Plant Physiology and Biochemistry 89 (2015) 64–75

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

A wheat lipid transfer protein (TdLTP4) promotes tolerance to abiotic and biotic stress in *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 11 September 2014
Accepted 13 February 2015
Available online 14 February 2015

Keywords:
Abiotic and biotic stresses
Gene expression
Jasmonic acid
Lipid transfer protein
Transgenic *Arabidopsis* plants
Wheat

ABSTRACT

Lipid transfer proteins (LTPs) are members of the family of pathogenesis-related proteins (PR-14) that are believed to be involved in plant defense responses. In this study, we report the isolation and characterization of a novel gene *TdLTP4* encoding an LTP protein from durum wheat *[Triticum turgidum L. subsp. Durum Desf.]*. Molecular Phylogeny analyses of wheat *TdLTP4* gene showed a high identity to other plant LTPs. Predicted three-dimensional structural model revealed the presence of six helices and nine loop turns. Expression analysis in two local durum wheat varieties with marked differences in salt and drought tolerance, revealed a higher transcript accumulation of *TdLTP4* under different stress conditions in the tolerant variety, compared to the sensitive one. The overexpression of *TdLTP4* in *Arabidopsis* resulted in a promoted plant growth under various stress conditions including NaCl, ABA, JA and H_2O_2 treatments. Moreover, the LTP-overexpressing lines exhibit less sensitivity to jasmonate than wild-type plants. Furthermore, detached leaves from transgenic *Arabidopsis* expressing *TdLTP4* gene showed enhanced fungal resistance against *Alternaria solani* and *Botrytis cinerea*. Together, these data provide the evidence for the involvement of *TdLTP4* gene in the tolerance to both abiotic and biotic stresses in crop plants.

1. Introduction

Lipid transfer proteins (LTP) are small basic proteins of about 9–10 kDa in size and may represent as much as 4% of the total soluble proteins. LTPs have been divided into two families, based on the length of the polypeptide of the mature protein. The LTPs that form the first family, namely LTP1, have molecular masses of approximately 10 kDa and are basic, with isoelectric points (pI) between 9 and 10 (Gasteiger et al., 2005). These LTPs have approximately 90–95 amino acids residues, of which eight are cysteines conserved in similar positions along the primary structure of the already characterized LTP1 family (Edstam et al., 2011). These eight cysteines, bound to each other to form four disulfide bridges that help the stabilization of the peptide tertiary structure (Han et al., 2001; Salcedo et al., 2007). The second family (LTP2) is formed of peptides that have molecular masses of approximately 7 kDa, possessing on average 70 amino acids; their other characteristics, such as a high pI, lipid transfer activity and another pattern of four conserved disulfide bridges, are shared with the LTP1 family (Castro et al., 2003). Both families present a signal peptide at the amino terminal region, which in general varies between 21 and 27 amino acids, for the LTP1 family (Suelves and Puigdomènech, 1997), and from 27 to 35 amino acids, for the LTP2 family (Garcia-Garrido et al., 1998). The excision of the signal peptide allows the targeting of the mature peptide of LTPs to cell secretory pathway where they are exported to the apoplast. Considering these localizations together with other findings, different functions are suggested for the role of LTP in the physiology of plants, such as their involvement in cutin synthesis (Han et al., 2001), β-oxidation (Tsuboi et al., 1992), somatic embryogenesis (Sterk et al., 1991; DeBono et al., 2009; Panikashvili et al., 2010). Expressions of LTPs are also inducible by environmental changes such as drought, cold, salt stress and also infection with bacterial and fungal pathogen (Jang

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http://dx.doi.org/10.1016/j.plaphy.2015.02.008
0981-9428/

et al., 2004). This have been shown for several additional nsLTPs from different species, including mungbean, bromegrass, sunflower, rice and *Arabidopsis* (Brotman et al., 2012). Further, nsLTPs are often found to be differentially expressed in various mutants. One example is the double mutant of the RNA binding proteins TSN1 and TSN2 (Tudor-SN) in *Arabidopsis*, where six nsLTPs are downregulated during normal conditions, salt stress or both. Another nsLTP from *Arabidopsis*, named DIR1, have been shown to be essential for long distance signaling in systemic acquired resistance (SAR) in *Arabidopsis* (Maldonado et al., 2002). The knockout mutant of DIR1 was shown to lack the ability to develop SAR in response to infection by *Pseudomonas syringae* and *Peronospora parasitica*.

Defensive role of LTPs has been proposed considering the ability of some members of this family, but not all, to inhibit the growth of plant pathogens (Molina and Garcia-Olmedo, 1993). Their extracellular distribution in the exposed surfaces in vascular tissue systems, high abundance and expression in response to infection by pathogens suggest that they are active plant-defense proteins (Blein et al., 2002). Interestingly, LTPs can inhibit the growth of fungal pathogens *in vitro* and they are capable of synergistically enhancing the antimicrobial properties of other antimicrobial peptides such as defensins and thionins (Marion et al., 2004). The relative activities of plant LTPs against pathogens vary, suggesting that they have distinct degrees of selectivity. Recent studies have demonstrated the antifungal potential of different LTPs. Transgenic rice expressing the homologous ns-LTP gene (Ace-AMP1) of *Allium cepa* showed antimicrobial activity towards *Magnaporthe grisea*, *Rhizoctonia solani* and *Xanthomonas oryzae pv. Oryzae* (Patkar and Chattoo, 2006). Transgenic wheat expressing Ace-AMP1showed enhanced antifungal activity against *Blumeria graminis f. sp. tritici* (Roy-Barman et al., 2006).

Signal molecules such as abscisic acid, salicylic acid, ethylene and methyl jasmonate are involved in the expression of LTP genes (Garcia-Garrido et al., 1998, Jang et al., 2004; Jung et al., 2005). Evidence that members of the LTP1 family are involved in defense responses came from studies showing that wheat LTP1 can compete for a high affinity receptor binding site in tobacco cells, with elicitin, an elicitor protein from oomycetes (Buhot et al., 2001). It was shown that a tobacco LTP1 could similarly act as a binding competitor for elicitin and that the strength of this interaction was increased by LTP in complex with jasmonic acid. Furthermore, treatment of plants with this complex lead to increased resistance against the oomycete *Phytophthora parasitica* (Buhot et al., 2004). Thus, it appears that, in this case, the LTP1 family and elicitin act on the same, currently unidentified, receptor in order to modulate plant defense responses.

In this study, we report the molecular cloning and expression analysis of a durum wheat TdLTP4 in two wheat varieties showing contrasting tolerance to drought and salinity. The effect of the overexpression of TdLTP4 gene on the tolerance of transgenic *Arabidopsis* plants to biotic and abiotic stresses is also discussed.

2. Materials and methods

2.1. Molecular cloning and in silico analysis of TdLTP4

Total RNA from durum wheat leaf was extracted using the RNeasy-mini Kit (Qiagen). To remove contaminating genomic DNA, total RNA (10 μg) was treated with DNase (Promega). DNase-treated RNA samples (0.5 μg) were reverse-transcribed using MMLV reverse transcriptase (Invitogen). The reverse transcription (RT) reaction was performed at 37 °C for 1 h using 2 μM oligo-dT_{18} primer. Two microliters of first strand cDNA were used as template for PCR amplification of the *TdLTP4*, using PFU as polymerase in a reaction solution prepared according to the instructions of the manufacturer (Fermentas). Primers, designed on the basis of the sequence of *Triticum aestivum nsLTP* (EF432573) gene were 5′- CTGCTCTTGCTCAGGTCGT-3′ and 5′-AGCAGTCGACCGAAGAGCTG-3'. After denaturation of the cDNA for 5 min at 94 °C, PCR amplification consisted in 35 cycles comprising successively 30 s at 94 °C, 30 s at 60 °C and 90 s at 72 °C, and a final extension for 5 min at 72 °C was done. Amplified products were cloned using the pGEM-T Easy vector system (Promega) and successful isolation of *TdLTP4* gene was confirmed by digestion and sequencing. The 5′ and 3′ regions of *TdLTP4* cDNA were verified using 5'- and 3′-RACE technique by using the 'First choice RLM Race kit' according to the instructions of the manufacturer (Ambion). The following primers were used (5′ to 3′): R5-TdLTP4: 5′-GCTCAAGG-CAGAGCTCACCTGA-3′; R3-TdLTP4: 5′-TCGTCGTCGCGAGAACTACG-3′. The right full length sequence of TdLTP4 was amplified using the right primers TdLTP4_Fw: 5′-GCTAGCTTGATCGAGATGGC-3′ and TdLTP4_Rv: 5′-CAGCAAGTGCTCGATCAGCG-3′, cloned in the pGEMT-easy vector and sequenced.

Open reading frames (ORFs) and amino acid sequences were deduced using ORF finder program and homology search were conducted using BLAST 2.0 program of the National Center of Biotechnology Information (NCBI). The software ClustalW (1.82) from Europe Biotechnology Information was employed for multiple sequence alignment of amino acid sequences of wheat LTP with other monocot LTPs retrieved from GenBank and subsequently a phylogenetic tree was constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with ClustalX 1.81. The reliability of the tree was measured by bootstrap analysis with 1500 trials and the phylogenetic tree was edited using molecular evolutionary genetics analysis and sequence alignment tool MEGA v 3.1 (Kumar et al., 2004). The theoretical calculation of isoelectric point and molecular weight were analyzed with 'ExPASy' using Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).

For molecular modeling, Maize ns-LTP (PDB code 1AFH) was used as the template. Modeling was done using molecular operating environment (MOE version 2001.07, Chemical Computing Group Inc., Montreal, QC, Canada). Ten intermediate homology models were built as a result of the permutational selection of different loop candidates and side chain rotamers. The intermediate models were averaged to produce the final model by Cartesian average. The validity of the model was tested using WHATCHECK and PROCHECK (Laskowski et al., 1993).

2.2. RNA extraction and RT-PCR

Total RNA was extracted from 200 mg of young leaves of the two durum wheat (*Triticum turgidum L. subsp. Durum [Desf.]*) lines (Mahmoudi, and Om Rabia3, are salt sensitive and tolerant genotypes respectively) and from seeds embryos using the Trizol method (*Invitrogen*). To remove contaminating DNA, RNA (10 μg) were treated with RNase-free DNase (Promega). DNase-treated RNA samples (0.5 μg) were reverse-transcribed as indicated above. Two microliters of the first strand cDNAs were used as templates for PCR amplification with a pair of gene-specific primers: 5′-ATGGCCCGTTCTGCTCTTG-3′ and 5′-TCAGCGAATCTTA-GAGCA-3′. A wheat actin gene fragment used as an internal control was amplified with the primers: 5′-GTGCCCATTTACGAAGGATA-3′ and 5′—GAAGACTCCATGCCGATCAT-3′. Samples were denatured for 5 min at 94 °C and then run for 35 cycles of 30 s at 94 °C, 45 s at 55 °C and 2 min at 72 °C with a final extension of 5 min at 72 °C. The PCR products were separated by agarose gel electrophoresis.

2.3. Generation of transgenic Arabidopsis plants

The full-length TdLTP4 open reading frame (ORF) was amplified

(A)

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JF799976 TdLTP4     MAR-SAVAQVVLVAVVA---AMLLAVTEAAVSCGQVSSALSPCISYAR-GNGASPSAACCSGVRSLASSARSTADKQAACKCI
CAA45210 Td         -------AQVMLMAVALVLMLAAVPRAAVAIDCGHVDSLVRPCLSYVQ--GGPGPSGQCCDGVKNLHNQARSQSDRQSACNCL
ABO28527 Ta         MAR-SALAQVVLVAVVA---AMLLAVTEAAVSCGQVSSALSPCISYAR-GNGASPSAACCSGVRSLVSSARSTADKQAACKCI
AAV28706 Ta         MAR-TAATKLVLVALVA---AMILAASDAAISCGQVSSALTPCVAYAK-GSGTSPSGACCSGVRKLAGLARSTADKQATCRCL
CAA42832 Hv         MAR----AQVLLMAAALVLMLTAAPRAAVALNCGQVDSKMKPCLTYVQ--GGPGPSGECCNGVRDLHNQAQSSGDRQTVCNCL
CAA85484 Hv         MAR-SAATQLVLVAMVA---AMLLVAADAAISCGQVSSALRPCISYAS-GNGGILPPACCSGVKRLAGAAQSTADKQAACKCI
AAB18815 Os         MAR-----AQLVLVALVAALLLAAPHAAVAITCGQVNSAVGPCLTYAR-G-GAGPSAACCSGVRSLFAAASTTADRRTACNCL
AAC18567 Os         MAR-AGHNKYVARVMVV----ALLLAARAPVTCGQVVSTWAPCIMYAD-GEGVAPTGGCCDGVRTLNSAAATTADRQTTCACL
CAA50660 Sb         MAR---LAVAIAVVAA-VVVVLAATTSEAAISCGQVSSAIALCLSYAR-GQGFAPSAGCCSGVRSLNSAARTTADRRAACNCL
CAA50661 Sb         MARSMKLAVAIAVVAAAAAVVLAATTSEAAVTCGQVSSAIGPCLSYAR-GQGSGPSAGCCSGVRSLNSAARTTADRRAACNCL
AAB06443 Zm         MAR-TQSAVAVAVVAAVLLLAAAATTSEAAITCGQVSSAIAPCLSYAR-GTGSGPSASCCSGVRNLKSAASTAADRRAACNCL
AAL30846 Si         MAP-MRKMQAVFALAMVFAAAALVASAAAAITCGQVASSLAPCIPYATGNANVMPSG-CCGGVRSLNNAARTSADRQAACRCL
AED97170 At         MAF-ALRFFTCFVLTVF-----IVASVDAAITCGTVASSLSPCLGYLS-KGGVVPPP-CCAGVKKLNGMAQTTPDRQQACRCL
Consensus           --------------------------------CG-VS-----CY--------------CCG----------VLAD---CCPC-
Clustal Consensus                                  ** *     *               **             *    * *

JF799976 TdLTP4     IPTKCGVSVPYAISSSVDCSKIR----------------
CAA45210 Td         IPPKCGVNLPYTISLNIDCSRV----------------
ABO28527 Ta         IPTKCGVSVPYAISSSVDCSKIR----------------
AAV28706 Ta         IPSKCGVSVPYTISASVDCSKIH----------------
CAA42832 Hv         IPSKCNVNVPYTISPDIDCSRFTERRSVKLVLSSSIHVEL
CAA85484 Hv         IPSMCGVSVPYAISASVDCSKIR----------------
AAB18815 Os         IPSKCGVSVPYTISASIDCSRVS----------------
AAC18567 Os         IPSKCGVNIPYAISPSTDCSRVH----------------
CAA50660 Sb         IPSKCGVSVPYTISTSTDCSRVS----------------
CAA50661 Sb         IPSKCGVSIPYTISTSTDCSRVS----------------
AAB06443 Zm         IPSKCGVSIPYTISTSTDCSRVN----------------
AAL30846 Si         IPGKCGVSVPFRISMSTDCNKVS----------------
AED97170 At         LPGKCGVSIPYPISTSTNCATIK----------------
Consensus           VPISC----------------------------------
Clustal Consensus   :*  *
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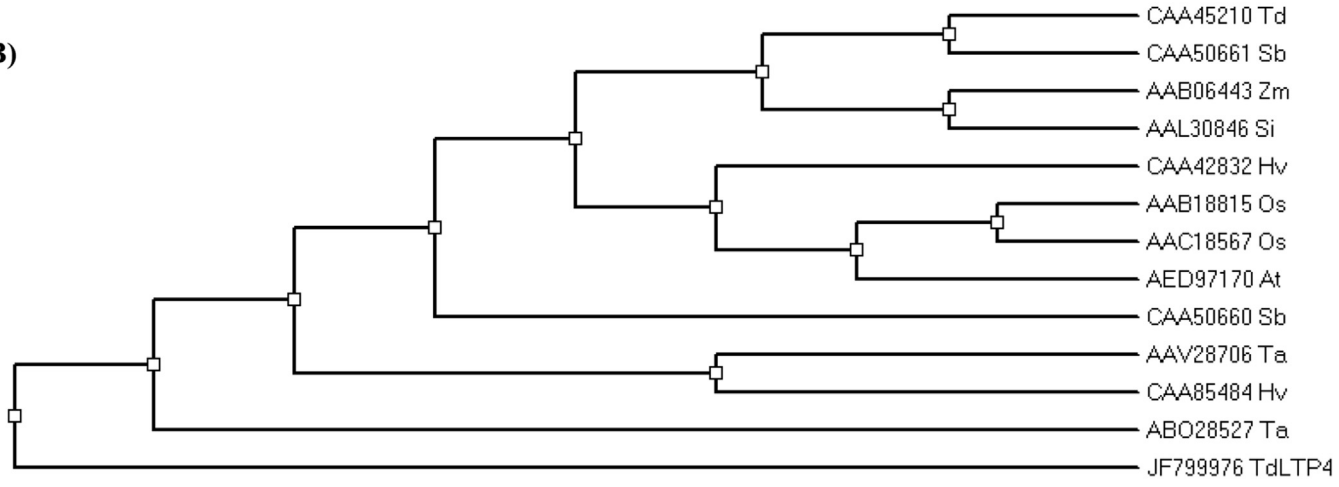
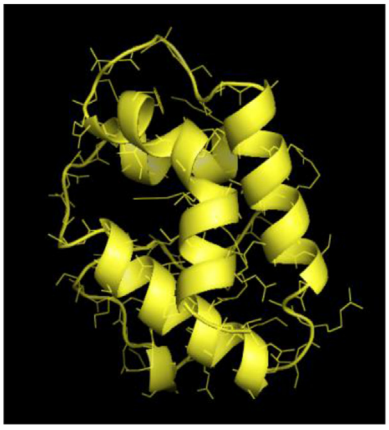
(B)



(C)



with PfuTurbo DNA polymerase (Stratagene; La Jolla, CA, USA) using forward (5′-GCCCATGGCCCGTTCTGCTCTTG-3′) and reverse (5′-GCCCATGGGCGA-ATCTTAGAGCA-3′) primers hybridizing to the 5′ and 3′ ends respectively and harboring *Nco*I restriction sites (underlined sequences). The resulting TdLTP4 ORF was cloned into the *Nco*I site of the pCAMBIA1301 plasmid. This vector contains the 35S promoter, the polyadenylation signal (3′NOS) and the hygromycine resistance gene (HPT) as a selectable marker, between the 35S promoter and terminator. *Agrobacterium tumefaciens (GV3101)*-mediated transformation was performed via the floral dipping technique of *Arabidopsis thaliana* (ecotype Columbia) (Clough and Bent, 1998). Transgenic plants were selected by planting seeds on plant nutrient agar plates supplemented with 10 mg/l hygromycine.

Transgenic lines were grown up to the T3 generation from which homozygous plants were isolated for further analyses. Genetic segregation data performed using the HPT gene gave rise to a 3:1 ratio confirming that this marker segregates as a single copy gene (data not shown).

The genome of transgenic plants was screened by PCR using a pair of Hygromycin gene primers: 5′-ATGAAAAAGCCTGAACTCAC-3′ and 5′-CTCTATTTCTTTGCCCTCG-3′.

The expression of TdLTP4 in transgenic lines was determined by RT-PCR using a pair of GFP gene specific primers: 5′-ATGAG-TAAAGGAGAAGAACT-3′ and 5′-GTATAGTTCATCCATGCCATG-3’. *Arabidopsis* actin gene fragment used as an internal control was amplified with the primers: 5′-GGCGATGAAGCTCAATCCAAACG-3′ and 5′-GCTCACGACCAGCAAGATCAAGACG-3′.

2.4. Plant material, growth conditions and stress treatments

Seeds of wild type *A. thaliana* (ecotype Columbia, Col-0) and transgenic plants were surface sterilized by treatment with ethanol 70% for 2 min and with 5% bleach for 15 min, washed five times with sterile water, and plated on Murashigue and Skoog (MS) agar medium (Murashigue and Skoog, 1962) under light/dark cycle conditions of 16/8 h at 22 °C. For seedling growth under stress treatments, one week-old seedlings were transferred to agar plates containing 100 mM NaCl, 20 μM ABA, 50 μM JA or 3 mM H_2O_2 and incubated for ten additional days. In the greenhouse, plants were grown in pots containing compost soil (leaf-mold: stable-litter: sand, 1:1:1) for 4–5 weeks (16/8 h daily light period, 23 °C temperature (night/day) and 60–70% relative humidity).

For durum wheat, two Tunisian cultivars Mahmoudi and Om Rabia3, sensitive and tolerant to salt stress, respectively, (Brini et al., 2009) were supplied by INRAT, Laboratory of Vegetal Physiology-Tunisia. Seeds of each line were sterilized in 0.5% NaOCl for 15 min, then washed three times with sterile water and placed on Petri dishes with a single sheet of Whatman #1 filter paper for germination. Three-days-old seedlings were transferred to containers with modified half-strength MS solution for 4 days, and then transferred to the same medium containing or not different stress solutions (100 mM NaCl; 50 μM JA; 20 μM ABA). All seedlings were grown in a glasshouse at 25 ± 5 °C, under photosynthetically active radiation of 280 μmol m^{-2} s^{-1}, 16 h photoperiod and 60 ± 10% relative humidity. After three days of stress exposure, Plant materials were harvested and used for RNA extraction and RT-PCR analysis.

2.5. Determination of survival rate, fresh weight and relative water content (RWC)

The survival rate assay was performed by plating 30 seedlings of transgenic and of wild type plants on MS medium with differents stress treatments. After four weeks of incubation, the number of survival of plants plated.

Fresh weights were determined immediately at the end of stress treatments. To determine the RWC, leaves were harvested and their fresh weight (FW) was determined immediately. They were floated on deionized water overnight at 4 °C and the turgescent weight (TW) was recorded. Finally, leaves were dried at 70 °C and the dry weight (DW) was recorded. Relative water content was calculated using the following formula:

$$RWC\ (\%) = (FW - DW/TW - DW) \times 100.$$

2.6. Leaf surface determination

UTHSCSA image tool is a free image processing and analysis program. It can acquire, display, edit and analyze images (http://www.ddsdx.uthscsa.edu/dig/itdesc.html). Total leaf area of *Arabidopsis* seedlings in plates was calculated in mm^2 using image tool program.

2.7. Root elongation test

Seeds of transgenic TdLTP4 lines or Wt were surface sterilized and plated on agar MS medium. Plates were placed vertically in a growth chamber under a 16 h light/8 h dark cycle at 22 °C. The root elongation was evaluated after three-week-old seedlings grown on standard MS agar plates containing 50 μM JA.

2.8. Ascorbate content determination

AsA contents were measured by HPLC using the HPLC Agilent 1100 series. A 0.5 g aliquot of Arabidopsis leaves was ground in 1% H_2SO_4 and the extract was filtered using 0.45 mm filters (Millipore). Then 100 ml of the samples were loaded on a Eurospher 100C18 (25 cm_4.6 mm_5 mm) reversed phase column. Elutions were performed in a buffer of 0.5%NaH_2PO_4/acetonitrile (93%:7%), pH 2.5, according to an isocratic mode. The flow rate was 0.7 ml min_1 and the detection was performed at 280 nm. The following dilutions of pure standard AsA (Aldrich) were employed 0.5, 0.25 and 0.1 mg ml_1.

2.9. Pathogenecity tests

To test the antifungal resistance of transgenic *Arabidopsis*, fungi were grown on PDA. Spores collected were diluted to 1×10^6 spores/mL and inoculated in the center of the detached *Arabidopsis* leaves, incubated at room temperature for 10 days. Leaves inoculated with water served as control. The extent of lesion was

Figure 1. (A) Amino acid sequence alignment of wheat TdLTP4, *Triticum durum* with other monocot LTPs. Td, *Triticum durum* (CAA45210); Ta, *Triticum aestivum* (ABO28527, AAV28706); Hv, *Hordeum vulgare* (CAA85484, CAA42832); Os, *Oryza sativa* (AAB18815, AAC18567); Sb, *Sorghum bicolor* (CAA50660, CAA56061); Zm, *Zea mays* (AAB06443) Si, *Setaria italica* (AAL30846); *Arabidopsis thaliana* (AED97170). The conserved amino acid residues are highlighted with asterisks. Conserved and semi-conserved substitutions are represented by colons and dots respectively. Numbering 1 to 8 represents eight conserved cysteins residues. (B) Phylogenetic tree of wheat TdLTP4, *Triticum durum* with other monocot LTPs. Td, *Triticum durum* (CAA45210); Ta, *Triticum aestivum* (ABO28527, AAV28706); Hv, *Hordeum vulgare* (CAA85484, CAA42832); Os, *Oryza sativa* (AAB18815, AAC18567); Sb, *Sorghum bicolor* (CAA50660, CAA56061); Zm, *Zea mays* (AAB06443) Si, *Setaria italica* (AAL30846); *Arabidopsis thaliana* (AED97170). (C) Predicted three-dimensional structural model of wheat TdLTP4 protein.

recorded and the difference between control and TdLTP-4 expressing transgenic plants was observed.

2.10. Confocal microscopy

Leaf samples were sliced with razor blades and mounted between slides and cover slip in water. The fluorescence of the GFP fusion proteins was observed with a Zeiss LSM confocal laser scanning microscope 510 using a 488 nm laser line of argon (Ar) laser, with the emission window set at 505e530 nm. Images were analyzed by using Zeiss LSM software, version 3.2.0.70. Images were recorded with picture size of 640 _ 424 pixels, arranged and labeled using PhotoFiltre. Cells were individually examined through a z series of images.

2.11. Statistical analysis

Data were analyzed using one-way analysis of variance and treatment mean separations were performed using Duncan's multiple range tests at the 5% level of significance.

3. Results

3.1. Isolation and sequence characterization of the wheat TdLTP4

The full-length cDNA of TdLTP4 was cloned and sequenced as described (see section 2). Sequence analysis of the TdLTP4 (GenBank accession no. JF799976) cDNA revealed an open reading frame (ORF) of 345 bp encoding a polypeptide that shares relatively high similarity with LTPs of cereals. Blast analyses showed that TdLTP4 shares 75% identity and 90% similarity with LTPs of *T. aestivum* (ABO28527), 66% identity and 84% similarity with the LTP of *Oryza sativa* (AAB18815), 86% identity and 92% similarity with LTP of *Hordeum vulgare* (CAA85484), 69% identity and 85% similarity with LTP of *Sorghum bicolor* (CAA50660), 76% identity and 86% similarity with LTP of *T. aestivum* (AAV28706), 65% identity and 85% similarity with LTP of *Zea mays* (AAB06443), 58% identity and 74% similarity with LTP of *Setaria italica* (AAL30846), 46% identity and 70% similarity with LTP of *A. thaliana* (AED97170).

The eight-cysteine motif appears to be a structural scaffold of conserved helical regions connected by variable loops. The position of the eight cysteine residues are conserved where the third and fourth cysteines are consecutives in the polypeptide chain and the fifth and sixth cysteines are separated by only one residue. Phylogenetic tree was constituted on the basis of amino acid sequence alignment of wheat TdLTP4 with other monocot LTPs (Fig. 1A and B).

The proposed three-dimensional structural model of wheat TdLTP4 is presented in Fig. 1C. The structure of wheat TdLTP4 using the method of “GORII” and “Chou and Fasman” showed the presence of 4 α-helices, 9 loops turns and also the presence of 8 conserved cysteine residues that are responsible for the formation of 4 disulfide bridges (Cys29-77, Cys39-54, Cys55-97, Cys75-111). The active site residues Gly30, Pro50 Ala52 and Cys55 present in the hydrophobic core may be suggested for catalyzing the reaction in lipid binding (Gomar et al., 1996).

3.2. Expression analysis of TdLTP4 cDNA in the two durum wheat varieties

We have studied the expression level of TdLTP4 cDNA in embryo seeds, leaves and roots of durum wheat cultivars Mahmoudi (sensitive) and Om Rabia3 (tolerant) exposed to different types of stresses (NaCl, JA; ABA). RT-PCR analysis of TdLTP4 expression showed that under standard conditions, the transcript accumulates to higher levels in embryos and leaves of the two genotypes compared to the roots (Fig. 2). In addition, TdLTP4 were higher in the tolerant one than in the sensitive one (Fig. 2). Using several types of stress, it seems that TdLTP4 can be induced (especially in roots of Om Rabia3) to different extents, by NaCl, JA and ABA (Fig. 2). Such results suggest that TdLTP4 may be involved in the response of wheat to various stress treatments.

3.3. Production of transgenic Arabidopsis plants overexpressing TdLTP4

In order to investigate the effect of TdLTP4 overexpression in *Arabidopsis*, the full-length ORF was cloned into *Nco*I site in the binary vector pCAMBIA1302 downstream of the 35S promoter (P35S) resulting in a translational fusion with GFP (TdLTP4::GFP). After *Agrobacterium*-mediated transformation of *Arabidopsis* plants and selection with the hygromycin, several transformants were produced. These transgenic lines were grown up to the T3 generation from which homozygous plants (for HPT marker) were selected. The genome of several transgenic plants was screened by PCR using HPT gene as template (conferring resistance to hygromycin) (Fig. 3A) and the expression level of TdLTP4 has been estimated on young leaves of five transgenic lines together with those of the control plants using GFP gene (Fig. 3B). Transgenic lines which showed low (L8), medium (L4) and high expression of transgene (L2) were chosen for physiolocal analysis.

To investigate *in vivo* the subcellular localization and the potential protective effects of TdLTP4 proteins in plant vegetative tissues, transient expression of green fluorescent protein (GFP)-fused TdLTP4 proteins was analyzed in agroinfiltrated *Arabidopsis* leaves. Confocal fluorescence imaging revealed that TdLTP4 is distributed in the peripheral cell layers and most abundant in the cell walls (Fig. 3C). The location can be explained by the presence of a potential signal peptide, deduced from all the sequences of LTP genes, able to drive the LTPs into cell membranes.

3.4. The overexpression of TdLTP4 enhances tolerance to salt, ABA and jasmonic acid treatments

Under standard growth conditions, the three transgenic lines were phenotypically indistinguishable from wild type plants (Fig. 4A). Transgenic lines (L2, L4 and L8) were tested for salt, ABA and Jasmonic Acid (JA)-stress tolerance. One week after germination, *Arabidopsis* seedlings were transferred to MS medium containing 100 mM NaCl or 20 μM ABA or 50 μM JA and plant survival was monitored. The transgenic plants overexpressing TdLTP4 are much more salt tolerant than wild type (Fig. 4A). In fact, Plants from TdLTP4 transgenic lines continue to grow well in the presence of 100 mM NaCl, whereas wild-type plants exhibit chlorosis and die after 10 days of salt-stress treatment. These results indicate that these transgenic plants have an enhanced ability to transiently survive the NaCl-stress treatment. When plants were subjected to 20 μM ABA, a clear difference was also observed between transgenic and control plants (Fig. 4A). After 10 days of ABA treatments, the transgenic lines continued to grow, albeit at a slower rate, whereas control plants exhibited chlorosis (Fig. 4A). When challenged with JA (50 μM), control plants showed growth inhibition whereas the transgenic lines survived and continued normal growth (Fig. 4A). The growth inhibition due to the different treatments was further analyzed by measuring the total leaf areas (TLA) on wild type and transgenic plants (Fig. 4B). In the absence of stress, similar TLA values were scored in transgenic and wild-type plants ($\approx 62\ mm^2$). These values decrease in the presence of NaCl, ABA and JA (Fig. 4B). At 100 mM NaCl, a reduction of 80% in the TLA value was registered in wild-type plants, whereas in transgenic lines, the

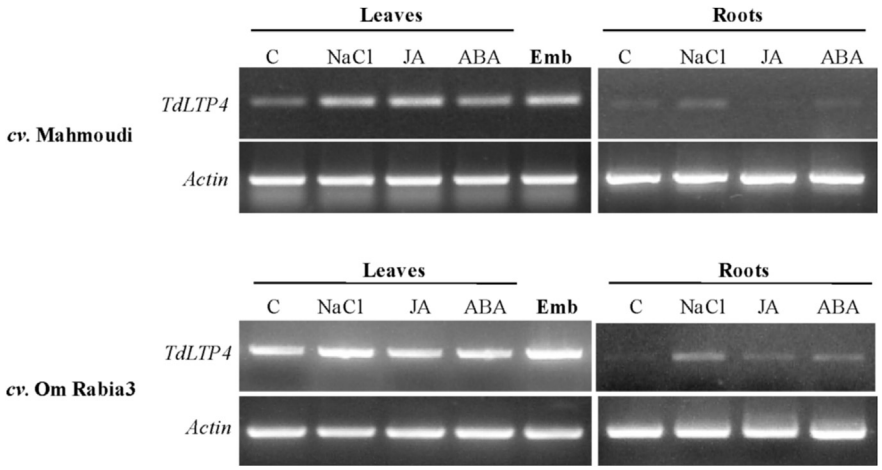


Figure 2. Expression level of TdLTP4 in tissues of salt-sensitive Mahmoudi and salt tolerant Om Rabia3 varieties. Lane 1, control tissues; lane 2: NaCl treated tissues; lane 3, JA treated tissues; lane 4, ABA treated tissues; Emb, Embryo tissues; A 380-bp Actin fragment was amplified by RT-PCR as internal control.

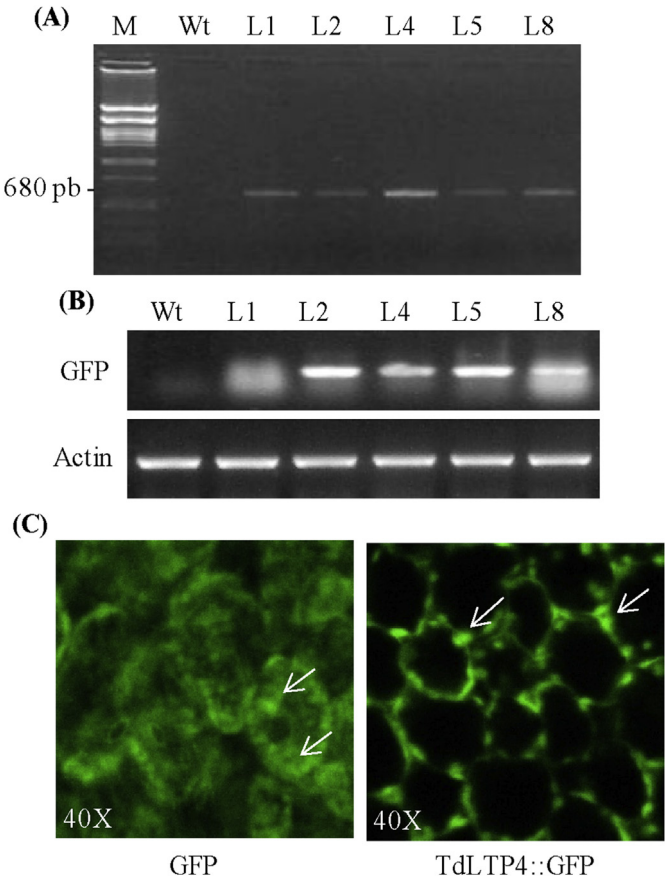


Figure 3. PCR, RT-PCR and subcellular localization of TdLTP4 in transgenic *Arabidopsis* lines. (A), Analysis by PCR of HPT gene in transgenic *Arabidopsis* lines. (B), Analysis by RT-PCR analysis of GFP expression in *Arabidopsis* transgenic lines. A 380-bp Actin fragment was amplified by RT-PCR as internal control. (C), Confocal microscopy images of TdLTP4 transgenic *Arabidopsis* leaves. Transgenic plants overexpressed GFP alone were used as a control.

TLA reduction was between 26% and 39%. At 20 μM ABA, the decrease in the TLA values was 78% and 30% in wild type and transgenic plants respectively. For plants treated with 50 μM JA, the decrease in the TLA values was 62% and 31% in wild type and transgenic plants respectively.

We also measured the shoot fresh weight (Fig. 3C) and the relative water content (RWC) (Fig. 4D) of the different transgenic lines in comparison with the control plants. In the absence of stress, similar shoots fresh weight and RWC were scored for all plants. However, when plants where treated with different stresses, a reduction of ~75% in fresh weight leaves and RWC were registered in the Wt plants (Fig. 3C and D). However, the reduction in fresh weight and RWC were slight in the transgenic lines treated with the different stresses, about ~25% (Fig. 4C and D).

3.5. The TdLTP4 transgenic plants are less sensitive to jasmonate

We quantified and compared the root elongation of TdLTP4 transgenic lines with that of Wt *Arabidopsis* plants treated with JA (Fig. 5A). The root elongation of Wt decreased dramatically with the addition of JA compared to transgenic lines (40%) (Fig. 5B). By contrast, the L2 displayed the highest growth rate which was slightly affected by JA (90%) (Fig. 5B). These data suggest an interaction between LTP and the defense signaling molecule jasmonic acid and the resulting complex is able to interact with a plasma membrane-located receptor (Buhot et al., 2004).

As it is well established that JA acts as a key signaling molecule in defense against pathogens and insects, it might be possible that TdLTP4 affects, via the down-regulation of some JAZ-encoding genes, these defense responses in *Arabidopsis*. To gain further insights into how the down-regulation of JAZ genes and the jai-like phenotype are connected in the LTP transgenic plants, we monitored the expression profiles of four JA-responsive genes (VSP2, LOX3, PR1 and PDF1.2) in the TdLTP4 lines. VSP2 (AT5G24770) and LOX3 (AT1G17420) are both markers for wound responses, whereas PR1 (AT2G14610) and PDF1.2 (AT5G44420) are pathogen-responsive genes (Table 1). As shown in Fig. 5C, the JA-induced expression of the VSP2 and LOX3 genes in the L2, L4 and L8 lines is moderately diminished compared with that observed in Wt plants. In contrast, the basal level of expression of PR1 is enhanced in TdLTP4 transgenic lines. In addition, PR1 and PDF1.2 show a higher expression level in TdLTP4 lines after JA treatment (Fig. 5C). The degree of induction of the PDF1.2 gene is remarkably higher in the transgenic lines (Fig. 5C). It seems that TdLTP4 may interfered with the JA signaling pathway, resulting in the higher JA induction of PR1 and PDF1.2 genes and compromised expression of VSP2 and LOX3 genes in the transgenic lines.

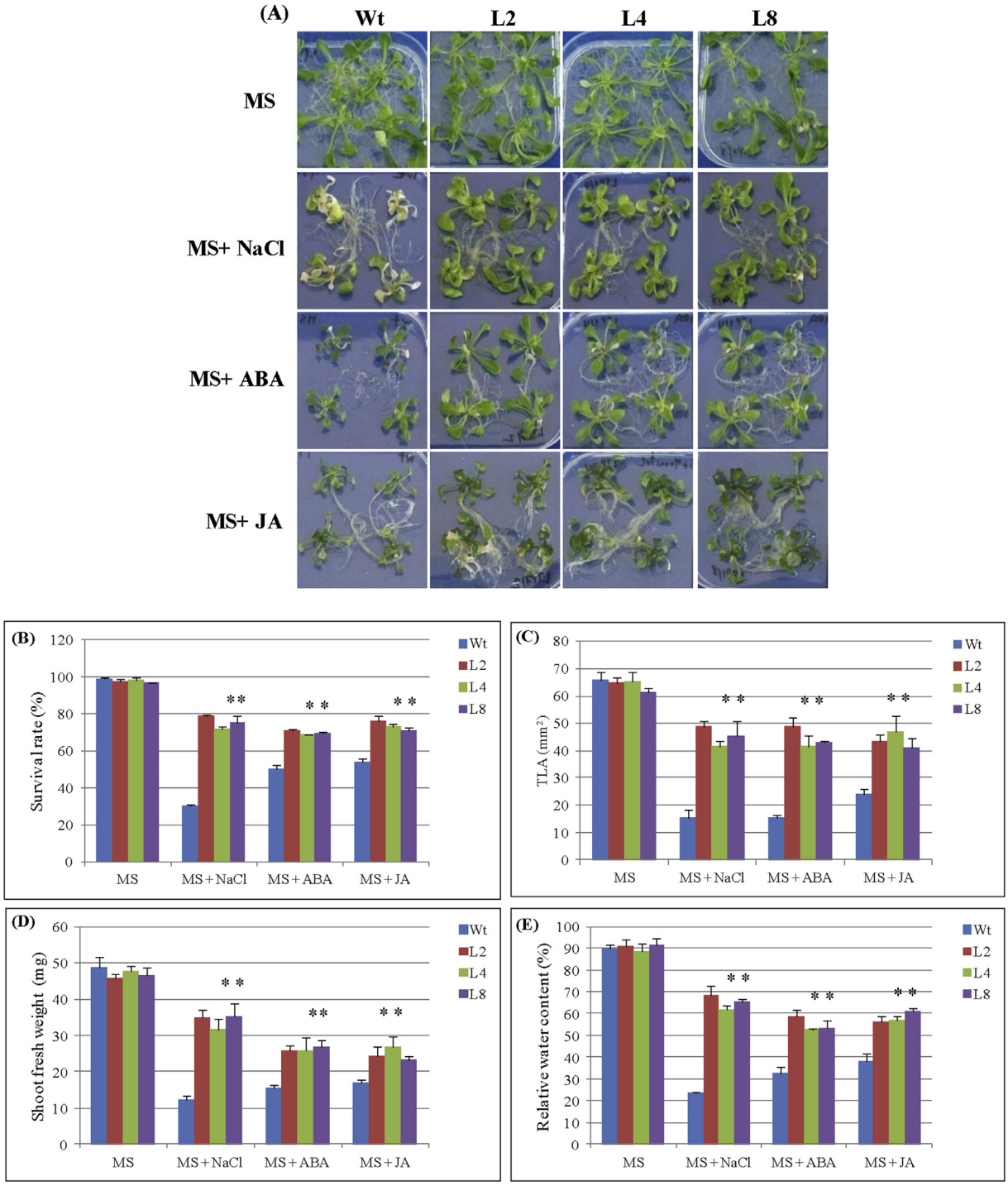


Figure 4. Effect of stress treatments on growth of wild type and transgenic *Arabidopsis* plants. (A), *Arabidopsis* plants grown in standard culture medium during one week (the first two true leaves have already emerged) were transferred to culture plates containing 100 mM NaCl, 20 μM ABA or 50 μM JA. The photograph was taken 10 days after transplantation. (B), Survival rate of *Arabidopsis* transgenic plants and control after 4 week of stress treatment. (C), Total leaf area (TLA) of individual leaves from wild type and transgenic *Arabidopsis* plants. Values are means of five replicates of one expanded leaf per plant. (D), Shoots fresh weight of *Arabidopsis* transgenic lines and control after 10 days of stress. (E), Relative water content (RWC) of transgenic and control plants after 10 days of stress. Values are mean ± SE (n = 5). Asterisks indicate significantly greater mean values compared to *Arabidopsis* control line ($P < 0.05$).

3.6. The TdLTP4 transgenic lines show enhanced tolerance to oxidative stress

HPLC was performed on ten days-old seedlings of TdLTP4 and Wt plants to measure the amounts of Ascorbic Acid (AsA). Compared with the Wt, our data show that the level of AsA is 1,5 fold higher in the L2 line (Fig. 6A and B). AsA is the major antioxidant in the plant cell, which acts either as a scavenger of toxic reactive oxygen species (ROS) including the superoxide radical (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), or as a substrate for a specific ascorbate peroxidase in the detoxification of H_2O_2.

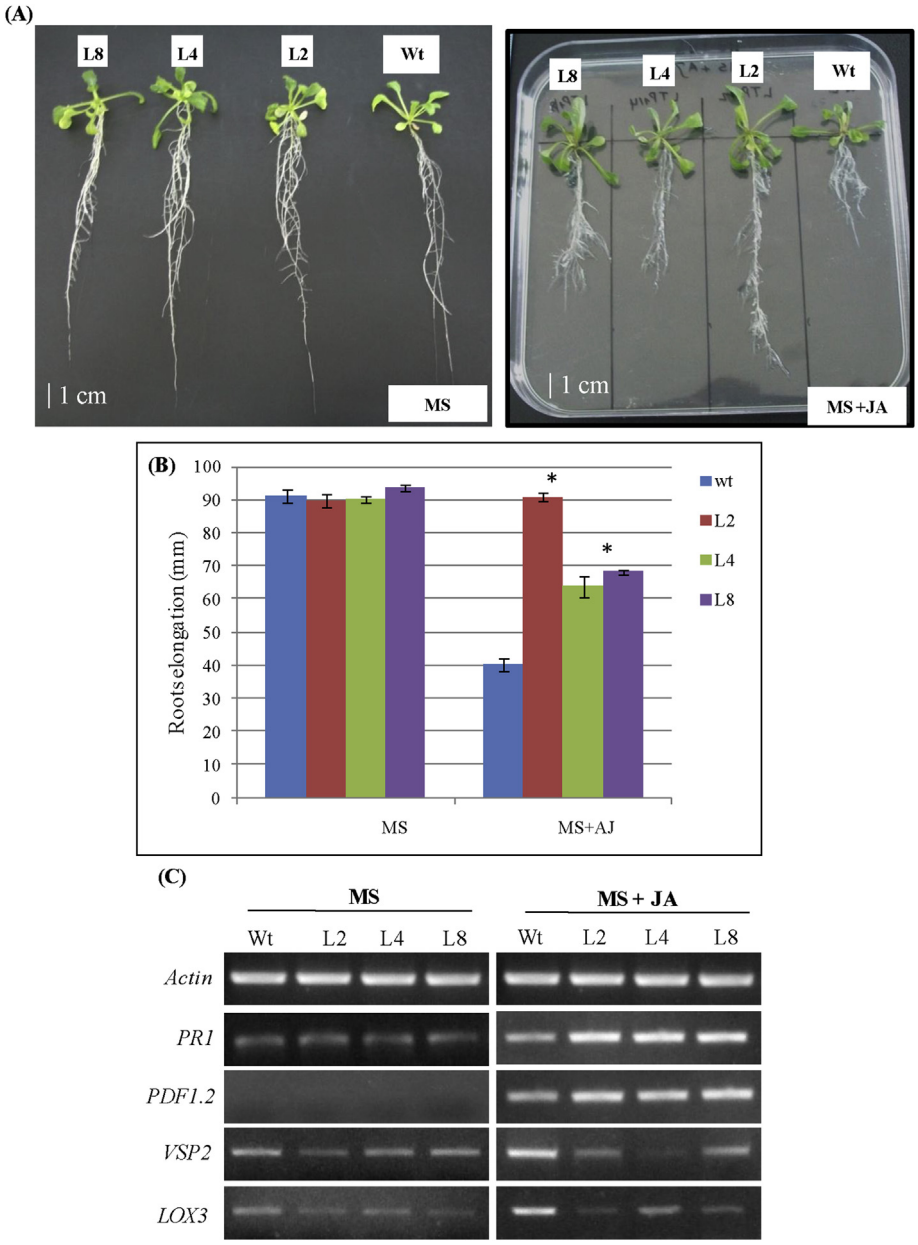


Figure 5. Sensitivity to JA of control and transgenic *Arabidopsis* lines. (A), Three-week-old seedlings were grown on standard MS agar plates containing 50 μM JA. (B), Effect of JA treatment on root growth of control and transgenic lines. Root lengths were measured on 3-week-old seedlings grown as in A. Values are mean ± SE (n = 4). Asterisks indicate significantly greater mean values compared to *Arabidopsis* control line ($P < 0.05$). (C), RT–PCR analysis of JA-regulated genes in control and transgenic lines. Fourteen-day-old seedlings were not treated (MS) or treated with 50 μM JA (MS + JA) during 6 h. A 380 bp Actin fragment was amplified by RT-PCR as internal control.

Table 1
Sequence of the primers used in RT–PCR analysis.

Gene accession no.	Primer name	Sequence
AT2G14610	PR1_F	CTCAAGATAGCCCACAAGAT
	PR1_R	CATTACTTCATTAGTATGGC
AT5G24770	VS_FR	CATCATAGAGCTCGGGATT
	VS_RV	GAAGGTACGTAGTAGAGTG
AT5G44420	PD_FR	CTAAGTTTGCTTCCATCATC
	PD_RV	CATGGGACGTAACAGATAC
AT1G17420	LOX_FR	CTACTATCCAAACCCGAA
	LOX_RV	CTCTCACCAATATACTCC

We therefore addressed the question of whether the enhanced accumulation of AsA in TdLTP4 transgenic lines leads to an improved tolerance to oxidative stress. For this purpose, we compared the effects of exogenous H_2O_2 on the growth of WT, L2; L4 and L8 plants. As illustrated in Fig. 6C, the growth inhibition in TdLTP4 lines is much less severe than in Wt plants, indicating that these transgenic lines may have enhanced tolerance to the oxidative stress caused by H_2O_2.

3.7. Effect of TdLTP4 overexpression in Arabidopsis on fungal resistance

As TdLTP4 exhibit antifungal activity *in vitro*, we were interested to investigate the fungal resistance of TdLTP4-expressing transgenic *Arabidopsis*. For this purpose, detached leaf fungal assays using phytopathogenic fungi were performed. The results of the

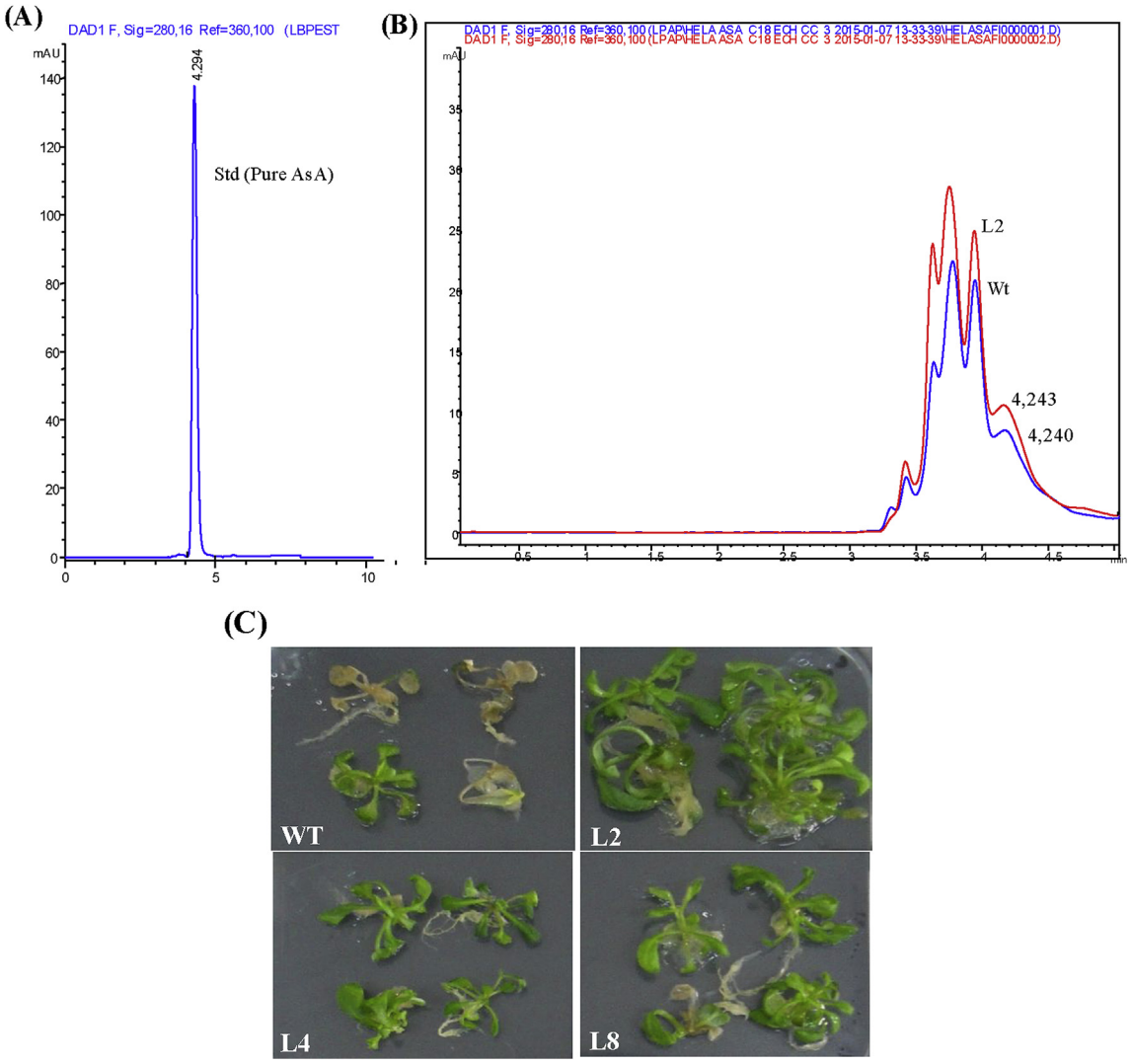


Figure 6. Ascorbate (AsA) content determination in Wt, L2 line of *Arabidopsis* by HPLC analysis and effect of stress by H_2O_2. (A) standard (pure AsA). (B) Levels of AsA in Wt, L2 line of *Arabidopsis*; Data are representative of three independent experiments. (C), Effect of *in vitro* stress treatments with 3 mM H_2O_2 on the growth of Wt and TdLTP4 transgenic *Arabidopsis* plants.

fungal infection assay using leaves from transgenic *Arabidopsis* plants revealed significant resistance against two phytopathogenic fungi *Botrytis cinerea* and *Alternaria solani* 10 days after inoculation (Fig. 7A). The leaves from the control plants showed spreading lesions (area 18–22 mm^2) around the site of contact (Fig. 7A and B). However, the leaves from LTP-expressing transgenic plants showed smaller and restricted lesion (area of lesion 3–7 mm^2) around the site of contact (Fig. 7A and B). These data outline the contribution of TdLTP4 in plant resistance to pathogenic fungi.

4. Discussion

Plant lipid-transfer proteins (LTPs) are abundant, small, lipid binding proteins that are capable of exchanging lipids between membranes *in vitro*. Despite their name, a role in intracellular lipid transport is considered unlikely, based on their extracellular localization. A number of other biological roles, including antimicrobial defence, signaling, and cell wall loosening, have been proposed, but conclusive evidence is generally lacking, and these functions are not well correlated with *in vitro* activity or structure (Ng et al., 2012).

Most importantly, LTPs may be important components of direct defense against fungal pathogens, and early evidence of this role came from *in vitro* screens of protein extracts from barley (*H. vulgare*) (Molina and Garcia-Olmedo, 1993), *A. thaliana* (Segura et al., 1993), and spinach (*Spinacia oleracea*) (Segura et al., 1993). Each of these isoforms inhibited growth of bacterial and fungal pathogens to varying degrees, depending on the isoform and the pathogen.

In this work, we have isolated and characterized a novel durum wheat lipid transfer protein called TdLTP4. The Phylogenetic analysis of amino acid sequences indicated that TdLTP4 had higher sequence identity and similarity to LTPs of wheat (ABO28527 and AAV28706) and barley (CAA85484) (Fig. 1B). TdLTP4 has a low sequence identity to AtLTP of *A. thaliana* (AED97170) and they belong to two different groups as showed in the Fig. 1B. Amino acid sequence analysis of TdLTP4 suggested its typical features of plant LTPs, viz., absence of tryptophan and phenylalanine, and conservation of eight cysteine residues that could form a network of disulfide bridges necessary for the maintenance of the tertiary structure of the molecule together with the central helical core, while the variable loops would provide the sequences required for

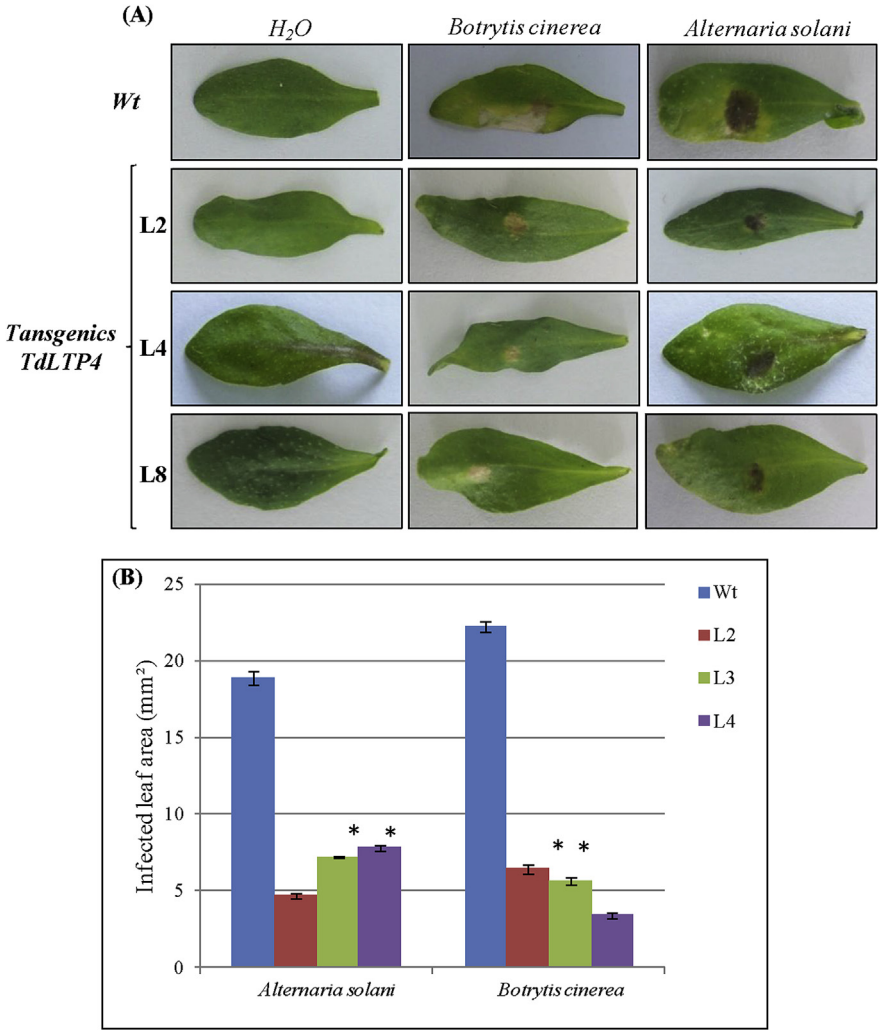


Figure 7. Transgenic resistance of *Arabidopsis* leaves expressing lipid transfer protein (*TdLTP4*). (A), Detached leaf from control *Arabidopsis* plant showed spreading lesions due to *Botrytis cinerea and Alternaria solani* infection. Detached leaf from TdLTP4-expressing *Arabidopsis* plant showed smaller and restricted lesion. A control of inoculation with water was used. Observations were made 10 days after inoculation. (B), Measure of infected leaf area of detached leaves from control and transgenic *Arabidopsis* plants. Values are means of five replicates of one expanded leaf per plant. Values are mean ± SE (n = 5). Asterisks indicate significantly greater mean values compared to *Arabidopsis* control line ($P < 0.05$).

the specific functions of the proteins (Jose-Estanyol et al., 2004). Three-dimensional structure of wheat TdLTP4 was simulated by the homology modeling tool MOE using the NMR structure of maize ns-LTP (1AFH) as template. Accordingly, the global fold involving four helical fragments connected by three loops and a C-terminal tail without a regular secondary structure stabilized by four disulfide bridges presumably formed due to the presence of eight cysteine residues were predicted in wheat TdLTP4. The striking feature of this structure is the existence of an internal hydrophobic cavity running through the whole molecule. As a whole, the global fold of this protein is similar to that of previously described LTP extracted from wheat seeds (Gincel et al., 1994).

The expression pattern of LTPs is complex, characterized by strong developmental and tissue specificity with distinct patterns of expression for the different genes. For example, a strong cell specificity, mainly in epidermal cells, has been observed in both maize seedling and carrot embryos (Sterk et al., 1991). Vroemen et al. (1996) showed that the AtLTP1 gene from *Arabidopsis thaliana* is highly expressed in embryo protoderm, which is the precursor of the plant epidermis. Using in situ hybridization, as well as by following AtLTP1 promoter-ß-glucuronidase transgene expression, it was observed that pattern formation in the embryo is reflected in the position-specific expression of this LTP gene (Vroemen et al., 1996).

The expression level of the wheat TdLTP4 gene was studied in both cultivars, Mahmoudi and Om Rabia3 under different stress conditions (NaCl; ABA and JA) and using different tissues (roots; leaves and embryos). Our data showed that TdLTP4 was induced by different stress treatments. This expression was clearly higher the embryo tissues of the two wheat varieties (Fig. 2). Developing embryos have been largely used as an experimental model to study desiccation tolerance, due to their ability to survive extreme water loss during the final maturation stage of development. Jang et al. (2004) showed that expression of the TaLTP1 gene was increased by NaCl treatment and water stress. Similar finding have been reported by several researchers (Torres-Schumann et al., 1992; Trevino and O'Connel, 1998).

Transcript accumulation of TdLTP4 was lower in leaves and roots of Mahmoudi than in Om Rabia3 (Fig. 2). The existence of differences in the patterns of expression of TdLTP4 between the two cultivars supports the idea that there is a relation between LTP and abiotic stress. This information will be useful in selecting material for future breeding programme.

Next, to elucidate the contribution of TdLTP4 to abiotic and

biotic stress, *Arabidopsis* transgenic plants overexpressing this gene were generated in this work. Subcellular localization of TdLTP4 shows that it was localized in the peripheral cell layers and most abundant in the cell walls of transgenic lines (Fig. 3C). The cellular location of LTPs has been found to be complex. Although immunolocalization studies on rape seeds indicated a uniform distribution, LTP appears to be concentrated in the peripheral cell layers of maize seedlings. It has also been reported that in Arabidopsis, and castor bean LTPs are mainly (or exclusively) located in the cell wall. These finding are consistent with the presence of a potential peptide signal in the sequence of LTPs.

Our findings show that the TdLTP4 transgenic lines are more tolerant than the wild type plants to salt stress and treatment with ABA. This enhanced tolerance was illustrated by higher growth rates. The growth inhibition due to salt stress or ABA of plants grown on sterile medium was much more pronounced on control than on transgenic plants (Fig. 4A). The chlorosis symptoms appearing under these conditions were also clearly delayed in the transgenic lines. Several reports previously described enhancement of stress tolerance following the expression of different LTP protein genes in plants. In barley, several LTP genes that contain a putative ABA-responsive element and a low temperature-responsive element are induced by low temperatures (White et al., 1994). The relationship between the putative protective role of LTPs and their differential expression in various stress conditions needs to be determined. In addition, the mechanisms by which LTPs ensure an enhanced tolerance to various stresses remains unknown.

As the generation of ROS is increased under stress conditions, AsA is believed to contribute actively in enhancing tolerance to various environmental stresses (Noctor and Foyer, 1998). As a scavenger of ROS, it is likely that the AsA accumulating in higher amounts in TdLTP4 lines has helped plants to withstand H_2O_2-mediated oxidative stress. This finding serves as a proof of concept and demonstrates that TdLTP4 overexpression contributes (perhaps via AsA) to reducing the accumulation of superoxide generated by stress.

Recently, the hypothesis that LTPs could be involved, in plant defense had emerged. It has been shown that a tobacco type I LTP (NtLTP1) binds the defense signaling molecule jasmonic acid (JA) and the resulting complex is able to interact with a plasma membrane-located receptor (Buhot et al., 2004). The authors also suggested that the formation of the complex provokes a conformational change on LTP that facilitates its recognition by the receptor. This LTP1-JA complex, when applied to the *Nicotiana tabacum* plant, induces long distance protection against *P. parasitica*. However, it has not been demonstrated whether the complex on the plasma membrane receptor is the requirement for the production of the mobile signal (Caaveiro et al., 1997).

We have tested the interaction between TdLTP4 and JA in transgenic lines overexpressed TdLTP4. Our results showed that transgenic lines are more tolerant than the wild type plants to JA (Figs. 4 and 5). This suggests that TdLTP4 might be involved in JA response and signaling since it induced highest protection level of Arabidopsis plantlets against *B. cineria* and *A. solani*. This is an agreement with the results previously reported for NtLTP1-JA complex in tobacco (Buhot et al., 2004).

The recent discovery of the JAZ family dramatically changed our understanding of JA signaling in *Arabidopsis*. It has indeed been shown that MYC2 transcription factor antagonistically regulates two JA signaling pathways controlling either pathogen or wounding responses. In this differential regulation, MYC2 represses pathogenresponsive genes (such as PR1 and PDF1-2) and activates genes involved in wound responses (VSP2 and LOX3) (Lorenzo et al., 2004). It is already well established that the JA insensitivity of *jai3* or *jai1* mutants is related to defects in MYC2-dependent expression of JA-responsive genes. It is hence possible that the expression of a number of MYC2- dependent genes is impaired in LTP-overexpressing lines, leading to a partial insensitivity to JA. In other words, TdLTP4 may have interfered with MYC2 function, resulting in the higher JA induction of PR1 and PDF1.2 genes and compromised expression of VSP2 and LOX3 genes in the transgenic lines.

Additional evidence has been provided through use of transgenic plants overexpressing LTPs. Transgenic rice containing the homologous ns-LTP gene (*Ace*-AMP1) of *A. cepa* showed antimicrobial activity towards *M. Grisea*, *R. Solani* and *X. Oryzae* pv. *Oryzae* (Patkar and Chattoo, 2006). A barley family 1 LTP overexpressed in *Arabidopsis* or tobacco (*N. tabacum*) led to enhanced resistance to *P. syringae* (Molina and Garcia-Olmedo, 1997). Findings from detached leaf fungal assays showed an antifungal resistance of transgenic plants expressing TdLTP4 (Fig. 7). Our results reported that TdLTP4 from wheat has antifungal activity against two phytopathogenic fungi (*B. cinerea* and *A. solani*) that attack economically important crops such as tomato, potato and grape. Thus, the wheat *TdLTP4* gene can be efficiently employed for the genetic engineering of crops for disease resistance.

Author's contribution

Conceived and designed the experiments: Faiçal Brini; Moez Hanin; Khaled Masmoudi and Abdelaziz Hmyene.

Performed the experiments: Hela Safi; Walid Saibi; Meryem Mrani Alaoui.

Analyzed the data: Faiçal Brini; Moez Hanin.

Contributed reagents/materials/analysis tools: Walid Saibi; Faiçal Brini.

Wrote the paper: Faiçal Brini; Moez Hanin.

Acknowledgments

This work was supported jointly by grants from the Ministry of Higher Education and Scientific Research, Tunisia and the Ministry of Higher Education and Scientific Research and Professional training of Morroco; Project code 08/TM/30.

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