the catalytic sites [12,13]. In turn, the typical Trxs are separated into several classes according to their primary structure and cellular localization. For instance, in *Arabidopsis* there are four chloroplastic Trxs proteins that are Trxs *f*-, *m*-, *x*-, and *y*-types, the mitochondrial Trxs *o*-type, and the cytosolic Trxs *h*-type [14]. Nevertheless, in poplar the protein Trx *h*-type, PtTrxh2, is present in the mitochondria, and it reduces alternative oxidase, producing its activation by pyruvate [15]. In wheat, the proteins TRXhA/B are located in the nucleus [16]. The chloroplastic Trxs *f*-type and the two Trx-like proteins, Trx-like 2 (TrxL2) and atypical Cys His-rich Trx (ACHT), deactivate several photosynthesis-related proteins in the dark, such as Rubisco activase and fructose-1,6-bisphosphatase, via their oxidation [17]. By contrast, depending on the light, Trxs activates these targets via the reduction of their disulfide bonds and in association with NADPH-Trx reductase C (NTRC). In addition to this Trx reductase system, Trxs are reduced in chloroplasts by the ferredoxin-dependent Trx reductase (FTR) [18]. It has been reported that the overexpression of NTRC and Trx *f*-type regulates the starch production in the transgenic tobacco plants [19]. So far, various functions are attributed to Trxs proteins depending on their distinct subcellular localizations and their different substrate specificities [11]. For example, in plant immunity, TRXh5 specifically reduces protein-SNO but not those regulated by S-nitrosoglutathione (GSNOR), producing an induction of the SA signaling pathway [20].

Regarding Trxs *h*-type, they were characterized from various plant species, and they are contributed not only in redox regulation through the reduction of disulfide bonds but also, they act as molecular chaperones, such as Trx of *Arabidopsis* [21,22] and TdTrxh2 of durum wheat [23]. Furthermore, Trxs *h*-type play a crucial role in tolerance to various abiotic stresses [24,25]. So far, the importance of Trx *h*-type proteins in response to biotic stress is reported in various studies. However, little is known about the role of Trx *h*-type in the response of durum wheat to *Fusarium graminearum*. The susceptibility of durum wheat to *Fusarium* attack poses a significant risk to its production in many regions [26,27]. *Fusarium* head blight (FHB), caused by *Fusarium graminearum* species, is a devastating disease of wheat and barley [28]. During plant–pathogen interactions, plants attribute mul-

multiple genes and activate sophisticated mechanisms to minimize the intensity of stress [29]. Among these genes, *Trxh* genes are crucial for plant defense against various pathogens, like *LmTrxh2* of *Lobularia maritima* and *AtTRX5* of *Arabidopsis* [30,31]. Despite the previous studies, the specific contribution of *TdTrxh2* to the wheat–pathogen response remains poorly understood. This research highlights the efficacy of the *TdTrxh2* protein as an antimicrobial agent and explores its contribution to the resistance of durum wheat against *F. graminearum*.

2. Results

2.1. The Induction of *TdTrxh2*'s Expression in Durum Wheat Under *F. graminearum* Infection Is Associated with H_2O_2 Accumulation

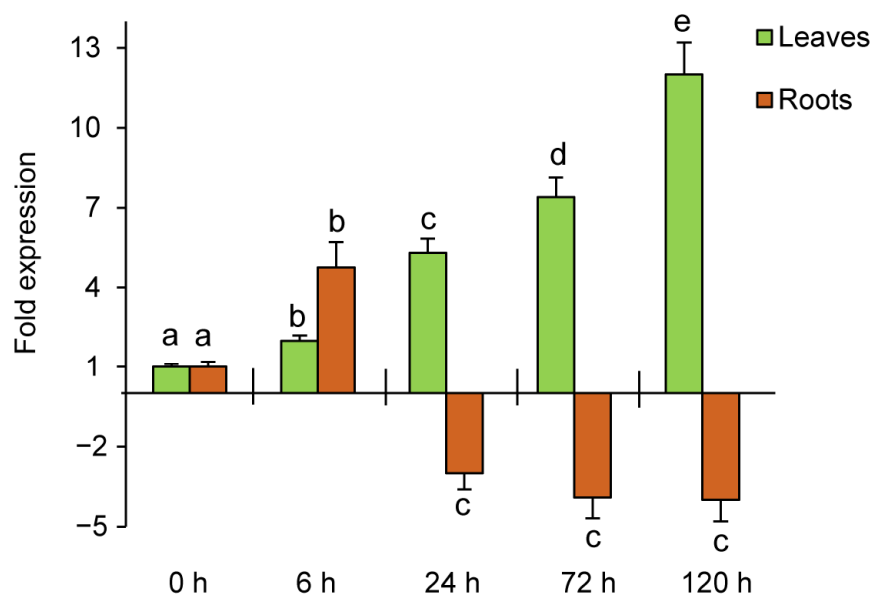
To evaluate the role of *TdTrxh2* in the response of durum wheat to *F. graminearum* infection, its expression profile was examined in leaves and roots of the cv. Om Rabiaa inoculated with *F. graminearum* for the short and long term (6, 24, 72, and 120 h). Interestingly, the results of qRT-PCR showed that *TdTrxh2* exhibits different expression patterns in these organs during stress. Indeed, during the first 6 h of inoculation, *TdTrxh2* was induced, especially in roots. However, the presence of *Fusarium* for one day or more repressed *TdTrxh2* expression in roots. By contrast, *TdTrxh2* was upregulated in leaves at different times of stress, and this induction reached its maximum after 120 h of inoculation, which was about 11-fold compared to the control condition (Figure 1A). Notably, the H_2O_2 levels increased significantly in roots exposed to *Fusarium* for 6 h, whereas in leaves the accumulation of H_2O_2 was observed after 24 h of inoculation. This accumulation was higher in leaves compared to root tissues exposed to *Fusarium* for five days (Figure 1B). Thus, these results suggested that the presence of *F. graminearum* induced the expression of *TdTrxh2* in roots and leaves under short- and long-term stress, respectively. Furthermore, the induction of *TdTrxh2* expression was accompanied by high H_2O_2 levels especially during long periods of stress, suggesting the involvement of this gene in response to oxidative stress produced by the *Fusarium* inoculation.

2.2. Analysis of the Promoter Regions of *TdTrxh2* and Three Paralogous *TdTrxh* Genes

To understand the functions of these four *TdTrxh* genes in biotic stress response, the *cis*-regulatory elements were retrieved and classified into two groups, which are hormone-responsive elements and those related to biotic stress. This analysis showed that most *TdTrxh* promoters contain ABA- and MeJA-responsive elements, with the exception of *TdTrxh2* and *TdTrxh9* promoters, respectively. Nevertheless, the elements related to gibberellic acid (GA), which are GARE- and TGACG-motifs, were present only in the *TdTrxh1* promoter region. Moreover, both *TdTrxh1* and *TdTrxh2* promoter regions contained elements related to ethylene (ET) response, while these elements were absent in *TdTrxh3* and *TdTrxh9* promoter regions. Besides, only *TdTrxh3* and *TdTrxh9* promoter regions harbored the TCA element that is implicated in the salicylic acid (SA) response (Figure 2). Concerning elements related to biotic stress, the WBOXNTERF3 element, which is implicated in wounding induction of the ERF3 gene [32], was detected in all these *TdTrxh* promoter regions, and the *TdTrxh2* promoter region harbored the highest number of this element compared to the other genes. By contrast, the WUN-motif that is related to wound response [33] was absent in all these promoter regions. Furthermore, the element GT1GMSCAM4 was present in almost all these promoter regions, except for the *TdTrxh2* promoter region. It has been reported previously that this element is involved in both pathogen and salt stress responses [34]. Nevertheless, *TdTrxh2* and *TdTrxh3* promoter regions contained TC-rich repeat, which is the *cis*-acting element involved in defense and stress responsiveness

(Figure 2). All these in silico data may shed light on the roles of these four *TdTrxh* isoforms in biotic stress and phytohormone pathways.

(A)



(B)

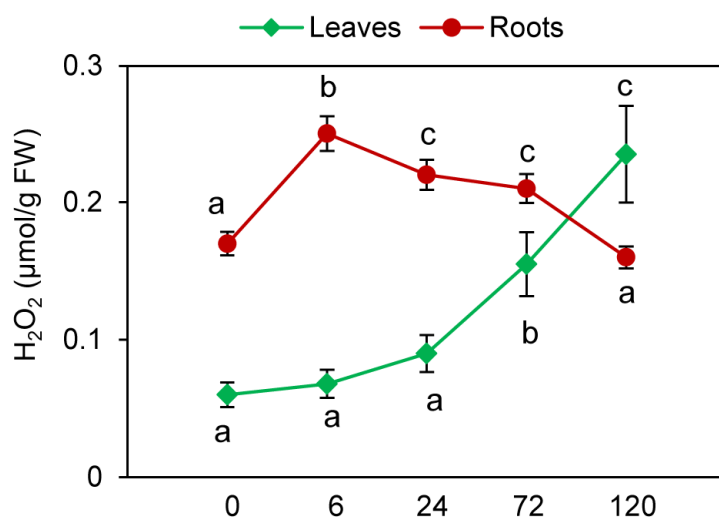


Figure 1. The induction of *TdTrxh2* gene expression under *F. graminearum* infection is associated with H₂O₂ accumulation. **(A)** Expression profile of *TdTrxh2* in leaves and roots of durum wheat infected by *F. graminearum* during 6, 24, 72, and 120 h. The expression of *TdTrxh2* in leaves and roots under the control condition (without *F. graminearum*) was adjusted to 1. The reference gene used in the qRT-PCR reactions was *TdActin*. Each qRT-PCR reaction was repeated three times, and each sample was the amount of three plants per treatment. Error bars indicate standard deviation of three biological replicates. Different letters indicate significant differences ($p < 0.05$) between control and stressed plants. **(B)** Determination of H₂O₂ levels in leaves and roots of the inoculated durum wheat seedlings with *F. graminearum* during 6, 24, 72, and 120 h. Error bars indicate standard deviation of three biological replicates. Different letters in each plant tissue (leaves or roots) indicate the significant differences ($p < 0.05$).

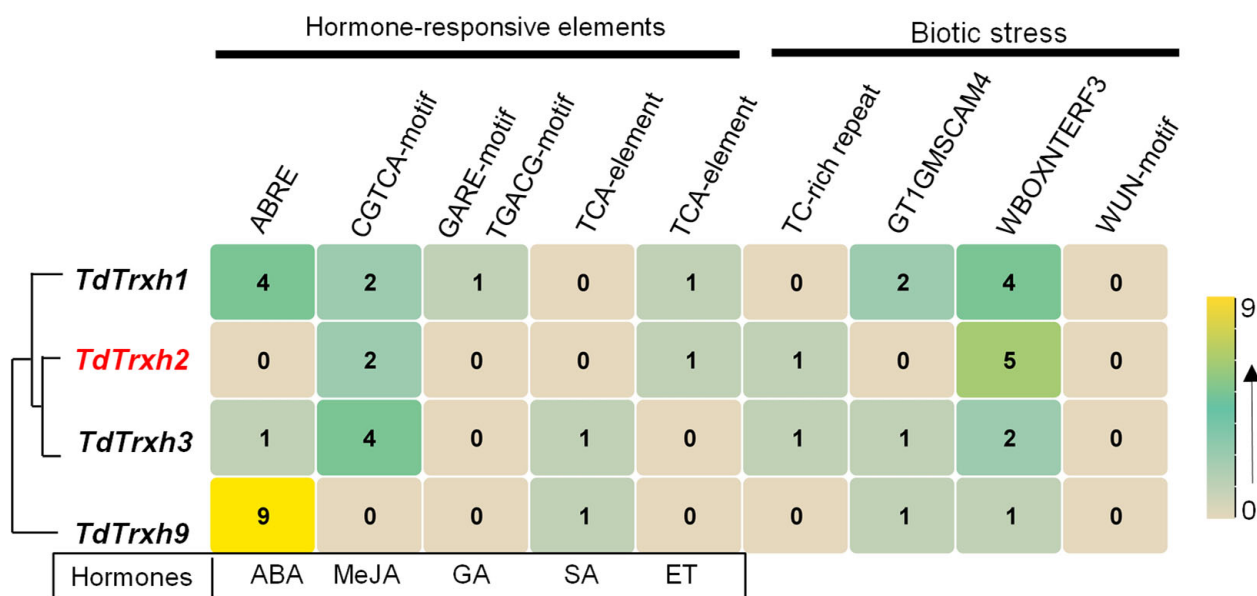


Figure 2. The *cis*-regulatory elements predicted in the promoter regions of *TdTrxh1*, *TdTrxh2*, *TdTrxh3*, and *TdTrxh9*. These elements are clustered in two different groups, which are hormone-responsive elements, including ABRE (ABA), CGTCA-motif (MeJA), GARE- and TGACG-motifs (GA), TCA element (SA), and ERE element (ET), and those related to biotic stress, including TC-rich repeat (*cis*-acting element involved in defense and stress responsiveness), GT1GMSCA (pathogen- and salt-induced gene), WBOXNTERF3 (wounding activation gene element), and WUN-motif (wound-responsive element). The figure was drawn using TBtools software version 1.x to present the putative *cis* elements that are spotlighted with different colors and numbers.

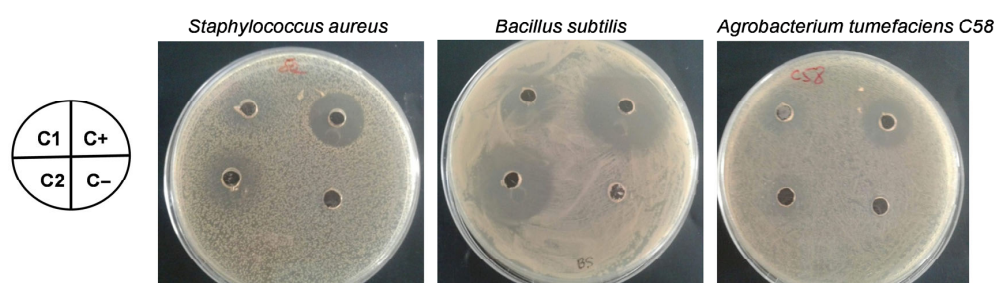
2.3. The Antimicrobial Activity of TdTrxh2 Protein

To gain insight into the inhibitory effect of the purified His-tagged TdTrxh2 protein against various bacterial and fungal strains, several parameters were identified, including the inhibition zones of bacterial and fungal strains' growth and the concentrations MIC, MBC, and MFC. The antibacterial activity of the His-tagged TdTrxh2 protein was assessed against Gram+ and Gram− strains and using the antibiotic Gentamicin (10 µg/well) as a positive control. The zones of inhibition for the antibiotic ranged from 14 to 25.5 mm. The data showed that the inhibition zones against Gram+ bacteria, which ranged from 16.5 to 23 mm, were higher than those against Gram− bacteria that were 12 and 13 mm (Table 1). Among the tested Gram+ bacteria, the greatest zone of inhibition was obtained against *Bacillus subtilis* (23 mm; Table 1; Figure 3A). Remarkably, the zones of inhibition were almost similar against the tested Gram− bacteria, about 12 to 13 mm (Table 1). On the other hand, the antibacterial activity of this protein was evaluated through the determination of the concentrations MIC and MBC. The results showed that the lowest MIC value (41 µg/mL) was obtained against *B. subtilis*. Moreover, the same MIC value was obtained in the case of the two Gram+ bacteria, *S. aureus* and *B. cereus*. However, the MIC value was eight-fold higher (332 µg/mL) toward the three Gram-negative strains *A. tumefaciens* C58 and B6 and *P. aeruginosa* compared to that obtained against *B. subtilis* (Table 2). The data showed an inverse relationship between the inhibition zone diameter and the MIC values. Indeed, the large inhibition zone observed correlates with a low MIC value, confirming the high potency of the purified protein against Gram+ strains, especially *B. subtilis*.

Table 1. Determination of the inhibition zone diameters (mm) of several bacteria strains using two concentrations of His-tagged TdTrxh2 protein, which are C1 (880 µg/mL) and C2 (1660 µg/mL). C+: reference antibiotic Gentamicin (10 µg/well).

Strains	C1	C2	C+
Gram-positive			
<i>S. aureus</i>	15 ± 0.01	18 ± 0.00	16 ± 0.03
<i>B. subtilis</i>	17 ± 0.01	23 ± 0.01	25.5 ± 0.02
<i>B. cereus</i>	15 ± 0.02	16.5 ± 0.02	20.5 ± 0.02
Gram-negative			
<i>A. tumefaciens</i> C58	11 ± 0.01	13 ± 0.01	15 ± 0.01
<i>A. tumefaciens</i> B6	9 ± 0.02	12 ± 0.01	15 ± 0.01
<i>P. aeruginosa</i>	11 ± 0.01	13 ± 0.01	14 ± 0.02

(A)



(B)

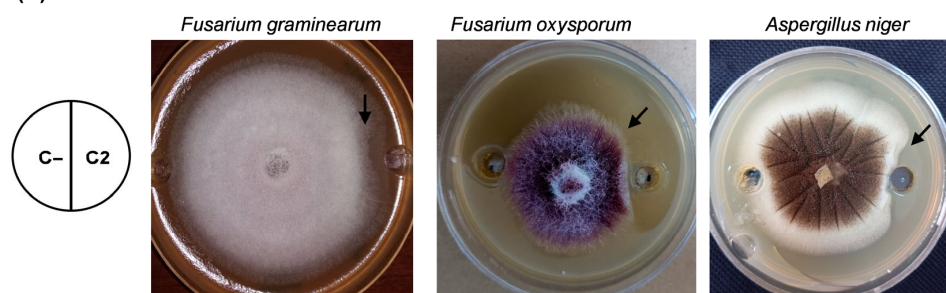


Figure 3. Growth inhibition zones of TdTrxh2 protein in different bacteria (A) and fungal (B) strains. C−: negative control. C+: Gentamicin at 10 µg/well. The purified His-tagged TdTrxh2 protein was added to two wells at different concentrations, which are C1: 880 µg/mL and C2: 1660 µg/mL. For the antifungal activity assay, experiments were done using the concentration C2.

Then, MBCs were determined, and the ratios MBC/MIC were calculated in order to distinguish the inhibiting and killing ability of the purified TdTrxh2 protein. It was proposed that a compound has a bactericidal effect when the MBC/MIC ratio is less than 4, while it has bacteriostatic effect when this ratio is more than 4 [35]. Thus, TdTrxh2 exhibited bactericidal properties against *S. aureus*, *B. subtilis*, and *B. cereus* (Table 2). In turn, due to the inability to kill 99% of the three Gram− bacteria, the MBC values were not determined. To evaluate the antifungal activity of the purified His-tagged TdTrxh2 protein, MIC and MFC were determined against various fungi (Figure 3B). The MIC values ranged from 83 to 332 µg/mL. The lowest MIC values were obtained in the cases of *F. culmorum* and *F. graminearum*, while MIC values were 4-fold higher against *A. alternata* and *B. cinerea* (Table 3). MFC values were not determined for these fungi except for *F. graminearum* and *B. cinerea*, where the value was 332 µg/mL and the MFC/MIC ratio was not more than 4

(Table 3). Thus, the results showed that TdTrxh2 exhibited fungicidal properties against *F. graminearum* and *B. cinerea*.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the His-tagged TdTrxh2 protein.

Bacterial Strains	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	MBC/MIC	Antibacterial Activity
Gram-positive				
<i>S. aureus</i>	166 ± 0.01	332 ± 0.05	2	Bactericidal
<i>B. subtilis</i>	41 ± 0.01	41 ± 0.00	1	Bactericidal
<i>B. cereus</i>	166 ± 0.03	166 ± 0.00	1	Bactericidal
Gram-negative				
<i>A. tumefaciens</i> C58	332 ± 0.04	>332	-	
<i>A. tumefaciens</i> B6	332 ± 0.03	>332	-	
<i>P. aeruginosa</i>	332 ± 0.01	>332	-	

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the His-tagged TdTrxh2 protein.

Strains	MIC ($\mu\text{g/mL}$)	MFC ($\mu\text{g/mL}$)	MFC/MIC	Antifungal Activity
<i>F. culmorum</i>	83 ± 0.01	>332	-	
<i>F. graminearum</i>	83 ± 0.01	332 ± 0.00	4	Fungicidal
<i>F. oxysporum</i>	166 ± 0.02	>332		
<i>A. niger</i>	166 ± 0.01	>332		
<i>A. alternata</i>	332 ± 0.03	>332		
<i>B. cinerea</i>	332 ± 0.01	332 ± 0.02	1	Fungicidal

2.4. Expression of TdTrxh2 Enhances Tolerance of the Transgenic Arabidopsis Lines to *F. graminearum* Infection

To explore the involvement of the *TdTrxh2* gene in response to *F. graminearum*, the detached leaves of the wild-type (Wt) and the two transgenic *Arabidopsis* lines TH3-1 and TH6-1 were exposed to *F. graminearum* for five days. Interestingly, leaves of the two lines exhibited a significant resistance against this fungus compared to those of the Wt plants (Figure 4A). Indeed, a spread lesion with an area of 80 mm^2 around the site of contact was observed in leaves of Wt plants, while the lesions were restricted in leaves of these two lines, about $10\text{--}30 \text{ mm}^2$ (Figure 4B). This finding highlighted the role of *TdTrxh2* in the response of durum wheat to *F. graminearum* attack.

2.5. Mechanism Underlying TdTrxh2's Response to *F. graminearum* Infection

To gain insight on the mechanism involved in the response of transgenic *Arabidopsis* lines to *F. graminearum*, the activities of ROS-scavenging enzymes and the expression profiles of a subset of SA- and ABA-related genes were analyzed and then compared to those of the Wt plants under control and stress conditions. The outcomes demonstrated that the presence of *Fusarium* enhanced the activities of the antioxidant enzymes in the transgenic lines as well as in Wt plants. Nevertheless, this induction was pronounced, especially in the transgenic lines exposed to fungal infection compared to the Wt plants. Indeed, CAT and POD activities increased intensively in the two lines inoculated with *Fusarium* compared to the Wt plants. However, SOD activity increased not only in transgenic lines but also in Wt plants under fungal infection (Figure 4C). Thus, the expression of *TdTrxh2* enhanced biotic stress tolerance of *Arabidopsis* plants via the activation of the two H_2O_2 -scavenging enzymes

CAT and POD. In parallel, the expression patterns of some SA- and ABA-related genes were analyzed to better understand the mechanism implicated in the *TdTrxh2* gene's response to biotic stress. As shown in Figure 5, the three genes *AtPR1*, *AtNPR1*, and *AtNPR4* were upregulated in leaves of the transgenic lines TH3-1 and TH6-1 exposed to fungal infection, whereas their expression remained lower in the Wt plants. Moreover, the qRT-PCR data showed that the expression of the *AtNCED3* and *AtDREB2A* genes that encode an enzyme in ABA biosynthesis and a transcription factor, respectively, were significantly induced in leaves of the two lines infected with *Fusarium* compared to the Wt plants. These results suggested that the involvement of *TdTrxh2* in response to *Fusarium* infection could be associated with the induction of a subset of genes that are related to salicylic acid (SA) and abscisic acid (ABA) pathways.

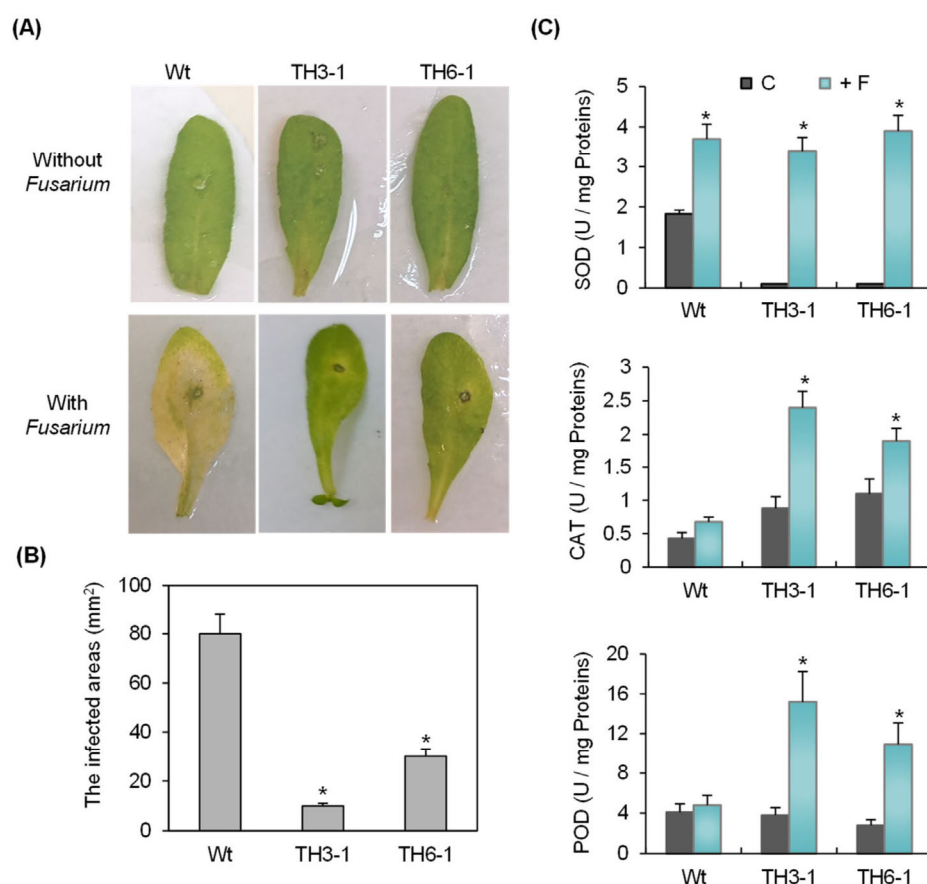


Figure 4. Tolerance of the two *Arabidopsis* lines TH3-1 and TH6-1 to *Fusarium graminearum*. **(A)** The detached leaves' fungal test was performed and then leaves were photographed after five days of fungal infection. **(B)** Determination of the infected area from the Wt plants and the two lines. Asterisk indicates significant differences ($p < 0.05$) between transgenic lines and Wt plants. **(C)** Regulation of ROS-scavenging enzymes in the transgenic lines TH3-1 and TH6-1 and Wt plants infected (F: with *Fusarium*) or not (C: control) with *F. graminearum* over five days. Error bars indicate standard deviation of three biological replicates. Asterisk indicates significant differences ($p < 0.05$) between transgenic lines and Wt plants.

2.6. Response of *TdTrxh2*-Expression *Arabidopsis* Lines to Phytohormone Treatments

Owing to the induction of the genes related to SA and ABA pathways in *TdTrxh2*-expressing *Arabidopsis* lines, we investigated the impact of the two phytohormones SA and ABA on the growth of the two lines TH3-1 and TH6-1. For this purpose, two-week-old seedlings of the two transgenic *Arabidopsis* lines and Wt plants were transferred to MS medium containing 10 μ M ABA and 50 μ M SA. After one week of phytohormones'

application, we observed a similar phenotype of all these seedlings under normal growth conditions. Nevertheless, the presence of SA or ABA meaningfully influenced the growth of Wt plants, whereas the two lines exhibited better growth parameters compared to Wt plants in the presence of these phytohormones, demonstrating that their aerial parts continued to grow under these conditions (Figure 6B). Moreover, SA and ABA did not inhibit the growth of the root systems of these lines, and their roots' elongation was about 5-fold higher compared to those of the Wt plants (Figure 6C). It is worth noting that the response of these transgenic plants to SA phytohormone was better than that under ABA treatment (Figure 6). Taken together, these results suggested that *TdTrxh2* confers tolerance of the transgenic lines to the exogenous application of SA and ABA phytohormones.

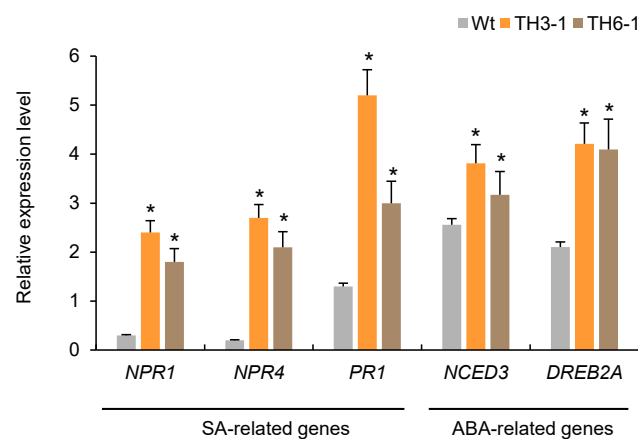


Figure 5. Expression profile analysis of a subset of SA- and ABA-related genes in the transgenic lines TH3-1 and TH6-1 and Wt plants infected with *F. graminearum* over five days. Error bars indicate standard deviation of three biological replicates. Asterisk indicates significant differences ($p < 0.05$).

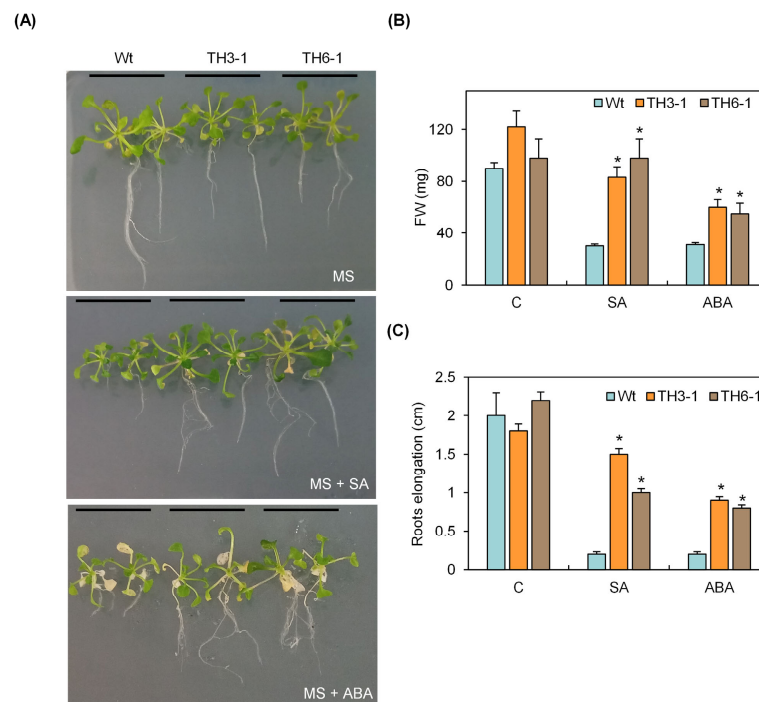


Figure 6. Response of *TdTrxh2*-expressing *Arabidopsis* plants to hormone treatments, which are 10 μ M ABA and 50 μ M SA. The effect of these hormones was compared to the control condition that consisted

of placing two-week-old seedlings in MS medium without SA or ABA. (A) The two-week-old seedlings of the two transgenic lines (TH3-1 and TH6-1) and the non-transformed plants (Wt) were put in MS medium containing or not 10 μ M ABA or 50 μ M SA to grow over one week. Then, the tolerance phenotype was evaluated by measuring the fresh weight (FW) (B) and the root elongation (C) of each seedling under each stress treatment. Asterisk indicates significant differences between transgenic lines and Wt plants in each stress treatment ($p < 0.05$).

3. Discussion

The plants' immune system involves a specific Trxs that possesses signaling and protective functions via the modulation of the redox status of target molecules [36]. Various studies report the role of Trxs not only in stress response but also in plant growth and development, like OsTRXh1 of rice that modulates the level of H₂O₂ under salt stress in *OsTRXh1*-overexpression plants, as well as its influence on plant development [37]. Although the role of Trxh proteins in biotic stress response is studied in various species, the function of durum wheat TdTrxh2 in response to biotic stress is still not clear until now. Thus, we aimed in this study to demonstrate the involvement of TdTrxh2 in the response to *F. graminearum* attack through the analysis of the transgenic TdTrxh2-expressing *Arabidopsis* plants and analysis of its expression in leaves and roots of durum wheat exposed to *F. graminearum* infection. It has been reported previously that TdTrxh2 is among the four Trxh isoforms of durum wheat that are involved in salinity tolerance via redox regulation [38]. The importance of Trx h-type in abiotic stress tolerance has been explored thoroughly in various plant species. For instance, *LhTRX-h3* and *AtTrx-h2* enhance drought tolerance of *Liriodendron* hybrid and salt tolerance of the transgenic *Brassica napus* plants, respectively [25,39]. On the other hand, Trxs h-type play a crucial role in defense responses during a compatible plant–pathogen interaction. In the plant–pathogen interaction, PAMPs are recognized by specific plasma membrane receptors to recognize the pathogen and initiate the PAMP-triggered immunity (PTI). This recognition is characterized by defense cascades' reactions and induces HR [40]. In tobacco, the PAMPs molecule, flg22 from *Xanthomonas axonopodis*, induces ROS-scavenging enzymes and PR genes' expression [41].

Bread wheat is more tolerant to *F. pseudograminearum* and *F. graminearum* than durum wheat, and, as a consequence, produces less yield loss [27,28]. Indeed, the strongest quantitative trait loci (QTL) associated with *Fusarium* crown rot (FCR) tolerance is *Qcrr.cpi.3B*, which is derived from the wild relative of bread wheat, *Triticum spelta* [42]. Moreover, in response to *Fusarium* infection, the differentially expressed genes are distributed on B and D subgenomes [43]. Moreover, the QTLs associated with FHB tolerance are more widely identified in bread wheat than in durum wheat [26]. In durum wheat, the QTLs associated with FHB tolerance are distributed on chromosomes 1B and 4B [44]. Moreover, Sari et al. [45] identified several QTLs associated with plant height and maturity during FHB resistance.

In the present study, we evaluated the effect of *F. graminearum* infection of durum wheat on TdTrxh2 expression in order to decipher its role in response to this fungus. During the response of wheat to *F. graminearum* infection, the majority of the genes were expressed between 48 and 72 h post-inoculation. These include encoding proteins involved in pathogen recognition, defense signaling, and resistance responses [46,47]. Our findings showed that TdTrxh2 was highly enhanced in leaves of durum wheat during 24, 72, and 120 h of inoculation. However, TdTrxh2 expression was downregulated in roots at 24 h post-inoculation (Figure 1). These data suggested that the TdTrxh2 expression level depended not only on the stage of the infection but also on the specific plant tissue. At a later stage of infection (72 h), the pathogen was colonized the subepidermal layers of the rachis, causing an extensive host cell breakdown and the emergence of arial mycelium [48]. Thus, in

long-term *Fusarium* inoculation, it seems that an oxidative stress is produced in the aerial part of wheat, which is explored by the high H_2O_2 levels during 120 h of inoculation and high expression level of *TdTrxh2*. By contrast, during the first six hours of pathogen infection, H_2O_2 was exhaustively accumulated in roots with the high accumulation of *TdTrxh2* transcripts (Figure 1). Interestingly, after 6 h post-inoculation, the H_2O_2 amount was reduced excessively in root tissues, accompanied by the downregulation of *TdTrxh2*. Thus, it appears that *F. graminearum* colonizes the durum wheat plants, reaching the aerial parts and triggering oxidative stress, as evidenced by high H_2O_2 accumulation and *TdTrxh2* induction. Similarly, it has been reported previously that the genes *PoTrxh3* and *h5* of *P. vulgaris* are upregulated in response to symbiosis and simultaneously with H_2O_2 accumulation [24].

On the other hand, we examined the predicted *cis* elements of *TdTrxh2* and then compared them to those of the three isoforms, *TdTrxh1*, *TdTrxh3*, and *TdTrxh9*. The outcomes showed that the *TdTrxh2* promoter region contains essentially the two hormone-related stressors, which are MeJA- and ET-responsive elements, and the biotic stress-related elements, WBOXNTERF3 and TC-rich repeat elements (Figure 2). The first element is primarily implicated with wounding stress [32], while TC-rich repeat element is involved in defense and stress responsiveness. It has been reported that TC-rich repeat element is present in the promoter regions of genes involved in response to various abiotic stresses, like high temperature, salinity, and drought [49,50]. Some of these *cis* elements have been described in the promoter regions of *Citrus sinensis* *CsTRXs*, and *CsTRXh4* harbors the highest number of regulatory elements [51]. The variability of the *cis*-regulatory elements in *TdTrxh* isoforms could be explained by their differential expression under different stresses. This is the case of *TRXh* genes of *Arabidopsis* that respond differentially to stress. For instance, the promoter region of *AtTRXh5* contains many copies of the W-box (TTGACC/T) and it is induced by wounds, which is not the case of *AtTRXh1*, *h2*, *h3*, and *h4*. Furthermore, *AtTRXh5* expression is enhanced by the bacteria *Pseudomonas syringae* via the positive regulation of the transcription factor WRKY6 [50]. There is also the *AtTRXh8* gene, which is strongly induced in response to wounds, several pathogens, and elicitors [52].

Despite the importance of GT1GMSCAM4 in pathogen and salt stress responses [34], it is worth noting that this element was absent from the *TdTrxh2* promoter region, suggesting the presence of other, as yet uncharacterized, regulatory elements.

Generally, the identified Trxh proteins from multiple plant species are among the components that crosstalk between abiotic and biotic stress, such as Trxh of *V. vulgaris* [24], *A. thaliana* [53], and *L. maritima* [30,54]. Likewise, the durum wheat *TdTrxh2* plays a major role in abiotic stress tolerance, including salt, osmotic, and cadmium toxicity stresses [23]. In the second part of this study, we evaluated the antimicrobial activity of *TdTrxh2* and finally the impact of its expression in transgenic *Arabidopsis* plants in response to *F. graminearum*. Our findings suggested that the *TdTrxh2* protein has an effective bactericidal and fungicidal agent against the tested pathogenic strains (Figure 3). This effect could be explained by the possible interaction of *TdTrxh2* with ROS-producing proteins, consequently leading to cell death. Similar to our protein, *LmTrxh2* of *L. maritima* possesses antibacterial activity against eight pathogenic strains [30]. Likewise, *AtTrxh5* and *OsTrxm* of *Arabidopsis* and *O. sativa*, respectively, have antifungal activity due to ROS accumulation [55,56]. Another example of plant antifungal protein is the protein *OsTDX*, a Trx-like protein, of *O. sativa* that inhibits the growth of various fungal pathogens [57]. In planta, the protective role of *TdTrxh2* protein against *F. graminearum* was proved via the activation of the major H_2O_2 -scavenging enzymes POD and CAT in leaves of *Arabidopsis* infected with this fungus (Figure 4). Likewise, the protein *TdTrxh2* activates both CAT and POD in transgenic *TdTrh2*-expressing *Arabidopsis* plants in response to abiotic stress [23], suggesting the major role of

TdTrxh2 in H₂O₂ homeostasis regulation. Furthermore, given the chaperon-like activity of TdTrxh2 [23], it is tempting to postulate that this protein stabilizes the stress-responsive proteins. Several studies highlight that *Trxh* genes improve tolerance of the transgenic plants to pathogen attack, such as *LmTrxh2* of *L. maritima* that confers tolerance of the transgenic tobacco plants to multiple pathogens [30], and *NtTrxh3* of *Nicotiana tabacum* whose overexpression enhances tolerance to tobacco mosaic virus and cucumber mosaic virus [58].

Our results suggest a potential correlation with TdTrxh2 protein and the SA and ABA signaling pathways to attenuate tolerance of the transgenic *Arabidopsis* plants. Previously, various studies supported the connection between Trx and phytohormone signaling pathways to control plant development and stress adaptation [59]. Some studies proved that Trxh proteins confer tolerance via the SA-associated defense signaling pathway, like bread wheat *TaTrxh1* that is induced by *Puccinia striiformis* f. sp. *tritici* infection and SA treatment [60]. In pepper plants, Trx proteins function in response against *Cucumber mosaic virus* by promoting the expression of the defense-related genes involved in the SA pathway, which are *NPR1* (non-expressor of PR gene 1), the activator of SA-mediated immune response, and *PR10* (pathogenesis-related) genes [61]. Here, we demonstrated that the tolerance observed in the *TdTrxh2*-expressing *Arabidopsis* lines against *F. graminearum* infection is associated with the upregulation of a subset of SA-mediated genes, including *NPR1*, *NPR4*, and *PR1*, and the two ABA-related genes, *NCED3* and *DREB2A* (Figure 5), suggesting a possible relation between TdTrxh2 protein and these signaling pathways. The protein NPR1 is a key node in SA signal transduction during SAR (systemic acquired resistance) response. The presence of NPR1 in the cytoplasm under SA induction is controlled by Trxs via S-nitrosylation to lead to disease resistance [62,63]. After infection, the interaction of NPR1 with NPR4 is disrupted by SA to lead to the activation of NPR1, which in turn interacts with the transcription factors TGA and WRKY to upregulate the expression of the target genes, including the antimicrobial *PR* genes [64,65]. In rice, OsTrxh2 and OsTrxh5 catalyze the dissociation of OsNPR1 into monomers, generating disease resistance via the activation of the *OsPR1a* gene. On the other hand, the protein OsTrxh2 is a direct target of XopI, which is an F-box effector of the bacteria *Xanthomonas oryzae*, consequently disrupting OsNPR1-mediated resistance [66]. In this study, we showed that the tolerance phenotype of the *TdTrxh2*-expressing *Arabidopsis* lines to *F. graminearum* infection is associated not only with the SA pathway but also the ABA signaling pathway, including *NCED3* and *DREB2A* proteins, which are involved in ABA biosynthesis and signaling, respectively [67,68]. The inactivation of *NCED3* of *N. tabacum* causes not only the reduction of ABA content but also affects plant growth and development, causing drought susceptibility as a consequence [69]. The phytohormone ABA interplays with other phytohormones like SA, jasmonic acid, and ethylene, contributing the HR reaction and adaptation in the long term [70,71]. Furthermore, the transcription factors are critically important in regulating diverse target genes and they are the convergence point for abiotic and biotic stress responses [72]. *DREB2A* regulates the expression of many drought-responsive genes in the ABA-independent pathway [73]. The ABA-independent pathway crosstalks with the ABA-dependent pathway in response to osmotic stress [74]. It is worth noting that the exogenous SA and ABA improve the growth of the transgenic *TdTrxh2*-expressing lines (Figure 6). Taken together, in this study, we demonstrated that TdTrxh2 plays a pivotal role in the plant defense response. However, its specific involvement in SA and ABA signaling pathways during host–pathogen interactions remains to be elucidated. Thus, further research is required to unravel the underlying mechanism of this regulation.

4. Materials and Methods

4.1. In Silico Analysis

Concerning the analysis of the promoter regions of the *TdTrxh2* gene and the three genes *TdTrxh1*, *TdTrxh3*, and *TdTrxh9*, the promoter region of each *TdTrxh* gene, about 1 Kb upstream of the ATG codon, was downloaded using the EnsemblPlants server (<https://plants.ensembl.org/index.html>) (accessed on 11 November 2024). Using the two website tools PlantCARE and PLACE [75,76], the regulatory *cis* elements involved in hormone and biotic stress responses were retrieved and then presented as a heatmap using TBtools software version 1.x [77]. The *cis* elements that were identified using the PLACE tool are WBOXNTERF3 and GT1GMSCAM4, while the others were retrieved using the PlantCARE database.

4.2. Plant Material and Stress Application

Seeds of the Tunisian durum wheat (*Triticum turgidum* Desf. ssp. *durum*), the cultivar Om Rabiaa (OR), were sterilized, as described by Tounsi et al. [78], and then placed on Petri dishes with a wet Whatman filter paper in the dark until the germination. Subsequently, they were transferred onto the Hoagland hydroponic solution in a growth room at 23 °C and 65% relative humidity, under light/dark conditions of 16 h light at 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 8 h dark, to grow for 10 days. After that, the control plants were separated from the stressed plants that were inoculated with *Fusarium graminearum* strain (2×10^7 spores/mL) in the Hoagland's solution, as detailed by Saidi et al. [79]. Before that, this strain was grown on potato dextrose agar (PDA) (Sigma-Aldrich Inc., St. Louis, MO, USA) plates for 7 days at 30 °C. After inoculation, all plants were kept in the high-humidity condition at 25 °C with a 16 h photoperiod. Leaves and root samples of the stressed and control plants were harvested at different times of stress for *TdTrxh2* expression profile analysis.

4.3. RNA Isolation and qRT-PCR Analyses

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and following the protocol described by Rio et al. [80] from leaves and roots of durum wheat inoculated with *F. graminearum* for 6, 24, 72, and 120 h in order to perform the qRT-PCR analysis. On the other hand, to analyze the expression profiles of the genes related to SA, which were *AtNPR1*, *AtNPR4*, and *AtPR1*, and those related to ABA pathways, which were *AtNCED3* and *AtDREB2A*, the total RNA from leaves of transgenic *Arabidopsis* TH3-1 and TH6-1 exposed or not to the fungal infection with *F. graminearum* was extracted using the same reagent and protocol described above. For each RNA sample, the residual genomic DNA was removed with 1 U of RNase-free DNase (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 37 °C. Then, the first-strand cDNA was synthesized using M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and the Oligo-dT primer (18 mer). The qRT-PCR preparation and reactions were performed as described by Feki et al. [81], with three technical repetitions for each sample and three biological repetitions for each experimental condition. Primers used in qRT-PCR reactions are described in Table S1. The durum wheat *actin* gene (*TdActin*) and the *Arabidopsis* ubiquitin 10 (*UBQ10*) were used as reference genes. All primers used in this analysis were obtained using Primer3web (version 4.1.0; <https://primer3.ut.ee/> (accessed on 5 January 2025)) to generate amplicon sizes less than 200 pb. The relative expression of each gene was calculated using the formula $2^{-\Delta\Delta\text{CT}}$ method mentioned in [82].

4.4. H₂O₂ Quantification

To determine the H₂O₂ levels in leaves and roots of durum wheat exposed to *Fusarium graminearum*, we followed the protocol described by Feki et al. [83].

4.5. Antibacterial Activity Tests

Previously, the His-tagged TdTrxh2 protein was expressed in the *E. coli* strain BL21 cells [23]. Then, its expression was induced after the addition of 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) during 6 h at 37 °C. The phase separation with the Triton X-114 method was followed to isolate the protein without bacteria endotoxins contamination. Finally, this protein was purified using the Ni-NTA column (ThermoFisher, Inc., USA), as described by Kamoun et al. [23]. In this study, the antibacterial activity of the His-tagged TdTrxh2 protein was tested against various bacteria strains: three Gram- strains, which were *Agrobacterium tumefaciens* (B6: ATCC 700368), *Agrobacterium tumefaciens* (C58: ATCC 27072), and *Pseudomonas aeruginosa* (ATCC 2785), and three Gram+ strains, which were *Bacillus subtilis* (ATCC 6633), *Bacillus cereus*, and *Staphylococcus aureus* (ATCC 25923). These strains were obtained from a local culture collection of the Centre of Biotechnology of Sfax (CBS), Tunisia. These bacteria were cultivated in LB agar at 37 °C for 12–24 h, while *Bacillus* species were incubated at 30 °C. To evaluate the antibacterial activity of TdTrxh2 protein, two methods were carried out, which were the agar diffusion method [30] and the microdilution method [84]. Here, 100 μ L of cell suspension was evenly spread onto the surface of LB agar. Four wells (6 mm in diameter) were carved in the agar using a sterile Pasteur pipette. Two wells were considered as positive and negative controls, containing Gentamicin (10 μ g/well) and buffer solution (Tris 100 mM and NaCl 50 mM), respectively. The purified His-tagged TdTrxh2 protein was poured into the two other wells, at two different concentrations, C1 (880 μ g/mL) and C2 (1660 μ g/mL). Finally, the plates were incubated at 37 °C for 24 h. The antibacterial activity was assessed by measuring the diameter of the circular zones of inhibition within the well. Tests were repeated three times.

In parallel, the antibacterial test was done by the broth microdilution method in 96-well microplates to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), as described by Ben Hsouna and Hamdi [84]. To determine the MIC that corresponds to the concentration of TdTrxh2 that completely inhibited bacteria growth, 10 μ L of cell suspension of different bacteria species was added to the corresponding well that contained 25 μ L of thiazolyl blue tetrazolium bromide (0.5 mg/mL; (Sigma-Aldrich Inc., St. Louis, MO, USA)) and the purified TdTrxh2-His protein (1660 μ g/mL). Finally, the microplates were incubated for 30 min at 37 °C. Concerning the MBC, it is defined as the lowest TdTrxh2 concentration that results in microbial death. As detailed by Ben Hsouna and Hamdi [84], MBCs were determined after the incubation of LB plates that were inoculated by 10 μ L from each well, at 37 °C for 24 h.

4.6. Antifungal Activity Tests

The antifungal activity of the His-tagged TdTrxh2 protein was tested against six fungi, which were *Aspergillus niger*, *Botrytis cinerea*, *Alternaria alternata*, *Fusarium graminearum*, *Fusarium oxysporum*, and *F. culmorum*. These strains were from the Centre of Biotechnology of Sfax (CBS), Tunisia. These strains were grown on potato dextrose agar (PDA; 1.5% agar) at 28 °C for 7 days. The antifungal activity was assessed by both the well diffusion and the broth microdilution methods. The well diffusion method for the antifungal test was performed as described by Magaldi et al. [85] with modifications. The PDA medium was inoculated by different fungi (*F. oxysporum*, *F. graminearum*, and *A. niger*) and kept at 28 °C for 48 h. After that, three wells (6 mm in diameter) were cut out of the agar. The negative control was the buffer solution (Tris 100 mM and NaCl 50 mM). TdTrxh2 purified protein was added and the inhibition zones were observed after 24 to 72 h of incubation at 28 °C. The broth microdilution method was carried out against various phytopathogenic fungi (*F. oxysporum*, *F. culmorum*, *F. graminearum*, *B. cinerea*, *A. niger*, and *A. alternata*) to determine the minimum fungicidal concentration (MFC) of TdTrxh2, as described by Ben Hsouna and

Hamdi [84]. MFC corresponds to the lowest concentration with no visible growth, with 99.5% killing of the original inoculum. These tests were performed in triplicate.

4.7. Pathogenicity Tests

To analyze the response of the transgenic *Arabidopsis* TH3-1 and TH6-1 lines to the *F. graminearum* strain, 5 μ L of spore suspension (10^5 spores/mL in 0.001% (v/v) of Silwet L-77 solution) was deposited in the center of each detached leaf, as described by Saidi et al. [79]. Control leaves were treated only with Silwet L-77 solution, and all the detached leaves were placed in the high-humidity condition at 25 °C. After 5 days of inoculation, leaves were photographed, and the infected leaf areas were determined using the image processing and analysis program UTHSCSA (<http://compdent.uthscsa.edu/dig/itdesc.html> (accessed on 15 June 2025)). Experiments were performed in triplicates.

4.8. ROS-Scavenging Enzymes' Activities

First, the proteins from the leaves (0.5 g) inoculated or not (control leaves) with *F. graminearum* were extracted, as described by Feki et al. [83]. Then, the protein concentration in each sample was determined according to Bradford [86]. The activities of the three ROS-scavenging enzymes CAT, SOD, and POD were determined following the protocols detailed by Feki et al. [83]. Briefly, CAT activity was determined by monitoring the disappearance of H_2O_2 , and one unit of CAT was defined as 1 μ mol/mL H_2O_2 decomposed per minute. SOD activity was evaluated using the photochemical NBT method. One unit of SOD was defined as the enzyme quantity required causing 50% inhibition of the rate of NBT reduction at 560 nm. POD activity was carried out by the guaiacol oxidation method. One enzyme unit of POD was defined as change in one unit of absorbance min^{-1} .

4.9. Evaluation of Transgenic *Arabidopsis* Lines' Tolerance to Hormones

The two transgenic *Arabidopsis* lines TH3-1 and TH6-1 expressing *TdTrxh2* were obtained previously by Kamoun et al. [23]. The seeds of these two lines, and the non-transformed plants (Wt plants), were sterilized and then germinated on MS agar medium. After 15 days of growth, seedlings were transferred to MS medium containing or not 10 μ M ABA and 50 μ M SA and kept growing under these stress conditions for one week. The tolerance phenotype was evaluated by determining the fresh weight (FW) and the root elongation of each plant under normal and stress treatments. Each test was performed in triplicate.

4.10. Statistical Analysis

The statistical software SPSS version 20 was used for statistical analysis of the results based on the ANOVA method. Means were compared using Student's *t*-test and Tukey's HSD test. The level of significance used for all statistical tests was 5% ($p < 0.05$).

5. Conclusions

In summary, we shed light on the role of the durum wheat *TdTrxh2* protein on biotic stress response via the analyses of expression patterns of the corresponding genes in leaves and roots of durum wheat exposed to fungal attack, its antimicrobial activity against various pathogens, and its functional characterization in the transgenic *TdTrxh2*-expressing *Arabidopsis* plants. Firstly, our results showed that *F. graminearum* infection led to the upregulation of *TdTrxh2*, with distinct temporal profiles across tissues, an immediate response in the roots, and a sustained, long-term induction in the leaves. Besides, this induction correlated closely with the accumulation of H_2O_2 in the affected tissues. On the other hand, the *in silico* analysis demonstrated the presence of elements related to ABA and MeJA hormones in wound and defense responses. Furthermore, the purified *TdTrxh2*

functions as a dual-action antimicrobial agent, effectively targeting both bacteria and fungi. Finally, the expression of *TdTrxh2* in *Arabidopsis* plants conferred tolerance to *F. graminearum* disease via the enhancement of H₂O₂-scavenging enzymes' activities and the induction of the expression of some SA- and ABA-related genes. Additionally, the application of exogenous ABA and SA improved the growth of transgenic lines. Consequently, *TdTrxh2* is speculated to act as a redox regulator within the SA and ABA signaling networks, potentially mediating the plant's transition toward pathogen tolerance. Taken together, our results suggested that *TdTrxh2* could be used for engineering of crops tolerant to *F. graminearum* attack and, consequently, improving crop yield.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants15040521/s1>. Table S1: Primer details used in qRT-PCR reactions. The reference genes are *UBQ10* in *Arabidopsis* and *TdActin* in wheat.

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