

# Identification and characterization of two members of the *FtsH* gene family in maize (*Zea mays* L.)

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**Abstract** Two full-length cDNAs, designated as *ZmFtsH2A* and *ZmFtsH2B*, were isolated from maize (*Zea mays* L.) by suppression subtractive hybridization coupled with *in silico* cloning approach. The predicted proteins of *ZmFtsH2A* and *ZmFtsH2B* both consisted of 677 amino acid residues and displayed high similarity to FtsH2 protease of *Arabidopsis thaliana*. DNA gel blotting analysis indicated that *AtFtsH2-like* genes exist as two copies in maize genome. The genomic sequences of *ZmFtsH2A* and *ZmFtsH2B* were cloned and the main difference was that the first intron of *ZmFtsH2B* was much longer than that of *ZmFtsH2A*. RT-PCR analysis revealed that both genes were constitutively expressed in all examined tissues and the expression level of *ZmFtsH2B* transcripts was higher than that of *ZmFtsH2A*. The responses of the two genes in maize seedlings to PEG, cold, high salt, and ABA treatments were compared, and the results showed that *ZmFtsH2B* transcription in leaves was markedly up-regulated by water deficit stress and ABA treatments while *ZmFtsH2A* constitutively expressed both in leaves and roots under all tested stressful conditions. Drought tolerance of transgenic tobaccos overexpressing *ZmFtsH2A* and *ZmFtsH2B* weren't improved compared to wild-type controls, which indicated that two genes might not be directly involved in plant drought tolerance or the number of functional FtsH heterocomplex might not be increased in this condition. Our current study provides fundamental information for the further investigation of the maize FtsH proteins.

**Keywords** FtsH protease · Expression pattern · Dehydration stress · *Zea mays*

## Introduction

Filamentation temperature-sensitive H (FtsH) is a membrane-anchored ATP-dependent metalloprotease originally identified in *Escherichia coli* [23, 31]; its homologs have subsequently been identified in many prokaryotic and eukaryotic organisms [13]. FtsH belongs to the AAA (ATPase associated with diverse cellular activities) protein family [17, 18].

Whereas all bacterial genomes contain a single *FtsH* gene, cyanobacteria have four such genes [16]. The *Arabidopsis thaliana* nuclear genome contains 12 such genes [28], and the rice (*Oryza sativa*) genome contains at least nine of them [37]. Thus, it appears that multiplication of *FtsH* genes correlates with the evolution of oxygenic photosynthesis and the trend is maintained in higher plants. In *Arabidopsis thaliana* three members of this family (*AtFtsH1–AtFtsH12*) are targeted to mitochondria and the other nine to chloroplasts, as shown by transient expression assays of green fluorescent protein (GFP) fusions [22]. Out of the nine chloroplast targeted isozymes, only *AtFtsH1*, 2, 5, and 8 were much reported. At the protein level, *AtFtsH2* is the most abundant FtsH protein in chloroplasts, followed by *AtFtsH5*, and then much lower accumulation levels of *AtFtsH8* and *AtFtsH1* [27]. Mutations in *AtFtsH2* cause severe leaf variegation and sensitivity to photoinhibition [3, 30]. Mutations in *AtFtsH5* lead only to slight variegation that disappears with development [20, 21], whereas inactivation of *AtFtsH8* and *AtFtsH1* in T-DNA insertion mutants does not lead to either leaf variegation or sensitivity to high

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light exposure [22]. A loss of *AtFtsH2* or *AtFtsH5* in *var2* or *var1* mutants is complemented by *AtFtsH8* and *AtFtsH1*, respectively. The spatial expression patterns of *AtFtsH1*, *AtFtsH2*, *AtFtsH5* and *AtFtsH8* are similar as revealed by promoter- $\beta$ -glucuronidase (GUS) fusions studies [36, 37]. The four FtsH exist in thylakoid membranes as a functional heterocomplex composed of type A (AtFtsH1 and AtFtsH5) and type B subunits (AtFtsH2 and AtFtsH8) [22, 36, 38]. Many researches demonstrate that the FtsH protease is involved in proteolytic removal of an oxidatively damaged and cleaved membrane protein. It has long been established that degradation of the D1 protein is inherent to the repair cycle of PSII from photoinhibition. Removal of damaged copies of D1 protein is a prerequisite for reassembly of the PSII complex with newly synthesized copies of D1. Recent reports suggest that lumen-located Deg 1 protease cooperates with the stroma-exposed proteases FtsH1-2-5-8 complex and Deg 2 in degrading D1 protein during repair from photoinhibition by cleaving lumen-exposed regions of the protein [2, 7, 11, 14]. And in addition, FtsH proteases play a key role in the repair of PSII under UV-B irradiation damage and moderate heat stress conditions [5, 12, 35].

Plant FtsH homologs exhibit various responses to stressful conditions. The *Nicotiana tabacum* DS9 gene (an *FtsH*-like gene) is constitutively expressed as a house-keeping gene in healthy leaves [26]. In contrast, the expression of *Arabidopsis AtFtsH1*, *AtFtsH5* and *AtFtsH2* is light-dependent [3, 14, 21]. In alfalfa (*Medicago sativa*), an *FtsH* gene is independently regulated either by low temperature or light [10]. Interestingly, a typical and specific heat-inducible *FtsH* gene has been cloned from tomato (*Lycopersicon esculentum*), whose expression is not induced by other stresses such as cold, drought, salinity or intense light [29]. Recent reports suggested that the chloroplastic FtsH11 protease plays a critical role in *Arabidopsis* thermo-tolerance [4]. Although a good deal of research has been conducted on the stress response of FtsH genes in plants, water-deficit stress inducibility of *FtsH* gene was not reported. We were interested in exploring the presence of water stress inducible *FtsH* in higher plants.

In the present study, we constructed a water stress inducible cDNA library using PCR-select cDNA subtraction with cDNAs from drought-treated maize leaves at flowering stage as driver and that from unstressed leaves as tester. Two *FtsH* cDNAs, named *ZmFtsH2A* and *ZmFtsH2B*, were identified by means of *in silico* cloning approach with the query probe EST from our library. The full-length genomic sequence corresponding to the two *FtsH* genes were cloned and the expression profiles of two genes in specific tissues and under various stressful conditions were also investigated.

## Materials and methods

### Plant materials and stress treatment

Maize (*Zea mays* L.) inbred line DH4866 was used in this study. Plants grew in flowerpots containing field soil under natural conditions. When the tassel spread out of the uppermost leaf, some plants were treated by drought stress for 7 days, and others were left under normal-watered conditions as control. During the period of the stress, the middle part of the top fully expanded leaves of the plants under water stress treatment and the control were taken at 8:00 am each day, and the leaf osmotic potential was measured by the Fiske<sup>®</sup> Micro-Sample Osmometer (Advanced instruments, Massachusetts, USA). The stressed plants were watered every day to maintain a similar water-deficiency state by adjusting water supply, according to the leaf osmotic potential. And the leaves osmotic potential of the stressed plants was hold to  $-0.4$  to  $-0.5$  Mpa, while the osmotic potential was  $-0.2$  to  $-0.25$  Mpa in the leaves of the control plants. During the stress, there were visible signs of water deficiency such as leaf rolling during the daytime, though at night the rolled leaves spread out. After 7 days of water deficiency by withholding water, the plants began to enter the flowering stage. The flag leaves of the plants were harvested at 168 h (7 day) of stress treatment, and frozen in liquid nitrogen immediately, stored at  $-80^{\circ}\text{C}$  for further analysis. Unstressed plants as controls were harvested at the same time as the stressed plants. And one sample consisted of the leaves from three plants under the same condition.

### Construction of SSH library

Total RNA was isolated, respectively, from samples by water-saturation phenol-guanidine isothiocyanate-chloroform method and mRNA was extracted from the RNA pool using an Oligotex<sup>™</sup> mRNA Purification Kit (QIAGEN, Maryland, USA). Forward-SSH (suppression subtractive hybridization) was performed using cDNA synthesized from treated plants as tester and that from the control plants as driver with the Clontech PCR-Select<sup>™</sup> cDNA subtraction Kit (Clontech, California, USA) according to the manufacturer's protocol. The cDNAs were digested with *Rsa*I and then ligated to different adapters. Two rounds of hybridization and PCR amplification were processed to normalize and enrich genes that are up-regulated by water stress. The PCR product from secondary PCR amplification of SSH was directly inserted into pGEM<sup>®</sup> T-easy vector (Promega, Wisconsin, USA) and transformed into *E. coli* DH5 $\alpha$  cells. After cultured overnight on LB medium with ampicillin, X-gal, and IPTG, white clones were picked out randomly, the insert cDNAs of the clones were assayed by PCR with the vector primers

(T7 and SP6) and sequenced by Sangon Company (Shanghai, China).

#### Isolation of two full-length AtFtsH2-like genes

A 192 bp EST (expressed sequence tag) was obtained by sequencing subtracted cDNA library of *Z. mays*. Following similarity searching from BLASTX at NCBI (<http://www.ncbi.nlm.nih.gov/blast>), the partial cDNA of putative FtsH protease gene fragment was identified. By *in silico* cloning analysis using the 192 bp EST as a query probe, two contig sequences, 2,510 and 2,430 bp in size, were obtained. According to the predicted sequence, two pairs of primers (for *ZmFtsH2A*, 5'-GCCTGACGAGTCTACA CAAGC-3' and 5'-TCTCGTTAATCTACGAGCAAG-3'; and for *ZmFtsH2B*, 5'-GCTC GCCTACTCTCCTC-3' and 5'-CACCATGAGTAAGTTACATCT-3') were designed to amplify two cDNAs including the complete ORF (open reading frame), respectively. PCR program involved 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min 20 s; final extension 72°C for 10 min. The amplified two cDNA fragments were inserted into pGEM<sup>®</sup> T-easy vector and sequenced.

Comparisons of cDNAs to genomic sequences of the *ZmFtsH2A*, *ZmFtsH2B* and homologous genes in *O. sativa* and *A. thaliana* were performed to design primers surrounding the postulated introns, and the corresponding introns were isolated. For the PCR, 200 ng of maize leaf genomic DNA was employed as the template and the introns were amplified with the primer sets as follows: for the first intron of *ZmFtsH2A*, 5'-GCCTGACGAGTCTACA CAAGC-3' and 5'-TTTGTCAAGATACTCAAGGAAC-3'; for the second intron of *ZmFtsH2A*, 5'-TCTTGGATTC CGCTTTAC-3' and 5'-ACTTTT GCTCTCCCGTC-3'; for the third intron of *ZmFtsH2A*, 5'-TTGACAGAATT GTGGCT GG-3' and 5'-AGTTGCTGCCTGGAGATG-3'; for the fourth intron of *ZmFtsH2A*, 5'-TG GTCTTACA TGGTTTATCC-3' and 5'-TGTTGTTTCTAATGTGCCT C-3'; for the first intron of *ZmFtsH2B*, 5'-GCTCGCCTAC TTCTACGCTC-3' and 5'-ACTTCCTTCTGGC ATCGC-3'; for the second intron of *ZmFtsH2B*, 5'-GGCTGACAT ATTGGATTC-3' and 5'-CCAGCTACAATTCTGTCA-3'; for the third intron of *ZmFtsH2B*, 5'-GGAAGGACA GCA ATTTCTTC-3' and 5'-TCACCTCAGGCT CTCCAA-3'; and for the fourth intron of *ZmFtsH2B*, 5'-GGTCTTGGT GGTAGAGCT-3' and 5'-CACTGTCTCCTTCTCAATG-3'. The amplified genomic fragments were inserted into pGEM<sup>®</sup> T-easy vector and sequenced as for the cDNA.

#### DNA gel blot and tissue-specific expression analysis

Genomic DNA of maize (*Z. mays*) leaves was extracted by the cetyltrimethyl ammonium bromide (CTAB) method.

RNase (Takara, Dalian, China) was used to digest RNA from genomic DNA. Genomic DNA (15 µg/sample) was digested overnight at 37°C with *KpnI*, *EcoRV*, *XbaI* and *EcoRI*, respectively, separated by 0.8% agarose gel electrophoresis and blotted onto Hybond-N<sup>+</sup> nylon membrane (Roche, Mannheim, Germany). A 953 bp probe of the *ZmFtsH2A* gene, amplified using primers 5'-TCTTGGATT CCGCTTTA C-3' and 5'-GACTTGACTTTAGGCAGG-3', was used for hybridization. Non-radioactive digoxigenin (DIG) probe labeling, hybridization and detection were carried out according to the procedure of the DIG SYSTEM protocol (Roche, Mannheim, Germany).

Total RNAs were extracted by Trizol reagent (Sangon, Shanghai, China) from different tissues of maize and treated with RNase-free DNase (Takara, Dalian, China). cDNA synthesis was performed with the RT (reverse transcription) reagent kit (Takara, Dalian, China) according to the manufacturer's protocol. Considering high sequence homology between *ZmFtsH2A* and *ZmFtsH2B*, two pairs of primers were designed corresponding to 3'-untranslated region (UTR) of the two genes. Gene-specific primers for *ZmFtsH2A* were 5'-TGGTAGACGACATGTAACCTGC-3' and 5'-TCTCGTTAATC TACGAGCAAGA-3'; and for *ZmFtsH2B*, 5'-CTAGCCTTGTAGGGGAGGAT-3' and 5'-ACATCTT ACATGTCGTGTACCG-3'. PCR program involved 95°C for 5 min; 33 cycles of 95°C for 40 s, 55°C for 40 s, 72°C for 30 s; final extension 72°C for 5 min. The maize  $\alpha$ -tubulin (5'-CACTGATGTTGCTGTCCTGC-3' and 5'-CGCTGTTGGTGATT TCGG-3') was used as the internal control.

#### Stress treatments and quantitative real-time RT-PCR assay

All plants used for treatment below were grown under normal conditions (16 h light/28°C, 8 h dark/19°C). For cold treatment, the 2-week-old seedlings grown at 28°C were shifted to 4°C. For water-deficit stress, high salt stress and ABA treatment experiments, the plants were grown for 2 weeks in liquid MS medium, and then treated with fresh liquid medium supplemented with 18% (W/V) polyethylene glycol (PEG) (MW 6,000), 200 mM NaCl, or 100 µM ABA, respectively. The samples were collected at indicated times (0, 1, 6, 12, and 24 h), and the roots and aerial parts were separated and frozen immediately into liquid nitrogen until required.

For QRT-PCR, total RNAs were extracted by Trizol reagent (Sangon, Shanghai, China) from samples and treated with RNase-free DNase I (Takara, Dalian, China). cDNA synthesis was performed with the RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocol. Real-time PCRs were done on a Chromo 4<sup>TM</sup> continuous

fluorescence detector (MJ research, Massachusetts, USA) with the SYBR<sup>®</sup> RT-PCR Kit (Takara, Dalian, China), in a 10  $\mu$ l reaction volume, which contained 5  $\mu$ l of SYBR<sup>®</sup> Green I PCR mix, 0.2  $\mu$ M of each forward and reverse primer, 1  $\mu$ l of diluted cDNA template, and appropriate amounts of sterile ddH<sub>2</sub>O. Amplification conditions were: 2 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Fold changes of RNA transcripts were calculated by the  $2^{-\Delta\Delta Ct}$  method [15] with maize  $\alpha$ -tubulin as an internal control. The entire experiments were repeated three times.

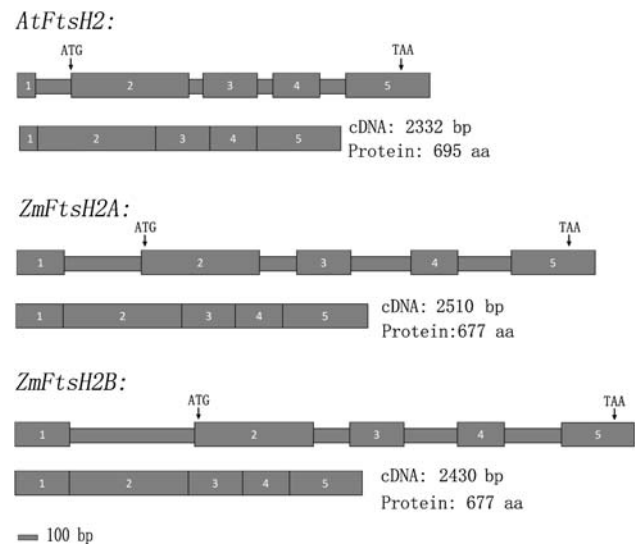
#### Plant transformation and selection of transgenic lines

To generate transgenic tobacco lines overexpressing the *ZmFtsH2A* or *ZmFtsH2B*, the whole length cDNA of the two genes were, respectively, subcloned into the binary vector pCAMBIA1300-35SMCSnos-*als*, which contained a constitutive CaMV35S promoter, a nos terminator and a mutational chlorsulfuron-resistance acetolactate synthase gene (*als*) from *Arabidopsis*, according to the standard molecular protocol. The recombinant binary plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by a freeze-thaw technique [8]. *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf discs was carried out following the procedure described previously [33]. Transformed shoots were selected on MS medium supplemented with 6-benzylaminopurine (1  $\mu$ g/ml), cefotaxime (400  $\mu$ g/ml) and herbicide chlorsulfuron (400  $\mu$ g/ml). Regenerated shoots were rooted on MS medium containing herbicide chlorsulfuron (400  $\mu$ g/ml). Transformants and WT tobaccos were grown in soil pots in a green house under normal conditions (16 h light/25°C, 8 h dark/19°C).

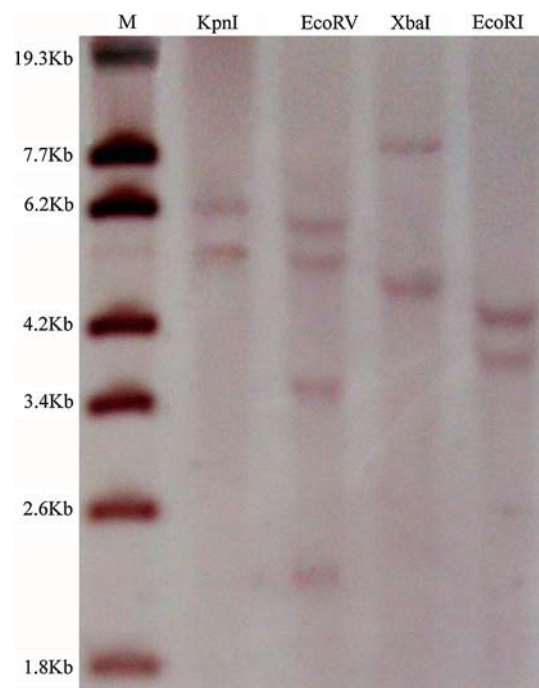
#### Drought tolerance assays of tobacco seedlings

Tobacco plants of wild type and transgenic lines were transferred to soil pots and watered regularly. After 4 weeks of growth, plants were subjected to drought stress treatment by stopping irrigation for 5 days. The activity of photosystem II, expressed as Fv/Fm, was determined on the third leaves of tobacco seedlings after dark adaptation for 30 min with a pulse modulation chlorophyll fluorometer (Hansatech, Norfolk, UK) at room temperature [25]. The total chlorophyll content of tobacco leaves was determined according to the method Arnon [1] described previously. The relative water content (RWC) in leaves was determined as Schonfeld et al. [24] described. Total soluble sugars in leaves were extracted with 80% ethanol and estimated by anthrone reagent using glucose as the standard [34]. And leaf cell membrane damage was determined

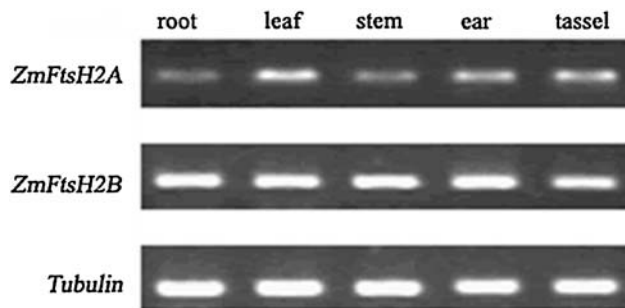
by measuring the ion leakage of cell membranes and MDA levels in plants, according to Gibon et al. [6] and Quan et al. [19], respectively.



**Fig. 1** Diagram of the structures of *AtFtsH2*, *ZmFtsH2A*, and *ZmFtsH2B* genes. Drawn to scale, larger boxes with numbers represent exons and the smaller ones represent introns. ORFs are also depicted. Scale bar = 100 bp



**Fig. 2** DNA gel blot analysis of *ZmFtsH2A* and *ZmFtsH2B*. *M* presents the  $\lambda$ DNA/*EcoT14 I* molecular weight marker; Genomic DNA (15  $\mu$ g/sample) is digested with *KpnI*, *EcoRV*, *XbaI* and *EcoRI*, respectively, followed by hybridization with the partial cDNA of *ZmFtsH2A*



**Fig. 3** Tissue specific expression of *ZmFtsH2A* and *ZmFtsH2B*. Expression of the *ZmFtsH2A* and *ZmFtsH2B* genes in different tissues, detected by RT-PCR with the maize  $\alpha$ -tubulin as a control

## Results and discussion

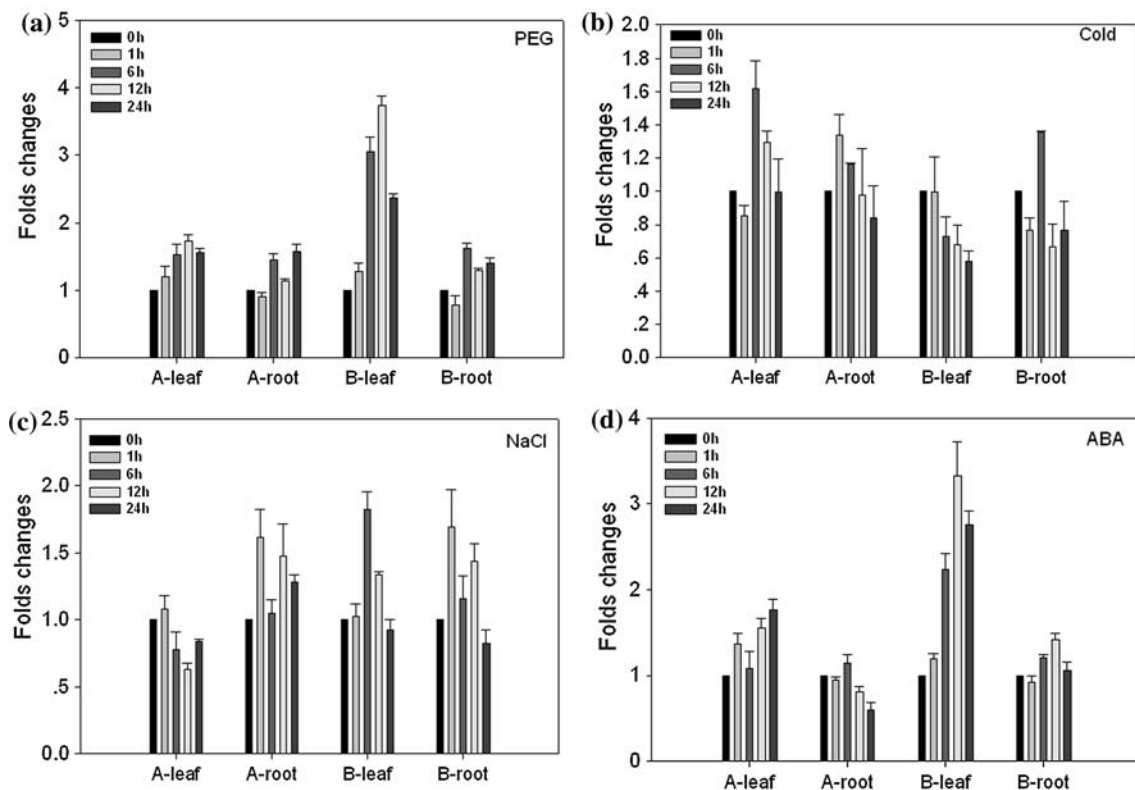
### Analysis of SSH library

To identify differential expression of genes related to water stress in maize leaves, a cDNA library was constructed by SSH with two mRNA populations of 7d-water-stressed and unstressed control flag leaves of *Z. mays*. A total of 940 white colonies picked out randomly from the library were

single-pass sequenced and 860 produced readable sequences represented 411 unique ESTs were obtained. All these unique ESTs have been submitted to the EST division of GeneBank with accession number FK933938-FK934348 and can be accessed at the NCBI EST database (<http://www.ncbi.nlm.nih.gov/dbEST>).

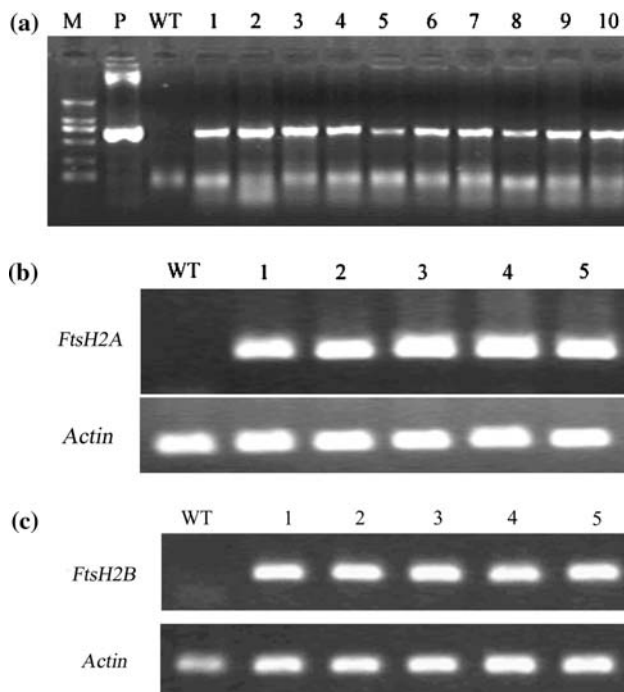
*ZmFtsH2A* and *ZmFtsH2B*: two *AtFtsH2*-like genes from maize

In this study the focus was on a 192 bp clone (NCBI No., FK934297) that showed strong sequence similarity to *Arabidopsis thaliana* *FtsH2* protease gene by searching GeneBank database using BLASTX. The maize 192 bp cDNA fragment was used as seed to search the NCBI expressed sequence tags database (dbEST) of *Z. mays*. Retrieved homologous ESTs were constructed into two contigs by CAP3 software [9]. One contig is 2,510 bp while the other contig is 2,430 bp long showing 95% identity to each other at the nucleotide acid level. The open reading frames (ORF) of the two contigs (named *ZmFtsH2A* and *ZmFtsH2B*, respectively) were determined by the program of NCBI ORF finder. And then two maize cDNAs with complete ORFs



**Fig. 4** Expression patterns of *ZmFtsH2A* and *ZmFtsH2B* under stresses. Real time PCR analyses were performed by using total RNA extracted from leaves and roots of 2-week-old seedling treated, respectively, with various stresses including PEG (a), cold (b), high salt (c) and ABA (d) at different time points (0, 1, 6, 12, and 24 h).

The fold change of RNA transcripts were calculated by the  $2^{-\Delta\Delta Ct}$  method with maize  $\alpha$ -tubulin as an internal control. *A-leaf*, *A-root*, *B-leaf* and *B-root* refer to expression of *ZmFtsH2A* and *ZmFtsH2B* in leaf and roots, respectively. Values are the means  $\pm$  SE of three independent biological replicates



**Fig. 5** Molecular characterization of the heterologous expression of *ZmFtsH2A* and *ZmFtsH2B*. **a** PCR analysis of transgenic tobacco plants with primers amplifying *als* gene: lane M, DNA marker DL2000; lane P, PCR result of plasmid pCPA; lane WT, wild type control; lane 1–5, FtsH2A gene transgenic plants; lane 6–10, FtsH2B gene transgenic plants. Analysis by RT-PCR of the heterologous expression of *ZmFtsH2A* (**b**) and *ZmFtsH2B* (**c**) in tobacco transgenic lines. WT wild type tobacco; A 216 bp actin fragment was amplified by RT-PCR as an internal control. Primers for detecting transgene expression were those have been used for expression analyses of *ZmFtsH2A* and *ZmFtsH2B*

were amplified by RT-PCR using two pairs of gene-specific primers designed based on the results of *in silico* cloning. Using the online bl2seq program (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>), a BLAST-based tool for aligning two sequences, the protein sequences similarity were obtained by paired comparing AtFtsH2 with the two maize putative FtsH proteases. The results showed that AtFtsH2 protein exhibited an amino acid identity of 85 and 87% with ZmFtsH2A and ZmFtsH2B, respectively. As the two FtsHs was 97% identical in their amino acids sequence and showed significant similarity to AtFtsH2 protease, the two cDNAs were designated as *ZmFtsH2A* and *ZmFtsH2B*. The full-length cDNA sequences of *ZmFtsH2A* and *ZmFtsH2B* were deposited in the GenBank database under the accession numbers EU257690 and EU257691, respectively.

*ZmFtsH2A* (2,510 bp) consisted of a poly (A) tail, a 3'-untranslated region of 241 bp and a 2,034 bp ORF encoding 677 amino acids. Similarly, *ZmFtsH2B* (2,430 bp) composed of a poly (A) tail, a 156 bp 3'-untranslated region and a 2,034 bp ORF encoding 677 amino acids. Analyses of

the two protein sequences with pI/Mw software ([http://expasy.org/tools/pi\\_tool.html](http://expasy.org/tools/pi_tool.html)) indicate that ZmFtsH2A has a predicted pI of 5.68 and a molecular mass (Mw) of 72.4 kDa and the theoretical pI/Mw of ZmFtsH2B is 5.69/72.5 kDa. Multiple sequence alignments was carried out at the amino acid level using ClustalW version 1.83 software with default parameters set as in the ClustalW web sever at EMBL-EBI (<http://www.ebi.ac.uk/Tools/clustalw/>). Both the central region of ZmFtsH2A and ZmFtsH2B contain two putative ATP binding sites (Walker-A, Walker-B) and the putative SRH (the second region of homology). The C-terminus of the ZmFtsH2A and ZmFtsH2B contain the putative zinc-binding motif HEXGH (where each X is a non-conserved amino acid residue) that is responsible for metalloprotease activity [32].

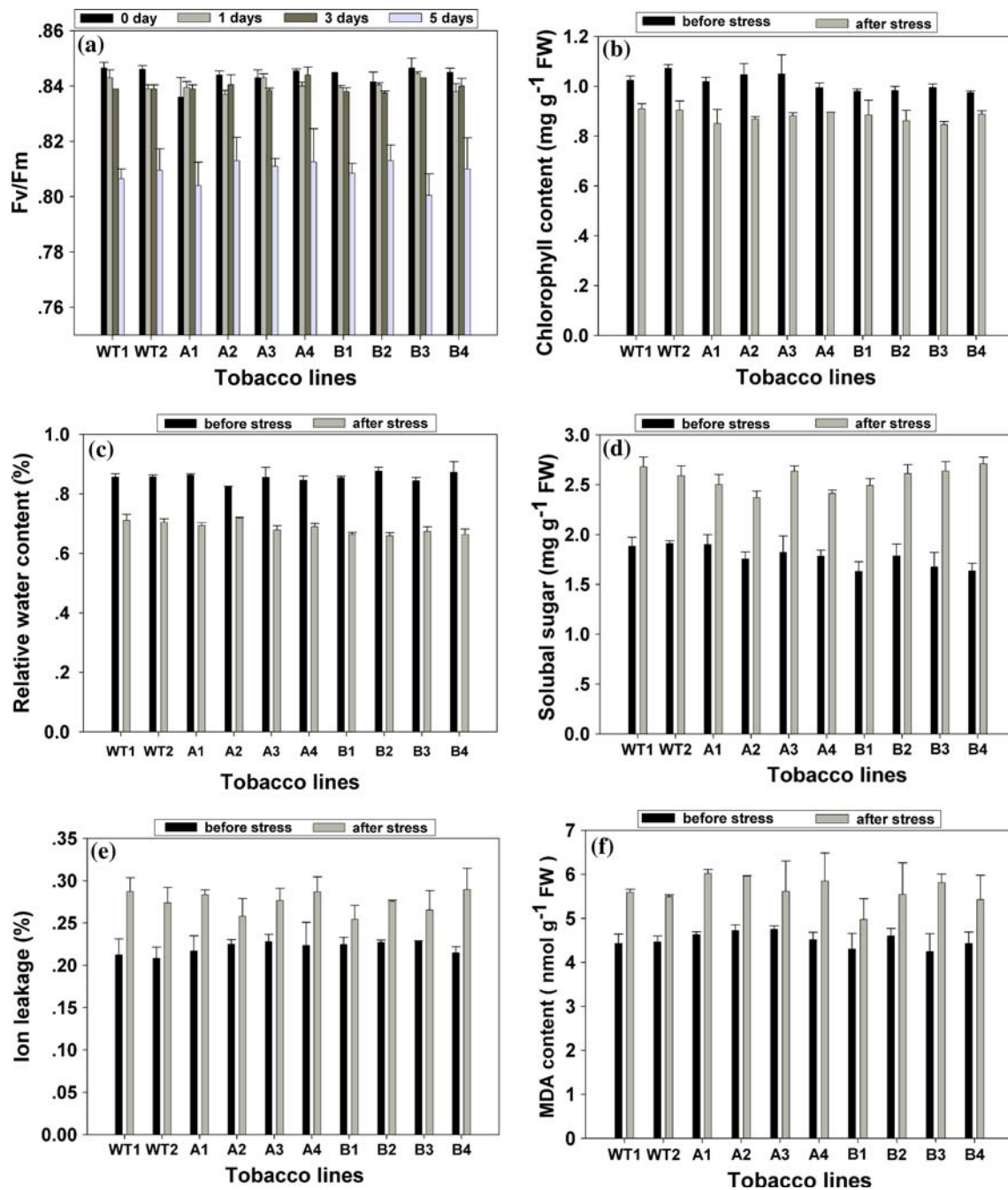
Using genomic DNA as template and primer sets specific to *ZmFtsH2A* and *ZmFtsH2B* cDNAs, a series of PCRs were operated and specific fragments produced were then sequenced. The comparison of these sequences with their cDNA counterparts in both genes showed three introns in the FtsH-encoding ORFs and one intron in their 5' untranslated leader sequences (Fig. 1). The complete genomic sequences corresponding to *ZmFtsH2A* and *ZmFtsH2B* were 4,420 and 5,187 bp in length, respectively. The maize genome database was queried and the results supported the exactness of the obtained genomic sequences (data no shown). And the main difference between the genomic sequences of the two maize members is that the first intron of *ZmFtsH2B* is much longer than that of *ZmFtsH2A* gene. The two maize genes showed an intron-exon structure comparable to that of AtFtsH2 (Fig. 1). The complete genomic sequences of *ZmFtsH2A* and *ZmFtsH2B* were deposited in the GenBank database under the accession numbers EU257692 and EU257693, respectively.

#### DNA gel blot analysis

To determine the gene copy number of *AtFtsH2*-like gene in maize genome, we performed genomic DNA gel blot analysis, using 953 bp partial *ZmFtsH2A* cDNA as the probe. There are no restriction sites of *KpnI*, *XbaI* and *EcoRI* within the probe region except for only one restriction *EcoRV* site of present in the probe region. Two hybridization bands were present on each lane except that four bands on *EcoRV* lane (Fig. 2), indicating that there were two copies of *AtFtsH2*-like genes in the genome of *Z. mays*.

#### Expression of ZmFtsH2A and ZmFtsH2B

To determine the tissue-specific expression patterns of *ZmFtsH2A* and *ZmFtsH2B* genes, semi-quantitative



**Fig. 6** Comparison of Fv/Fm **a** Chlorophyll content, **b** relative water content, **c** total soluble sugar content, **d** membrane ion leakage, **e** MDA content, **f** in leaves of tobacco seedlings before stress and after 5 days of drought treatment. The values are mean  $\pm$  SE of three replications

RT-PCR was performed in which 0.5  $\mu$ g of total RNA from various tissues was used as the template. Specific combinations of primers were used to selectively amplify *ZmFtsH2A* or *ZmFtsH2B* and  $\alpha$ -tubulin as a control of the expression levels. The results indicated that both *ZmFtsH2A* and *ZmFtsH2B* were constitutively expressed in leaf, stem, root, ear and tassel of maize under normal growth conditions (Fig. 3). On the other hand, two maize genes showed differential expression levels. The *ZmFtsH2B* message RNA

was considerably abundant, whereas *ZmFtsH2A* transcript was found at a much lower level in the tissues (Fig. 3). The relative transcript levels of the two genes in maize organs reflect the relative abundance of each message in the *Z. mays* EST database (results not shown).

The expression profiles of the *ZmFtsH2A* and *ZmFtsH2B* under ABA treatment and different abiotic stress conditions were analyzed by quantitative real-time RT-PCR. As shown in Fig. 4, *ZmFtsH2A* and *ZmFtsH2B*

showed differential expression patterns, according to the tissues and stress treatments considered. The expression of *ZmFtsH2A* was not altered at significant levels both in leaves and roots of maize seedlings under all tested stressful conditions, while the transcript level of *ZmFtsH2B* only increased up to more than twice folds in leaves of maize seedlings subjected to ABA treatment and water deficit stress with 18% PEG. It was worth noticing that the transcription of *ZmFtsH2B* was induced at 6 h by exogenous ABA in leaves and kept a relative high transcript level. However, *ZmFtsH2B* transcripts did not accumulated to a significant level in roots under ABA treatment. This result indicates that *ZmFtsH2B* transcription seems to be regulated through ABA-dependent signaling pathway, but the pathway still needs further study. Altogether, the results suggest that *ZmFtsH2A* was constitutively expressed and the transcript level of *ZmFtsH2B* was up-regulated by water stress and ABA treatment in maize leaves.

#### Drought tolerance analysis of transgenic tobaccos

The tobacco leaf discs were transformed via *Agrobacterium tumefaciens* with the *ZmFtsH2A* or *ZmFtsH2B* gene, respectively, under the control of cauliflower mosaic virus (CaMV) 35S promoter. For transgenic plants selection, the genomic DNA was isolated from tobacco leaves by the cetyltrimethylammonium bromide (CTAB) method, and PCR assays were performed with the primers designed for the *als* gene from T<sub>0</sub> generations. And the expression of two heterologous genes in T<sub>0</sub> transgenic plants was confirmed by RT-PCR analysis, respectively (Fig. 5). Four lines from each construction and two wild type (WT) lines generated in parallel were chosen for drought tolerance studies.

Under normal growth conditions, no phenotypic variation could be observed among *ZmFtsH2A* or *ZmFtsH2B* transgenic tobaccos and WT plants. And all the examined plants wilt to the same level under drought stress conditions. The physiological parameters, such as Fv/Fm, RWC, total soluble sugars, MDA level and total chlorophyll content of tobacco leaves, were measured, and the results showed that the drought tolerance of transgenic tobaccos overexpressing the *ZmFtsH2A* or *ZmFtsH2B* were not improved compared with wild type controls, which indicated that maize FtsH2 might not be directly involved in plant drought tolerance (Fig. 6), or the number of functional heterocomplex composed of type A and type B subunits might not be increased although the overexpressing the *ZmFtsH2A* or *ZmFtsH2B* in transgenic tobaccos plants was achieved.

In summary, two novel full-length *FtsH* genes of *Z. mays* were cloned and characterized. It is the first *FtsH* protease genes isolated from maize. Bioinformatics analysis showed that the deduced *ZmFtsH2A* and *ZmFtsH2B* was highly homologous to the FtsH2 protease of

*Arabidopsis thaliana*, and two FtsHs contained all conserved residues displayed by plant FtsH protein family. Both *ZmFtsH2A* and *ZmFtsH2B* were ubiquitously expressed in all tested tissues and *ZmFtsH2B* transcript level was much higher than that of *ZmFtsH2A*. Real-time PCR assays showed that *ZmFtsH2B* was up-regulated by water stress and ABA treatments, whereas *ZmFtsH2A* constitutively expressed under all tested stressful conditions. To investigate the physiological functions of two FtsH proteases, the full length cDNAs of *ZmFtsH2A* and *ZmFtsH2B* driven by 35S promoter were transformed into tobaccos. And the results of drought tolerance analysis of transgenic tobaccos indicated that maize FtsH2 might not be directly involved in plant drought tolerance. The cloning and characterization of *ZmFtsH2A* and *ZmFtsH2B* genes will be helpful to understand the detailed functions or roles of FtsH family members in *Z. mays*.

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