



A novel thaumatin-like protein from durum wheat, TdPR-5, is homologous to known plant allergens, responsive to stress exposure, and confers multiple-abiotic stress tolerances to transgenic yeast

Rania Djemal, Ons Bahloul, Habib Khoudi *

Laboratory of Plant Biotechnology and Improvement, University of Sfax, Center of Biotechnology of Sfax, Route Sidi Mansour, Km 6 B.P. 1177, 3018 Sfax, Tunisia

ARTICLE INFO

Editor: A Rooney

Keywords:

Thaumatococcus
Heavy metals
Abiotic stress
Durum wheat
Allergens

ABSTRACT

Thaumatococcus-like proteins (TLPs) are highly complex group of Pathogenesis-Related proteins (PRs) recognized as human allergens. They are known to rapidly accumulate in high levels following stresses. Several members of TLPs were isolated from different plant species, but few were described in durum wheat. In this study, a PR-5 member, *TdPR-5*, was isolated from a Tunisian variety of durum wheat. Phylogenetic analysis showed that *TdPR-5* protein shared high homology with TLPs isolated from several plant species. Allergenic assessment of *TdPR-5*, using Allermatch tool, revealed that *TdPR-5* had significant identity with TLPs, known as plant allergens. qRT-PCR analysis revealed that *TdPR-5* is robustly induced by drought, salt and heavy metals such as cadmium (Cd), copper (Cu) and zinc (Zn). Notably, *TdPR-5* induction is concomitant with the induction of the durum wheat transcription factor *TdSHN1* shown previously to control the expression of tobacco PR-5 gene when overexpressed in transgenic tobacco plants. Maximum fold-change in relative expression of *TdPR-5* and *TdSHN1* genes following stress treatments reached 6.5 times (mannitol 1 h) and 10 times (Cu 24 h), respectively. Heterologous expression of *TdPR-5* in transgenic yeast conferred resistance to multiple abiotic stresses. These results indicated that *TdPR-5* may play an important role in multiple abiotic stress tolerance in wheat. This study also provides valuable information regarding food safety. In fact, overexpression of strong transcription factors such as *TdSHN1* or exposure of plants to stressful conditions can lead to an increase in the quantity of allergens in crops thereby increasing the risk for consumers with known allergy or sensitization.

1. Introduction

During their life cycle, plants are constantly challenged by both biotic and abiotic stresses. These stresses represent a major impediment to crops production and can lead to up to 70% yield loss in the case of abiotic stress (Thakur et al., 2010). Exposure of plants to high levels of heavy metals leads to their toxic accumulation which decreases growth and reproduction of plants and threatens human health via food chain (Khoudi et al., 2012). Climate change is expected to exacerbate the situation. Due to their sessile nature, plants have elaborated many sophisticated mechanisms to withstand these adverse conditions. These mechanisms involve multi-gene networks whose action leads to significant changes in the expression level of thousands of genes among which chaperones, detoxification enzymes, various classes of proteases and PRs (Kayum et al., 2016).

PR-5 proteins belong to the class of PR protein family 5. These

proteins act as first line of the plant defensive system and are composed of diverse functional and structural antimicrobial proteins induced following pathogen attack (Hamamouch et al., 2011). Later, studies showed that PRs are also involved in many important functions such as abiotic stress tolerance, hormone signaling, programmed cell death, enzyme activity, seed germination and fruit maturation (Farrakh et al., 2018). Due to the fact that PR proteins respond to both biotic and abiotic stresses, they are identified also as stress proteins (Midoro-Horiuti et al., 2001). Based on their sequence similarities, PRs were divided into 17 families (van Loon et al., 2006). Due to evolutionary conservation of molecular processes governing defense reactions in plants, high structural and sequential similarities was revealed between PRs derived from various plant species (Liu et al., 2010). PR-5 proteins are known as thaumatococcus-like proteins (TLPs) due to their amino acid sequence similarity (40–70%) to thaumatococcus; a protein exhibiting a high sweetening capacity first discovered in *Thaumatococcus daniellii* (Eden et al., 1982).

* Corresponding author.

E-mail address: hkhoudi@hotmail.com (H. Khoudi).

<https://doi.org/10.1016/j.plgene.2022.100360>

Received 17 December 2021; Received in revised form 18 April 2022; Accepted 5 May 2022

Available online 10 May 2022

2352-4073/© 2022 Elsevier B.V. All rights reserved.

Based on their isoelectric point (pI), PR-5 proteins can also be divided into three groups: acidic PR-5, basic (osmotin) and neutral osmotin-like (OLPs) (van Loon and van Strien, 1999). In addition, according to their molecular mass, TLPs can be subdivided further into long (MM of 21–26 kDa) or small TLPs (MM of 15–18 kDa). Despite the fact that large TLPs were described in monocots, dicots and conifers, small TLPs were described particularly in monocot species (Fierens et al., 2007).

TLPs exhibit an original three dimensional structure characterized by the presence of three domains: domain I or central domain composed of two β -sandwich with 11 β -sheets surrounded by domain II consisting of 3 α -helical structure and two β -sheets and domain III composed of a junction loop and β -sheet (Liu et al., 2010; Barre et al., 2015). They are also characterized by the presence of 10 to 16 conserved cysteine residues involved in the formation of 5 to 8 intra-molecular disulfide bounds responsible of protein stability and its resistance to temperature changes and pH extremes (Petre et al., 2011; Barre et al., 2015). PR-5 proteins can have various subcellular localizations such as vacuole and intercellular space (Wang et al., 2013).

Increasing evidence suggests that several members of PRs are allergenic to humans (Ferreira et al., 2004). According to the International Union of Immunological Societies, one-fourth of known allergens are PR proteins (Hoffmann-Sommergruber, 2002). Based on their mode of entry into human body, PRs are classified into food and airborne allergens. Several PR-5 proteins are pollen allergens such as Jun a 3 from mountain cedar while others belong to the group of food allergens such as Pru av 2 from cherries or Cap a 1 from bell pepper and Mal d 2 from apple (Fuchs et al., 2006; Anliker et al., 2002; Krebitz et al., 2003). In wheat, a thaumatin-like protein was associated to baker's respiratory syndrome (Salcedo et al., 2011). PRs are also involved in cross reactivity among allergens derived from different plants.

Structural features present in allergens determine the allergenicity of a given protein. These features are known as allergenic determinants or epitopes and are involved in the binding to B and T cells (Pomés, 2010). The fact that allergens from different species share similar structural features allowed their identification by antibodies which leads to IgE cross reactivity. In addition, the advent of powerful computational methods that make use of machine learning, sequence alignment and epitope mapping techniques makes it now possible to analyze, *in silico*, protein allergenicity and identify epitopes (Jamakhani et al., 2018).

Due to their importance in plant defense, brewery industry, and their allergenic proprieties, PR-5 proteins have long been the subject of intensive research in many laboratories worldwide. For instance, many PR-5 genes were isolated from different species such as *Prunus domestica* (El-Kereamy et al., 2011), *Brassica rapa* (Ahmed et al., 2013), *Zea mays* (Liu et al., 2011), *Triticum aestivum* (Li et al., 2015) and *Ocimum basilicum* (Misram et al., 2016). The overexpression of TLPs in transgenic plants conferred disease resistance such as resistance to downy mildew as well as to several phytopathogenic fungi (Misram et al., 2016). In addition, they can confer resistance to abiotic stress. In fact, overexpression of Arabidopsis PR-5 gene improved drought tolerance (Liu et al., 2013).

PRs are induced not only following biotic stresses but also by a wide range of abiotic stressors. The link between PRs and abiotic stress was first established by Singh et al. (1989) who reported high levels of PR-5 proteins in young tobacco leaves subjected to salt stress. Both osmotin and OLPs accumulate following abiotic stress where they mediate compartmentation of ions and solutes. In Arabidopsis, PR-5 was shown to be induced by Cd stress (Semane et al., 2010). The expression of PR-5 genes, following abiotic stress, is mediated by transcription factors such as members of the ethylene response transcription factors (AP2/ERF). In fact, *ERF1* and *JERF3*, which belong to the ERF family of transcription factors, are involved in stress response and modulate the expression of PRs (Tsutsui et al., 2009). In addition, we have recently demonstrated that overexpression of *TdSHN1* (an AP2/ERF transcription factor of durum wheat) in transgenic tobacco, upregulates the expression of the tobacco PR-5 gene (Djemal and Khoudi, 2016; Djemal and Khoudi,

2019).

Durum wheat (*Triticum turgidum* ssp. *durum*) is one of the most economically important cereal crops and is widely cultivated in hot and dry regions such as the South Europe and North Africa. In the Mediterranean region, durum wheat is consumed as semolina and pasta. In Tunisia, durum wheat is one of the most cultivated cereals (60%) (Ayed-Slama et al., 2018). Although PR-5 genes were isolated and characterized from different plant species, no homologous genes were described in durum wheat. In this study, we isolated and characterized a novel PR-5 gene member from a Tunisian durum wheat variety. We also evaluated, *in silico*, its potential allergenicity and determined its expression profile following several abiotic stresses including salt, drought and heavy metals. Moreover, we evaluated its effect on abiotic stress tolerance in transgenic yeast.

2. Materials and methods

2.1. Plant material and stress treatments

One durum wheat (*Triticum turgidum* ssp. *durum*) variety (Oum Rabia), provided by the Tunisian Agronomic Institute, was used in the present study. This variety is widely cultivated in Tunisia and is considered as one of the most productive varieties. Seeds were surface sterilized using commercial bleach for 15 min followed by several washes with sterile water. Thereafter, seeds were germinated on wet Whatman filter sheets placed in petri dishes for 10 days. To isolate wheat PR-5 gene sequences, germinated seedlings were used for genomic DNA extraction. To determine the expression profile of durum wheat PR-5 gene following abiotic stress, germinated seedlings were subjected to salt stress (200 mM) and mannitol (300 mM) as well as to heavy metals: CuSO₄ (8 mM), ZnSO₄ (8 mM) and CdCl₂ (100 μ M) and used for RNA isolation.

2.2. Nucleic acids isolation

Wheat seedlings, prepared as mentioned above, were used for genomic DNA and RNA isolations. For DNA isolation, 200 mg of leaf tissue were grounded in liquid Nitrogen to obtain fine powder. The latter, was suspended in 1 ml of extraction buffer (1 M Tris-HCl pH: 8, 5 M; NaCl, 0.5 M; EDTA pH: 8 and 10% SDS) and the mixture was incubated at 65 °C for 1 h. Thereafter, 1 ml of chloroform/isoamyl alcohol (24/1) was added and samples were subjected to agitation for 30 min. Samples were then centrifuged for 30 min at 3000 rpm and 1 ml of cold absolute ethanol was added to the supernatant. The mixture was incubated at –20 for 30 min and the precipitated DNA was recovered using sterile pipette tips and washed twice using cold ethanol (70%) and resuspended in sterile water.

Total RNA isolation was performed following the Trizol method (Invitrogen) and using 200 mg of leaf material. To prevent DNA contamination, total RNA was treated with RNAase-free DNase and used for cDNA synthesis. Reverse transcription was performed for 1 h at 37 °C using MML-V reverse transcriptase (Invitrogen) and oligo-dT following manufacturer's instructions.

2.3. Primers design and amplifications of PR-5 sequences in durum wheat

To design PCR primers for PR-5 gene amplifications using genomic DNA, homologous protein and nucleotide sequences derived from bread wheat (*T. aestivum*), rice (*oryza sativa*), barley (*Hordeum vulgare*) and durum wheat (*T. turgidum*) were retrieved from GeneBank of NCBI. First, a protein sequence alignment was performed between the four PR-5 proteins: rice PR-5 (accession: EU267986.1), bread wheat (accession: KJ764822.1), barley (accession: NIAShv1025J11) and durum wheat (accession: VAI81953.1) and conserved regions were identified. A second alignment between nucleotide sequences of the proteins of bread wheat, durum wheat and barley was performed to identify conserved

regions at nucleotide sequence level. Conserved regions at the N-terminal and C-terminal and having 18 or more nucleotides of size, were selected and used for primers design. The designed primers were designated: TP-FW and TP-REV (Supplementary Table S1). PCR amplifications were performed using Oum Rabia genomic DNA as template and the following PCR conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min. An extension period of 10 min at 72 °C was also used at the end of these cycles. PCR fragments of expected size were purified from agarose gel using commercial purification kit and cloned in pGEM-T easy vector. Positive clones were identified using blue/white selection and enzymatic digestion and clones with the expected sizes were sequenced using ABI prism automated sequencer.

2.4. Quantitative RT-PCR analysis

To determine the expression profiles of *PR-5* and *TdSHN1* genes in durum wheat, a quantitative RT-PCR was performed using reverse transcribed RNA derived from seedlings exposed to different treatments as described above. Samples were subjected to amplifications using CYBR Green fluorescent dye following the manufacturer recommendations. qRT-PCR analysis was performed using the 96-well plate with the CFX 96 Touch TM Real-Time PCR System. Amplification reactions consisted of a total volume of 10 µl, containing 5 µl 2 × SYBR @ Select Master Mix, 0.5 µl of each primer (10 µM), 1 µl of RNase-free water and 3 µl cDNA. Amplification conditions were as following: 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. Samples were amplified in triplicate and the $2^{-\Delta\Delta CT}$ method was used for data analysis. The *Actin* gene of wheat (accession: AY663392.1) was used as reference gene for normalization. The primer sequences used in this study are: TP-FW and TP-REV, *TdSHN1* specific primers: SHN1-FW and SHN-REV and the wheat *Actin* primers wheat actin FW and wheat actin RV (Supplementary Table S1).

2.5. Expression of *TdPR-5* gene in yeast and determination of abiotic stress tolerance

The confirmed *PR-5* gene sequence was cut from pGEM-T easy vector using *EcoRI* restriction enzyme and cloned in the same site in yeast expression vector pYES2 downstream of the GAL promoter. The obtained recombinant vector was transformed in yeast strain W303 using lithium acetate method (Elble, 1992). Recombinant clones were identified by colony-PCR and one clone harboring *PR-5* gene in the correct orientation, relative to the GAL promoter, was used for the following experiments. To assess the effect of *PR-5* gene on abiotic stress tolerance in yeast, two methods were used: solid and liquid culture. For solid culture, yeast cells harboring pYES2-*TdPR-5* and pYES2 empty vector were inoculated into Sc-Ura liquid medium supplemented with 2% glucose and incubated O/N at 30 °C with shaking. The following day, optical densities (OD) at 600 nm of the cultures were measured and adjusted to OD of 0.4 in 10 ml Sc-Ura liquid medium supplemented with 2% galactose and incubated for 36 h at 30 °C to induce expression of *TdPR-5* gene. Thereafter, the OD of the induced cultures were measured and adjusted to 1 into 200 µl solutions of Sc-Ura supplemented with 5 M NaCl, 30% PEG or 20 Mm H₂O₂ to simulate salt, osmotic and oxidative stresses, respectively and incubated for 36 h at 30 °C. For cold stress, yeast cells were re-suspended in Sc-Ura medium and incubated at −20 °C for 24 h in ethanol bath. Control culture without stress was set using equal number of cells as used for stress culture but the cells were cultured in 200 µl of sterile water and incubated for 36 h at 30 °C. At the end of stress treatments, cells were diluted 1, 10, 100 and 1000 times and 2 µl aliquots from each dilution were spotted on Sc-Ura solid medium supplemented with 2% glucose and incubated at 30 °C for 72 h. For heavy metal stress, liquid culture was used. For this, a pre-culture was prepared in liquid medium YNB-glucose using single colony of recombinant yeast and maintained O/N at 30 °C with agitation.

Thereafter, these pre-cultures were used to inoculate YNB medium supplemented with 2% galactose and kept O/N at 30 °C to induce expression of *TdPR-5* gene. The OD of cells was determined and adjusted to 0.2 with YNB-galactose supplemented with 100 µM CdCl₂, 10 mM ZnSO₄ or 100 µM CuSO₄. Cultures (three cultures per clone and per treatment) were incubated for 24 h at 30 °C and their OD was measured.

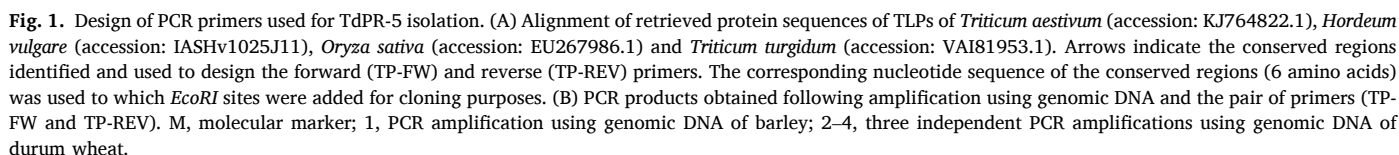
2.6. Bioinformatics analysis

Blast tool to search against non-redundant gene (nr) and protein database available at NCBI (www.ncbi.nlm.nih.gov) was used to identify putative *PR-5* gene. The identification of ORF and amino acids sequence was performed using ORF finder available at (<https://www.ncbi.nlm.nih.gov/orffinder/>). Multiple DNA and protein sequence alignment were performed using program ClustalW available from (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and Parline sequence alignment for primers design. Phylogenetic tree was obtained using neighbor joining method algorithm available in MEGA-X software. The three-dimensional structure of the *TdPR-5* protein was predicted using the SWISS-MODEL available at (<http://swissmodel.expasy.org>). Molecular weight and isoelectric point (pI) were predicted using expasy software available at (http://web.expasy.org/compute_pi/). *TdPR-5* protein subcellular location was predicted using Softberry, available at (<http://www.softberry.com/berry.phtml>). To search for glycosylation and phosphorylation sites, NetNglyc and netphos-3.1b available, respectively, at NetPhos (<http://www.cbs.dtu.dk/services/NetPhos/>) and (<http://www.cbs.dtu.dk/cgi-bin/>) were used. Transmembrane analysis was performed using TMHMM bioinformatics software available at (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). To predict the presence of signal peptide, SignalP-5.0 software available at (<http://www.cbs.dtu.dk/services/SignalP/>) was used. To check *TdPR-5* allergenicity, allermatch tool available at (<http://www.allermatch.org/database.html>) was used and BepiPred software available at (<http://www.cbs.dtu.dk/services/BepiPred/>) for epitope prediction.

3. Results

3.1. Isolation of genomic sequence of *TdPR-5* gene

In order to isolate the genomic sequence of durum wheat *PR-5* gene, we performed protein sequence alignment between *PR-5* proteins of bread wheat, durum wheat, rice and barley. This alignment revealed the presence of an extensive conservation of amino acid sequences between the three proteins of bread wheat, durum wheat and barley (Fig. 1A). The conservation of sequence at amino acids level was also maintained at the nucleotide sequence level (data not shown). Two conserved regions at N- and C-terminal regions, having a size of at least 18 nucleotides, were chosen to design the two primers: TP-FW and TP-REV (Fig. 1A). These primers were used in PCR amplifications of genomic DNA isolated from Tunisian durum wheat variety Oum Rabia. A fragment of the expected size (520 pb) (Fig. 1B) was successfully obtained, purified and cloned in pGEM-T easy vector. Five positive clones were selected and sequence analysis revealed that they were identical. The search for homologous sequences at GeneBank of NCBI revealed that durum wheat *PR-5* protein, isolated in this study, has indeed a TLP signature (Fig. 2A) and is highly homologous to the thaumatin-like *PR-5* sequences from other species with highest identity of 100% with *T. aestivum* thaumatin-like protein Ta-TLP (accession: AF384146.1), 99% to *Triticum urartu* hypothetical protein TRIUR3_28396 (accession: EMS68875.1), 96% to *H. vulgare* pathogenesis-related protein 1C (accession: P32938.1), 95% to thaumatin-like protein PWIR2 of *Aegilops tauschii* (accession: XP_02174573.1), 68% to *Z. mays* thaumatin-like protein precursor (accession: ACG34013.1), 65% to *Oryza sativa* thaumatin-like protein (accession: ACA50508.1) and 70% to *Brachypodium distachyon* thaumatin-like protein (accession: XP_010234031.1). The durum wheat *PR-5* gene was designated, here, as *TdPR-5*. It



Signal 5.0 online software revealed that TdPR-5 has a signal peptide of 20 aa in length (Fig. 3). The presence of signal peptide suggests that TdPR-5 protein is a secretory protein localized to the extracellular space. Indeed predicted cellular localization of TdPR-5, using softberry, revealed that it is predominantly extracellular. This prediction was also supported by the results of the analysis performed using TMHMM bioinformatics software (Fig. 4). TdPR-5 was also analyzed using NetNglyc and netphos-3.1b softwares. These analyses revealed the absence of glycosylation site(s) in TdPR-5 but the presence of several phosphorylation sites such as FYDISVIDG and DFSCSTGDA.

It is well known that PR proteins can act as airborne or ingestant food

The expression profiles of *TdPR-5* and *TdSHN1* transcription factor genes were determined in leaves derived from seedlings of the original variety Oum Rabiaa (from which both genes were isolated) and subjected to salt and mannitol stresses. As illustrated in Fig. 6A, the expression of *TdPR-5* and *TdSHN1* were modulated by both stresses. In fact, compared to control condition, the level of *TdPR-5* transcript was strongly induced after 1 h of both salt (4.4-fold upregulation) and mannitol treatments (6.5-fold upregulation) (Fig. 6A and Table 2).

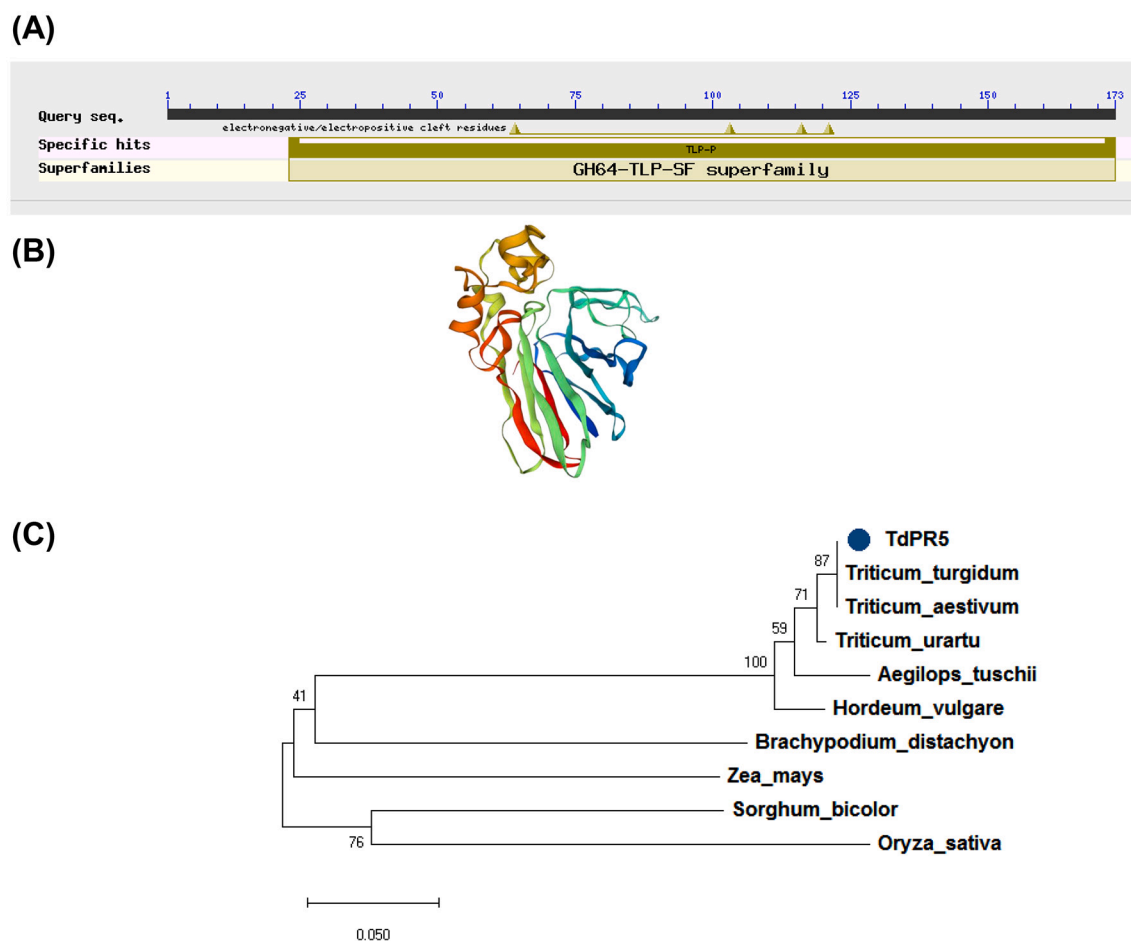


Fig. 2. Analysis of TdPR-5 protein. (A) The conserved functional catalytic domain GH64-TLP-SF (glycoside hydrolase family 64 and TLP superfamily) of TdPR-5 revealed by BLASTP search of database using TdPR-5 as query. (B) 3D model of TdPR-5 produced using protein structure homology-modeling server (SWISS-MODEL). Shown are α -helix and β -strands structures organized into the three characteristic domains of thaumatin-like proteins (C) Neighbor-joining phylogenetic tree of TdPR-5 with selected TLPs from different cereals: *Triticum aestivum* (accession: AF384146.1), *Triticum urartu* (accession: EMS68875.1), *Hordeum vulgare* (accession: P32938.1), *Aegilops tuschii* (accession: XP_02174573.1), *Zea mays* (accession: ACG34013.1), *Oryza sativa* (accession: ACA50508.1), *Brachypodium distachyon* (accession: XP_010234031.1), *Sorghum bicolor* (accession: XP_002443621.1) and *Triticum turgidum* (accession: VA181953.1). The tree was built by the maximum likelihood method with 1000 bootstraps values using MEGAX software. TdPR-5 is indicated by blue circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thereafter, the expression level decreased after 6 h and increased again after 24 h of stress (Fig. 6A). In the case of *TdSHN1* transcription factor, the maximum fold-change in relative expression was achieved at 1 h following both salt and mannitol treatments (6-fold and 5-fold upregulation, respectively) (Table 2 and Supplementary Fig. 1A). Thereafter, *TdSHN1* expression decreased at 6 h and 24 h for both salt and mannitol treatments (Supplementary Fig. 1A).

3.4. Expression of TdPR-5 and TdSHN1 transcription factor genes under heavy metal stress

Heavy metals are among the most important abiotic stresses of the plant kingdom and are the cause of yield reduction and health problems to humans. Thus, the expression of *TdPR-5* in the leaves of *Oum Rabiaa* following heavy metal stress was determined. At 1 h, the expression of *TdPR-5* was strongly induced (4-fold upregulation) following ZnSO_4 treatment (Fig. 6B). At 6 h treatment, the expression of *TdPR-5* was strongly induced following CuSO_4 treatment while it decreased, compared to 1 h, in the case of ZnSO_4 treatment and was not significantly different from the control in the case of CdCl_2 treatment (Fig. 6B). After 24 h, the expression of *TdPR-5* continued its increase and reached 5-fold upregulation in the case of CuSO_4 treatment. In the case of CdCl_2 treatment, *TdPR-5* expression was significantly higher than control after

24 h treatment (2.5-fold upregulation compared to control) (Fig. 6B). The expression of *TdSHN1* transcription factor was also modulated by different heavy metals. The induction of expression of *TdSHN1* was highest under the following treatments: CdCl_2 (24 h, 7-fold upregulation), CuSO_4 (24 h, 10-fold upregulation) and ZnSO_4 (1 h, 4-fold upregulation) (Table 2).

3.5. Expression of TdPR-5 in yeast cells imparts multiple abiotic stress tolerances

Yeast eukaryotic system is a rapid and easy to use model to gain some insights into heterologous gene function. In this study, we used this system to evaluate the function of *TdPR-5* gene in abiotic stress tolerance. For this, *TdPR-5* gene was put under the control of the galactose-inducible GAL1 promoter and transferred into yeast strain W303. Under control conditions, yeast cells harboring either the empty vector pYES2 or recombinant pYES2-*TdPR-5* showed no obvious differences in growth (data not shown). However, the growth of transgenic yeast harboring pYES2-*TdPR-5* plasmid was visibly better than that of yeast cells harboring the empty vector following salt, PEG, cold and hydrogen peroxide treatments (Fig. 7A).

Liquid culture method was also used for the evaluation of heavy metal stress tolerance conferred by the expression of *TdPR-5* gene in

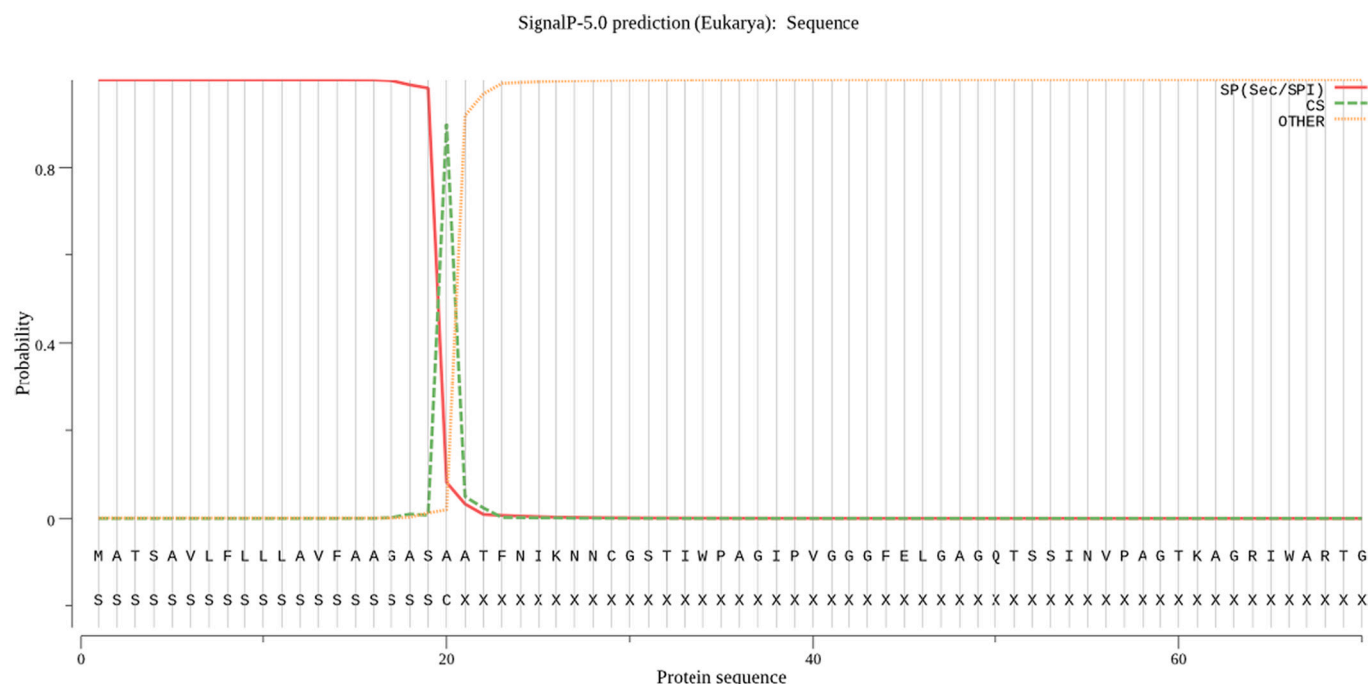


Fig. 3. Signal peptide prediction of TdPR-5 using free online SignalP-5.0 prediction tool. SignalP-5.0 predicts the presence of signal peptides and the location of their cleavage sites in proteins. The results for TdPR-5 are as follows: Signal peptide likelihood was around 0.999. Predicted location of peptide cleavage site (probability 0.8987) was between position 20 and 21. SP (Sec/SPI): type of signal peptide predicted; CS: the cleavage site; other: the probability that the sequence does not have any kind of signal peptide.

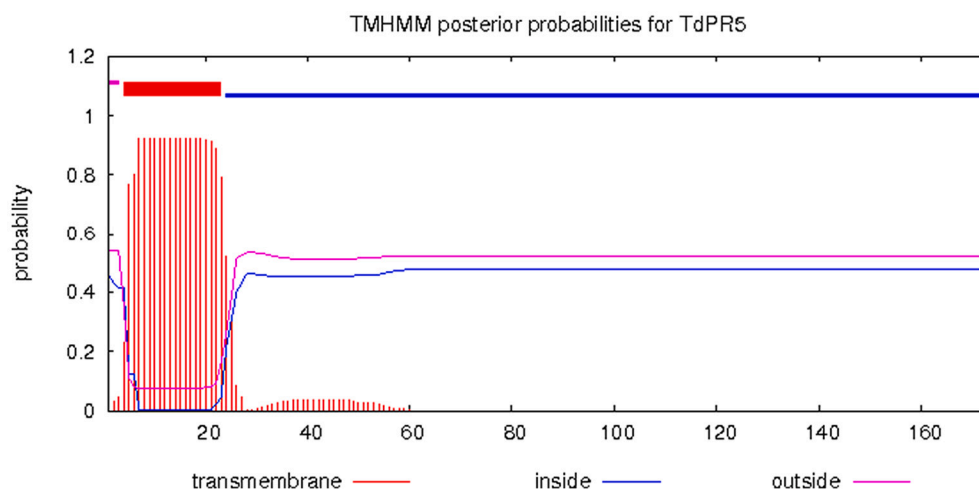


Fig. 4. Predicted transmembrane topology of TdPR-5. TMHMM predicts transmembrane helices in proteins. The full-length protein sequence of TdPR-5 was subjected to prediction by the free online TMHMM server v2. TdPR-5 has one predicted TMH with expected number of amino acids of 19.16618. According to the online guide this number is larger than 18 which indicate that TdPR-5 is likely transmembrane protein or harbors a signal peptide. The expected number in the transmembrane helices in the first 60 amino acids of TdPR-5 is 19.16431 which indicate that the predicted TMH in TdPR-5 is a signal peptide.

transgenic yeast. Under control conditions, growth of transgenic yeast was slightly higher than that of the empty vector (OD: 1.2-fold higher than the empty vector) (Fig. 7B). The growth of transgenic yeast harboring *TdPR-5* gene was significantly better than that of yeast harboring empty vector when subjected to heavy metal treatments. In fact, the growth of *TdPR-5* transgenic yeast was more important than that of the empty vector: CuSO_4 (OD: 2.3-fold higher than the empty vector), ZnSO_4 (OD: 2.4-fold higher than the empty vector) and CdCl_2 (OD: 1.8-fold higher than the empty vector) (Fig. 7B).

4. Discussion

TLPs belong to a large, very complex gene family which plays important roles in plant defense as well as in several developmental processes (Liu et al., 2010). Members of this family are known to

respond to different biotic and abiotic stresses. The overexpression of TLPs in transgenic plants imparts both biotic and abiotic stress tolerances (Liu et al., 2013; Singh et al., 2013; Subramanyam et al., 2012). Importantly, several TLPs are allergenic proteins which can represent a serious concern to human health. Despite their importance, little is known about TLPs of durum wheat; an economically important crop in the Mediterranean region and in many regions in the world (Zaim et al., 2017). In this study, we isolated and characterized a TLP gene from a Tunisian durum wheat variety and evaluated its involvement in abiotic stress response and its allergenic potential.

To isolate and characterise TLP-encoding genes from different species, researchers used one of the following simple strategies: 1) design of primer pairs based on the conserved motifs existing in DNA/RNA sequences and 2) the use of public databases containing genomic or transcriptomic sequences. In this study, we followed the strategy 1 and

Table 1

Allergic evaluation of TdPR5 protein using Allermatch tool.

I. Identity of the best hit (percent identical amino acids in the aligned 80-amino-acid sliding window).

II. The number of hits the input sequence had with this allergen.

III. The percentage of windows analyzed for this input sequence hitting this allergen.

IV. Results of a fasta alignment of the complete input sequence against this database sequence.

The first number is the percentage of identity. The second number is the length of sequence over which fasta aligned.

Protein ID	Species name	I	II	III	IV
P27357	<i>Triticum aestivum</i> (bread wheat)	97.5	94	100	95.4/153
Q69CS6	<i>Jinuperus rigida</i> (needle juniper)	63	92	97.8	46.8/154
P81295	<i>Jinuperus ashei</i> (white cedar)	63	90	95.7	41.8/201
Q69CS3	<i>Cupressis sempervirens</i> (cypress)	63	87	92.5	41.8/201
Q9AR60	<i>Capsicum annuum</i> (bell pepper)	60	83	88.3	40.5/205
P8333	<i>Prunus persica</i> (peach)	53.7	92	97.8	42.6/155
E3SU11	<i>Olea europaea</i> (olive)	53.1	94	100	45.4/152
I6SHZ9	<i>Malus domestica</i> (apple)	50	71	75.5	40.0/125

took advantage of the extensive sequence homology of TLP proteins between diverse cereal species to design primers which allowed the isolation of a durum wheat TLP member; *TdPR-5*. Amino acid sequence alignment between four TLPs from cereal species: rice, bread wheat, durum wheat and barley, revealed conserved regions at N and C terminals (Fig. 1A). This conservation was also maintained at nucleotide

sequence level. The designed primers targeting the conserved regions allowed the successful isolation of *TdPR-5* gene using genomic DNA of Tunisian durum wheat variety. *TdPR-5* represents the first TLP gene identified in durum wheat so far and is 100% identical, at protein level, to *Ta-TLP* gene from *T. aestivum*. However, when nucleotides sequences of *TdPR-5* and *Ta-TLP* genes were compared, a single nucleotide difference was detected at position 516 bp: C in *TdPR-5* instead of T in *Ta-TLP* gene sequence. This difference did not change the amino acid sequence (cysteine in both cases). Phylogenetic analysis revealed that *TdPR-5* grouped with TLPs of cereal origin (Fig. 2C). TLPs are ubiquitous in several genomes ranging from mosses to cereals, which indicate their complex evolutionary path (Paterson et al., 2009).

PR-5 proteins are grouped into small and long TLPs based on their molecular weight. Long TLPs have molecular weights between 22 and 26 kDa and small TLPs have molecular weights between 15 and 18 kDa. Since *TdPR-5* protein has a molecular weight of 17.58 kDa, it can be classified as small TLP. *TdPR-5* shares similar structural features and ultracellular localization as previously reported TLPs (Singh et al., 2013). In fact, *TdPR-5* contains a predicted N-terminal signal peptide which suggests that *TdPR-5* is a secreted protein (Fig. 3). In addition, *TdPR-5* predicted ultracellular localization is extracellular which is consistent with the presence of a signal peptide (Fig. 3). Due to the fact that most of secreted proteins contain glycosylation site(s), it is surprising that *TdPR-5* lacks this site. However, it contains several serine phosphorylation sites which may have functional relevance.

Due to the fact that *TdPR-5* is a member of PR protein family and that

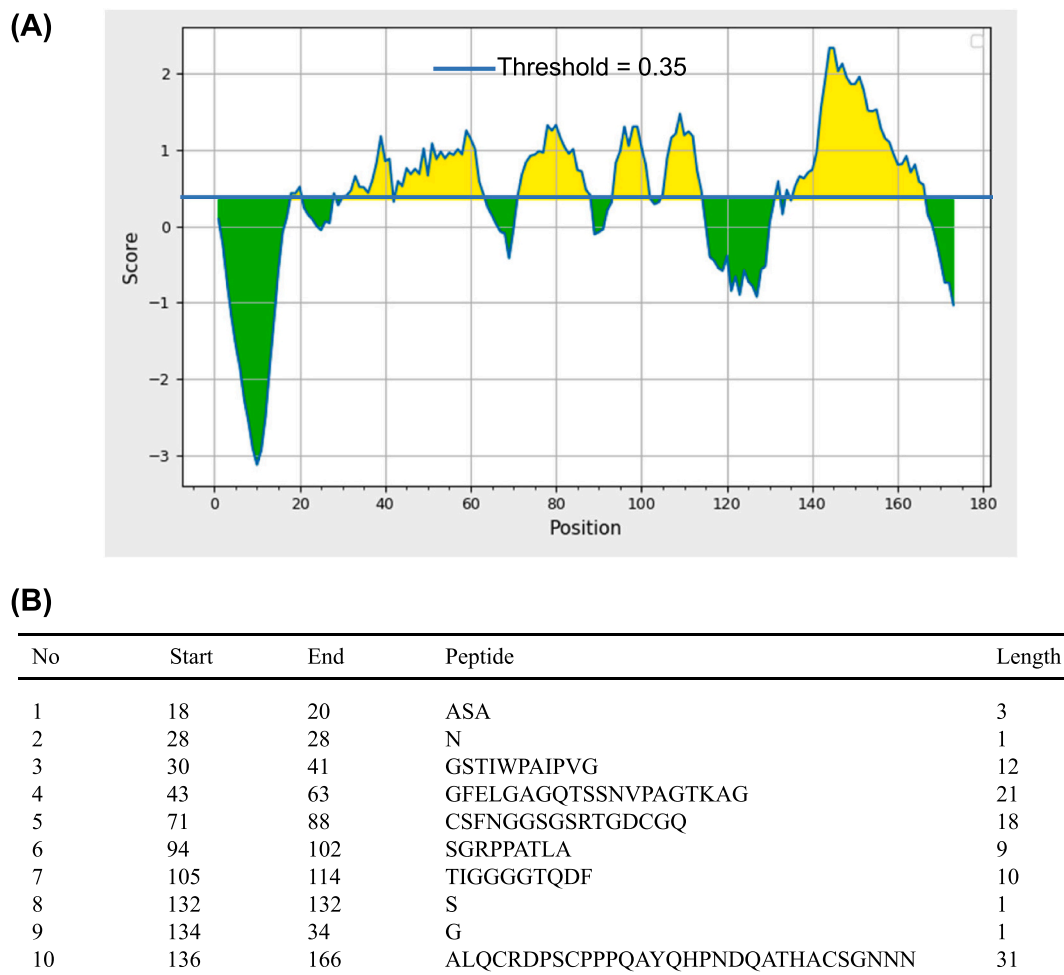


Fig. 5. Prediction of B-cell linear epitopes in *TdPR-5* protein using BepiPred web server. (A) Yellow picks represent predicted epitope residue positions. (B) Table listing positions and sizes of 10 predicted linear epitopes in *TdPR-5* protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

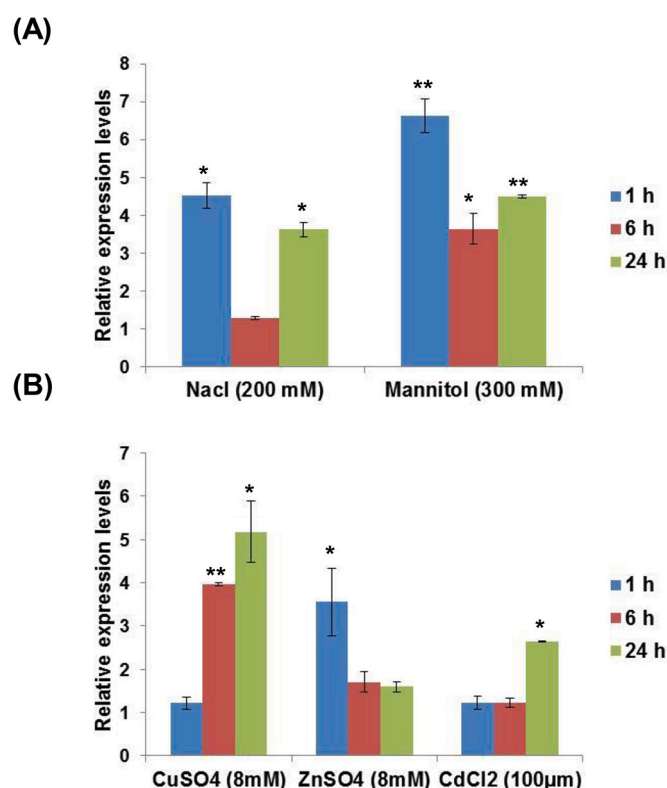


Fig. 6. Expression profile analysis of *TdPR-5* gene under different abiotic stresses. Seeds of durum wheat variety Oum Rabiaa were germinated on wet Whatman filter sheets placed in petri dishes for 10 days. Thereafter, germinated seedlings were subjected to salt stress (200 mM) and mannitol (300 mM) as well as to heavy metals: CuSO₄ (8 mM), ZnSO₄ (8 mM) and CdCl₂ (100 μM) for 1 h, 6 h and 24 h. (A) Expression profile following salt and mannitol treatments. (B) Expression profile following heavy metal treatments. The wheat *Actin* gene (accession: AY663392.1) was used here as internal control. Data represent the mean and SD. *, ** denotes statistically different at ($p < 0.01$), following Student's *t*-test.

Table 2

Maximum fold-change in relative expression value of *TdPR5* and *TdSHN1* transcription factor genes following different stress treatments.

Gene	Stress type				
	Salt (1 h)	Mannitol (1 h)	Cd (24 h)	Cu (24 h)	Zn (1 h)
<i>TdPR5</i>	4.4 x	6.5 x	5 x	5.8 x	3.3 x
<i>TdSHN1</i>	6 x	5.5 x	7 x	10 x	4 x

many members of this family are strong allergens capable of triggering IgE-mediated immunological responses in humans, it is important to explore the allergenic proprieties of *TdPR-5* protein. In addition, regulatory agencies require allergenicity evaluation of expressed proteins to guarantee full safety to the consumer. The structural conservation of PR proteins across the plant kingdom can cause serological cross-reactivity with other PR proteins involved in respiratory, contact and ingestant food allergy (Hauser et al., 2010). This can lead, in the most dramatic scenarios, to life-threatening anaphylactic shock. To address this concern, we used an *in silico* approach to assess the allergenic potential of *TdPR-5* protein. Results revealed that *TdPR-5* has indeed potential allergenicity like many other homologous proteins, in particular that of bread wheat with which it shares 94.5% identity (Table 1 and Fig. 5). Recently, Izumi et al. (2021) reported that the thaumatin-like protein of cherry (Pru av 2) was able to trigger percutaneous sensitization in BALB/c mice. Based on these studies, it is of paramount importance to study the accumulation of human allergenic proteins such as *TdPR-5*

protein in crops.

PR-5 proteins are very important and required for the establishment of the plant defense against both biotic and abiotic stresses. As demonstrated here, *TdPR-5* gene expression was strongly induced by different abiotic stresses including heavy metals (Fig. 6B and Table 2). Wheat is one of cereal crops that fulfill the major food requirement worldwide. In Tunisia, for example, durum wheat is one of the most economically important crops and is also the most cultivated cereal (60%). Previous studies have established that wheat-derived products are the major source of heavy metal intake in humans particularly Cd. In addition, wheat accumulates more Cd than other cereals (Rizwan et al., 2012). In the present study, we showed that exposure of durum wheat to heavy metal robustly induces the expression of *TdPR-5* gene. Our results are in line with previous reports which indicated that the levels of PR-5 proteins increase following plant exposure to heavy metals such as mercury, lead, Cu and Cd (Didierjean et al., 1996; Walliwalagedara et al., 2010; Semane et al., 2010). Leng et al. (2015) reported that the expression of thirteen PR-5 genes of grapevine was upregulated following exposure to Cu.

Members of the AP2/ERF transcription factor family are known to play an important role in the expression of PR proteins (Tsutsui et al., 2009). PR-encoding genes contain GCC box in their promoter regions which allows the AP2/ERF transcription factors to bind and modulate their expression. We have previously shown that *TdSHN1* transcription factor binds to GCC box (Djemal and Khoudi, 2015). Interestingly, overexpression of *TdSHN1*, in transgenic tobacco, induced the expression of the endogenous tobacco PR-5 gene (Djemal and Khoudi, 2019). In the present study, the expression of *TdPR-5* gene seems to be correlated to the expression of *TdSHN1* transcription factor. In fact, as shown in Table 2 and supplementary Fig. 1, all the stress treatments led to a robust induction of both *TdPR-5* and *TdSHN1* genes.

Yeast expression system is commonly used to identify plant genes involved in abiotic stresses. In the present study, the heterologous expression of *TdPR-5* gene led to significant resistance of recombinant yeast to multiple abiotic stresses namely, salt, osmotic and oxidative stresses (Fig. 7A). Interestingly, transgenic yeast harboring *TdPR-5* gene was also found to be resistant to Cd, Cu and Zn (Fig. 7B). Many plant genes were found to confer abiotic stress tolerance to yeast. Importantly, these genes were also shown, later, to confer tolerance to transgenic plants. To identify candidate genes for salt tolerance, Nakahara et al. (2015) performed a yeast functional screen of cDNA library of *Salicornia europaea*; one of the most salt-tolerant plant species. Interestingly, one of the identified genes encoded a protein highly homologous to TLPs and conferred salt tolerance to yeast. In another study, Shim et al. (2009) reported the isolation of wheat *TaHSFAa* gene using yeast system. *TaHSFAa* conferred abiotic stress tolerance both to yeast and transgenic rice plants. Although several studies showed that PR-5 proteins confer abiotic stress tolerance when overexpressed in yeast, little is known about their effect on heavy metal stress tolerance. In the present study, we showed that *TdPR-5* gene is able to confer Cd, Cu and Zn tolerances to transgenic yeast. Experiments to evaluate the effect of overexpression of *TdPR-5* gene in transgenic plants on heavy metal tolerance are underway.

The quality and yield of agricultural products is closely related to environmental factors and genetic background. Environmental factors include both biotic and abiotic components having a significant impact on plant growth and yield. Importantly, it was reported that allergen content in soybean varieties grown in various locations exhibited significant allergen variability (Stevenson et al., 2012). Another study showed that the amount of LTPs in non-transgenic corn increased in un-irrigated locations compared to irrigated ones (Ariyaratna et al., 2009). On the other hand, the production of transgenic plants by overexpression of strong transcription factors can have a significant impact on the expression of specific allergens. An increase in the quantity of a given allergen in transgenic crops can lead to increasing risk for consumers with known allergy or sensitization. Therefore, it is worthwhile

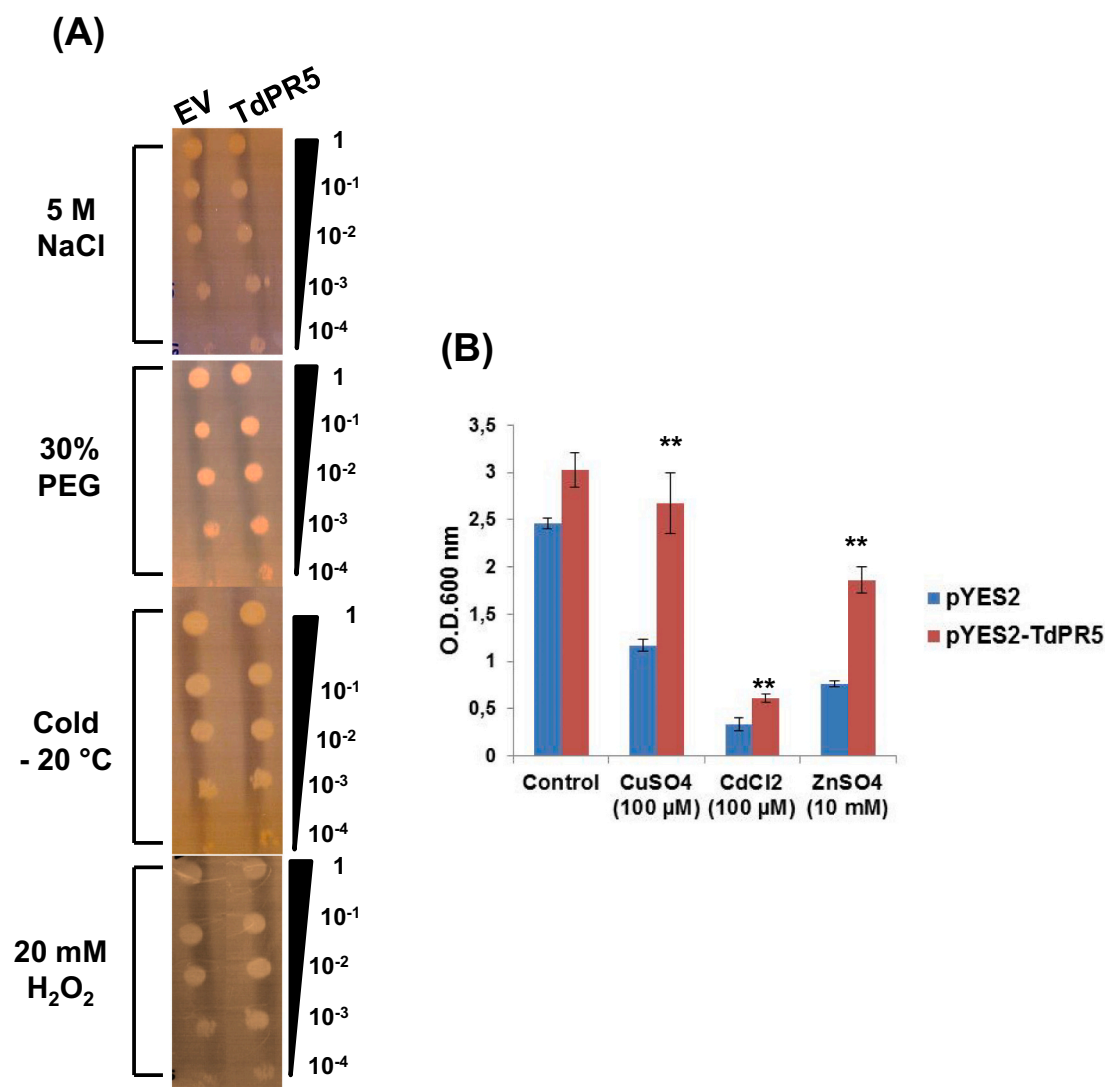


Fig. 7. Analysis of stress tolerance of TdPR-5 in yeast strain W303. (A) Yeast cells harboring pYES2-TdPR-5 and pYES2 empty vector were pre-cultured in Sc-Ura liquid medium supplemented with 2% glucose O/N at 30 °C. The following day, cultures were transferred to Sc-Ura liquid medium supplemented with 2% galactose and TdPR5 gene was induced for 36 h at 30 °C. Thereafter, the OD of the induced cultures were adjusted to 1 into 200 μl solutions of Sc-Ura supplemented with 5 M NaCl, 30% PEG or 20 mM H₂O₂ to simulate salt, osmotic and oxidative stresses, respectively and incubated for 36 h at 30 °C. For cold stress, yeast cells were re-suspended in Sc-Ura medium and incubated at -20 °C for 24 h in ethanol bath. For Control culture equal number of cells were cultured in 200 μl of sterile water and incubated for 36 h at 30 °C. Following these treatments, serial dilutions of yeast cells were spotted on Sc-Ura solid medium supplemented with 2% glucose and grown for 3 days at 30 °C. (B) A pre-culture was prepared in liquid medium YNB-glucose using single colony of recombinant yeast and incubated O/N at 30 °C. Thereafter, these pre-cultures were used to inoculate YNB medium supplemented with 2% galactose and kept O/N at 30 °C to induce expression of TdPR-5 gene. The OD of cells was determined and adjusted to 0.2 with YNB-galactose supplemented with 100 μM CdCl₂, 10 mM ZnSO₄ or 100 μM CuSO₄. Cultures were incubated for 24 h at 30 °C and their OD was measured. Shown is graphic presentation of the OD_{600 nm} of yeast cells. ** denotes statistically different at (p < 0.01) following Student's t-test.

to evaluate the safety of plants derived from the overexpression or induction by stress of genes such as *TdSHN1* and *TdPR-5*.

Credit author statement

Conceptualization; H. Khoudi and R Djemal; Investigation and Methodology; R. Djemal, O Bahloul and H Khoudi; Supervision; H Khoudi; Validation; H Khoudi and R Djemal; Writing - review & editing; R Djemal, O Bahloul and H Khoudi.

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.

Acknowledgments

This work was supported by grant from the Tunisian Ministry of Higher Education and Scientific Research (Contrat Programme LBAP).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plgene.2022.100360>.

References

- Ahmed, N.U., Park, J.J., Jung, H.J., Kang, K.K., Lim, Y.P., Hur, Y.K., Nou, I.S., 2013. Molecular characterization of thaumatin family genes related to stresses in *Brassica rapa*. *Sci. Hortic.* 152, 26–34.
- Anliker, M.D., Borelli, S., Wüthrich, B., 2002. Occupational protein contact dermatitis from spices in a butcher: a new presentation of the mugwort-spice syndrome. *Contact Derm.* 46, 72–74.
- Ariyaratna, H., Pramod, S.N., Goodman, R.E., 2009. The abundance of lipid transfer protein (LTP), the major food allergen of corn, varies between hybrids and growth conditions. *J. Allergy Clin. Immunol.* 123 (2), S28.
- Ayed-Slama, O., Bouhaouel, I., Chamekh, Z., Trifa, Y., Sahli, A., Ben Aissa, N., Slim Amara, H., 2018. Genetic variation of salt-stressed durum wheat (*Triticum turgidum* subsp. durum Desf.) genotypes under field conditions and gynogenetic capacity. *J. Genet. Eng. Biotechnol.* 16, 161–167.
- Barre, A., Caze-Subra, S., Gironde, C., Bienvenu, F., Brienvu, J., Roug, P., 2015. Allergénicité des protéines édulcorantes. *Rev. Fr. Allergol.* 55, 363–371.
- Codex Alimentarius Commission, 2003. Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants and Appendix IV, Annex on the assessment of possible allergenicity, 25th Session, Rome, Italy 30 June–5 July, 2003, pp. 47–60 http://ftp.fao.org/es/esn/food/guide_plants_en.p.
- Didierjean, L., Frendo, P., Nasser, W., Genot, G., Marivet, J., Burkhard, G., 1996. Heavy metal-responsive genes in maize: identification and comparison of their expression upon various forms of abiotic stress. *Planta*. 199, 1–8.
- Djemal, R., Khoudi, H., 2015. Isolation and molecular characterization of a novel WIN1/SHN1 ethylene-responsive transcription factor TdSHN1 from durum wheat (*Triticum turgidum* L. Subsp. durum). *Protoplasma* 252, 1461–1473.
- Djemal, R., Khoudi, H., 2016. TdSHN1, a WIN1/SHN1-type transcription factor, imparts multiple abiotic stress tolerance in transgenic tobacco. *Environ. Exp. Bot.* 131, 89–100.
- Djemal, R., Khoudi, H., 2019. Combination of the endogenous promoter-intron significantly improves salt and drought tolerance conferred by TdSHN1 transcription factor in transgenic tobacco. *Plant Physiol. Biochem.* 139, 435–445.
- Eden, L., Hesling, L., Klock, R., Ledebauer, A.M., Meat, J., Toonen, M.Y., 1982. Cloning of cDNA encoding the sweet tasting plant protein thaumatin and its expression in *Escherichia coli*. *Gene*. 18, 1–12.
- Elble, R., 1992. A simple and efficient procedure for transformation of yeasts. *Biotechniques*. 13, 18–20.
- El-kereamy, A., El-Sharkawy, I., Ramamoorthy, R., Taheri, A., Errampalli, D., Kumar, P., Jayasankar, S., 2011. *Prunus domestica* pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. *PLoS One* 6, 17973.
- Farrakh, S., Wang, M., Chen, X., 2018. Pathogenesis-related protein genes involved in race-specific all-stage resistance and non-race specific high-temperature adult-plant resistance to *Puccinia striiformis* f. sp. tritici in wheat. *J. Integr. Agric.* 17, 2478–2491.
- Ferreira, F., Hawranek, T., Gruber, P., Wopfner, N., Mari, A., 2004. Allergic cross-reactivity: from gene to the clinic. *Allergy* 59, 243–267.
- Fierens, E., Rombouts, S., Gebruers, K., Goesaert, H., Brijs, K., Beaugrand, J., Volckaert, G., Van Campenhout, S., Proost, P., Courtin, C.M., Delcour, J.A., 2007. TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family. *Biochem. J.* 403, 583–591.
- Fuchs, H.C., Bohle, B., Dall'Antonia, Y., Radauer, C., Hoffmann-Sommergruber, K., Mari, A., Scheiner, O., Keller, W., Breiteneder, H., 2006. Natural and recombinant molecules of the cherry allergen Pru av 2 show diverse structural and B cell characteristics but similar T cell reactivity. *Clin. Exp. Allergy* 36, 359–368.
- Hamamouch, N., Li, C.Y., Seo, P.J., Park, C.M., Davis, E.L., 2011. Expression of Arabidopsis pathogenesis-related genes during nematode infection. *Mol. Plant Pathol.* 12, 355–364.
- Hauser, M., Roulias, A., Ferreira, F., Egger, M., 2010. Panallergens and their impact on the allergic patient. *Allergy, Asthma Clin. Immunol.* 6, 1–14.
- Hoffmann-Sommergruber, K., 2002. Pathogenesis-related (PR)-proteins identified as allergens. *Biochem. Soc. Trans.* 30, 930–935.
- Izumi, E., Hidaka, S., Hiroi, A., Kinugasa, S., Yano, E., Zaima, N., Moriyama, T., 2021. Thaumatin-like protein (Pru av 2) is a cherry allergen that triggers percutaneous sensitization in mice. *Foods*. 10, 134.
- Jamakhani, M., Lele, S.S., Rekadwad, B., 2018. *In silico* assessment data of allergenicity and cross-reactivity of NP24 epitopes from *Solanum lycopersicum* (tomato) fruit. *Data Brief*. 21, 660–674.
- Kayum, M., Kim, H.T., Nath, U., Park, J., Kho, K., Cho, Y., Nou, I., 2016. Research on biotic and abiotic stress related genes exploration and prediction in *Brassica rapa* and *B. oleracea*: a review. *Plant Breed. Biotech.* 4, 135–144.
- Khoudi, H., Maatar, Y., Gouiaa, S., Masmoudi, K., 2012. Transgenic tobacco plants expressing ectopically wheat H⁺-pyrophosphatase (H⁺-PPase) gene TaVP1 show enhanced accumulation and tolerance to cadmium. *J. Plant Physiol.* 169, 98–103.
- Krebitz, M., Wagner, B., Ferreira, F., Peterbauer, C., Campillo, N., Witty, M., Kolarich, D., Steinkellner, H., Scheiner, O., Breiteneder, H., 2003. Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. *J. Mol. Biol.* 329, 721–730.
- Leng, X., Jia, H., Sun, X., Shangguan, L., Mu, Q., Wang, B., Fang, J., 2015. Comparative transcriptome analysis of grapevine in response to copper stress. *Sci. Rep.* 5, 17749.
- Li, X.Y., Gao, L., Zhang, W.H., Liu, J.K., Zhang, Y.J., Wang, H.Y., Liu, D.K., 2015. Characteristic expression of wheat PR-5 gene in response to infection by the leaf rust pathogen, *Puccinia tritici*. *J. Plant Interact.* 10, 132–141.
- Liu, J.J., Sturrock, R., Ekramodoullah, A.K.M., 2010. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. *Plant Cell Rep.* 29, 419–436.
- Liu, W.X., Zhang, F.C., Zhang, W.Z., Song, L.F., Wu, W.H., Chen, Y.F., 2013. Arabidopsis Di19 functions as a transcription factor and modulates PR1, PR2, and PR-5 expression in response to drought stress. *Mol. Plant* 6, 1487–1502.
- Liu, L., Zhang, Z., Zhao, M., Wang, J., Lin, H.J., Shen, Y.O., Pan, G., 2011. Molecular cloning and characterization of pathogenesis-related protein 5 in *Zea mays* and its antifungal activity against *Rhizoctonia solani*. *Afri. J. J.* 10, 19286–19293.
- Midoro-Horiuti, T., Brooks, E.G., Goldblum, R.M., 2001 Oct. Pathogenesis-related proteins of plants as allergens. *Ann. Allergy Asthma Immunol.* 87 (4), 261–271. [https://doi.org/10.1016/S1081-1206\(10\)62238-7](https://doi.org/10.1016/S1081-1206(10)62238-7) (PMID: 11686417).
- Misram, R.C., Sandeep Kamthanm, M., Kumar, S., Ghosh, S., 2016. A thaumatin-like protein of *Ocimum basilicum* confers tolerance to fungal pathogen and abiotic stress in transgenic Arabidopsis. *Sci. Rep.* 6, 25340.
- Nakahara, Y., Sawabe, S., Kainuma, K., Katsuhara, M., Shibasaki, M., Suzuki, M., Yamamoto, K., Oguri, S., Sakamoto, H., 2015. Yeast functional screen to identify genes conferring salt stress tolerance in *Salicornia europaea*. *Front. Plant Sci.* 6, 920.
- Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, K., Hellsten, U., Mitros, T., Poliakov, A., 2009. The Sorghum bicolor genome and the diversification of grasses. *Nature* 457, 551–556.
- Petre, B., Major, I., Rouhier, N., Duplessis, S., 2011. Genome-wide analysis of eukaryote thaumatin-like proteins (TLPs) with an emphasis on poplar. *BMC Plant Biol.* 11, 1–16.
- Pomés, A., 2010. Relevant B cell epitopes in allergic disease. *Int. Arch. Allergy Immunol.* 152, 1–11.
- Rizwan, M., Meunier, J.D., Miche, H., Keller, C., 2012. Effect of silicon on reducing cadmium toxicity in durum wheat (*Triticum turgidum* L. cv. Claudio W.) grown in a soil with aged contamination. *J. Hazard. Mater.* 209–210, 326–334.
- Salcedo, G., Quirce, S., Diaz-Perales, A., 2011. Wheat allergens associated with Baker's asthma. *J. Invest. Allergol. Clin. Immunol.* 21, 81–92.
- Semane, B., Dupae, J., Cuypers, A., Noben, J.P., Tuomainen, M., Tervahauta, A., Kärenlampi, S., Van Bellegheem, F., Smeets, K., Vangronsveld, J., 2010. Leaf proteome responses of *Arabidopsis thaliana* exposed to mild cadmium stress. *J. Plant Physiol.* 167, 247–254.
- Shim, D., Hwang, J.U., Lee, J., Lee, S., Choi, Y., An, G., Martinoia, E., Lee, Y., 2009. Orthologs of the class A4 heat shock transcription factor *HsfA4a* confer cadmium tolerance in wheat and rice. *Plant Cell* 12, 4031–4043.
- Singh, N.K., Nelson, D.E., LaRosa, P.C., Bracker, C.E., Handa, A.K., Hasegawa, P.M., Bressan, R.A., 1989. Osmotin: a protein associated with osmotic stress adaptation in plant cells. Environmental stress in plants. Springer Berlin Heidelberg, Germany.
- Singh, N.K., Kumar, K.R.R., Kumar, D., Shukla, P., Kirti, P.B., 2013. Characterization of a pathogen induced thaumatin-like protein gene *AdTLP* from *Arachis diogeni*, a wild peanut. *PLoS One* 8, e83963.
- Stevenson, S.E., Woods, C.A., Hong, B., Kong, X., Thelen, J.J., Ladics, G.S., 2012. Environmental effects on allergen levels in commercially grown non-genetically modified soybeans: assessing variation across north america. *Front. Plant Sci.* 3, 196.
- Subramanyam, K., Arun, M., Mariashibu, T.S., Theborel, J., Rajesh, M., Singh, N.K., Manickavasagam, M., Ganapathi, A., 2012. Overexpression of tobacco osmotin (*Tbom*) in soybean conferred resistance to salinity stress and fungal infections. *Planta*. 236, 1909–1925.
- Thakur, P., Kumar, S., Malik, J.A., Berger, J.D., Nayyar, H., 2010. Cold stress effects on reproductive development in grain crops: an overview. *Environ. Exp. Bot.* 67, 429–443.
- Tsutsui, T., Kato, W., Asada, Y., Sako, K., Sato, T., Sonoda, Y., Kidokoro, S., Yamaguchi-Shinozaki, K., Tamaoki, M., Arakawa, K., Ichikawa, T., Nakazawa, M., Seki, M., Shinozaki, K., Matsui, M., Ikeda, A., Yamaguchi, J., 2009. DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in Arabidopsis. *J. Plant Res.* 122, 633–643.
- van Loon, L.C., van Strien, E.A., 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55, 85–97.
- van Loon, L.C., Rep, M., Pieterse, C.M.J., 2006. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162.
- Walliwalagedara, C., Atkinson, I., van Keulen, H., Cutright, T., Wei, R., 2010. Differential expression of proteins induced by lead in the dwarf sunflower *Helianthus annuus*. *Phytochemistry*. 71, 1460–1465.
- Wang, L., Yang, L., Zhang, J.X., Dong, J., Yu, J., Zhou, J., Zhuge, Q., 2013. Cloning and characterization of a thaumatin-like protein gene *PeTLP* in *Populus deltoides* x *P. euramericana* cv. 'Nanlin89'. *Acta Physiol. Plant.* 35, 2985–2998.
- Zaim, M., El Hassouni, K., Gamba, F., Filali-Maltouf, A., Belkadi, B., Sourour, A., Amri, A., Nachit, M., Taghouti, M., Bassi, F.M., 2017. Wide crosses of durum wheat (*Triticum durum* Desf.) reveal good disease resistance, yield stability, and industrial quality across Mediterranean sites. *Field Crop Res.* 214, 219–227.