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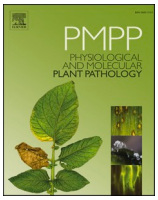

Functional validation of *Lobularia maritima* thioredoxin-h2 protein for its ability to combat bacterial and fungal infections

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ABSTRACT

Thioredoxins (Trxs) belong to a family of multifunctional redox proteins that is critical for maintaining and regulating the cellular redox environment during plant cell growth. Also, they are important for the development of plant's response to biotic and abiotic stress; however, the specific biological functions of h-type thioredoxins (Trxhs) in plants have not been fully elucidated. Herein, we investigated the role of LmTrxh2, a specific member of the Trxh family, in response to various biotic stress simulants, including mechanical wounding, exposure to jasmonic acid (JA), picolinic acid (PA), salicylic acid (SA), ethephon (ETP), and hydrogen peroxide (H_2O_2). We observed that LmTrxh2 transcripts were significantly upregulated upon exposure to these stress simulants. The characterization of enzymatic activity revealed that the recombinant LmTrxh2 protein functions as a disulfide reductase. While the role of Trx proteins in redox regulation is well known, their involvement in antimicrobial activity is still unexplored. Therefore, we assessed the antimicrobial effect of LmTrxh2 towards various microorganisms and observed a concentration-dependent inhibition of microbial growth. The minimum inhibitory and minimum bactericidal concentrations and the diameters of the inhibition zones were 40–1250 μg/mL, 40–1250 μg/mL, and 12.5–32.5 mm, respectively. In addition, we used previously developed *LmTrxh2*-transgenic tobacco lines and found that they showed enhanced resistance to fungal infections triggered by *Fusarium graminearum* and *Aspergillus niger*. This resistance was associated with an upregulation of known defense-related genes. Overall, our findings suggest that *LmTrxh2* is responsive to multiple biotic stress simulants and plays a critical role in the basal resistance of plants to pathogen infections. These results highlight the potential of *LmTrxh2* in the development of strategies to protect crops from various stress factors and emphasize its importance in the adaptation of plants to different stress conditions.

1. Introduction

Being sessile from their nature, plants are vulnerable to several environmental stress factors that affect their growth, development, and survival. However, plants use various defense mechanisms to protect themselves from stress agent and increase their resistance. Generally, pathogenic infections cause cellular and apoplectic accumulation of reactive oxygen and nitrogen species (ROS and RNS), such as nitric oxide (NO), superoxide (O_2^-) and its specific dismutation product, namely hydrogen peroxide (H_2O_2) [1]. Despite the key role of these

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molecules in cell signaling, their accumulation lead to nitro-oxidative stress, which in turn leads to the formation of undesirable oxidative thiol modifications [2–5]. Specifically, at the cellular level, thiol oxidation is a complex chemical process that affects many essential biological processes. Cellular thiol oxidation is known to interact and interfere with important mechanisms, such as epigenetic regulation, signaling processes, oxidative stress, cell growth and development [6]. Therefore, to protect the cellular equilibrium, a balance between the production and degradation of ROS must be tightly regulated. To maintain the growth, metabolism, and general development of the plant, it is necessary to intercept ROS and control the damage caused by ROS molecules [7,8].

Unfavorable cellular conditions lead to the activation of various antioxidant and protective mechanisms, mainly consisting of the production of different antioxidant enzymes, such as catalase, ascorbate or glutathione peroxidases (APX and GPX, respectively), monodehydroascorbate reductases (MDAR), dehydroascorbate reductases (DHAR), or peroxiredoxins (PRX), all of which act as H_2O_2 scavengers and deactivators [4–9]. Specifically, the activity of defense enzymes could be inhibited by high levels of oxidative stress. In particular, thiol oxidation of antioxidant enzymes is an important component of redox regulation in cells and is essential for many physiological processes. Thus, oxidation of the thiol groups of antioxidant enzymes, including glutathione and enzymatic thiols, can lead to their inactivation, thus altering cell signaling pathways and contributing to oxidative damage. The oxidation of thiols is also essential for the control of several different enzymatic activities and changes in protein structure. In this regard, it has been reported that the oxidation of protein thiol groups modifies their structure and conformation and can even alter the affinity of the protein for its substrate, leading to a change in physiological activity [10]. Trx is an important enzymatic component of the ROS machinery [8]. In this context, increasing evidence suggests that thioredoxins (Trxs), a peculiar class of enzymes, are able to protect the antioxidant enzymes from inactivation caused by thiol oxidation. Specifically, several *in vitro* proteomic studies have identified antioxidant enzymes as potential substrates for the cytosolic and mitochondrial Trx family members from various terrestrial plants and green algae [11–17].

Trx proteins are ubiquitous acidic tiny proteins [18] that are identified in organelles and eukaryotes [19–21]. The components of this class of proteins share the same highly conserved amino acid sequence of the active site. There are six types (f, m, x, y, h, and o) of plant Trx proteins that differ in their amino acid sequences and cellular distribution [22,23]. Members of each family are thought to regulate different biological processes for numerous proteins [24–30].

Plants respond to microbial infections by activating several defense responses. One of the most important plant defense mechanisms is the activation of the R gene-mediated response. This process refers to the activation of defense mechanisms in plants triggered by the recognition of microbe-derived molecules by their corresponding resistance (R) gene. This recognition leads to a cascade of signal transduction, including protein phosphorylation, ion flux, reactive oxygen species (ROS) production, and the intracellular accumulation of salicylic acid (SA). SA plays an important modulatory role in the upregulation of proteins related to pathogenesis (PR) production [31]. In addition, it was also reported that JA and ethylene are two important hormones involved in plant defense responses. In particular, JA is involved in the activation and regulation of defense responses to both necrotrophic and herbivorous pathogens, whereas ethylene is involved in the defense responses to necrotrophic pathogens, herbivory, and abiotic stresses [32,33]. These plant hormones also regulate early signaling processes in the defense cascade, leading to the generation of pathogenesis-related compounds and stimulation of the plant's immune system [34].

Lobularia maritima, commonly called sweet alyssum, is an annual herbaceous flowering plant widely distributed in temperate regions around the world [35]. This plant has been demonstrated to have notable health benefits due to the presence of several bioactive compounds, such as flavonoids, polyphenols, anthocyanins, and isothiocyanates [36]. This plant is known to alleviate various diseases and contributes to maintain healthy cholesterol and blood sugar levels.

In light of this latter consideration, the main goal of the present work is to investigate the effects of the treatment performed using several biotic stress factors, including wounding, jasmonic acid (JA), picolinic acid (PA), salicylic acid (SA), ethephon (ETP), and hydrogen peroxide (H_2O_2), on the expression of the *LmTrxh2* gene, a specific member of the Trxh family.

Previously, Ben Saad et al. [37] had identified the first *Trxh2* gene of the halophyte plant *L. maritima* and named it *LmTrxh2*. The *LmTrxh2* gene was found to be regulated by several stress factors, and its expression increased the tolerance of genetically engineered tobacco lines to the previously mentioned stress factors [37–39]. Therefore, the antimicrobial potential of LmTrxh2 was also evaluated by calculating the inhibition zone (IZ), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MCB). *LmTrxh2*-overexpressing transgenes showed increased basal resistance to pathogen infection in tobacco leaves, which was associated with constitutively increased expression of known defense-related genes. Our data show for the first time that *LmTrxh2* is involved in modulating defense responses to biotic stress, in addition to its previously demonstrated role in abiotic stress responses.

2. Materials and methods

2.1. Plant materials and biotic stress experiments

The saline marshes near the Chebba region, Mahdia in Tunisia, were the source of the seeds of *L. maritima*. Seed sterilization and germination were prepared as detailed earlier by Ben Romdhane et al. [40]. Then, seedlings were left to grow in a nutrient solution for four weeks, as reported by Ben Saad et al. [41], and were subjected to different biotic stress treatments. The middle part of the leaf was wounded by creating an incision using a scalpel blade. Chemical treatment was performed by leaf treatment with PA (10 mM), JA (100 μM), and SA (100 μM). Then, the leaves that were detached from *L. maritima* were placed for 1 h in dionized water in beakers (to remove any probable influence of wound stress) and then treated with 100 μM ETP. H_2O_2 was added at a dose of 10 mM by dipping the roots of the seedlings in the H_2O_2 solution. Control seedlings were maintained under normal conditions without any stress. Fully developed leaves were collected and directly frozen in LN_2.

2.2. Transcriptomic analyses

The total RNA was extracted from *L. maritima* plants subjected to various stress treatments by TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. The synthesis of the cDNA first-strand was induced from 2 μg of total RNA using the M-MLV reverse transcriptase enzyme (Invitrogen) and then diluted five times before the qPCR amplification. The cDNA was amplified using the Light Cycler 480 (Roche, Basel, Switzerland) with thermal cycling detailed by Ben Romdhane et al. [42]. Then, at the end of the experimental cycle, the melting curve was measured and analyzed to assure that it was a single amplification. Finally, thresholds of the cycles (CT) of the triplicate PCRs were averaged and used to quantify the transcripts. The primer pairs were designed using Primer 3 software [43] to ensure that the amplification was specific for the *LmTrxh2* gene and the housekeeping gene *ubiquitin 10* mRNA (*UBQ10*: At4g05320) [37,38]. The relative expression ratio of the *LmTrxh2* gene was calculated using the $2^{-\Delta\Delta Ct}$ method [44]. Each RT-qPCR that corresponded to each sample was performed three times, and for the experimental conditions, three biological repetitions were conducted. The expression analysis of a set of tobacco stress-related genes ((*NtPR1*a, X12485.1), (*NtPR2*, M60460.1), (*NtHIN1*, Y07563), (*NtLOX1*, X84040.1), (*NtACS6*, AF392978), and (*NtNPR1*, DQ837218.1)) in NT and transgenic tobacco plants was conducted as

mentioned previously. The expression levels of the genes were normalized compared to the tobacco house-keeping gene *Actin* (*NtACT*: XM_016633661) [38,39] (Table S1).

2.3. LmTrxh2 expression and purification of recombinant protein in E. coli

The LmTrxh2 protein isolated from *L. maritima* was identified by Ben Saad et al. [35]. PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) was used to amplify the full-length open reading frame of *LmTrxh2* (ORF) with primers that correspond to the 5′ and 3′ ends and *Eco*RI restriction sites. The *LmTrxh2* ORF was cloned into the pET28a vector and transformed into *E. coli* BL21 cells. Then, proteins that were recombinant were induced with 1 mM IPTG at 30 °C for 6 h and purified with HisLink™ Protein Purification Resin according to the manufacturer's instructions (Promega). The quantification of proteins was performed by the Bradford [45] assay; the standard that was used was bovine serum albumin. SDS-PAGE was applied to check the purity and correct the size of the recombinant proteins.

2.4. LmTrxh2 activity assay

Trx activity was studied with a purified recombinant LmTrxh2 protein, in an insulin reduction assay as reported by Holmgren [46]. For this purpose, purified LmTrxh2 protein was mixed and then incubated with 0, 2.5, 5, or 10 μM of purified LmTrxh2 protein. Each measurement of LmTrxh2 activity at 650 nm was repeated three times.

2.5. Acquisition of test microorganisms and cell culture

Purified and authentic bacterial and fungal cultures were purchased from the American Type Cell Culture (ATCC) and a local culture collection of the Centre of Biotechnology of Sfax, Tunisia, including gram-positive and gram-negative bacteria. Bacterial strains (*Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 14579, *Micrococcus luteus* ATCC 1880, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 1911, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella enterica* ATCC 43972) were cultured as previously mentioned by Ben Hsouna et al. [47–50].

2.6. Agar diffusion method

The assays for the determination of antibacterial effects were conducted using the method previously mentioned by Ben Hsouna et al. [50]. LmTrxh2 was dissolved in di-methyl sulfoxide/water (1/1) and sterile water to obtain a final dose of 10 mg/mL. Then, 50 μl of this solution was added to each well, and the plates were incubated at 37 °C for 24 h. Carboxyline (10 μg/well) and DMSO served as positive and negative controls, respectively. To measure the strength of the antimicrobial activity, the diameter of the circular zones of inhibition around the well was evaluated. Each test was repeated three times.

2.7. Determination of MIC and MBC

The minimum inhibitory concentration (MIC) of LmTrxh2 was evaluated as previously reported by Ben Hsouna et al. [47,51]. Sterile 96-well microplates were used to conduct the assays. As a positive growth control, wells consisting of the test microorganisms that were incubated in the growth medium were prepared. On the other hand, the negative control contained di-methyl sulfoxide/water (1/9) mixture. Then, sterile cover plates were placed on the plates. Plates, therefore, were incubated for 24 h at 37 °C.

To indicate the microorganisms' growth, 25 μl of the indicator solution of thiazolyl blue tetrazolium bromide (MTT) (0.5 mg/mL) dissolved in sterile water was added to the wells and incubated at 37 °C for 30 min. This compound tetrazolium salt is colourless and has a key role as an electron acceptor, and biologically active microorganisms act by reducing it to a red-coloured formazan product. Once there was an inhibition of microbial growth, the solution in the well remained clear after incubation with MTT. DMSO and water were used as negative controls. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of LmTrxh2 needed to kill the test microorganisms. Each experiment for the measurement of MIC and MBC was repeated in triplicate.

2.8. Pathogenicity tests

Potato dextrose agar (PDA) plates were used to grow *Fusarium graminearum* and the *Aspergillus niger* strain (CTM 10099). For each detached leaf, 5 μL of spore suspension (10^7 spores/mL in 0.001 % (v/v) of Silwet L77 solution) was placed in the center of the leaves. As controls, leaves were inoculated with Silwet L-77 solution. At five days post inoculation (dpi), the leaves were photographed. The infected leaf area was quantified using ImageJ software (https://imagej.nih.gov/ij///index.html). Each experiment was repeated three times.

2.9. Data analysis

Statistical SPSS Windows (V. 12) software was used to analyze the obtained data. Data are represented as the mean ± s. e.m. of three independent replicates. To compare differences in the means' value between the groups, one-way analysis of variance (ANOVA) was employed followed by Tukey's test.

3. Results

3.1. Expression of the LmTrxh2 gene in response to biotic stress simulators

To explore the role of *LmTrxh2* in host defense responses, the level of expression facing wounding, pathogen elicitor-PA- and signaling molecules -SA, JA, H_2O_2 and ETP- in the leaves of *L. maritima* exposed or not to these stresses for 1, 3, 12, 24 and 48 h was examined by qRT-PCR. *LmTrxh2* showed the highest induction up to approximately 4-fold 3 h after wounding, and then the transcript level rapidly decreased and became insignificant (Fig. 1). PA was used as an elicitor known to stimulate the defense response in plants [52]. Interestingly, we observed earlier expression of the *LmTrxh2* gene within 3 h after treatment with PA, which persisted up to 24 h, followed by a reduction after 48 h (Fig. 1).

The *LmTrxh2* gene was induced by phytohormones, particularly by the SA, JA, and ETP (Fig. 1), which are crucial signal modulators in the responses of plants to pathogens [53]. Since SA has a crucial role in systemic acquired resistance (SAR) in plants, the level of expression of *LmTrxh2* showed an enhancement (3-7-fold) followed by a decline. Jasmonates are signaling molecules that are important for the initiation and maintenance of defense responses in different plants. The highest *LmTRxh2* transcript accumulation was recorded at 24-h after treatment, which was three-fold higher than that of the control treatment and maintained at a high level. Remarkably, *LmTrxh2* transcript levels were rapidly enhanced 1 h after treatment with ETP and remained at a high level, increasing 5-fold, followed by a decrease (Fig. 1). H_2O_2 has an important role in triggering the defense response in different stress situations in plants. The *LmTrxh2* gene was induced by the H_2O_2 treatment after 1 h and maintained its high levels for up to 24 h, followed by a steady reduction (Fig. 1). Hence, the present results suggest that *LmTRxh2* reacts to biotic stress simulants and enhances tolerance to pathogens in plants.

3.2. Overexpression, purification of recombinant LmTrxh2 protein and Trx activity

The nucleotide sequence of *LmTrxh2* was amplified with gene-

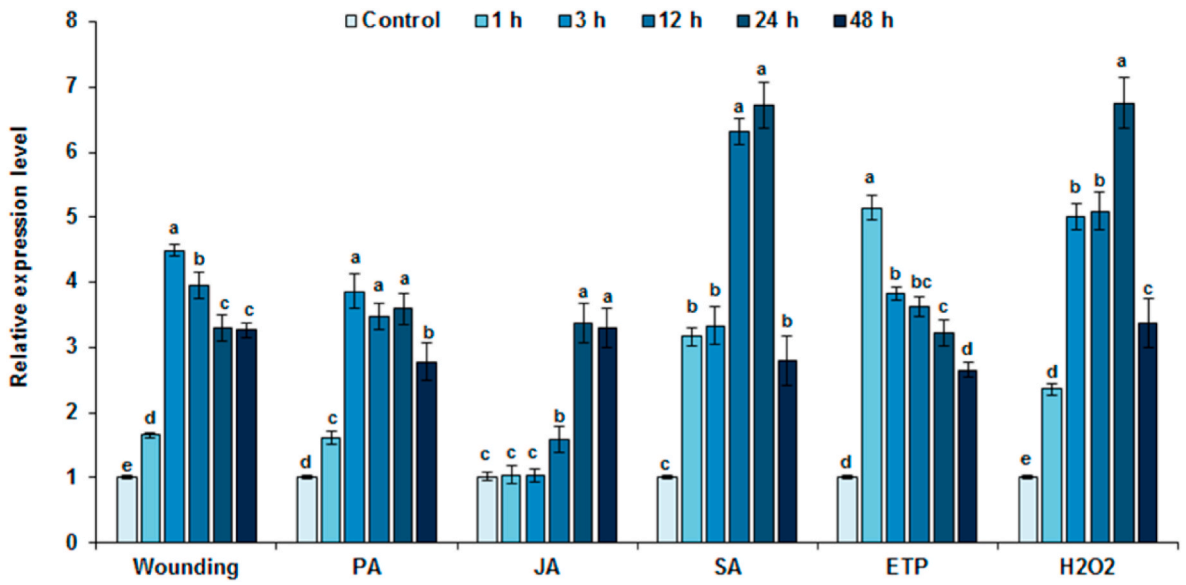

Fig. 1. Analysis of the expression profiles of LmTrxh2 gene in leaves of treated L. maritima plants. The UBQ10 gene was used as an internal control. Different letters indicate significant differences ($P < 0.05$).

specific primers, and then the coding *LmTrxh2* sequence was inserted into pET28a (+) and expressed in *E. coli*. SDS-PAGE analysis showed that the induced cells had a notable band with a molecular weight of ~15 kDa compared to the non-induced cells (Fig. 2a). The recombinant LmTrxh2 protein was purified, and the His tag was cleaved and removed. The particular property of Trx proteins has been shown to have a disulfide reductase function [46,54]. Using DTT as a reducing agent, the disulfide bonds that connect the insulin A and B chains could be reduced by Trx. Through the increase in the absorbance to 650 nm, the formation of insoluble B chain precipitates could be detected photometrically [46]. A significant increase in turbidity at 650 nm was recorded immediately after the addition of recombinant LmTrxh2 protein compared to the negative control (DTT alone) (Fig. 2b), which indicates a decrease in insulin as previously described by Holmgren [46]. Trx activity increased in the reaction mixture as the concentration of recombinant LmTrxh2 protein was increased from 2.5, 5, and 10 μM (Fig. 2b).

3.3. Antibacterial activity

The antibacterial activity of LmTrxh2 was tested by measuring the diameter of the inhibition zone (IZ) (Fig. 3) and then, the calculation of the MBC and MIC values. As presented in Table 1, LmTrxh2 displayed different levels of antibacterial capacity toward all evaluated strains. The recorded IZs ranged from 32 to 12 mm. Of all tested gram-positive bacteria, the largest IZ was measured against *Listeria monocytogenes* (32.5 mm), followed by *Bacillus cereus* (32 mm) and *Enterococcus faecalis* (32 mm). Among the gram-negative bacteria, the largest inhibition zone was measured against *Pseudomonas aeruginosa* (32 mm). The diameter of the inhibition zone showed by the positive control prepared with

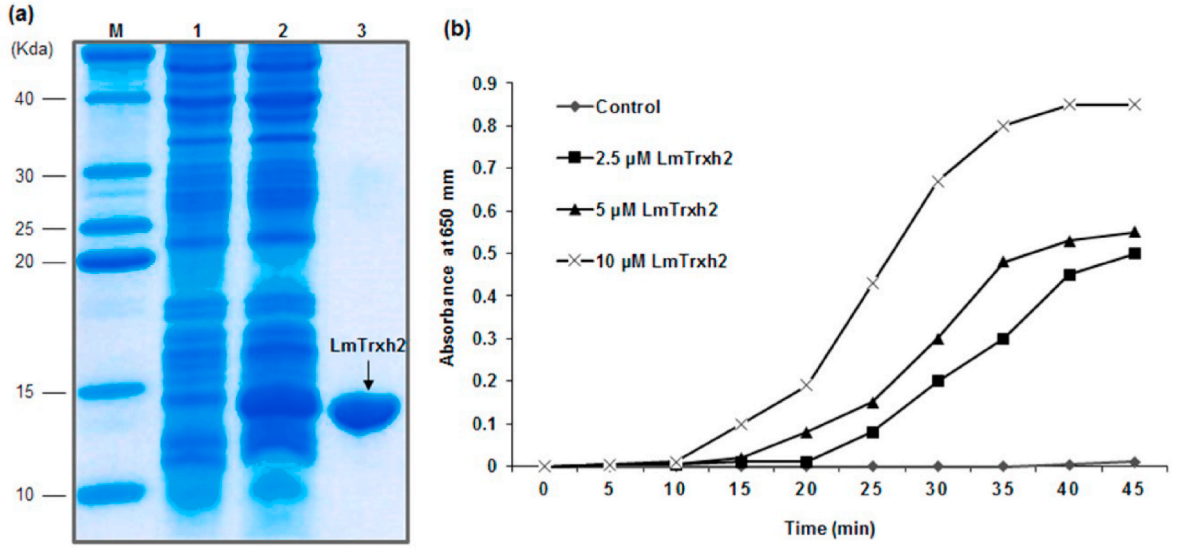

Fig. 2. Purification of LmTrxh2 recombinant protein and thioredoxin activity assay. (a) SDS-PAGE of LmTrxh2 recombinant protein: Crude extract of Pet28a-LmTrxh2 recombinant *E. coli* before (Lane 1) and after IPTG induction (Lane 2). Purified recombinant protein LmTrxh2 (Lanes 3). Molecular weight markers (M) are shown in kilodaltons in the left pane. (b) The activity of the LmTRxh2 protein determined by insulin disulfide reduction assay: precipitation of insulin upon reduction was monitored as a turbidity increase at 650 nm.

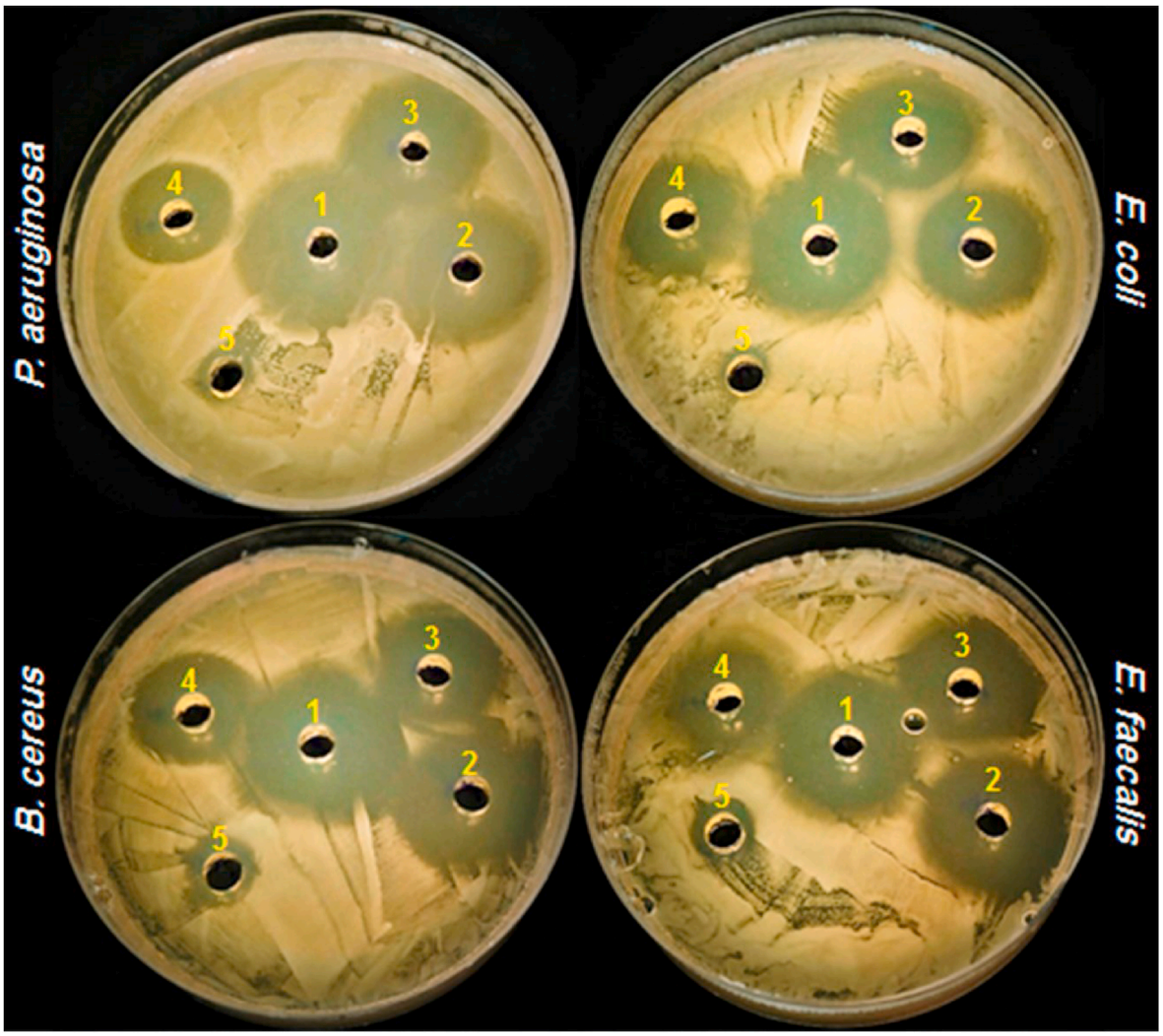

Fig. 3. Growth inhibition zones of LmTrxh2 in different bacterial strains: *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, and *Enterococcus faecalis*. (1): LmTrxh2 (2.5 mg/well); (2):1/2 diluted LmTrxh2 (1.25 mg/well); (3):1/4 diluted LmTrxh2 (0.41 mg/well); (4): reference antibiotic carboxyline; (5): Negative control.

Table 1
Antibacterial capacity of LmTrxh2.

Bacterial strains	Inhibition zones diameter (mm)			
	LmTrxh2	1/2	1/4	Carboxyline
Gram-positive				
B. cereus (ATCC 14579)	32 ± 0.14	28 ± 0.14	27.5 ± 0.35	23 ± 0.5
S. aureus (ATCC 25923)	18 ± 0.28	15 ± 0.42	12.5 ± 0.21	13 ± 0.1
E. faecalis (ATCC 29212)	32 ± 0.28	29 ± 0.14	28 ± 0.42	28 ± 1.2
M. luteus (ATCC 1880)	26 ± 0.3	27.5 ± 0.35	21.5 ± 0.35	19 ± 0.2
L. monocytogenes (ATCC 1911)	32.5 ± 0.21	29 ± 0.14	27.5 ± 0.35	21 ± 0.3
Gram-negative				
P. aeruginosa (ATCC 9027)	32.5 ± 0.21	28.5 ± 0.7	28 ± 0.6	22 ± 0.6
E. coli (ATCC 25922)	31 ± 0.14	28.5 ± 0.21	28 ± 0.42	29 ± 1.1
S. enterica (ATCC 43972)	32 ± 0.14	29 ± 0.14	28 ± 0.42	21 ± 0.7

Values are given as mean ± S.D. of triplicate experiments.
Diameter of inhibition zones of including diameter of disc 6 mm.
LmTrxh2 (2.5 mg/well).

carboxyline (10 μg/well) ranged from 29 to 13 mm; as assumed, no inhibitory effect toward the tested bacteria was recorded for the negative control. An important antibacterial capacity toward the eight evaluated bacteria was recorded for LmTrxh2 (Fig. 4), with MIC values between 1250 and 40 μg/mL (Table 2). Furthermore, LmTrxh2 strongly inhibited the development of *Listeria monocytogenes* and *Pseudomonas aeruginosa* with MICs of 40 μg/mL. It is known that these bacteria have a high level of virulence, and their infection is difficult to treat with conventional antibiotics, especially the multidrug resistant ones.

In the present investigation, the growth of *Listeria monocytogenes* was significantly inhibited by LmTrxh2. The MBC values of most strains tested correspond to the MICs, so the MIC/MIC ratio was equal to 1. Thus, our sample acts in the same way on gram-positive and gram-negative bacteria through bactericidal power.

3.4. *LmTrxh2*-tobacco plants displayed improved tolerance to diseases

Owing to the high level of *LmTrxh2* transcripts revealed in response to all the evaluated treatments in the present study, we investigated the potential of *LmTrxh2* on plant biotic stress tolerance in transgenic tobacco. Constitutive expression of *LmTrxh2* in transgenic tobacco lines was previously functionally linked to abiotic stress tolerance [37–39]. Two homozygous *LmTrxh2* transgenic lines (Tr1 and Tr2) have

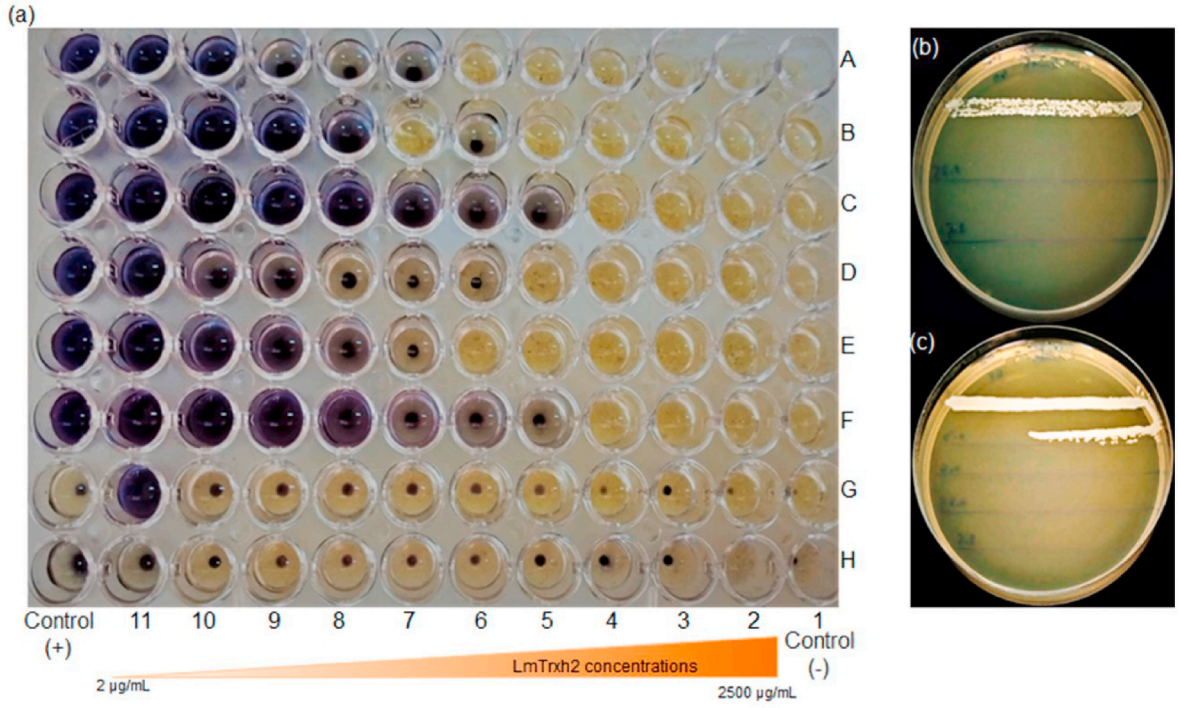

Fig. 4. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in LmTrxh2. (a) The determination of the MIC. From (A) to (H): the different bacterial strains. From (1) to (11): Negative control and decreased concentrations (2500 to 2 µg/mL). The determination of BMC determination against *Salmonella enterica* (b) and *Staphylococcus aureus* (c) by the streak method.

Table 2
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of LmTrxh2.

Bacterial Strains	CMI (µg/mL)	CMB (µg/mL)	CMB/CMI	Interpretation
Gram-positive				
B. cereus (ATCC 14579)	55 ± 0.06	55 ± 0.06	1	Bactericidal
S. aureus (ATCC 25923)	1250 ± 0.0	1250 ± 0.0	1	Bactericidal
E. faecalis (ATCC 29212)	154 ± 0.25	304 ± 0.41	2	Bactericidal
M. luteus (ATCC 1880)	1250 ± 0.0	1250 ± 0.00	1	Bactericidal
L. monocytogenes (ATCC 1911)	40 ± 0.04	40 ± 0.04	1	Bactericidal
Gram-negative				
P. aeruginosa (ATCC 9027)	40 ± 0.04	40 ± 0.04	1	Bactericidal
E. coli (ATCC 25922)	300 ± 0.0	300 ± 0.19	1	Bactericidal
S. enterica (ATCC 43972)	65 ± 0.04	65 ± 0.04	1	Bactericidal

moderate and high levels of LmTrxh2 protein, respectively [37]. To this end, fungal experiments were performed on detached leaves with the necrotrophic pathogens *Fusarium graminearum* and *Aspergillus niger*. Symptom development was assessed after 5 dpi, and the infected leaf area was analyzed. One-month-old transgenic plants Tr1, Tr2, and NT were infected with the two fungi. Fig. 5a and b shows that fungal necrosis was the highest in the detached leaves of the line NT. However, in the transgenic lines (Tr1 and Tr2), the disease progression was slower, and lesions were confined to a smaller area in the detached leaves compared to the plants of NT. Our results revealed that transgenic tobacco overexpressing *LmTrxh2* have enhanced basal resistance to virulent pathogens such as *Fusarium* and *Aspergillus*.

To have a better idea about the mechanism of action of *LmTrxh2* in response to biotic stress, we investigated the expression profiles of six biotic stress-responsive genes: *PR1a* (marker gene for SA signaling), *PR2* (marker gene for MeJA signaling), *NPR1* and *HIN1* (marker genes for plant defense), *LOX1* (marker for wound response), and *ACS6* in NT and transgenic tobacco lines by qPCR before and after 3 dpi *Fusarium* and *Aspergillus* infections. As shown in Fig. 6, the gene transcript profile showed no difference between NT and the two transgenic lines before infection. However, the expression levels of all related genes, except ACS6, which is involved in ethylene biosynthesis, were significantly higher in the transgenic plants than in the plants from the NT plants after 3 dpi of infection. Therefore, these results suggest that LmTrxh2 may have induced the activation of the expression of pathogenesis-related genes in transgenic tobacco through the downregulated expression of SA or JA signaling genes and the upregulation of genes involved in the JA signaling pathway, resulting in an enhanced resistance to phytopathogens, which could be related to the crosstalk of the SA, MeJA, and ET signaling pathways.

4. Discussion

Thioredoxin is a critical factor that has a key role in conferring host resistance and in the activation of various other plant regulators. Trx is an important regulatory component of plant defense responses and is crucial for the modulation of the redox status of the molecules involved in plant defense pathway [55]. Although its function is well studied in several plant species [56], both the main mechanism of *LmTrxh2* gene action and its role in the plant defense pathways against biotic stress are still ambiguous. In the present study, we aimed to study the possible role of *LmTrxh2* from *L. maritima* in stress tolerance. Our results suggest that the *LmTrxh2* gene is modulated by biotic stress stimulators and plays an important role in the regulation of signaling pathways that are implicated in the defense response and enhance the basal resistance. The novel role of LmTrxh2 that we discovered here deepens our understanding of plant responses to various environmental stresses. In our study, the level of *LmTrxh2* was upregulated toward biotic stress simulators such as wounding, pathogenic elicitors (PA), and signaling

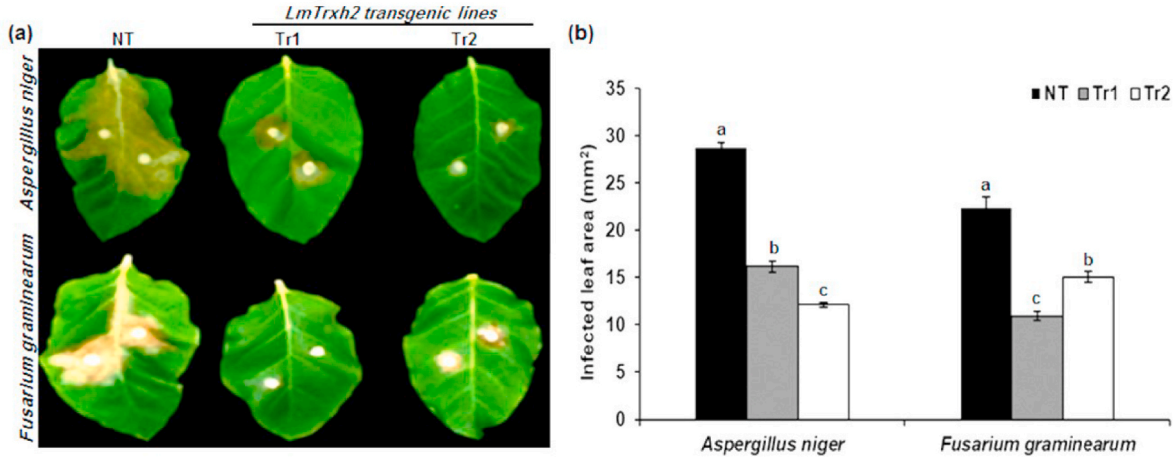

Fig. 5. Resistance of transgenic of tobacco leaves expressing LmTrxh2 gene. (a) Detached leaf from NT and LmTrxh2 transgenic tobacco plants after 5 dpi. (b) Determination of infected leaf area of detached NT and transgenic tobacco leaves. Values are means of three replicates of one expanded leaf per plant. Different letters indicate significant differences ($P < 0.05$).

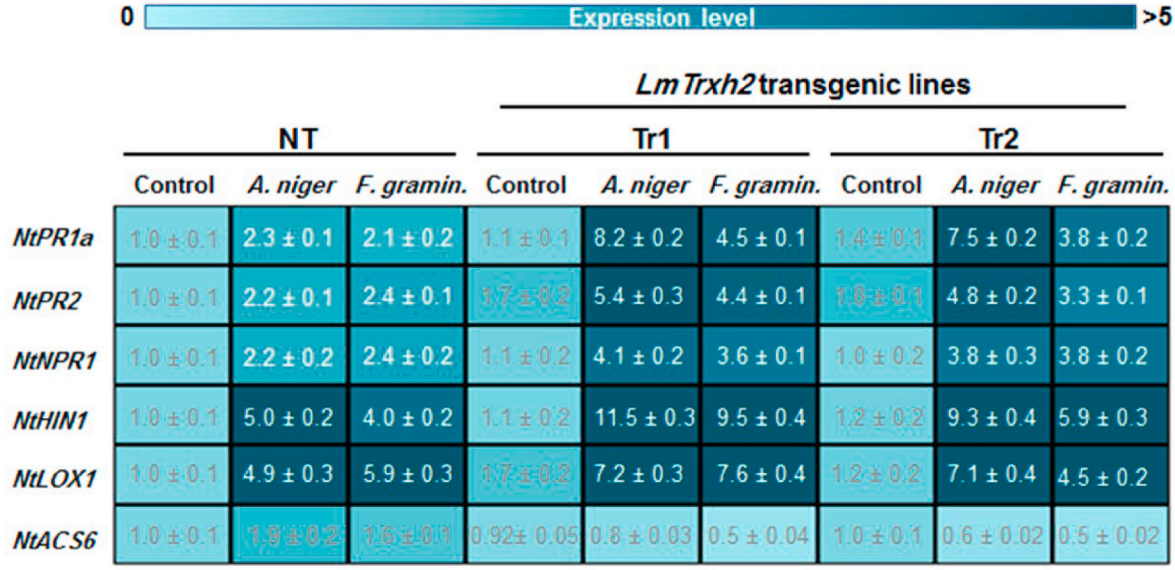

Fig. 6. Heatmap of defense-related genes (NtPR1a, NtPR2, NtHIN1, NtLOX1, NtACS6, and NtNPR1) expression profile in transgenic tobacco lines (Tr1 and Tr2) and NT plants before and after fungus infection. Values in each cell are mean ± SEM (n = 3).

molecules (JA and ETP). Most Trxs that are located in chloroplasts are implicated in the process of photosynthesis and in the response to stress and hormone signaling [57]. MeJA is an inducer of the antioxidant system in plants when there is a danger ROS production level [58]. Along with Trxs, MeJA may be very important in the regulation of ROS homeostasis during pathogen attack. Similar to previous findings, the *Trxh5* gene was strongly activated in response to pathogenic attacks as well as wounding, abscission, and ABA treatments [24,59]. Recent studies prove that the *TaTrxh1* gene expression is significantly induced in response to *P. striiformis f. sp. tritici* infection, whereas the overexpression of *TaTrxh1* leads to cell death [60].

Antimicrobials-resistant bacteria constitute an important global problem these days. This occurrence entails the necessity of increased costs in treatment, which is troublesome. Also, higher mortality and morbidity rates among the affected ones are reported, which is detrimental to the economy and health care system [61,62]. LmTrxh2 appears to be an effective bactericidal agent against the eight foodborne pathogenic strains tested, with very interesting characteristics that can be used in different areas of the resistant bacteria treatment. These findings were similar to those reported for *Triticum durum*-derived protein including annexin 12 (TdAnn12) and lipid-transfer protein (TdLTP4) [50,51]. The bactericidal and bacteriostatic effects were equally dependent on whether the strains tested were gram-negative or gram-positive, which is directly related to the differences in the architecture and molecular components of the peripheral wall [63]. In agreement with LmTrxh2 antibacterial activity, TdLTP4 and TdAnn12 show the highest inhibitory activity against gram-negative bacteria.

ROXY18/GRXS13 genes transcription was triggered by pathogens, including *Botrytis cinerea*, as previously shown [64]. *Trxh8* and *Trxh5* genes are upregulated in response to biotic and abiotic stresses [4]. Out of the eight members of the *Trxh-h* group, only *Trxh5* was upregulated under stress conditions [24,59,65]. Thus, thioredoxin *Trxh5* promoted the SA-induced gene expression dependently to the conformational state of *NPR1* (Non-Expresser of PR gene 1) [66]. NPR1 in the oligomeric form with disulfide cross-linking between conserved cysteine residues remains in the cytoplasm. Under the salicylic acid signaling response, the pathogen-inducible *Trxh*5 and *Trxh*3, which are constitutively expressed enzymes, help to reduce the disulfide bonds of NPR1. The monomeric

NPR1 activates the SA-inducible defense-related genes [65], whereas thioredoxin *Trxh*5 reduces the protein-SNO molecule of the S-nitrosylated NPR1 [28].

Additionally, the *LmTrxh2* transcript is highly induced by PA, a potent mammalian macrophage-mediated agent that induces tumor cell apoptosis [67] to protect plant cells from PA-induced cell death. It is very common that ETP- and PA-responsive *LmTrxh2* was highly expressed very early following treatment. This finding suggests that *LmTrxh2* has a putative role in improving plant resistance to fungal pathogens. The identification of the enzymatic activity of the recombinant LmTrxh2 protein has shown that it has a disulfide reductase function that acts by reducing intermolecular disulfide bonds [46,54]. Previous reports have shown that recombinant AtTrx-h5 protein has an inhibitory effect on the growth of conidia germination in 7 filamentous fungal cells and proliferation in 3 pathogenic yeast cells [68]. Song et al. [69] reported that the antioxidant response via *Trx-1* in *Salmonella* occurred independently of canonical thiol disulfide oxidoreductase enzyme activity and acted through the induction of intracellular SPI2 (*Salmonella* pathogenicity island 2) gene transcription independently of thiol-disulfide exchange. This gene transcription activation is crucial for the resistance of *Salmonella* to both ROS generated by NADPH phagocyte oxidase and oxygen-independent lysosomal host defense.

In addition, the overexpression of *LmTrxh2* in the transgenic lines showed an enhanced tolerance to the pathogenic fungi *Fusarium gramenarium* and *Aspergillus niger* compared to the NT lines. Other investigations have emphasized the implication of Trx proteins in the defense against biotic stress. Trx-h3 is very important in defending plants against oxidative stress [70,71]. Trx-h5 is implicated in the plant's defense response and the modulation of the *NPR1* gene structurally and functionally [65,72]. Recently, the induction of the *AtTrxh5* gene was reported to occur when plants are attacked by different viral infections [73]. In fact, the level of *AtTrxh5* is upregulated after pathogen attack, and its expression is linked to the generation of a hypersensitive reaction (HR) during an incompatible interaction. Nevertheless, the upregulation of *AtTrxh5* is not specific to a virulent strain since it is also observed following infection with a virulent pathogen at a later time point that corresponds to disease onset [74]. In fact, when overexpressed, several *Trx* genes improve tolerance to tobacco/-cucumber mosaic virus, e.g., *NtTrxh3* [75]. OsTrxm, a protein found in rice, was characterized as exhibiting antifungal action that can be generated by the substitution of amino acids at crucial locations [76].

Plant hormones, such as SA, JA, and ET, and their interplay are also very important in conferring resistance to biotrophic and necrotrophic pathogens [77]. Herein, we revealed that *LmTrxh2* enhanced the expression of the *NtPR1a* (Pathogenesis-related protein 1) and *NtNPR1* genes in transgenic tobacco lines, where *PR1a* is implicated in systemic resistance [78]. Similarly, *AtTrxh5* was found to enhance the SA-induced expression of immune genes by modulating the conformational structures of the SA-responsive co-activator *NPR1* [28]. Since SA and JA are known to function as key signaling molecules in the defense response of plants to pathogens and insects, it is possible that *LmTrxh2* acts by enhancing the expression of JAZ-encoding genes in tobacco (i.e., *LOX1*).

In addition, the downregulation of *ACS6*, an important gene for ET signal transduction, was recorded in the transgenic lines compared to the NT plants. The overexpression of *ACS2*, an enzyme of ET biosynthesis, in rice enhanced the tolerance to necrotrophic (*Rhizoctonia solani*) as well as biotrophic (*M. oryzae*) fungi [79]. It has been shown previously that ERF TF expression, which is involved in the biosynthesis of ET, increases the plant's tolerance to necrosis inducing fungi, such as *Botrytis cinerea* and *Plectosphaerella cucumerina* in transgenic *Arabidopsis* [80]. The interplay of the SA and JA/ET signaling pathways could confer resistance to *Fusarium gramenarium* and *Aspergillus niger*, with associated marker genes expressed at low or high levels in transgenic plants after infection.

The molecular mechanism of stress-related gene expression and signal transduction is very complex and is the result of a synergy among multiple genes and multiple pathways. Therefore, we hypothesized that *LmTrxh2* might be involved in a complex regulatory network that affects both the gene expression and stress tolerance in plants. In *Arabidopsis thaliana*, AtTrxs are pathogen-inducible and contribute to plant defense via the expression of defense responsive *PR* genes. In addition, the two reactive cysteine residues are found in the conserved motif of Trxs, which play post-translational regulatory roles in a number of cellular processes, such as oxidative stresses and plant pathogen interactions. In a recent *in silico* prediction study of the *LmTrxh2* structure conducted by Ben Saad et al. [37], it was reported that the catalytic Trx motif encompasses two catalytic cysteine residues (59 and 62). NPR1 is an important molecule that is SA-responsive and undergoes conformational changes upon oxidative modifications under conformational signaling, during the plant defense. When a pathogen attacks, the SA level increases, NPR1 disulfide bonds are quickly reduced, and NPR1 monomers are translocated to the nucleus, thereby activating SA-responsive genes of the defense response [66,81]. The NPR1 protein interacts with TGA transcription factors, and TGA1 and TGA4 contain conserved cysteine residues, which play a crucial role under oxidative stress generated by plant pathogen interactions. These findings allow us to conclude the correlation between redox-based cysteine modifications and help in the expression of genes involved in plant disease resistance.

5. Conclusions

In this study, we functionally analyzed the role of *L. maritima LmTrxh2* in plants under various biotic stresses. Our results suggest that *LmTrxh2* positively regulates the biotic stress response. The purified protein is effective when directly used against pathogens. This may have potential as a lead compound for the development of antimicrobial agrochemicals or a potent therapeutic agent. Additionally, the overexpression of *LmTrxh2* in transgenic tobacco increased the resistance to *Fusarium graminarium* and *Aspergillus niger* infections via the activation of *PR* genes and stress-responsive genes. Our results imply that *LmTrxh2* may enhance biotic stress tolerance in tobacco and could be exploited for engineering stress-tolerant crops.

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CRediT authorship contribution statement

Rania Ben Saad: Writing – original draft, Methodology, Investigation, Conceptualization. **Walid Ben Romdhane:** Writing – review & editing, Validation, Software, Formal analysis. **Wirginia Kukula-Koch:** Writing – review & editing, Validation. **Bouthaina Ben Akacha:** Writing – original draft, Investigation, Formal analysis, Data curation. **Narjes Baazaoui:** Writing – review & editing, Funding acquisition. **Mohamed Taieb Bouteraa:** Methodology, Data curation. **Yosra Chouaibi:** Data curation. **Anis Ben Hsouna:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Maisto:** Writing – review & editing, Validation. **Miroslava Kačániová:** Validation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2023.102206.

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