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A wheat lipid transfer protein 3 could enhance the basal thermotolerance and oxidative stress resistance of *Arabidopsis*

Fei Wang [a,b,1], Xin-shan Zang [a,b,1], Muhammad Rezaul Kabir [a,b], Ke-lu Liu [a,b], Zhen-shan Liu [a,b], Zhong-fu Ni [a,b], Ying-yin Yao [a,b], Zhao-rong Hu [a,b], Qi-xin Sun [a,b], Hui-ru Peng [a,b,*]

[a] State Key Laboratory for Agrobiotechnology, Key Laboratory of Crop Heterosis and Utilization (MOE), Key Laboratory of Crop Genomics and Genetic Improvement (MOA), Beijing Key Laboratory of Crop Genetic Improvement, China Agricultural University, Beijing 100193, China
[b] National Plant Gene Research Centre (Beijing), Beijing 100193, China

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ABSTRACT

Wheat (*Triticum aestivum L.*) is one of the major grain crops, and heat stress adversely affects wheat production in many regions of the world. Previously, we found a heat-responsive gene named *Lipid Transfer Protein* 3 (*TaLTP3*) in wheat. *TaLTP3* was deduced to be regulated by cold, ABA, MeJA, Auxin and oxidative stress according to cis-acting motifs in its promoter sequences. In this study, we show that *TaLTP3* is responsive to prolonged water deficit, salt or ABA treatment in wheat seedlings. Also, *TaLTP3* accumulation was observed after the plant suffered from heat stress both at the seedling and the grain-filling stages. TaLTP3 protein was localized in the cell membrane and cytoplasm of tobacco epidermal cells. Overexpression of *TaLTP3* in yeast imparted tolerance to heat stress compared to cells expressing the vector alone. Most importantly, transgenic *Arabidopsis* plants engineered to overexpress *TaLTP3* showed higher thermotolerance than control plants at the seedling stage. Further investigation indicated that transgenic lines decreased H_2O_2 accumulation and membrane injury under heat stress. Taken together, our results demonstrate that TaLTP3 confers heat stress tolerance possibly through reactive oxygen species (ROS) scavenging.

1. Introduction

High temperature during the grain-filling stage causes deleterious effects on the yield and quality of crop products (Peng et al., 2004). Heat stress has many adverse impacts on plants, including injury of membrane stability, production of reactive oxygen species (ROS) and imbalance of cell homeostasis. Plants adopt various mechanisms to relieve these damages: the HSFs (Heat Shock Factors) and HSPs (Heat Shock Proteins) are well known to play vital roles in the HSR (Heat Shock Response); otherwise, the Mitogen Activated Protein Kinase (MAPK) and Calcium Dependent Protein Kinase (CDPK) cascades are activated and the compatible solutes can be generated (Wahid et al., 2007). In other words, the transcriptome, proteome, metabolome and lipidome of plants are reprogrammed to counter the heat stress. A previous study analyzed the genome-wide expression profiles of wheat grown under high temperature and found a large number of genes that responded to heat stress. The *non-specific Lipid Transfer Proteins* (*nsLTPs*) were identified as being responsive to heat stress in different patterns at different time points (Qin et al., 2008).

Plant nsLTPs are of low molecular weight and basic proteins that have the ability to exchange lipids between membranes in vitro (Yeats and Rose, 2008). There is growing evidence that nsLTPs are involved in many biological roles, such as anther development, stress resistance and different signaling pathways. A lipid transfer protein *CaMF2* (*Capsicum annuum*) is specifically expressed in flower buds of the male fertile line and plays a vital role in pollen development (Chen et al., 2011). *OsC6* encodes a lipid transfer protein in rice (*Oryza sativa*), which is widely distributed in anther tissues and plays a crucial role in regulating postmeiotic anther development (Zhang et al., 2010). Several findings described that LTPs contribute to plant tolerance to environmental stresses, especially to biotic stress. The OsLTP110 protein could inhibit the germination of *Pyricularia oryzae* spores in vitro (Ge et al., 2003) and overexpression of the *LTP* gene could bring a substantial resistance to biotic stresses. For instance, transgenic tobacco overexpressing either the *TaLTP3F1*, *HvLTP2* (*Hordeum vulgare*) and *NtLTP1* (*Nicotiana tabacum*) genes showed fungal, bacterial and aphid resistance, respectively (Choi et al., 2012; Kirubakaran et al., 2008; Molina and García-Olmedo,

Abbreviations: IWGSC, International Wheat Genome Sequencing Consortium; ABRE, ABA responsive element; GARE, gibberellin responsive element; MS, Murashige and Skoog medium; ROS, reactive oxygen species; NBT, nitroblue tetrazolium; H_2DCFDA, 2,7-dichlorodihydrofluorescein diacetate.

* Corresponding author at: College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China.
E-mail addresses: wangfeihh@163.com (F. Wang), dulzxs@163.com (X. Zang), rezaulw@yahoo.com (M.R. Kabir), liukelu731@gmail.com (K. Liu), shanzhenliu@163.com (Z. Liu), wheat3392@cau.edu.cn (Z. Ni), yaoyingyin@163.com (Y. Yao), hzr803@163.com (Z. Hu), qxsun@cau.edu.cn (Q. Sun), penghuiru@cau.edu.cn (H. Peng).

[1] Fei Wang and Xin-shan Zang contributed equally to this work.

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2003). In addition, *nsLTP* genes could respond to abiotic stresses including NaCl, drought, cold and wounding (Cameron et al., 2006; Torres-Schumann et al., 1992; Yubero-Serrano et al., 2003), and recently it has been shown that *ZmLTP3* and *AtLTP3* could improve plant survival under salt and drought stresses, respectively (Guo et al., 2013; Zou et al., 2013). Finally, nsLTPs function in signaling transduction pathways. For example, the transcription of *CaLTP1* (*C. annuum*) was induced by ABA, Ethylene, MeJA and SA in pepper leaves (Jung et al., 2003), and ZmLTP (*Zea mays*) was found to bind CaM in a calcium-independent manner (Li et al., 2008). To sum up, these results provide new insight into the physiological function of LTPs.

The *nsLTPs* in wheat are a big family and play important roles in many physiological processes. The mRNAs and the proteins of the *nsLTPs* would accumulate in the wheat grains whether grown under moderate or severe high temperature (Altenbach et al., 2008). In a previous study, l56 wheat putative *nsLTPs* genes were identified by comparative analyses of rice *nsLTP* genes and wheat (*Triticum aestivum*) EST sequences. The 122 wheat non-redundant *nsLTPs* were organized into eight types and 33 subfamilies based on phylogenetic analysis (Boutrot et al., 2008). The *nsLTP* gene family is much bigger and more complex in *Triticum* than in the *Arabidopsis* and *Oryza* genomes. Wang et al. (2010) classified the *TaLTP* genes into several groups according to the transcript profile: *TaLTP1*, *2*, *7*, *8*, and *10* clustered in the same group, which were significantly induced by drought and cold treatments, but no notable responses to salinity treatment were observed. *TaLTP5*, *6*, and *11* belong to other groups and showed no detectable response to abiotic stresses in seedlings. Also, the expression of *TaLTP* genes is developmentally regulated in seeds (Boutrot et al., 2005). Different responsive profiles of *TaLTP* genes were identified in plant organs by detecting promoter activity fused with GUS in transgenic *Arabidopsis* or *Oryza* (Boutrot et al., 2007). The variable expression profile of *TaLTPs* suggested that *TaLTPs* may be involved in multiple biological processes.

The *TaLTP3* belongs to the Type 1 (9 kDa) nsLTPs, and responds to many environmental stresses. After *Hessian fly* infection, an increase in *TaLTP3* mRNA in wheat was observed, which suggested that the *TaLTP3* gene products might work in wheat–*Hessian fly* interactions (Jang et al., 2005; Saltzmann et al., 2010). Also, the transcripts of *TaLTP3* were stimulated by heat, darkness, SA, ethylene and H_2O_2 (Jang et al., 2005; Wang et al., 2010). Although it has been reported that *TaLTP3* could respond to several biotic and abiotic stresses, its function has not been identified hitherto. In this study, the coding and promoter region of *TaLTP3* were isolated from 'Chinese Spring', and the expression pattern of *TaLTP3* in the seedlings treated by various stresses was studied. A transgenic approach was used to annotate the function of *TaLTP3* in heat stress.

2. Materials and methods

2.1. Plant materials and stress treatments

The wheat genotype 'Chinese Spring' (CS) was used in this study. The seeds were first surface-sterilized in 1% sodium hypochlorite and then soaked in the water. After sprouting, the seedlings were transferred into petri dishes and cultured in a growth chamber at 22 °C under a 16-h-light/8-h-dark photoperiod. For abiotic stress, the 10-day-old seedlings were irrigated with 20% PEG6000, 100 mM NaCl, 100 μM ABA separately or heat stressed at 40 °C. All the treatments started at the beginning of the light period. The leaves were collected and immediately frozen in liquid nitrogen, then stored at −80 °C for further use. Heat stress on the mature plants was performed by exposing the plants (10 days post-anthesis, DPA) to a high temperature of 40 °C, the flag leaves and ears were harvested and stored at −80 °C for RNA analysis.

Arabidopsis thaliana ecotype Col-0 was used as control, together with the *TaLTP3* overexpressing lines, and the plants were grown on Murashige and Skoog (MS) medium [0.8% plant agar (w:v), 3.0% (w:v) sucrose, pH 5.8] in a growth chamber at 22 °C under a 16-h-light/8-h-dark photoperiod. The 7-day-old plants were used for RNA analysis.

2.2. Expression analysis by quantitative RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen), and 2 mg of total RNA was used to synthesize first-strand cDNA using oligo (dT) 15 primer with M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green I premix (Takara, Japan) on a BIO-RAD real-time PCR detection system as described by Qin et al. (2008). Three biological experiments were repeated and the threshold cycles (Ct) of each test target were averaged for triplicate PCR reactions. The relative quantification of target gene transcript levels was performed using the comparative Ct method by the following formula: Relative expression level $= 2^{-\triangle Ct}$, where $\triangle Ct = Ct_{\text{target gene}} - Ct_{\text{actin.}}$ The primer pairs used for qRT-PCR are shown in Table 1.

2.3. Cloning of the CDS and the promoter region of TaLTP3

The CDS of *TaLTP3* was amplified using the specific PCR primers (Table 1). The amplicons were inserted into the pGEM T-vector (Promega) for sequencing. To analyze the promoter sequence of *TaLTP3*, the CDS sequences were used to blast with the wheat genome sequence in the IWGSC (International Wheat Genome Sequencing Consortium) and the 1203 bp nucleotides upstream of the ATG were used for analyzing.

2.4. Subcellular localization of TaLTP3

The subcellular localization of TaLTP3 was performed in *Nicotiana benthamiana* leaves. The full-length CDS of *TaLTP3* was fused with a GFP reporter gene and driven by the CaMV 35S promoter. The constructs with *35S:GFP* or *35S:GFP-TaLTP3* were then introduced into the *Agrobacterium* strain GV3101. Meanwhile, another *Agrobacterium* strain carrying a P19 protein was used to suppress gene silencing (Voinnet et al., 2003). The infiltration of *N. benthamiana* was performed as described previously (Wroblewski et al., 2005). Transformed leaves were observed under a Confocal Laser Scanning Fluorescence Microscopy (LSFM, Carl Zeiss) by a laser of 488 nm.

2.5. Genetic transformation and thermotolerance assay in yeast

The CDS of TaLTP3 was cloned into the yeast expression vector *pYES2* (Invitrogen, USA) behind the *GAL1* promoter, which is induced by

Table 1
The primer pairs used in this study.

Primers	Sequences (5′–3′)	Function
TaLTP3-F	CCATGGCTCGTCTCAACAG	CDS amplification
TaLTP3-R	ATGCACGTCGGAAGGATTAT	
TaLTP3-QF	GTGCGGGCAGGTGGACT	qRT-PCR
TaLTP3-QR	CTGGTGGCGAGGCTCTTG	
TaLTP3-PF	TTCTCCTCCCCACTCTTACC	Promoter amplification
TaLTP3-PR	CCTCCTCCACACAACGATAC	
TaLTP3-BamHI	CGCGGATCCGTTGACATTGTTGCAGTTGG	Subcellular location
TaLTP3-EcoRI	CCGGAATTCCCATGGCTCGTCTCAACAG	vector construction
TaLTP3-KpnI	GGGGTACCACCATGGCTCGTCTCAACAG	Transgenic *Arabidopsis*
TaLTP3-XbaI	GCTCTAGAATGCACGTCGGAAGGATTAT	or yeast vector construction
TaActin-F	GGAATCCATGAGACCTAC	qRT-PCR
TaActin-R	GACCCAGACAACTCGCAAC	
ATHsa32-QF	CTTCGTAGTGGTCCTTCAGC	AT4G2132, qRT-PCR
ATHsa32-QR	TAAACACCGTTCAGCCTTTC	
AThsp101-QF	GATGGAGAAGAAGGTGGTGA	AT1G74310, qRT-PCR
AThsp101-QR	GAAAGCTCAAGTCATACAA	
ATACT8-QF	TGCAGACCGTATGAGCAAAG	AT1G49240, qRT-PCR
ATACT8-QR	CCGTCATGGAAACGATGTCT	