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Molecular cloning and characterization of a novel H^+-translocating pyrophosphatase gene in *Zea mays*

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Abstract
A cDNA encoding a putative H^+-translocating pyrophosphatase (H^+-PPase) has been cloned from *Zea mays* by suppression subtractive hybridization (SSH) coupled with *in silico* cloning approach. The isolated 2974 bp full-length cDNA named *ZmGPP* contains a single 2400 bp open reading frame encoding a putative protein of 799 amino acids. The predicted protein has 16 transmembrane domains and is significantly similar to Golgi apparatus resident type-II H^+-PPase from *Arabidopsis thaliana*. DNA gel blotting analysis shows that *ZmGPP* is a low-copy gene. Organ expression pattern analysis reveals that *ZmGPP* expressed highly in leaf and tassel, followed by in stem, root, and ear. The Real-time RT-PCR assays showed that the expression of *ZmGPP* was up-regulated both in shoots and roots of maize seedlings under dehydration, cold and high salt stresses. Those results suggest that the *ZmGPP* product may play an important role in abiotic stress tolerance of *Z. mays*.

Keywords: *H^+-PPase,* in silico *cloning, Golgi apparatus, RT-PCR,* Zea mays

Introduction

The H^+-translocating pyrophosphatase (H^+-PPase) is an electrogenic proton pump that acidifies intracellular compartments in plant cells and unicellular eukaryotes (Rea and Poole 1993). Two distinct biochemical subclasses of H^+-PPases have been characterized to date: type I vacuolar H^+-PPase stimulated by K^+ (Kim et al. 1994; Docampo and Moreno 2001) and type II H^+-PPase insensitive to K^+ located in the Golgi apparatus (Drozdowicz et al. 2000; Mitsuda et al. 2001). Typified by AVP1 (type I H^+-PPase) from *Arabidopsis thaliana*, all characterized plant vacuolar H^+-PPases share greater than 80% sequence identity in their amino acid sequences and catalyze K^+-stimulated H^+ translocation (Maeshima 2000; Drozdowicz and Rea 2001). The type I H^+-PPase is a single subunit protein located in the vacuolar membrane (Maeshima 2000). It belongs to the fourth class of electrogenic proton pump in addition to the P-, F-, and V-type ATPases. The proton pumping reaction couples with the hydrolysis of PPi. V-PPase acidifies vacuoles together with vacuolar H^+-ATPase in the plant cell (Rea and Poole 1993). The proton-motive force generated by the vacuolar ATPase and vacuolar pyrophosphatase can drive secondary transporters, such as the Na^+/H^+ antiporter and Ca^{2+}/H^+antiporter, as well as organic acids, sugars, and other compound transporters to maintain cell turgor (Lincoln 1992). Overexpression of the vacuolar H^+-PPases in transgenic *A. thaliana* and *Nicotiana tabacum* resulted in increased plant tolerance to drought and salt stresses (Gaxiola et al. 2001; Gao et al. 2006; Guo et al. 2006).

In higher plants only one type II H^+-PPase (AVP2) gene of *A. thaliana* has been cloned and reported by now (Drozdowicz et al. 2000). The AVP2 protein has only 35% amino acid sequence identity to the vacuolar H^+-PPase encoded by AVP1. The use of green fluorescent protein (GFP)-tagging has suggested the

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presence of AVP2 in the Golgi apparatus of *A. thaliana*. Matsuoka et al. (1997) reported that the acidic environment generated by Golgi resident V-ATPase is important for the sorting of soluble vacuolar proteins. It is likely that the type-II H^+-PPase functions as an H^+ pump of GA in combination with GA resident V-ATPase for generating the acidic environment (Mitsuda et al. 2001).

Although the existence of H^+-PPase in the Golgi apparatus of maize roots has been demonstrated by immunoelectron microscopy (Oberbeck et al.1994), the corresponding gene has not been identified. In this report, we focus on the cloning of an AVP2-like proton pumping pyrophosphatase gene candidate for Golgi apparatus resident H^+-PPase of *Zea mays* and the analysis of its expression pattern in various tissues and under different treatments.

Materials and methods

Growth of maize seedlings and stress treatments

Sterilized germinated seeds from maize (inbred DH4866) were transferred to a 25°C growth chamber and incubated with 70% relative humidity. Then seedlings used for subtractive cDNA library construction were treated with 18% PEG 6000 for 24 h. For gene expression analysis, the seedlings of maize were treated as follows. In the treatments of high salt, dehydration stress or ABA treatment, the seedlings of maize were grown in the liquid medium that contained full-strength Murashige and Skoog salts and 200 mM NaCl, 18% PEG 6000 or 100 μM ABA, pH 5.7 under normal conditions (16-h light/28°C, 8-h dark/19°C). For cold treatment the plants grown at 28°C were shifted to 4°C. For dehydration and cold stresses, leaves and roots samples were collected at the various periods (0,0.5,1,6,12 and 24 h) and for high salt and ABA treatments, samples were collected at 0,1,2,6,12 and 24 h. All samples were frozen in liquid N_2 until required.

Construction of subtracted cDNA library

Total RNA was extracted from samples by water-saturation phenol–guanidine isothiocyanate–chloroform method and mRNA was purified from total RNA using an Oligotex™ mRNA Purification Kit (QIAGEN). The substracted cDNA library was constructed using a Clontech PCR-Select™ cDNA subtraction Kit (CLONTECH) according to the manufacturer's protocol. A mix of cDNAs that were reverse-transcribed from 2 μg mRNA of maize seedlings treated with 18% PEG for 1 and 24 h, was used as a tester and the cDNA from untreated seedlings used as a driver. 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 the differentially expressed cDNAs.

Screening of subtracted cDNA library

The PCR product from secondary PCR amplification of suppression subtractive hybridization (SSH) was directly inserted into pGEM® T-easy vector (PROMEGA) and transformed into *Escherichia coli* DH5α cells. After cultured overnight on LB medium with ampicillin, X-gal, and IPTG, 100 white clones were picked out randomly and the insert cDNAs of the clones were assayed by PCR with the vector primers (T7 and SP6) and sequenced by Sangon Company. A 764 bp cDNA clone was identified as partial cDNA of putative H^+-PPase by searching using BLASTx at NCBI (http://www.ncbi.nlm.nih.gov/blast).

Gene cloning

The maize 764 bp cDNA was used as seed to search the NCBI EST database of *Z. mays*. Retrieved homologous ESTs were constructed into contigs by CAP3 (Huang 1996). The open reading frames (ORF) of these contigs sequence were determined by the program of NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). According to the predicted sequence, a pair of primers P1 (5′-GCTTGACGGCAAAAGGACC-3′) and P2 (5′-CCTCATCTACCTGGTCGCATA-3′) were designed to amplify H^+-PPase cDNA including the complete encoding region. PCR program involved 95°C for 5 min; 30 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 2 min; final extension 72°C for 10 min. The amplified 2.6 kb cDNA fragment was inserted into pGEM® T-easy vector and sequenced.

The 3′-end of cDNA was obtained according to the BD SMART™ RACE cDNA Amplification kit manual (CLONTECH). Gene specific primer R1 (5′-GGTTACTGGCGACACGGTTGGAGA-3′) was designed based on the result of sequencing. PCR conditions were as follows: 95°c for 5 min, 5 cycles of 95°c for 1 min and 72°c for 2 min; 6 cycles of 95°c for 1 min, 72°c for 1 min (dropping 1°c every cycle), 72°c for 2 min; 30 cycles of 95° for 1 min, 65°c for 1 min, 72°c for 2 min; additional extension 72°C for 10 min. The amplified cDNA fragment was inserted into pGEM® T-easy vector and sequenced. All primers were designed by Primer Premier 5.0 software.

DNA gel blot analysis

Genomic DNA of maize (*Z. mays*) leaves was extracted by the cetyltrimethyl ammoniun bromide (CTAB) method (Sambrook et al. 1989). RNase (TAKARA) was used to digest RNA from genomic DNA. Genomic DNA (15 μg/sample) was digested overnight at 37°C with EcoRI, KpnI, EcoRV and

BamHI, respectively, separated by 0.8% agarose gel electrophoresis and blotted onto Hybond-N^+ nylon membrane (ROCHE). The 764 bp cDNA fragment of *ZmGPP* was used as the probe for hybridization. Probe labeling (DIG), hybridization and detection were carried out according to the procedure of the DIG SYSTEM protocol (ROCHE).

Tissue-specific expression analysis

Total RNA was isolated from different tissues of maize (*Z. mays*) as described previously (McCarty 1986). RNase-free DNase I (TAKARA) was used to digest the genomic DNA from the total RNA. About 500 ng total RNA from each sample was used for the synthesis of the first-strand cDNA for RT-PCR using SuperScript™ RT-PCR kit (TAKARA). Primers P3 (5′-GTTACTGGCGACACGGTTG-3′) and P4 (5′-ACATTCGGGGTCCACATC-3′) were designed based on the result of 3′-RACE to amplify the fragment of 439 bp. The maize *α-tubulin* (T1: 5′-CACTGATGTTGCTGTCCTGC-3′ and T2: 5′-CGCTGTTGGTGATTTCGG-3′) was used as the control.

Quantitative RT-PCR assay

For Real-time RT-PCR, total RNAs were extracted by Trizol reagent (SANGON) from leaves and roots samples and treated with RNase-free DNase I (TAKARA). cDNA synthesis was performed with the RT reagent kit (TAKARA) according to the manufacturer's protocol. Real-time quantitative RT-PCRs were done on a Chromo 4™ continuous fluorescence detector (MJ RESEARCH) with the SYBR® RT-PCR Kit (TAKARA), in a 10 μl reaction volume, which contained 5 μl of SYBR® Green I PCR mix, 0.2 μM of each forward and reverse primer, 1 μl of diluted cDNA template, and appropriate amounts of sterile ddH_2O. 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 $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001) with maize *α-tubulin* as an internal control. The entire experiments were repeated three times.

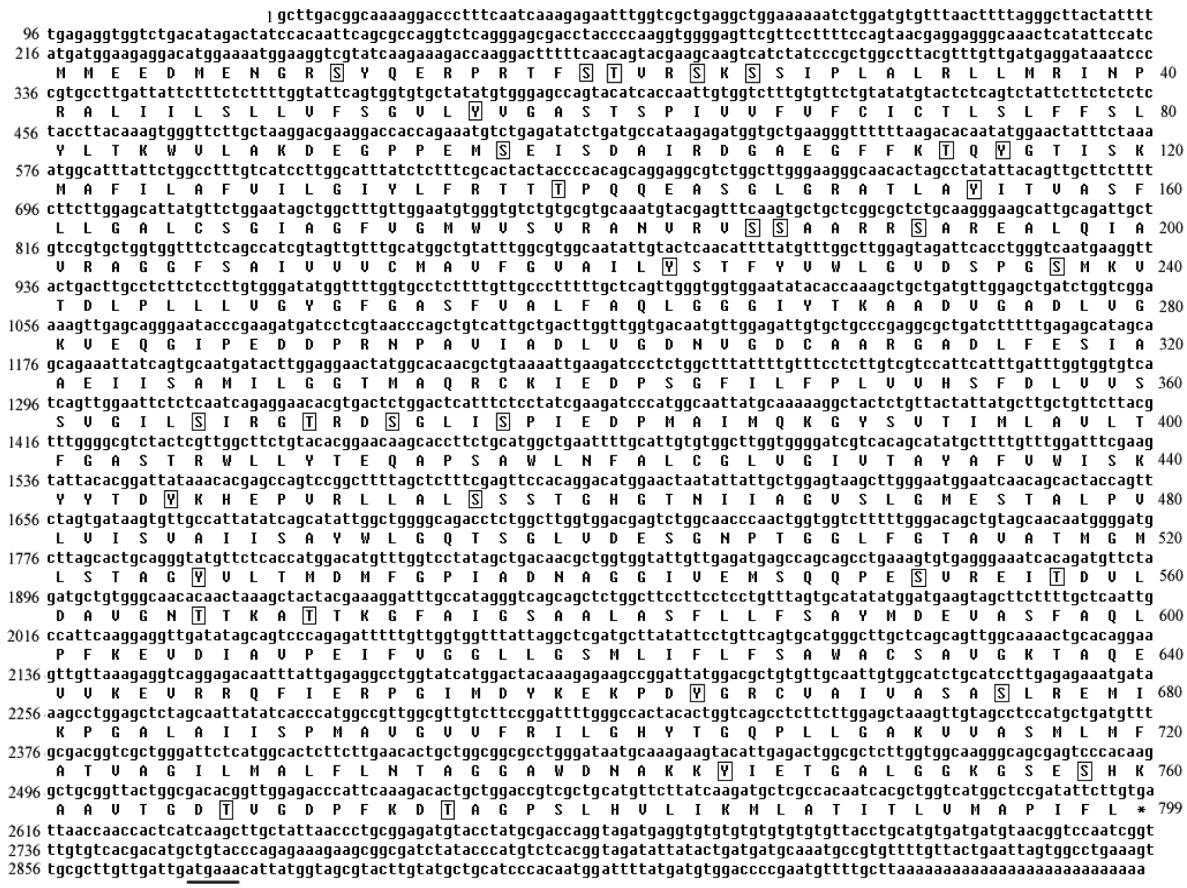

Figure 1. Nucleotide and derived amino acid sequences of putative ZmGPP. The number on the left indicates the position of the first nucleotide, while those on the right represents the position of the last amino acid, in the respective row. The cDNA sequence has a 2400 bp open reading frame, which encodes a 799 amino acid protein. The stop codon is indicated by an asterisk (*), and a polyadenylation signal (ATGAAA) is underlined. The 33 potential phosphorylation sites in the mature protein are boxed.

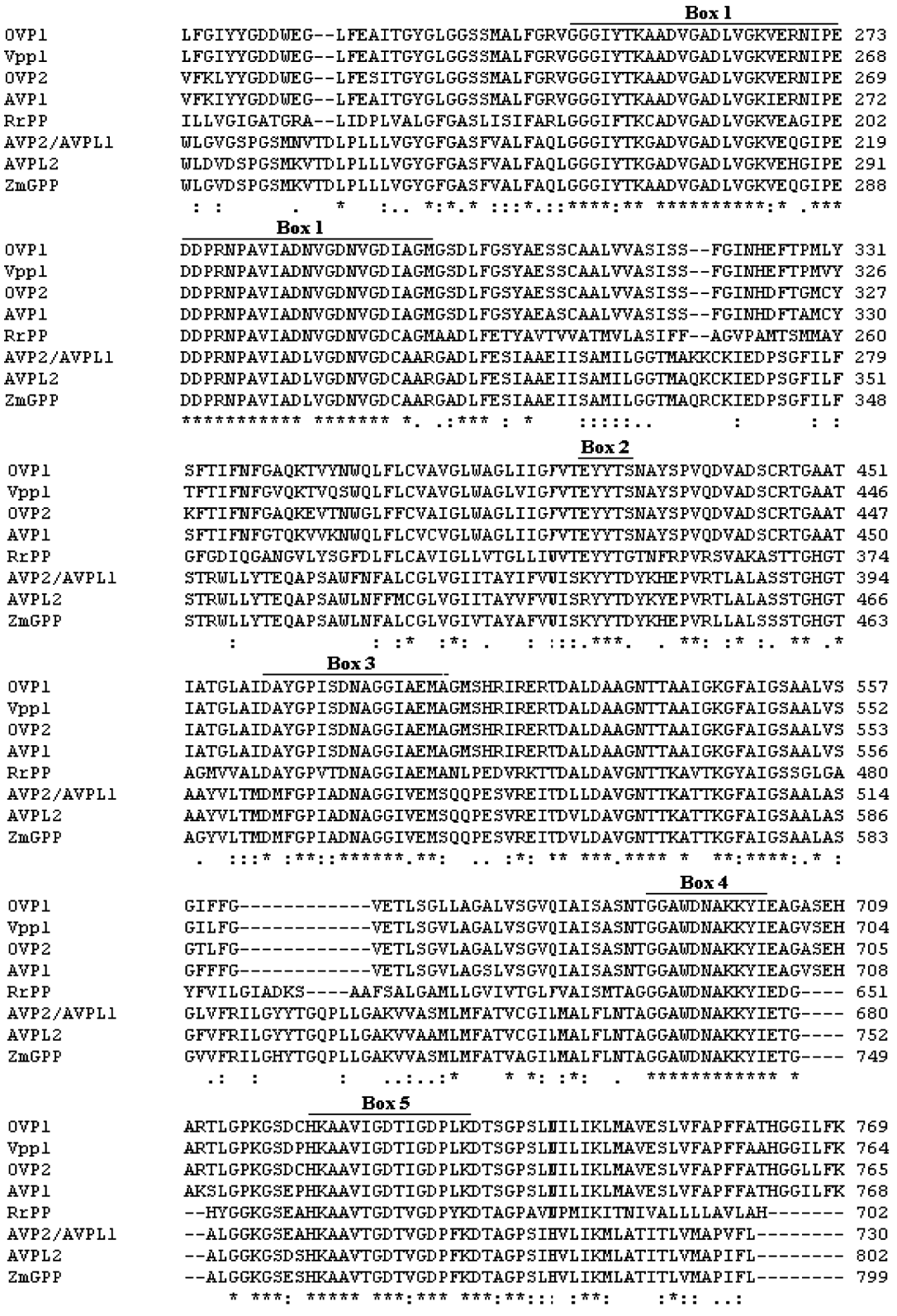

Figure 2. The alignment of the deduced amino acid sequence of *ZmGPP* and H^+-PPases from other species by the Clustal W program. The amino acid sequences are as follows: Vpp1 and ZmGPP from *Z. mays*; AVP1, AVP2/AVPL1, and AVPL2 from *A. thaliana*; OVP1 and OVP2 from *O. sativa*; and RrPP from *Rhodospirillum rubrum*. An asterisk indicates that the residues in that column are identical in all sequences; a colon indicates that conserved substitutions have been observed; and a full stop indicates that semi-conserved substitutions are observed. There are five conserved amino acid sequence boxes marked by lines above the amino acid sequence shown in the sequence alignment which have been reported to be used in identification of H^+-PPase homologues by Drozdowicz and Rea (2001).

Results

Cloning of a cDNA encoding an H^+-PPase from Z. mays

A 764 bp cDNA fragment was obtained by screening subtracted cDNA library of *Z. mays*. Following similarity searching from BLASTx at NCBI, the partial cDNA of putative H^+-PPase cDNA fragment was identified. The 764 bp cDNA fragment was used as the seed to search the NCBI EST database of *Z. mays*. Retrieved homologous ESTs were constructed into a 1511 bp contig which has full 3′ end of ORF. Then we used the 1511 bp contig sequence to search the NCBI non-redundant (nr) database of *Oryza sativa*. The *O. sativa* homologous sequence (GenBank accession no.AK070310) of the 1511 bp sequence was obtained. Then 5′ end sequence of AK070310 was used as a new seed to search the NCBI EST database of *Z. mays*. Retrieved homologous ESTs were constructed into an 898 bp contig which has full 5′ end of ORF.

By *in silico* cloning, we got an 898 bp contig which has full 5′ end of ORF and a 1511 bp contig which has full 3′ end of ORF, respectively. Then primers P1 and P2 were designed based on the two contigs respectively, and a 2684 bp putative H^+-PPase cDNA containing the complete ORF was amplified. The 3′ end of the cDNA, 474 bp in length, was obtained by the SMART RACE cDNA method. The complete sequence of the putative H^+-PPase cDNA, 2974 bp, was acquired by overlapping the two sequences. The isolated full-length cDNA has a significant similarity to Golgi apparatus resident type-II H^+-PPase from *A. thaliana*, therefore it was named *ZmGPP*. The full-length cDNA of *ZmGPP* was registered in GenBank (accession no. EF051578).

ZmGPP gene sequence analyses

Conceptual translation shows that the full-length cDNA contains a 2400 bp ORF encoding a putative H^+-PPase of 799 amino acids including a start codon (Met) and a stop codon. The 5′ UTR is 215 bp long, and the 3′ UTR of 359 bp contains a polyadenylation signal, ATGAAA, 71 bp upstream of the poly (A) tail. The deduced amino acid sequence contains 16 transmembrane domains predicted by TopPred software (http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred.html). Analysis of the protein sequence with pI/Mw software (http://www.expasy.org/tools/pi_tool.html) indicates that the protein has a predicted molecular mass of 84,682.92 Da and a pI of 5.53. Further analysis of the protein sequence by NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos) shows that it has 33 possible phosphorylation sites (16Ser + 9Thr + 8Tyr). SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) suggests that the protein has no signal sequence. The full-length nucleotide and deduced amino acid sequences are shown in Figure 1.

As ascertained by searching at NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), the putative ZmGPP protein contains a 735 amino acid conserved region (aa: 61–795) that belongs to the H^+-PPase family. Alignment of the deduced protein sequence of ZmGPP with H^+-PPase from other species was performed using the ClustalW program (Figure 2). The results indicated that the ZmGPP protein shared 89% identity with the AVP2 protein of *A. thaliana* at the amino acid level, and the five conserved domains reported by Drozdowicz and Rea (2001) were also

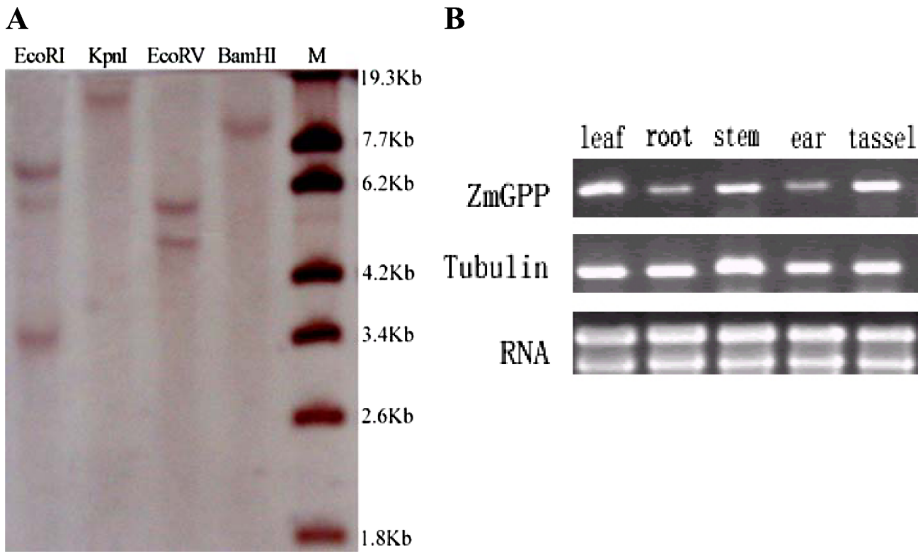

Figure 3. DNA gel blot analysis and tissue-specific expression analysis of *ZmGPP* gene. (A) M presents the λDNA/EcoT14 I molecular weight marker, genomic DNA (15 μg/sample) is digested with EcoRI, KpnI, EcoRV and BamHI, respectively, followed by hybridization with the partial cDNA of *ZmGPP*. (B) Expression of the *ZmGPP* gene in different tissues, detected by RT-PCR with the maize *α-tubulin* as a control.

present in the deduced amino acid sequence of ZmGPP.

DNA gel blot analysis of ZmGPP

To determine the gene copy number of *ZmGPP* gene in maize genome, we performed genomic DNA gel blot analysis, using 764 bp partial *ZmGPP* cDNA as the probe. Less than three hybridization bands were present in each line (Figure 3A), indicating that the *ZmGPP* was a low-copy gene in the genome of *Z. mays*.

Expression of ZmGPP

RT-PCR analysis indicated that the *ZmGPP* mRNA was constitutively expressed in leaf, stem, root, tassel and ear under normal growth conditions. However, the fully expanded leaf and tassel showed higher expression level of *ZmGPP* than other tissues (Figure 3B).

The expression of the *ZmGPP* gene in leaves and roots under stressful conditions and ABA treatment was analyzed by Real-time RT-PCR. The results showed that the gene expression of *ZmGPP* varied under different treatments (Figure 4). *ZmGPP* accumulated to significant levels both in leaves and roots under dehydration and cold treatments. However, under NaCl treatment, the transcript level of *ZmGPP* in leaves did not change significantly while that in root increased up to more than three times compared with untreated plants. It is worth noticing that the transcription of ZmGPP was induced quickly

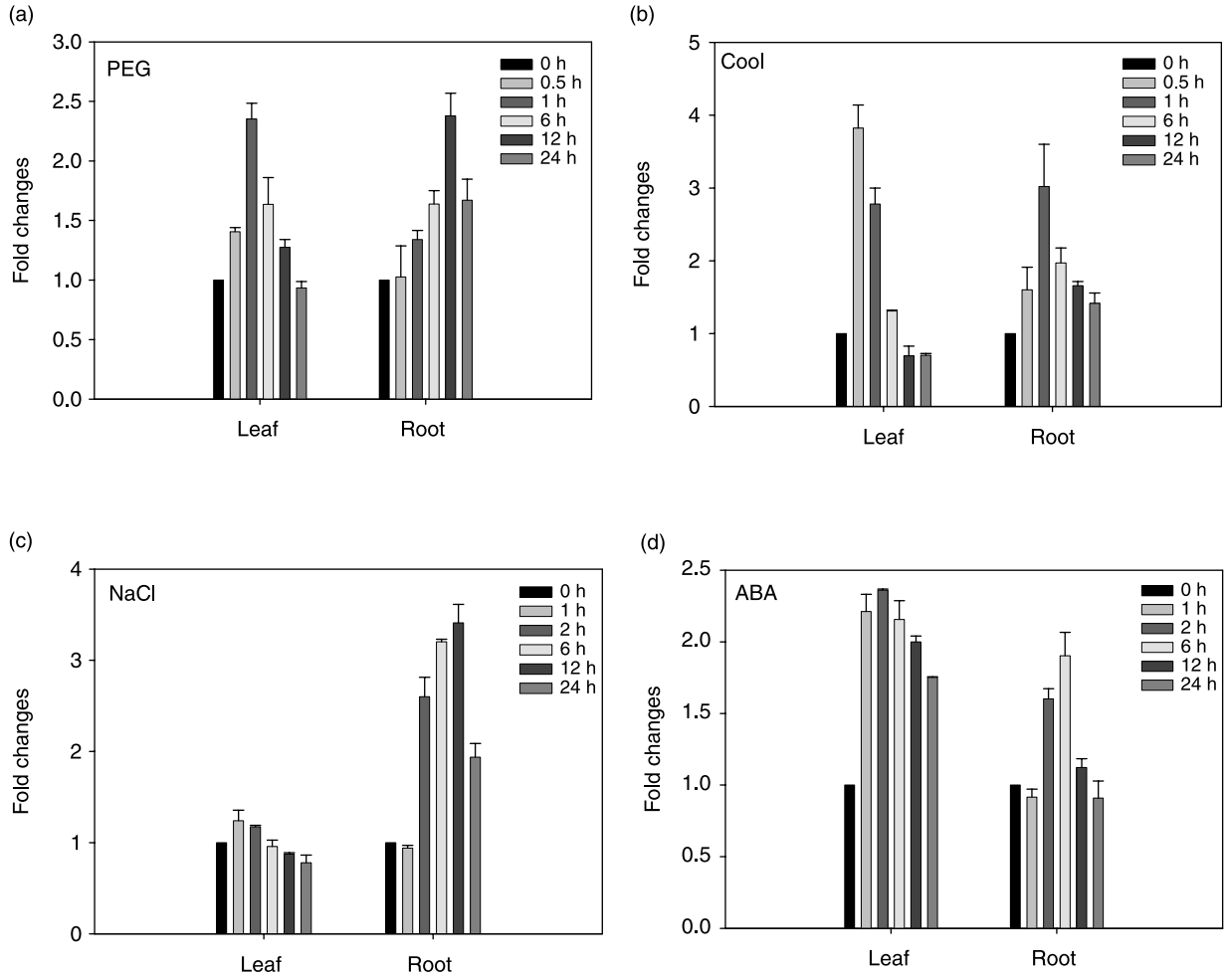

Figure 4. Expression analysis of *ZmGPP* in response to abiotic stresses and ABA. Total RNA was isolated from the leaves and roots of *Z. mays* at six different time points. Expression patterns of *ZmGPP* were analyzed by Real-time RT-PCR with *α-tubulin* as internal control. The fold change in expression of *ZmGPP* is shown in A, B, C and D, respectively. (A) expression pattern of *ZmGPP* under dehydration stress (18% PEG 6000), the transcript level of *ZmGPP* increased up to more than twice in 1 h leaves and 12 h roots, respectively; (B) expression pattern of *ZmGPP* under low temperature (4°C), the transcript level of *ZmGPP* increased up to more than three times in 0.5 h leaves and 1 h roots; (C) expression pattern of *ZmGPP* under salt stress (200 mM NaCl), the transcript level of *ZmGPP* increased up to more than three times in 6 and 12 h roots while it did not change significantly in leaves; (D) expression pattern of *ZmGPP* under ABA treatment (100 μM ABA), the transcription of *ZmGPP* was induced quickly in leaves by exogenous ABA and kept a high transcript level.

in leaves by exogenous ABA and kept a relative high transcript level. However, *ZmGPP* did not accumulate to a significant level in roots under ABA treatment.

Altogether, the results suggest that the expression of *ZmGPP* is up-regulated by these abiotic stress conditions, though in different patterns and ZmGPP may play an important role in abiotic stress tolerance of *Z. mays*.

Discussion

SSH is a powerful technique to screen differentially expressed genes, and is widely used in cloning of plant genes induced by stresses (Zheng et al. 2004; Qi et al. 2005). But the isolated fragment in SSH is partial cDNA digested by restriction enzyme. *In silico* cloning is a novel eukaryotic gene cloning strategy developed with genomic plan and EST plan. It is easy to obtain the full length of differentially expressed genes by combining SSH and *in silico* cloning together. Most of ESTs are sequences of the ends of cDNAs. As for long cDNA sequence, it is possible that full length cannot be obtained for the lack of middle sequence information. However, the completed *O. sativa* genome database can be used to assist us with cloning genes of *Z. mays*.

In this study, only 1511 bp long contig was obtained by *in silico* approach using the seed of the 764 bp EST sequence from SSH library. This contig was not complete and lacked its 5′ end. So its homologous gene of *O. sativa* was found and used to search the NCBI EST database of *Z. mays* as a new seed. Then an 898 bp contig containing its 5′ end was obtained. So the possible 5′ and 3′ sequence of the gene was clear although the sequence between them was still unknown. Primers were designed according to the results of *in silico* cloning and full length for the cDNA was obtained by RT-PCR. In addition, its 3′ UTR was cloned by 3′-RACE method.

The presumed protein of the cDNA we obtained has only 39% amino acid sequence identity to the maize vacuolar H^+-PPase encoded by *Vpp1* (GenBank accession no. AJ715528) (Wisniewski and Rogowsky 2004), but shares 89% identity to Golgi apparatus resident type-II H^+-PPase (AVP2) from *A. thaliana*. So we regarded it as a candidate gene for Golgi apparatus H^+-PPase of *Z. mays* and named it *ZmGPP*. The predicted primary structure of ZmGPP protein has the typical features of H^+-PPase enzyme.

RT-PCR analysis revealed that the expression of *ZmGPP* gene was higher in leaf and tassel compared with other tissues. Analysis of *ZmGPP* transcription by quantitative Real-time RT-PCR showed that it was induced by dehydration, cold and high salt stresses. These results suggest that *ZmGPP* gene may play an important role in abiotic stress tolerance. While for ABA treatment, transcript accumulation was higher in leaves than in roots and not accumulated to significant levels both in leaves and roots. This result indicates that whether *ZmGPP* transcript expression is regulated through ABA-dependent signaling pathway remains uncertain and still needs further study.

Acknowledgements

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