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Short Communication

RT-PCR amplification and *in-silico* analysis of some drought-responsive putative boiling soluble proteins (hydrophilins) related genes from *Triticum aestivum* L.

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Wheat is second most important food crop grown all over worldwide. However, various abiotic stresses like drought, salt, heat and cold affect wheat productivity. Among all, drought stress is the major abiotic stress factor spreading across the world (Shinozaki and Shinozaki, 2007). Therefore, developing wheat cultivars tolerant to abiotic stresses is essentially required. PBW 175 is well known water stress tolerant cultivar. However, very little is known about molecular basis of tolerance in PBW 175. Therefore, understanding molecular basis of water stress tolerance in PBW 175 will lead to enhanced water stress tolerance in wheat. Plants perceive and respond to water stress by altering physiological, biochemical, and transcriptional processes (Parry *et al*., 2005). A substantial number of genes, gene products and pathways associated with drought response have been identified using a variety of experimental approaches (Gowda *et al*., 2004), yet the function of majority of the genes with altered expression remains unknown (Bray, 2004). Numerous putative genes under drought stress showing enhanced expression have been uncovered by genome wide expression studies (Seki *et al*., 2002). Among many genes that respond to drought stress and which are manipulated by biotechnological approaches, some encode enzymes involved in metabolism, some are active in signalling or in the transport of metabolites or in regulating internal cellular energy (Shinozaki and Shinozaki, 2007). Besides, all the drought responsive regulatory proteins, when plants are exposed to osmotic or treated with ABA, a novel set of proteins accumulates, which although represent just 0.2% of the total genome in plants, however, they play an important role in the stress adaptation in plants. These are typified by high degree of hydrophilicity, enabling them to remain in solution even after boiling the extracts *in vitro. In silico* analysis of BSPs from several kingdoms like : plant, bacteria and fungi have revealed the conservation of lysine rich regions in these proteins, thus , suggesting an evolutionary role for these cellular boiling soluble proteins during water – deficits (Garay- Arroyo *et al*., 2000). So, it can be concluded that hydrophilins have evolved independently in different protein families and in different organisms, but with the similar goal of protecting specific functions under water stress like conditions. Because plant responses to abiotic stress are complex and multigenic, the function of many of the stress-induced genes is still a matter of conjuncture. Although several classes of hydrophilin like dehydrins and heat-shock proteins (HSPs) have been documented, the role of hydrophilins is not well documented. During the last few years, bioinformatic approaches have contributed significantly to unravel stress responses. The advent of new technologies, especially after the 'post-genomic' era, allowed foresights into the complexity of plant responses (Oono *et al*., 2003). Hence, several water stress responsive transcripts can be identified/annotated *in-silico*, and hopefully they can provide valuable information for understanding the mechanisms of the abiotic stress tolerance. Therefore, considering the importance of computational analysis, in the present study, some putative drought-responsive boiling soluble proteins related genes were isolated by RT-PCR followed by *in-silico* analysis in *Tritcum aestivum* cv. PBW 175 in order to find out some stress related signature sequences. *In-silico* analyses of putative ESTs and their annotation represent a powerful tool for understanding mechanisms involved in water stress tolerance and its manifestation at function level of unknown genes.

The seeds of *Triticum aestivum* L. cvs. PBW 175 (drought tolerant)(Sairam and Srivastava, 2001, Mallick *et al*., 2009) were surface sterilized and thoroughly rinsed with

deionized water and imbibed for 6 h. After imbibition, seeds were placed in petri plates containing sterile filter sheets, moistened with water. The plates having seeds were incubated at 25 ±1°C in a seed germinator and allowed to grow for three days. Water stress was imposed to 3- day old seedlings for 48 h by withholding water supply. The drought stressed samples were harvested and kept at -80^0 C for further analysis.

Total RNA was extracted from the drought stressed seedlings of drought tolerant cv. PBW 175 using User Manual Nucleospin RNA plant isolation (Macherey Nagel, Duren, Germany) kit according to the manufacturer's protocol. DNase-treated RNA sample (l µg) was reverse-transcribed using M-MLV reverse transcriptase (Roche, Germany). The reverse transcription (RT) reaction for the cDNA synthesis was performed at 65^{0}C for 1 h using 2.5 µM oligo-dT (Roche Kit, Germany) in the thermocycler. The cDNA so formed was diluted by adding 20 µl of sterile water and then stored at -20 ^{0} C for RT –PCR. To check if the cDNA was properly synthesized, 26S rRNA was amplified by PCR conditions. PCR was used to amplify the cDNA of *Triticum aestivum* using some specifically designed conserved primer sets from different boiling soluble genes using Primer 3 and Primer- Blast software (Table 1). One microliter of cDNA was used as a template for PCR amplification with a pair of gene-specific primers in a final reaction mixture of 20 µl containing PCR buffer (1X), dNTP mixture (0.1 mM), 1.5 mM $MgCl_2$, 4 picomoles of each forward and reverse primer, and 0.5 units of DNA polymerase (Merck, USA). The cycling conditions consisted of an initial denaturing step at 95°C for 5 min, followed by 40 cycles at 94°C for 45 sec (DENATURATION), 48°C(*TADHN1*), 52^{0}C(*TAWCS120a*, *TAWCS120b*),54^{0}C(*TADreb1a*) for 45 sec (ANNEALING) and 72°C for 1 min (EXTENSION) and the final elongation step at 72°C for 10 min. PCR amplified DNA was purified using Qiagen extraction kit (France) and eluted in 20 – 30 µl of TE. The genes were sequenced and successfully submitted to GenBank of EMBL sever with accession nos: *TADreb1a*: LN813033 (*Triticum aestivum dehydration-responsive element-binding* (*DREB*) protein genes), *TADHN1*: LN813034 (*Triticum aestivum* dehydrin) *TAWCS120a*: LN813036; *TAWCS120b*: LN813037 (*Wheat Cold Shock 120 gene*).

Table 1. Primers used to amplify *T. aestivum* boiling stable proteins related genes.

Gene name	Accession number	Forward Primer	Reverse primer
TaDREB1	AF305376	5'GAATGGATCCCGGAAAGCAC3'	5'GGGAATGAACCAAGCCACAG3'
TADHN	AY574032	5'GGTCTCAAGGGAAGGAAGCT3'	5'CCTTTCTTCTCCTCCTCGGG3'
Wcs120	M93342	5'GAGAACCAGGCACACATCG3'	5'GGTTCCAGCGTGACCCTG3'
Wcs120	M93342	5' ACTTGGTCGTTGGAGGAGAG3'	5'GCTGCGTCTGTCTCTTGGAT3'

The sequences of *TADreb1a*; *TADHN1*; *TAWCS120a*; *TAWCS120b* were retrieved from EMBL and subjected to ORF bioinformatic tool scan at NCBI. The predicted protein sequences from + ORF frames were subjected to protein functional analysis by detecting signature sequences using SMART tool (http://smart.embl-heidelberg.de/). The sequences were further validated by using some other tools like PFAM (pfam.xfam.org/), INTERPRO SCAN (www.ebi.ac.uk/Tools/pfa/iprscan/) and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Homology search was performed using BLAST-P tool in order to analyse stress related signature sequences (www. ncbi. nlm. nih. gov). Conserved regions analysis among various protein homologues were carried out using CLUSTAL-W tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic tree was constructed based on aligned gene sequences from various plants using NJ method by MEGA 4 tool (Tamura *et al.*, 2007). Sub-Cellular localization of stress related final selected sequences was predicted by PSORT (psort.hgc.jp/), SLP-local (sunflower.kuicr.kyoto-u.ac.jp/~smatsuda/slplocal.html), Target-P (www.cbs.dtu.dk/services/TargetP/) CELLO (cello.life.nctu.edu.tw/) online tools, respectively.

Water stress is the major stress which affects wheat productivity and results in the alternation of number of genes. Since it is multigenic trait hence there are many genes involved in drought stress tolerance and thus identification and annotation of stress responsive genes is required for improving wheat under water stress. This study was primarily aimed at identifying boiling soluble proteins (hydrophilins) related genes linked to drought stress. Using specific gene primers from hydrophilins genes, 4 ESTs were generated from 3- day old drought stressed seedlings using RT-PCR (Fig.1).

These stress-related genes were designated as *TADreb1a, TADHN1, TAWCS120a* and *TAWCS120b.* These sequences were processed by NCBI BLAST which identified homologues boiling stable genes from various plant species, which has been proved to play a functional role

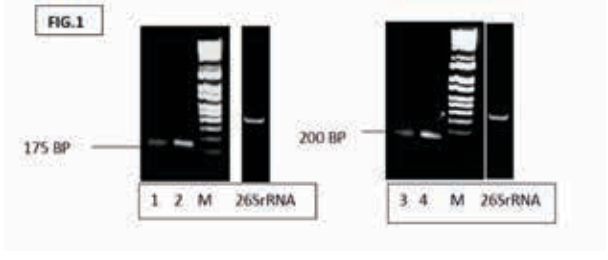

Figure 1: RT-PCR amplification of boiling soluble proteins related genes . 1: *TADreb1a*, 2:*TAWCS120b*, 3: *TADHN1,* 4: *TAWCS120a.* M: 100 bp ladder. A 500 bp ethidium bromide-stained 26S rRNA was used as an internal control to check whether the cDNA was properly synthesized.

in dehydration tolerance in plants (data not shown). Phylogenetic studies of *TADreb1a,* and *TADHN1,* clearly indicated the existence of major clades, indicating a divergent and independent evolution of boiling soluble encoding genes in different plant species (data not shown). In order to detect any signature sequence related to stress, function analyses of + (1-3) frames of *TADreb1a, TADHN1,TAWCS120a* and *TAWCS120b* genes was performed by SMART tool. SMART analysis resulted in the identification of several signature sequences in almost all the frames of *TADreb 1a, TADHN1, TAWCS120a* and *TAWCS120b.* However, among all the 3 frames (+ 1-3), only one frame from each gene showed potential stress–related signature sequences as indicated in Table 2. For example: 1 signature sequence was present in *TADreb1a*, 3 in *TADHN1* and 2 in *TAWCS120a*, respectively. Notably, no stress related signature sequence was detected in *TAWCS120b* gene. By virtue of having stress related

Table 2. Stress related signature sequences of *TADHNI, TADrebla, TAWCS 120a*

Frame	Sequence	Subcellular location	Number of domains	IDs of domains	Signature Sequence
			TADreb1a; LN813033		
+3	EGGPENSNCAYRGV RQRTWGKWVAEIRE PNRGNRLWLGSFP	Nuclear Or Cytosol	5	1) **Smart** : AP2 domain (SM00380) 2) **Interproscan** : AP2/ERF domain (IPR001471) 3) **PFAM** : AP2 (CL0081, PF00847) 4) **CDD** : AP2 (cd00018)	1)AYRGVRQRTWGKWVAEIREPNRGNRLWLGS FP (32aa)
			TADHN1;LN813034		
+2	GEHVTGLPAPAAPAS VQTHHDTDVVVEKI DGDVKTEAAPAVPE EEKK	Nuclear Or Cytosol	3	1) **Smart** : Low complexity region SCOP domain d1ka1a_ YL1_C domain (SM000993) 2) **Interproscan** : Not detected 3) **PFAM** : Not detected 4) **CDD**: Not detected	1)VKTEAAPAVPEEEKK (15 aa) 2)HDTDVVVEKIDGDVKTEA (18 aa) 3)VTGLPAPAAPASVQTHHDTDVVV (23 aa)
			TAWCS120a ; LN813036		
+1	GDTRRPLVPRHAGT ATHGAPATGGQRAA GSRWN	Mitochondrial	2	1) **Smart** : Low complexity region SCOP domain d1dmga_ 2) **Interproscan** : Not detected 3) **PFAM** : Not detected 4) **CDD** : Not detected	1)HAGTATHGAPATGG (14 aa) 2)DTRRPLVPRHAGTATHGAPATGGQRAAGS (29aa)

Table 3. BLAST-P analysis of stress related signature sequences of TADHNI, TADrebla, TAWCS120a that share 100% identity/ query coverage and minimum E-value with different proteins from various plants.

	Signature sequence	Protein name and organism	Accession number	Max score	Total score	Query cover	E value	Identity
TADreb1a; LN813033								
1)	AYRGVRQRTWGK WVAEIREPNRGNR LWLGSFP (32 aa)	Dehydration – responsive element binding protein 1 [*Hordeum vulgare*]	ACY68199.1	76.6	76.6	100%	5e-16	100%
TADHN1;LN813034								
1)	VKTEAAPAVPEEE KK (15 aa)	Cold acclimation protein WCOR410c [*Triticum aestivum*]	AAB18202.1	49.0	49.0	100%	2e-05	100%
2)	HDTDVVVEKIDGD VKTEA (18 aa)	cold acclimation protein WCOR410 [*Agropyron cristatum*]	AEJ88292.1	59.6	59.6	100%	6e-09	100%
3)	VTGLPAPAAPASV QTHHDTDVVV (23 aa)	Cold acclimation protein WCOR410c[*Triticum aestivum*]	AAB18202.1	73.2	73.2	100%	3e-13	100%
TAWCS120a,LN81303 6								
1)	HAGTATHGAPAT GG (14 aa)	dehydrin-/LEA group 2-like protein [*Lophopyrum elongatum*]	AAC05921.1	43.5	196	100%	0.001	100%
2)	DTRRPLVPRHAGT ATHGAPATGGQR AAGS (29aa)	dehydrin-/LEA group 2-like protein [*Lophopyrum elongatum*]	AAC05921.1	43.5	197	51%	0.004	100%

motifs in *TADreb 1a, TADHN1, TAWCS120a* genes, it can be inferred that wheat cv. PBW 175 has unusual capacity to tolerate drought stress under water limiting conditions. Signature sequences were further validated through other bioinformatic tools such as PFAM, INTERPRO SCAN and CDD. Sub-cellular localization studies indicated that *TAWCS120a* was localised in mitochondria whereas *TADreb1a*, and *TADHN1* were present in nucleus or cytosol (Table 2). BLAST-P analysis of each stress-related signature sequence from *TADreb1a, TADHN1, TAWCS120a* revealed substantial homology to various stress regulated proteins like DREB and COR's from plant species (Table 3). Transcription factors like DREB have gained much attention, owing to their involvement in the regulation of many downstream stress-related genes by binding to DRE/CRT cis-acting elements in the promoter regions of these genes that play a key role in producing a cascade of responses to abiotic stimuli (Kasuga *et al.*, 2004). COR proteins were originally designated in gp-II LEA/dehydrin family (Shih *et al.*,2008). Like LEAs, it can be speculated that *TADreb1a, TADHN1, TAWCS 120a* proteins may be implicated in regulation of drought stress tolerance in cv. PBW 175.

To summarize, data presented here represent a substantial contribution towards understanding the role of boiling soluble proteins related genes in relationship with drought stress at molecular level. These candidate genes can be useful in molecular breeding schemes by using various wheat cultivars for drought stress improvement. Further studies at expression level using RT-qPCR and miRNA (micro-RNA) is underway in order to find out good candidates for functional studies in wheat under drought stress.

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