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Real time and *In-Silico* Based Analysis of Heat Stress Responsive Transcription Factor MBF1c from Wheat

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Abstract

Heat stress adversely affect total yield of wheat, to cope up with stress plants respond by overxpressing their heat stress related genes and transcription factors. In this study we have isolated highly heat responsive transcription factors TaMBF1c (Multibridging factor) from wheat. This gene was isolated based on real time based fold change expression under control and heat stressed conditions. TaMBF1c shown 14.2 and 19.5 fold upregulation at 37°C and 42°C. Further, deep *in-silico* studies have shown their role related to heat stress response. This transcription factor would be a powerful genomic resource for developing abiotic stress tolerant crops.

Key words: *Wheat, heat stress, Transcription factor, real time, MBF1c, in-silico*

1. Introduction

Global climate change have a negative effect on earth, the temperature is increasing day by day. As a result, plants have to face lot of environmental fluctuations. These fluctuations usually comes in the form of drought and heat stress. Of them, heat stress in most devastating which reduces the yield production in major food crops. Wheat is one of the most staple food crop in the world but heat stress is having a negative effect on its yield. The negative impacts include programmed cell death, production of ROS (Reactive Oxygen Species), breakdown of thermo-labile proteins, overexpression of HSPs (Heat Shock Proteins) and HSFs (Heat stress specific transcription factors) (Xue and McIntyre 2011, Mittler et al 2012, Grover et al 2013). Heat stress protection genes like HSPs are expressed to a very high levels to combat heat stress. Hsfs (Heat shock factors) bind to heat shock elements with a consensus sequence of GAAnnTTCnnGAA which are present in promoter region of many HSPs (Xue et al, 2014) Of these, MBF1c (Multiprotein bridging factor1c) is one of the several important heat stress specific transcription factor which plays an important role in providing heat stress tolerance.

MBF1c acts as transcriptional co-activator and make a bridge between TBP (TATA-Box Binding Protein) and DNA binding regulators (Millership et al 2004). It has been shown that MBF1c regulates heat stress response in *Arabidopsis* (Suzuki et al. 2008). It is also suggested that its overexpression has improved tolerance against both abiotic (osmotic, heat,) and biotic stress response in plants (Suzuki et al 2005). TaMBF1a from wheat was also induced by biotic stress (stripe rust pathogen) (Zhang et al 2005). A hypothetical model has been suggested which explains the role of TaMBF1c on heat induction, MBF1c acting as bridge between DREB2 (Dehydration Responsive Elemental Binding Protein), HsfA1b (Heat stress specific transcription factor A1b), HsfA2 and TFs (Transcription factors), SA (Salicylic acid) ultimately providing thermotolerance to wheat (Qin et al. 2015).

Abiotic stress severely retards plant growth especially in case of cultivated plants leading to crop yield. Thus, mechanisms to gain insights to develop abiotic stress resistance in wheat is necessary for its improvement. In this study we have taken abiotic stress related TF from wheat

which was cloned, sequenced and analyzed through real time PCR. *In-silico* (protein secondary and 3D structure prediction, disorderness and phylogenetic relationship) based studies which have proved its role in giving heat stress tolerance in wheat.

2. Materials and methods

2.1 Plant material and heat stress conditions

Wheat cv C306 (heat tolerant) were given heat stress at 37°C and 42°C at grain filling stage for 4h in a BOD (Biological Oxygen Demand) incubator by increasing temperature at the rate of 1°C/10 min. Plants grown under normal temperature conditions (25°C) were used as control. Leaf samples were collected following heat stress treatment, frozen immediately in liquid nitrogen and stored at -80°C for future use.

2.2 Total RNA isolation and RIN values observation

Total RNA was isolated from leaves using spectrum plant total RNA kit (Sigma, USA). Any kind of genomic DNA contamination was removed using On column DNaseI digestion (Sigma, USA) according to the manufacturer's instructions. RNA was analyzed on 1.2% TBE gel and quantitatively checked on Nano drop spectrophotometer (Thermo scientific, USA).

2.3 cDNA prep, semi-q and real time PCR analysis

For cDNA preparation 1µg of total RNA was taken from control as well as stressed samples. SuperscriptIII first strand cDNA synthesis kit (Invitrogen, USA) was used for cDNA preparation as per manufacturer's instructions. Semi quantitative PCR was carried out in 50 µl reaction mixture having 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 5 µM forward/reverse primer each, 20 ng of cDNA as template and 5 units of Taq polymerase. List of the primers used in the study are shown in Table 1.

Table 2: List of primers used in the study

Name	5′ to 3′ sequence	Length	Amplicon
flTaMBF1c For	ATG CCG ACG GGC AGG ATG AGC	21	471
flTaMBF1c Rev	TCA CTT TGT CCC GGC GGG CGC	21	
qTaMBF1c For	CGGAAGCTGGACGAGA TGAC	20	105
qTaMBF1c Rev	CGCCTGGCTCCATCCTT TC	19	
qTaactin For	GAAGCTGCAGGTATCC ATGAGACC	24	125
qTaactin Rev	AGGCAGTGATCTCCTT GCTCATC	23	

The PCR reaction was carried in a thermal cycler (Biorad, USA), following steps were taken: Initial denaturation at 94°C for 3 min, 30 cycles for 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, with a final extension of 10 min at 72°C. The amplicons were analyzed on 1.5% TAE gel. For real time PCR, the 20 µl reaction mixture consisted of 1X Sybr green dye (Roche, Germany), 5 µM of forward/reverse primer and 20 ng of template. The reaction was carried out in real time PCR machine (Roche, Germany), having following program: Initial denaturation at 95°C for 3 min, 40 cycles for 95°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec. *TaActin* gene was used as internal control for normalization the transcripts of target genes. The reactions were kept in triplicates, fold change expression was calculated according to $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001).

2.4 Full length gene isolation

Primers for amplifying full length CDS were designed using IDT software tool (www. idtdna.com/) and based on the gene sequence available at NCBI database. TaMBF1c accession no is GQ370008. PCR was carried out in a thermal cycler (Biorad, USA), following steps were taken: Initial denaturation at 94°C for 3 min, 30 cycles for 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, with a final extension of 10 min at 72°C. The amplicons were analyzed on 1.5% TAE gel, further ligated to pGEM-T vector (Promega, USA) and transformed to *E. coli* DH5α cells. The colonies were screened using vector back bone specific primers flanking the insert. The positive clones were inoculated in 5 ml Luria broth consisting of 100 µg/ml ampicillin and incubated overnight at 37°C, 200 rpm. The recombinant plasmid was isolated using mini prep kit (Qiagen, USA). The

isolated plasmid was analyzed for restriction analysis using *Eco*RI enzyme to check the presence of insert.

2.5 *In-silico* based studies

The deduced amino acid from CDS was predicted for theoretical pI, molecular weight, location inside the cell, total number of positively and negatively charged residues, molecular formula, extinction coefficient, instability index, aliphatic index, hydropathicity index. The secondary structure prediction was done using PSIPRED software tool (http://bioinf.cs.ucl.ac.uk/psipred/). The site of glycosylation and phosphorylation was also predicted using PSIPRED software tool. The disordered state of the secondary structure was predicted by using Phyre2 software tool (http://www.sbg.bio.ic.ac.uk/phyre2). For normalized QMEAN4 score and 3D protein structure prediction SWISS MODEL tool was used (https://swissmodel.expasy.org/). Further, for distant evolutionary relationship phylogenetic tree was prepared using MEGA7 software tool (www.megasoftware.net/). The boostrap value was set at 1500 and tree was prepared using neighbour-joining method based on nucleotide as well as amino acid sequence.

3. Results

3.1 RNA isolation, semi qPCR and Real time PCR

The isolated total RNA showed 28S and 18S rRNA bands at respective place. cNDA preparation showed smear in the range in 500 bp to 10 kb (Fig. 1 A-B).

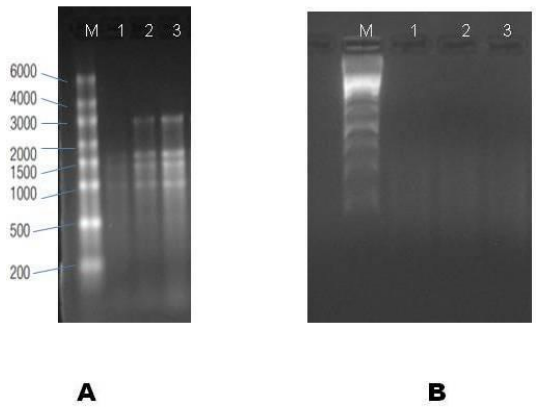

Fig. 1 A-Total RNA preparation from control and heat stressed plants. M is the RNA riboruler. B-cDNA preparation from the isolated total RNA from control and stressed plants. M is the 1 kb DNA ladder.

Fold change expression of TaMBF1c was 14.2 and 19.5 at 37°C and 42°C in wheat cv C306. The semi qRT-PCR results were in accordance with the real time PCR results as expressed by the band intensity on agarose gel (Fig. 2 A-B).

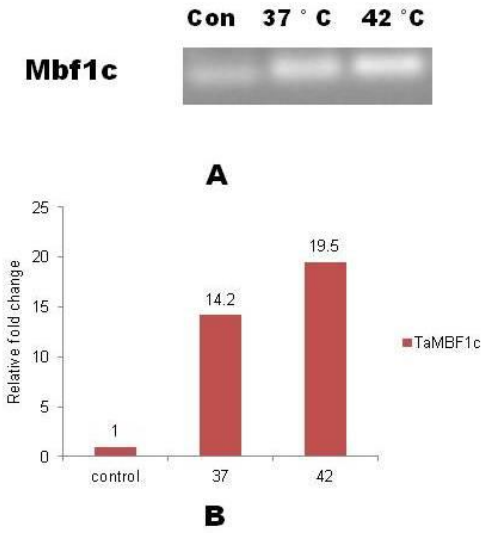

Fig. 2 A-Semi q-PCR of TaMBF1c. B-Real time PCR of TaMBF1c under heat stressed conditions.

3.2 Full length gene isolation

The amplicon TaMBF1c showed a clean band at just below 500 bp marker. After transformation in bacterial cells the colony PCR confirmed the presence of insert. Further plasmid isolated from positive clones were confirmed by restriction analysis, in which pGEM-T backbone and insert of TaMBF1c were clearly seen (Fig.3).

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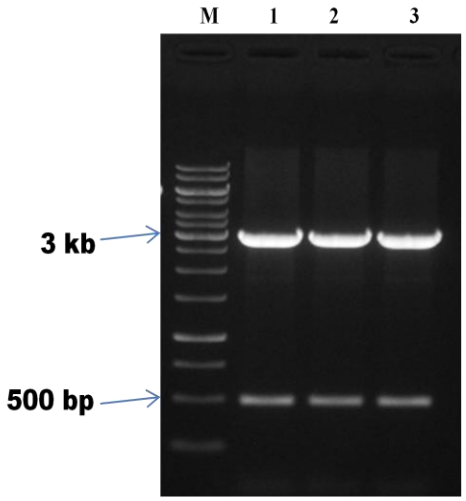

Fig. 3 Restriction digestion analysis of three cloned TaMBf1c amplicon in pGEM-T plasmid. M is the 1 kb DNA marker.

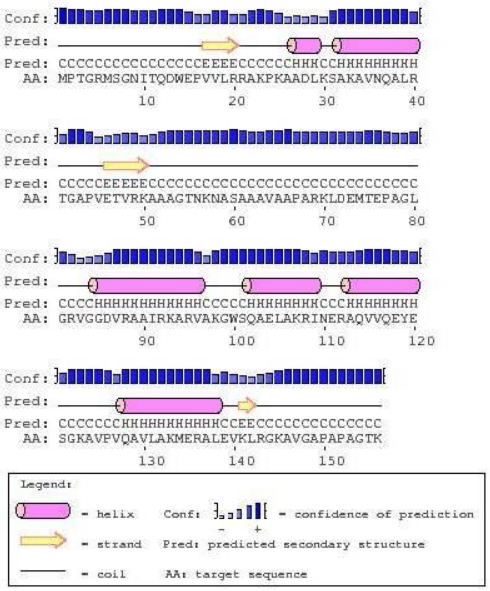

Fig. 4 Secondary structure prediction of TaMBF1c

3.3 *In-silico* studies of TaMBF1c

The protein parameters of TaMBF1c are shown in Table 2.

Table 2. Protein parameters of TaMBF1c

Parameters	**TaMBF1c**
Theoretical pI	10.70
Molecular weight	16326.92 Da
Total number of positively charged residues	29
Total number of negatively charged residues	14
Molecular formula	$C_{706}H_{1200}N_{226}O_{209}S_4$
Extinction coefficient	12490
Absorbance 0.1% (=1g/L)	0.765
Instability index	43.50 (Unstable)
Aliphatic index	80.90
Hydropathicity index	-0.376
Expected location inside the cell	Cytoplasm, extracellular matrix, chloroplast, nucleus

Secondary structure of TaMBF1c had 6 α helices (51%), 3 β strands (3%) and 11 coils, the disorderness of secondary structure was 53% (Fig. 4).

The phylogenetic relationship revealed that our TaMBF1c was closer to *Triticum aestivum*, *Hordeum vulgare*, *Brachypodium distachyon* (Fig. 5).

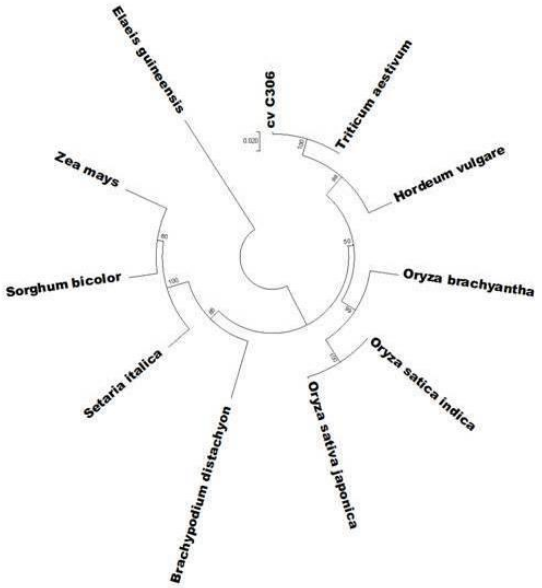

Fig. 5 Phylogenetic relationship of TaMBF1c with that of other plant species.

4. Discussion

To the best of our knowledge, this study is the first report describing real time expression analysis and *in-silico* based studies of highly heat stress responsive transcription factor in Indian bread wheat cv. C306. Chen and Li, 2017 have shown the expression of *BdMBF1c* gene under heat stress in *Brachypodium distachyon* leaves at seeding and heading-stage which was 2751.3 and 358.9 folds. Our *TaMBF1c* showed fold change of 14.2 and 19.5 under heat stress conditions of 37°C and 42°C respectively in cv. C306. Qin et al (2015) showed that overxpression of *TaMBF1c* was able to provide heat stress tolerance to transformed yeast cells and transgenic rice, the rice plants could tolerate even upto 48°C. Six downstream genes were upregulated as a result of its overexpression, till date only hypothetical pathway of MBF1c is known, so further deep insights related to molecular biology and *in-silico* studies are required. These studies would provide information regarding thermotolerance and other abiotic stress tolerance for deciphering heat stress signalling pathway. Bechtold et al (2013) have shown the regulatory role of HsfA1b in controlling the expression of MBF1c by interacting with HSE1b motif present in the promoter region of MBF1c. Overexpression of HsfA members from different plant species have proven to provide upregulation of heat inducible genes in transgenic plants (Yokotani et al, 2008; Liu and Charng, 2013. Our TaMBF1c can be further deployed to raise thermotolerant transgenic crops. The *in-silico* based studies would further help in identification of site to changed at the gene level for better functionality of engineered protein. The phylogeny of TaMBF1c in this study have shown their close relatedness to other poaceae members *Hordeum vulgare* and *Brachypodium distachyon.*

So, the molecular understanding and *in-silico* based studies of this heat stress responsive TF would provide basis for understanding the wheat-heat interaction. Further, climate smart crops can be raised by introducing this TF in important cereal crops with the help of transgenic technology. TaMBF1c can be further modified by site-directed mutagenesis at a particular domain for better functionality of the expressed protein. The diversity of functional protein can be improved by using alternative site splicing (Staiger and Brown, 2013).

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