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Characterization of a drought inducible trithorax-like H3K4 methyltransferase from barley

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Abstract

Histone H3 lysine 4 methylations catalyzed by histone lysine methyltransferases (HKMTs), like the *Arabidopsis thaliana* ATX1 and ATX2, are important epigenetic modifications related to chromatin decondensation and gene activation. In order to study this epigenetic mechanism in monocot cereal plants, we performed homology searches of *ATX1* and *ATX2* against the *Brachypodium distachyon* L. Beauv and rice (*Oryza sativa* L. spp. *japonica*) genomes, discovering single homologues for each cereal crop representing both *Arabidopsis* sequences. Using this information, we employed the rolling circle amplification - rapid amplification of cDNA ends (RCA-RACE) method to isolate, clone and characterize *HvTX1* from RNA extracted from barley (*Hordeum vulgare* L.) tissues and studied its expression during seed development and under drought stress. The cloned cDNA sequence contained a 3 093 bp ORF homologous to *ATX1* and *ATX2*. Characterization of the translated *HvTX1* transcript sequence revealed the multi-domain nature of the putative protein, including all conserved regions characteristic for ATX1 and ATX2. By comparative genomic analysis and homology searches in EST databases we located, with high probability, the gene coding for *HvTX1* on the barley chromosome 5H. Constant elevation of *HvTX1* expression was observed during seed development. Expression of *HvTX1* after drought stress was analyzed by quantitative real-time polymerase chain reaction (qPCR) in two different barley cultivars with varying drought stress tolerance, revealing *HvTX1* drought-induction in a tolerance-specific manner.

Additional key words: *Arabidopsis thaliana,* ATX, *Brachypodium distachyon,* epigenetic modifications, histone, HKMT, *Hordeum vulgare*, *Oryza sativa.*

Introduction

Histone lysine methyltransferases (HKMTs) are involved in a large array of biological functions (Chen *et al.* 2010b). Almost all HKMT families containing SET domains, are included in four distinct SET-domain groups (Dillon *et al.* 2005), and may be involved in either activation (*e.g.* TrxG) or repression (*e.g.* PcG) of transcription. PcG repressors and TrxG activator proteins are parts of multi-protein complexes that form an epigenetic switch which controls transcription levels by altering the structure of chromatin (Schwartz *et al.* 2010). The trithorax family (TRX, class III SET-domain) proteins are responsible for methylation of lysines on H3, mainly H3K4, of many developmental genes (Schuettengruber *et al.* 2007). Methylation of H3K4 is a

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Abbreviations: ATX - trithorax protein family in *Arabidopsis thaliana*; C-SAC - SET-associated cysteine-rich motif; DAF - days after fertilization; DAST - domain associated with SET in trithorax; FLC - FLOWERING LOCUS C; FYRC - F/Y-rich C-terminus; FYRN - F/Y-rich N-terminus; H3K4 - histone 3 lysine 4; HAT - histone acetyltransferase; HDAC - histone deacetylase; HKMT - histone lysine methyltransferase; IMF - immature flower; MADS - acronym referring to the genes *MCM1*, *AGAMOUS*, *DEFICIENS*, *SRF*; PcG - polycomb group; PHD - plant homeo domain; qPCR - quantitative real-time polymerase chain reaction; RCA-RACE - rolling circle amplification - rapid amplification of cDNA ends; SET - trithorax; TRX - trithorax family; TrxG - trithorax group.
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key epigenetic mark that requires the interaction of several modifiers, including histone acetylases (Ruthenburg *et al.* 2007). Generally, trimethylation of H3K4 is required for the active transcription of genes in plants, while dimethylation is independent from transcriptional activation (Zhang *et al.* 2009). In *Arabidopsis* and rice the dimethylated and trimethylated states (H3K4me2,3) are located on the promoters and the 5'-ends of transcribed gene regions (Li *et al.* 2008, Zhang *et al.* 2009).

Over the past decade, knowledge on discovery and characterization of *Arabidopsis* homologues of the trithorax-like genes, involved in both gene activation (Avramova 2009) and silencing (Jacob *et al.* 2009) is gradually accumulating. Two sister groups have been identified forming the TRX proteins in *Arabidopsis*, one including ATX1 and ATX2 and the other containing ATX3, 4 and 5 (Avramova 2009). The very similar trithorax homologues ATX1 and ATX2 both target H3K4, but utilize different mechanisms for their distinct functions (Avramova 2009). By H3K4 trimethylation, ATX1 regulates the activation of the floral repressor MADS-box transcription factor FLC and thus the transition from vegetative to reproductive development (Pien *et al.* 2008). ATX2 may have the same or different target specificity with ATX1 and has an established role in H3K4 dimethylation also in targets where H3K4me3 may not be a prerequisite for gene activation (Avramova 2009). ATX1 and ATX2 are multi-domain proteins with highly similar architecture, containing several signature motifs (Avramova 2009). The site of methylation catalysis is the SET protein-protein interaction domain, capable of catalyzing histone lysine mono-, di-, or trimethylation of several lysines in the histones H3 and H4 (Dillon *et al.* 2005). Moreover, a characteristic SET-associated cysteine-rich motif (C-SAC or post-SET domain) is observed in all ATX proteins (Baumbusch *et al.* 2001). The ATX-specific motif PGDXXWXK characterizes the plant-specific PWWP domain, which has been suggested to be a methyl-lysine recognition motif involved in protein-protein interactions, that plays a role in plant cell growth and differentiation (Wang *et al.* 2009). Two Class III SET-specific domains, which unique for ATX1 and ATX2 while lacking for ATX3, ATX4 and ATX5, are Tudor and domain associated with SET in trithorax (DAST; Avramova 2009). Tudor domains recognise methylated amino acids and are implicated in chromatin binding (Adams-Cioaba and Min 2009). DAST is usually split in two phenylalanine/tyrosine-rich (FYR) motifs and has distinct protein arrangement in plants. It is considered specific to higher eukaryotes, possibly implicated with multicellularity (Avramova 2009). Other distinct features of ATX1 and ATX2 is that they contain one plant homeo domain (PHD) finger, while the other TRX proteins carry two such domains (Avramova 2009). Among other functions, PHD fingers are specialized in binding on methylated lysines and have been shown to identify histone H3K4 trimethylation (Adams-Cioaba and Min 2009). Recently, the PHD finger in ATX1 has been implicated as an active regulator in drought stress responses (Ndamukong *et al.* 2010).

Overall, plant TRX proteins have multiple and complex roles, but very little is known about them in cereals. The recent sequencing of the wild grass model plant *Brachypodium* offers a new means for the study of epigenetic mechanisms in agriculturally important cereal crops (Garvin *et al.* 2008). Among the most important crops cultivated globally are wheat and barley. Present population dynamics and major global climate changes and mainly the escalating drought conditions, make crop production increasingly challenging and require ways to boost production while decreasing water requirement (Feuillet *et al.* 2008, Ashraf 2010). The focus of breeding is now shifting to the identification of significant traits that have optimum potential to increase overall yield (Tester and Langridge 2010). Plant stress responses are regulated through the action of various multifunctional genes, with roles in signal sensing and transduction, transcriptional regulation and functional gene activation (Hu *et al.* 2010). Among them, many transcription factors are actively involved with both ABA dependent and independent abiotic stress signaling (Agarwal and Jha 2010). Histone modifiers, including histone lysine methyltransferases (Kapazoglou *et al.* 2010), are such major regulators of plant development and adaptation that may be used efficiently as diagnostic markers for breeding or they can be exploited further for enhanced crop improvement (Feuillet *et al.* 2008, Tsaftaris *et al.* 2008). All barley putative epigenetic modulators (HDAC, HAT and PcG proteins) isolated and studied by our group so far were responsive to abiotic stress (Demetriou *et al.* 2009, 2010, Kapazoglou *et al.* 2010, Papaefthimiou *et al.* 2010, Papaefthimiou and Tsaftaris 2011). It is important to focus our research studies in barley into unraveling the effect of environmental stresses on histone modifiers.

In order to study the TRX histone H3 lysine 4 methyltransferases in monocot cereal plants we identified, cloned and characterized *HvTX1* in barley, a putative trithorax Class III SET domain methyltransferase homologue of *ATX1* and *ATX2*. The expression of the gene was evaluated during seed development and under drought stress.

Materials and methods

Plants and cultivation: Two commercial barley (*Hordeum vulgare* L.) cultivars, moderately drought tolerant Caresse and drought tolerant Demetra, were kindly provided by the Cereal Institute at the National Agricultural Research Foundation of Greece (www.nagref.gr). For gene isolation, seeds (fertilized ovary) and immature (unfertilized) flower (IMF) from cv. Caresse were collected by hand during the 2008

spring period. All tissues were stored in -80 °C for subsequent RNA extraction, cDNA synthesis, sequencing and qPCR analysis.

Seeds from both cultivars were germinated on filter paper wet with distilled water in darkness at 22 ºC for 2 d. The young seedlings were transferred to 0.60 dm^3 plastic pots with a 3:1 soil (black and blond sphagnum) and *Perlite* mixture (*Terrahum*, Geeste, Germany). An open hydroponic-type arrangement was used for the experimental setup consisting of 48 pots from each cultivar, in two independent replicates, which were constantly irrigated with tap water (pH 6 - 7). Three seedlings inside each pot constituted one individual sample. After 7 d, the water stress was induced by withholding watering of 24 pots for each cultivar for 10 d (until all shoots and roots stopped growing and wilting became apparent). The other 24 pots were used as controls and were kept in well-watered conditions. Seedlings were harvested 1st, 3rd, 6th and 10th day in the morning and shoots were stored in -80 °C until further use for RNA extraction, cDNA synthesis and qPCR analysis.

RNA isolation and cDNA synthesis: Total RNA was extracted using the *Nucleospin*® RNA plant kit (*Macherey-Nagel*, Düren, Germany), according to manufacturer instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in 50 μm^3 total volume using 1 µg of total RNA for first strand cDNA synthesis, 1 µg of 3'RACE adapter primer (5´-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3´) (*Invitrogen*, Carlsbad, CA, USA), 1 mM dNTPs and 200 *U M-MuLV* reverse transcriptase (*Invitrogen*). Alternatively, the cDNA synthesis reaction with RNA extracted from tissues collected during the drought experiment was done with 1 µg of random *HEX* primers (5´-NNN NNN-3´) (*VBC Biotech*, Vienna, Austria). A negative control was also included in all cDNA synthesis experiments, where no reverse transcriptase was added.

RCA-RACE cloning: Detection, isolation and cloning of the *HvTX1* mRNA from Caresse was initiated with specific primer sets (Table 1) designed according to partial EST sequences from the *Affymetrix barley1 GeneChip contig_11967* (Close *et al.* 2004). In order to obtain the complete *HvTX1* cDNA sequence (3093 bp), RCA-RACE was used as described by Polidoros *et al.* (2006). Briefly, pooled RNA from shoot, immature flower, seeds 2 - 4 and 5 - 10 d after flowering (DAF) were used as template for circular cDNA libraries. All reactions were performed using a *Mastercycler Ep* gradient (*Eppendorf*, Hamburg, Germany). For obtaining the complete cDNA sequence, combinations of all forward and reverse primers designed for *HvTX1* (Table 1) were used for both cDNA fragment isolation/ amplification and sequence verification. PCR cycling conditions were: denaturation at 94 °C, 2 min; 40 cycles of 94 °C, 30 s; annealing temperature (Table 1), 1 min; extension 72 °C, 3 min, and final extension at 72 °C, 10 min. The *pCR2.1 TOPO* vector (*Invitrogen*) was used for cloning PCR fragments of interest according to the manufacturer's protocol. Selected clones were picked and commercially sequenced. Obtained sequences were assembled and analyzed with the *BioEdit* sequence alignment editor (Hall 1999) and the full-length cDNA sequence was deposited at *GenBank* (accession number HM152530).

Sequence and comparative genomic analyses: The HvTX1 transcript sequence was analysed for *CpG islands* with the software tool *CpG Island Searcher v. 1.3* (Takai and Jones 2003); calculation settings included a 200 bp window with 1 bp shifts for identification of *CpG islands* within sequence ranges with GC content higher than 50 % and observed/expected ratio higher than 0.6. The obtained HvTX1 open reading frame (ORF) sequence was analysed and translated into amino acid sequence using the online tool transeq (http://www.ebi.ac.uk/tools/emboss/transeq/). The exploration of protein domain architectures was achieved by alignment and sequence comparisons using the *InterPro* database (Hunter *et al.* 2009); domains with the highest homology (E-value $\geq 10^{-2}$) were accepted. Comparisons of amino acid sequences were done with *SIM4* (Florea *et al.* 1998) and visualized with *LALNVIEW* (Duret *et al.* 1996) and *DOG 1.0* (Ren *et al.* 2009). Estimation of evolutionary divergence between HvTX1, its rice and *Brachypodium* sequence homologues and representative sequences of all ATX groups, obtained from *ChromDB* (www.chromdb.org), was performed by pairwise distance analysis conducted using the Poisson correction method in *MEGA4* (Tamura *et al.* 2007). A total of 294 positions were included in the final dataset after deletion of all positions containing gaps and missing data.

Comparative analyses of *HvTX1* against the *Brachypodium* and rice genomes were performed using both online and offline tools available at *Gramene* (Jaiswal 2006), *PlantGDB* (Dong *et al.* 2005), *Plaza* (Proost *et al.* 2009) and *HarvEST* (Close *et al.* 2007). Local *BLAST* search of the transcript was performed with *BioEdit* against mapped wheat ESTs and 13 barley EST datasets of various tissues and stresses available at the Clemson University Genomics Institute (CUGI, www.genome.clemson.edu/projects/barley/).

Relative expression analyses: Quantitative real-time PCR (qPCR) analyses were performed with an *Opticon 2* real-time PCR system (*MJ Research*, Waltham, MA, USA). Six developmental stages were studied, including the immature flower (IMF), and at 1 - 3, 3 - 5, 5 - 10, 10 - 15, 15 - 20 DAF. Shoots were used harvested several times during drought stress experiment, as described above. Each reaction was performed in triplicate using a *Platinum SYBR Green qPCR SuperMix UDG* kit (*Invitrogen*). Primer pairs were carefully designed manually, to allow for best quantitation of transcript expression differences. A 254 bp sequence fragment was targeted and isolated from *HvTX1* with the primer set

Table 1. Forward (F1 - FH) and reverse (R1 - RH) primers used in cDNA construction and qPCR experiments.

Primer name	Sequence 5' - 3'	Ta [ºC]	Target gene
F1	TGGTGAAGCAGAAGACCTTACCC	60	*HvTX1*
F2	ATTGAGGAACTGCGGTGCTGCC	60	
F3	TGGATAAGCAGGGATACGATGAGC	60	
F4	GCAAGAAGCACAGACAACCA	55	
F5	GGCCAACAACAACCTCAAGT	55	
F6	TTCGGCCATTCACTATTTCC	55	
F7	TGTTCCTTGCTACAGGCACAGC	60	
FC	TTAAGCGGAGGGTTGTACTG	55	*Hsdr4*
FHvActin	GCCGTGCTTTCCCTCTATG	57	*actin*
R1	AGGGTAAGGTCTTCTGCTTCACC	60	*HvTX1*
R2	TCAGAAAGGGCACCGCCTGCTTC	60	
R3	CGGCCATCTGTTGTAGGTTT	55	
R4	TGGTTGTCTGTGCTTCTTGC	55	
R5	ATGCTCATCCCCAAGAACAC	55	
R6	ATGGGCTGTGCCTGTAGCAAGG	60	
R7	AGGGTAAGGTCTTCTGCTTCACC	60	
RC	TTGTCTTCATACCTACCAAGC	55	*Hsdr4*
RHvActin	GCTTCTCCTTGATGTCCCTTA	57	*actin*

F3 - R2 (Table 1). A barley actin (*HvActin*) transcript fragment was isolated with primer sets FHvActin - RHvActin (Table 1), and used as the endogenous control. The drought responsive wild barley gene *Hsdr4* (Suprunova *et al.* 2007), was included in the qPCR analysis as a control indicator of drought by amplifying a fragment with a carefully designed primer pair (FC-RC, Table 1). The qPCR reactions were performed in 20 μm^3 reaction volumes containing 0.2 ng cDNA, 0.1 pmol each primer, 1× *Platinum SYBR Green qPCR Supermix-UDG* (*Invitrogen*, Paisley, UK) and sterile dH_2O. General thermocycler conditions were 50 °C for 2 min; 95 °C for 2 min; then 42 cycles of 95 °C for 20 s; annealing temp (Table 1), for 15 s; 72 °C for 15 s; a final extension at 72 °C for 10 min and plate read at 82 °C. To identify and verify the PCR products a melting curve was performed from 65 °C to 95 °C with readings every 0.2 °C and a 10 s hold between observations. Relative quantification and statistical analyses were done by pair wise fixed reallocation randomization tests, using the *REST-XL* software (Pfaffl *et al.* 2002). For characterization of *HvTX1*, the statistical analyses were carried out in respect to differences observed between the IMF and corresponding developmental stages of the seed. For the drought stress experiments, statistical analyses were done in respect to differences observed between seedling of well-watered controls and those subjected to water withdrawal. Additional gene expression data were mined from the barley gene expression atlas with accession number BB3 (Schreiber *et al.* 2009) available on the online database *PLEXdb* (www.plexdb.org). The median log intensity of transcript hybridization data was normalized using the *MAS5.0* method.

Results

In order to identify the putative barley *ATX1*/*ATX2* homologues and study their involvement in drought stress, homology searches of *ATX1* and *ATX2* against barley EST databases and the closely related *Brachypodium* and rice genomes were conducted. Only one homologue corresponding to both *Arabidopsis* sequences was identified in *Brachypodium* (Bradi4g08510) and in rice (Os09g04890). The corresponding barley homologous transcript *HvTX1* was isolated and cloned from cv. Caresse using the RCA-RACE method (Polidoros *et al.* 2006). The assembled *HvTX1* cDNA sequence contains a 3093 bp ORF encoding for the 1 029 aa putative barley HKMT (Fig. 1).

CpG islands were detected on the 5' of the coding region of *HvTX1* (656 bp, 67.8 % GC content), and spanning the coding sequence of the PHD domain (201 bp, 51.7 % GC content) in *HvTX1* (Fig. 2).

The closest protein-coding gene homologues of *HvTX1* obtained by similarity analysis search against grass plant databases were the sequences from *Brachypodium* (Bradi4g08510, 89 % similarity) and rice (Os09g04890, 83 % similarity) (Fig. 1, Table 2). Pairwise distance analysis of the HvTX1, Bradi4g08510 and Os09g04890 amino acid sequences with members of the *Arabidopsis* TRX family, highlighted a much higher evolutionary relatedness with the ATX1/ATX2 group proteins (0.31 pairwise distance, Table 2). Sequence similarity of HvTX1 to both ATX1 and ATX2 (Fig. 1) was comparable (59.11 and 59.92 % respectively), with single nucleotide polymorphisms occurring randomly in equal proportions on the nucleotide sequences (54 %).

Alignment of the HvTX1 amino acid sequence with the equivalent rice, *Brachypodium* and *Arabidopsis* ATX1/ATX2 group amino acid sequences, revealed both highly conserved as well as unique regions in the sequence of the barley homologue (Fig. 1). A *Tudor* domain is localized within amino acid positions 173 - 231 (IPR002999). The PWWP domain (IPR000313) is localized on the N-terminus within amino acids 266 - 343. DAST is also detected on HvTX1. Specifically, the terminus FYRN (IPR003888) is localized within amino acids 412 - 466 and the FYRC domain (IPR003889) within amino acids 470 - 555. PHD finger domain (IPR001965) is localized within amino acids 571 - 620. The SET domain (IPR001214) is localized near the C-terminus of the translated transcript, located within amino acids 866 - 990. The associated post-SET (C-SAC) module (IPR003616) is detected immediately downstream from SET, spanning the region between 990 - 1006 aa.

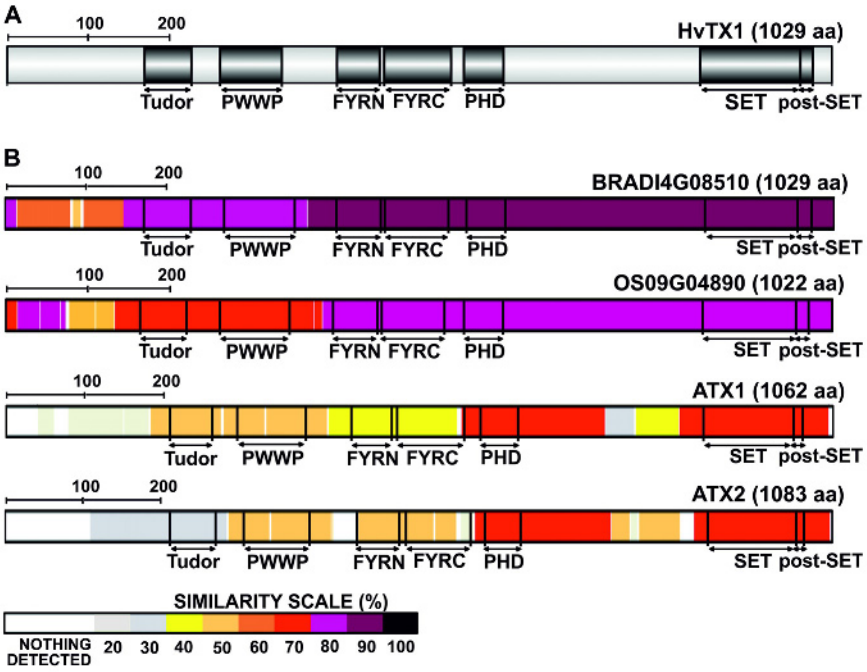

Fig. 1. *A* - Schematic diagram presenting the precise protein domain architecture of the inferred HvTX1 (1029 aa). Conserved domains with E-value ≥ 10^{-2} were considered. Scale represents 100 aa. *B* - Schematic diagrams illustrating the protein domain architecture of the *Brachypodium* (Bradi4g08510) and rice (Os09g04890) ATX1/ATX2 group homologues, and the *Arabidopsis* ATX1 and ATX2 proteins. Conserved domains with E-value ≥ 10^{-3} were considered. Scales represent 100 aa. Each diagram is colour coded on the basis of local alignment with the HvTX1 amino acid sequence. Similarities are represented by *rectangles* indicating segments coloured according to the degree of identity to HvTX1, as indicated by the colour-coded scale.

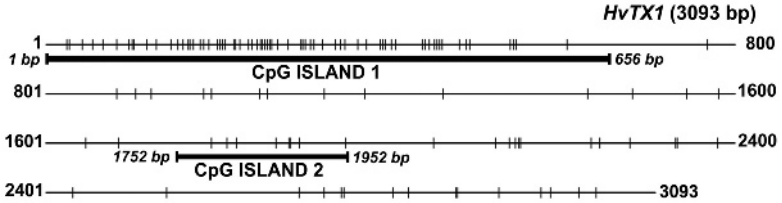

Fig. 2. Schematic diagrams of two CpG islands identified and aligned on the *HvTX1* ORF sequence. CpG island 1 has 67,8 % GC content, stretching from 1 to 656 bp. CpG island 2 has 51.7 % GC content and 201 bp length (1752 - 1952 bp) and is spanning the coding sequence of the PHD domain. CpG dinucleotides are indicated as *short lines crossing* the ORF sequence, which is illustrated as a *continuous stretch*.

Table 2. Pairwise distance analysis estimates of evolutionary divergence between the HvTX1 amino acid sequence, its rice and *Brachypodium* sequence homologues and representative member amino acid sequences of all ATX family groups obtained from *ChromDB* (www.chromdb.org).

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
HvTX1 [1]	-							
Bradi4g08510 [2]	0.06							
Os09g04890 [3]	0.08	0.10						
ATX1 (SDG27)[4]	0.31	0.33	0.29					
ATX2 (SDG30)[5]	0.31	0.32	0.28	0.17				
ATX3 (SDG14)[6]	1.12	1.10	1.13	1.15	1.12			
ATX4 (SDG16)[7]	1.12	1.13	1.13	1.10	1.11	0.40		
ATX5 (SDG29)[8]	1.13	1.14	1.15	1.11	1.11	0.40	0.10	-

Comparative genomic analysis was conducted in order to localize the genomic homologue of the barley *HvTX1* gene transcript on the genomes of *Brachypodium* (5 chromosomes, Bd1-5) and rice (12 chromosomes, Os01-Os12). The *HvTX1* homologue genomic sequences are made up of 25 exons and 24 introns and are located on Bd4 on *Brachypodium* (Bradi4g08510, with coordinates (5')-7591624-7575866-(3') bp); and Os09 on rice (Os09g04890, with coordinates (5')-2614721-2602074-(3') bp). Local similarity search on EST datasets led to the identification of one wheat 384 bp EST sequence (BE591512), highly similar to *HvTX1* (97 %), localized on the 5A, 5B and 5D wheat chromosome sets.

The relative expression study of *HvTX1* was undertaken in comparison to the IMF in cv. Caresse. Continuous induction of the barley transcript expression

was monitored throughout seed development (Table 3). *HvTX1* was up-regulated by almost three-fold during the final measured stage (15 - 20 DAF). The normalized expression data of the barley sequence assembled as contig_11967, which is closely homologous to *HvTX1* (99 % sequence similarity), indicate highest transcript intensities for the germinating seed coleoptyle and immature inflorescence in cv. Morex. The lowest transcript intensities were monitored on leaves and on pre-anthesis tissues (bract and anther).

During the drought stress, soil pH was roughly 5.5 - 7.0 and the soil moisture was initially 80 - 84 %. The experiment was ceased on day 10, when wilting was apparent in most (> 90 %) seedlings of both cultivars and soil moisture was very low (Table 4). The known drought responsive barley gene *Hsdr4* (Suprunova *et al.* 2007) used as control was steadily expressed in both cultivars for the first 6 days and was distinctly upregulated by the tenth day of the experiment (Table 4) positively indicating the successful establishment of drought by our treatment.

The *HvTX1* expression pattern was characterized by low expression during the first 6 d and subsequent up-regulation in a cultivar-specific manner on the 10th day. Soil moisture decreased to ~ 57 % soil water content (WC) in Caresse pots and ~ 38 % in Demetra pots by day 6 (Table 4).

Table 3. Cycle threshold (C_T) values of *HvTX1* and the internal control gene *HvActin* and the mean fold change in expression ($\Delta\Delta C_T$) of *HvTX1* in comparison to the IMF, at various time points in seed development of the barley cultivar Caresse. Statistical significance for $\Delta\Delta C_T$ ($P \leq 0.05$) is indicated by an *asterisk*. Expression data of the *HvTX1* homologue sequence assigned as contig_11967 are demonstrated by their transcript log intensity value means in tissues of the barley cv. Morex (data from cv. Morex selected from other sources were included for comparative purposes). DAF - days after fertilization.

Caresse Tissue	C_T *HvActin*	*HvTX1*	$\Delta\Delta C_T$	Morex tissue germinating seed	Log intensity *contig_11967*
IMF	28.84	29.76	1.000	coleoptyle	9.047
	29.4	29.39		radicle	8.234
	29.34	30.00		embryo	8.383
1 - 3 DAF	27.35	27.71	1.159 ± 0.001	seedling	
	27.49	27.89		root	8.292
	27.73	27.90		crown	8.899
3 - 5 DAF	30.88	31.85	1.338 ± 0.103	leaf	8.058
	30.41	29.78		pre-anthesis tissues	
	31.05	31.02		immature inflorescence	9.747
5 - 10 DAF	30.90	31.33	1.686 ± 0.001	floral	8.093
	31.50	31.50		pistil	8.731
	31.30	30.18		anthers	8.173
10 - 15 DAF	29.73	29.02	2.445 ± 0.001*	seed development	
	29.79	28.83		5 DAP caryopsis	8.940
	29.91	29.28		10 DAP caryopsis	8.800
15 -20 DAF	31.64	30.80	2.875 ± 0.001*	16 DAP caryopsis	8.463
	31.73	30.68		22 DAP embryo	8.594
	31.76	30.65		22 DAP endosperm	8.364

Table 4. The mean fold change ($\Delta\Delta C_T$) in expression of *HvTX1* and *Hsdr4* at various time points during drought stress treatment of the barley cultivars Caresse and Demetra. Results of the two independent replicates are displayed separately. Soil water content at each time point is demonstrated for the control and stressed plants.

Day	$\Delta\Delta C_T$ Caresse		Demetra		Soil water content [%] Caresse		Demetra	
	Hsdr4	*HvTX1*	*Hsdr4*	*HvTX1*	control	stressed	control	stressed
1st	1.060	0.894	0.882	0.904	84.950	82.202	82.576	81.029
	1.376	0.841	1.159	1.251	82.963	82.543	84.000	80.542
3rd	0.651	0.669	0.893	0.887	83.428	77.961	83.449	68.393
	0.959	0.854	0.849	0.867	82.966	80.174	83.306	66.722
6th	0.987	0.794	1.150	0.771	84.370	56.037	82.803	34.952
	0.911	0.615	1.185	0.794	82.079	56.980	82.868	38.721
10th	2.456	2.979	4.979	5.264	83.922	16.306	83.791	7.902
	2.400	2.591	4.393	5.225	84.675	20.063	83.943	9.841

Discussion

The putative trithorax-like HKMT from barley designated HvTX1 was isolated, cloned and characterized. By comparative analyses of the *Arabidopsis ATX1* and *ATX2* genes with genomes of the cereal plants *Brachypodium* and rice, a single ATX1/ATX2 group homologue sequence was obtained from each cereal. We thus presumed that only one homologue would also exist in barley and perhaps wheat. The 3093 bp transcript sequence of the isolated barley homologue *HvTX1* exhibits close and comparable resemblance to both *ATX1* and *ATX2* sequences with single nucleotide polymorphisms occuring in equal proportions (54 %). Evolutionary divergence estimation of the amino acid sequences supports the relatedness of HvTX1 to the ATX1/ATX2 group members only (Table 2). Moreover, HvTX1 contains all ATX1/ATX2 group specific modules and the overall domain architecture and sequence analyses confirmed that it is likely a putative trithorax-like protein (Fig. 1, Table 2). Specifically, the conserved modules identified on HvTX1 are *Tudor*, *PWWP*, *DAST* (*FYRN*, *FYRC*), *PHD*, *SET* and post-*SET*. Among them, *Tudor* and *DAST* are unique for the ATX1/ATX2 group. *SET*, *PHD* and *PWWP* are conserved methyl-lysine recognition modules, indicating the possible involvement of HvTX1 in dynamic multivalent binding interactions (Ruthenburg *et al.* 2007). Indeed, plant transcriptional activation is a dynamic process controlled *via* an epigenetic code by separate mechanisms, with tissue, gene and even residue specificity (Ahmad *et al.* 2010). Such regulatory functions are the di- and tri-methylation of H3K4, controlled by ATX1 and ATX2, and involved in the activation process of plant homeotic genes (Avramova 2009).

In *Arabidopsis* the mono-methylated state of H3K4 was associated with CG DNA methylation (Zhang *et al.* 2009). In rice, the ratio of H3K4me2/me3 combined to DNA methylation levels was directly correlated to transcriptional activity and each modification preferentially marked different groups of genes (Li *et al.* 2008). The same study showed that all three modifications mainly occur just downstream from the transcription start site of rice genes, suggesting a role in transcriptional initiation and elongation (Li *et al.* 2008). Extensive DNA methylation occuring on cytosine bases of eukaryotic genomes is involved, among other functions, in selective silencing of transcription initiation and elongation (Gehring and Henikoff 2007). Transcription start sites of many eukaryotic genes associate with *CpG islands*, which are regions characterized by exceptionally high frequencies of non-methylated cytosine/guanine base pairs (Gardiner-Garden and Frommer 1992). Such regions may also be essential for binding of transcription factors to specific sites, as is the case for the recruitment of a H3K36 demethylase (Blackledge *et al.* 2010). In barley, the observed frequency in the whole genome of gene-associated CpG islands is 30 %, much lower than that observed in *Arabidopsis* (80 %) and rice (78 %) that have smaller genome sizes (Ashikawa 2001). In the present work we identified a highly condensed CpG island on the 5' end of *HvTX1*, which is a probable target for epigenetic modifications, that may additionally regulate the expression of the gene (Benayoun and Veitia 2009).

The genomic sequences of the rice and *Brachypodium* ATX1/ATX2 group homologues are located on chromosomes Os09 (Os09g04890) and Bd4 (Bradi4g08510) respectively. Syntenic studies have previously shown a large number of orthologous loci shared by Bd4 and the wheat and barley chromosomes 4 and 5 respectively. In addition, syntenic and comparative genomic mapping studies have revealed large numbers of orthologous pairs between Os09 and the highly syntenic barley and wheat chromosomes 5 (Moore *et al.* 1995, Stein *et al.* 2007, Salse *et al.* 2008). Moreover, *HvTX1* is highly similar (97 %) to a wheat EST, which has been mapped on the wheat chromosome 5. It is thus very probable that the barley gene encoding for *HvTX1* is located on the barley chromosome 5H and that a highly syntenic homologue gene also exists in wheat.

Relative expression analysis of *HvTX1* was undertaken for the major stages in barley seed development, including the pre-storage and endosperm cellularization (0 - 5 DAF), the intermediate (5 - 10 DAF) and the storage (10 - 20 DAF) phases (Rolletschek *et al.* 2004). Expression of *HvTX1* was constantly up-regulated throughout the 20-d period studied reaching a maximum of 3 fold in the storage phase (Table 3). Expression data of the *HvTX1* homologue transcript sequence in cv. Morex assigned as *contig_11967* indicate highest transcription in the germinating seed coleoptyle and immature inflorescence. The lowest transcription was monitored in leaves and in pre-anthesis tissues (bract and anther). Since our work shows the existence of a single homologue in cereals for both *ATX1* and *ATX2*, we may assume that the barley *HvTX1* transcript expression in seed might be related to modulation of the gradual activation of target genes while seed development moves through consecutive stages. In our recent work describing three HAT proteins from the GNAT-MYST family and HTMases from the PcG-2 family on barley, we recorded expression patterns similar to *HvTX1* exhibited by *HvELP3* and *HvMYST*, two putative key histone acetyltransferases (Papaefthimiou *et al.* 2010) and *HvSu(z)12b*, a putative key histone PcG methyltransferase (Kapazoglou *et al.* 2010). In accordance with our findings, large-scale transcriptomic analyses revealed the increasing accumulation of various transcripts during seed development and maturation in maize (Prioul *et al.* 2008) and in barley, including many transcription factors (Sreenivasulu *et al.* 2008). Fewer genes involved with signal transduction and energy were preferentially expressed during late seed development, whereas genes encoding storage proteins were increasingly expressed in

both plants up to at least 21 DAF (Prioul *et al.* 2008, Sreenivasulu *et al.* 2008). Our sequence and comparative genomic analyses results have indicated that the barley trithorax-like transcript *HvTX1* is most possibly encoding for a H3K4 methyltransferase. We may thus assume that the barley *HvTX1* expression in seed is related to modulation of the gradual activation of target genes at least for the first 20 d. We can also suggest its active implication with the promotion of storage protein transcription during late development.

Water availability is a major limiting factor of crop productivity and the complete understanding of the molecular mechanisms underlying drought tolerance in plants in general and specifically in barley, would greatly promote the challenge of breeding for high and stable yield (Feuillet *et al.* 2008). The photosynthetic rate in barley is significantly reduced under drought, most possibly accompanied by metabolic impairment (Robredo *et al.* 2010). The *HvTX1* studied in this work is probably located on chromosome 5H, which have been implicated with drought responsiveness suggested by several quantitative trait loci (QTL) studies in barley (Moore *et al.* 1995, Teulat *et al.* 2001, 2003, Farshadfar *et al.* 2008, Chen *et al.* 2010a). Expression of *HvTX1* under drought stress was evaluated by removing pots with seedlings from water source, thus imitating environmental conditions of gradual establishment of drought. Indeed, slower rates of soil water withdrawal correlate with slower development of water stress in barley (Robredo *et al.* 2010), and thus the gradual establishment of stress related mechanisms. Under severe drought stress by the 10^{th} day of the experiment, when both barley cultivars were probably on the same developmental stage, *HvTX1* expression increased by about 5 fold in Demetra (drought-tolerant) and 3 fold in Caresse (intermediate drought-tolerance). Since severe drought stress was already established, this difference in transcript responsiveness is possibly linked to cultivar drought tolerance. In agreement with our results, drought stress experiments during the flowering stage in barley have shown gene expression variability, also related to drought tolerance of the barley cultivar (Guo *et al.* 2009). In addition, large scale microarray studies of transcript abundance under drought stress in barley leaves have indicated variability of gene expression, relative to their function (Ozturk *et al.* 2002, Talame *et al.* 2007). The homologous sequence ATX1 is involved in diverse and dynamic interactions during dehydration stress (Ding *et al.* 2011), where the PHD finger has been signified as a probable regulator (Ndamukong *et al.* 2010). In *Arabidopsis*, drought-induced expression of stress-responsive genes was associated with an increase in H3K4 tri-methylation and H3K9 acetylation (Kim *et al.* 2008). Moreover, the same study showed that in response to drought stress H3K4me3 enrichment was established after full activation of stress-responsive genes (Kim *et al.* 2008). Overall, histone modification levels, including H3K4 methylation, can be very predictive for the expression of a plethora of genes (Karlić *et al.* 2010).

In conclusion, the complete cDNA containing the ORF encoding for HvTX1, the barley putative ATX1/ATX2 homologue, was cloned and characterized in this work. *HvTX1* transcript expression was elevated during seed development and under drought stress. These results expand the studies of epigenetic modifiers in barley, and would contribute to further our understanding of seed development and drought stress adaptation in barley and other temperate cereals.

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