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Molecular Cloning of a Cotton Phosphatase Gene and Its Functional Characterization

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Abstract—In this study, we report isolation of a phosphatase gene designated *GhHL1* from cotton and its functional characterization. *GhHL1* transcripts were detected in all cotton tissues examined. Southern blotting analysis indicated that it exists in multiple-copies. Biochemical analysis showed that GhHL1 was Mg^{2+}-dependent and cation-sensitive. Purified recombinant GhHL1 protein dephosphorylated both 3′,5′-bisphosphate nucleotide and inositol 1,4-bisphosphate, demonstrating dual 3′,5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. Overexpression of *GhHL1* complemented yeast *hal2* mutant and enhanced yeast growth under elevated NaCl or LiCl, showing a role in salt tolerance associated with ionic stress response. Taken together, these results show that *GhHL1* is a functional and good candidate gene, which might be used to improve salt tolerance in plants.

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Key words: cotton, phosphatase, salt tolerance, enzyme activity, functional characterization

Cotton (*Gossypium hirsutum*) is an important source of vegetable oil, paper, biofuel, and other secondary products, although its primary value resides in its cotton fibers (lint) used in textile products. Soil salinity is a major constraint on cotton production worldwide, and yet the exact basis for salt inhibition of cotton growth and yield, and the relative importance of ionic versus osmotic stress caused by salt ions is only partially known. Previous work shows that cotton plants growing under salt stress exhibit higher activities of the antioxidative enzymes superoxide dismutase, peroxidase, and glutathione reductase, and that this stress leads to increased membrane damage, lower photosynthetic rate, lower stomatal conductance, and lower chlorophyll content [1]. Ectopic expression in cotton of a Na^+/H^+ antiporter encoded by *Arabidopsis AtNHX1* [2] and a 14-3-3 protein encoded by *Arabidopsis AtGF14λ* [3] was shown to confer salt tolerance as measured in yield, though the exact functions of these gene products in cotton are still uncertain. Work is still needed to identify and analyze these and other gene determinants of cotton salt stress tolerance as a means to both illuminate mechanisms underlying salt response in cotton and to elucidate new strategies for engineering cotton stress protection [4, 5].

Recent studies have revealed a group of homologous genes necessary for salt tolerance in diverse organisms. The first of these genes, *HAL2*, was cloned from *Saccharomyces cerevisiae* and later shown to be a salt-sensitive 3′(2′),5′-bisphosphate nucleotidase (PAPase) that dephosphorylates 3′(2′),5′-bisphosphate nucleotide (PAP) and 3′(2′),5′-bisphosphate nucleotide sulfate (PAPS) [6, 7]. The accumulation of PAP is toxic to cells, suggesting a direct correlation between PAP accumulation and growth inhibition by salt stress deactivation of PAPase [7-9]. Biochemical analyses also reveal that many HAL2 homologs exhibit inositol polyphosphate 1-phosphatase (IPP1ase) activity responsible for catabolizing inositol 1,4-bisphosphate (IP2) and inositol 1,4,5-trisphosphate (IP3) and suppressing stress response path-

Abbreviations: FDD-PCR, fluorescence differential display-polymerase chain reaction; IP2, inositol 1,4-bisphosphate; IP3, inositol 1,4,5-trisphosphate; IPP1ase, inositol polyphosphate 1-phosphatase; PAP, 3′(2′),5′-bisphosphate nucleotide; PAPase, 3′(2′),5′-bisphosphate nucleotidase; PAPS, 3′(2′),5′-bisphosphate nucleotide sulfate.

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ways [10-13]. Furthermore, many *HAL2* homologous genes have also been identified from various organisms such as *CysQ* from *E. coli* [14], *SAL1/FIERY1 (FRY1)/HOS2*, *SAL2*, *AHL*, *RHL*, and *ZmDP1* from various plant species [15-19], and *RnPIP* and *HsPIP* from mammals [10, 11]. Taken together, the results suggest that the *HAL2* homologous genes may have important biological functions in response to salt stress.

The HAL2 homolog SAL1/FIERY1/HOS2 (SAL1) of *Arabidopsis thaliana* was shown to have a quite specific function as a negative regulator in salt and abscisic acid signaling pathways by mediating catabolism of the secondary messenger (IP3). Through its IPP1ase activity, SAL1 hydrolyzes inositol IP3, yet only 4% as efficiently as it hydrolyzes the PAP substrate [13, 16, 18]. The *hos2* mutant allele (having a single amino acid substitution A70V) completely lost SAL1 protein activity during cold temperature stress, yet it retains normal activity in warmer conditions. By comparison, the *fry1* null mutant allele in this gene exhibits defective cold stress response, but also more general defects in drought, salt, and abscisic acid stress response [13, 20]. *DHAL2*, a yeast *HAL2* homolog isolated from the halotolerant yeast *Debaryomyces hansenii*, encodes two protein isoforms, a cytosolic form and membrane-bound form [12], with the latter form showing (conversely from SAL1) higher IPP1ase than PAPase activity. Overexpression of *DHAL2* in the membrane-bound form in yeast confers significantly higher LiCl tolerance than does overexpression of the cytosolic *HAL2* [12], and only the membrane-bound DHAL2 is detected in high salt stress conditions. Taken together, these studies reveal that although the expression of *HAL2* homologs exhibits comparable biochemical function in different species, there are significant differences in the expression of various *HAL2* homologs and their effects on stress response based on species-type and protein localization.

To advance our efforts toward cotton improvement for salt tolerance, we isolated a highly expressed cDNA from cotton using fluorescence differential display-polymerase chain reaction (FDD-PCR) that showed high similarity with a well-known salt tolerance determinant from yeast *HAL2* [21]. Here we report the isolation and functional analysis of this cotton *HAL2* homolog designated as *GhHL1*.

MATERIALS AND METHODS

Plant materials, yeast strain, and chemicals. Cotton (*Gossypium hirsutum* cv. CRI 12) seeds were germinated and plants grown at 28°C in a growth room with a photoperiod of 16 h light and 8 h darkness. The *Saccharomyces cerevisiae* strain used in this study was JRM4 kindly provided by Dr. Pedro L. Rodriguez Egea (Universidad Politecnica, Spain). The yeast was grown on minimal growth medium (SD) containing 2% glucose, 0.7% yeast nitrogen base without amino acids, and 50 mM MES adjusted to pH 6.0 with Tris. The SD media were supplemented with 100 μg/ml methionine to produce SDM media. Commercially available PAPS contained 4 moles of lithium per mole of PAPS. All the chemicals used here were purchased from Sigma (USA).

Cloning of the *GhHL1* gene. A 990-bp fragment preferentially expressed in developing cotton fiber was obtained through FDD-PCR [21]. Sequence analysis showed that this fragment shared high similarity with yeast *HAL2* and its homologs at both the nucleotide and amino acid levels, but lacked a part of the coding sequence at the 5′ end. To obtain the putative full-length cDNA, mRNA was prepared from 10-dpa (days post anthesis) fibers with polyA Attract System 1000 (Promega, USA) and then the adapter-ligated cDNA library was constructed. A rapid amplification of the 5′ cDNA end (5′-RACE) was performed with the Marathon cDNA Amplification kit (Clontech, USA) and the primer (RA: 5′-TGAGGAAGGATGAAGCCTGCC-3′) was designed based on the 3′ end sequence. A database search was performed using the BLAST program of NCBI (http://www.ncbi.nlm.nih.gov/blast/), and yeast *HAL2*-homologous sequences of various species were downloaded from GenBank. Multiple sequence alignments were carried out with DNAMAN software (version 4.0, Lynnon Biosoft Company). Homology modeling was completed at the website of http://www.expasy. org/swiss-mod/SWISS-MODEL.html.

Isolation of cotton genomic DNA and Southern blot analysis. Total genomic DNA was isolated from leaf tissue of cotton using the CTAB method [22]. Ten milligrams of genomic DNA was digested with different restriction enzymes—*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I. The treated samples were fractionated on a 0.8% agarose gel and then blotted onto nylon membranes (Hybond-N^+; Amersham, USA) by capillary transfer. The full-length cDNA of *GhHL1* coding region was amplified using Pfu polymerase and used as a probe. The probe was labeled using a random primer labeling kit (TaKaRa, China). Probes for *GhHL1* were prepared from the PCR products, which were amplified by specific primers (GhHL1-F: 5′-ATGTCTTATGATAAAGAACTG-3′ and GhHL1-R: 5′-TCACAAAGATGAAGCTTTCTC-3′). The hybridization was carried out according to Huang and Liu [23].

Real-time PCR analysis. Total RNA was extracted from all organs of mature plants including stems, inflorescences, siliques, cauline leaves, rosette leaves, and roots as well as seed and embryo according to Wan and Wilkins [24]. RNA was treated with DNase I enzyme before reverse transcription. The first strand cDNA was synthesized according to the Promega manual (A3500). Real-time PCR was performed in DNA Engine Option 2 (MJ Research) with SYBR® Green I dye (Tiangen, China) using gene specific primers (RT-GhHL1-F: 5′-

GTCTTCCACACAAAGGGTATCG-3′ and RT-GhHL1-R: 5′-GGTTGGTGACAATGATGCCTGT-3′). The amplified products of cotton UBQ7 were used to normalize the amount of template cDNA [25]. The primers were RT-GhUBQ7-F: 5′-AGGCATTCCACCTGACCAAC-3′ and RT-GhUBQ7-R: 5′-GCTTGACCTTCTTCTT-CTTGTGC-3′. Each reaction was processed in triplicate. Real-time experiments were carried out at least twice with biologically independent samples.

Expression and purification of GhHL1 protein. To express *GhHL1* in *E. coli*, the coding region of *GhHL1* was amplified with the following primers: 5′-GCGCCATATGTCTTATGATAAAGAACTG-3′ (the *Nde*I site is underlined) and 5′-GCATGAATTC**TCA**GTGATGATGATGATGATGCAAAGATGAAGCTTTCTCC-3′ (the *Eco*RI site is underlined and the stop codon is in bold). This primer contains a polyhistidine coding sequence (double underlined) that binds metal and facilitates the purification of the expressed proteins. PCR products were amplified from plasmid templates using Pfu polymerase (Tiangen), digested with *Nde*I and *Eco*RI, and ligated into *Nde*I/*Eco*RI-digested pT7-7 vector to generate construct pT7-GhHL1. This construct was further confirmed by sequencing and then transformed into *E. coli* strain BL21(DE3) and selected on LB-kanamycin plates (50 μg/ml). Overnight cultures were re-inoculated and grown until $A_{600} = 0.8$. The cells were induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20°C for 4 h. The cells were pelleted by centrifugation for 20 min at 12,000 rpm and resuspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole). After sonication and centrifugation at 4°C for 15 min at 12,000 rpm, the supernatant was passed over a Ni^{2+}-NTA column previously equilibrated with lysis buffer. Unbound protein was washed out using wash buffer (50 mM Tris, pH 8.0, 1000 mM NaCl, 40 mM imidazole). His-tagged protein was eluted with elution buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 150 mM imidazole). The purified protein was used for enzyme assay.

Enzyme assay. Phosphatase assay was applied to quantify the inorganic phosphate released from substrate using the malachite green method with minor modifications [7]. Briefly, the standard assay was conducted in a total volume of 100 μl reaction mixture including 50 mM Tris-MES (pH 7.5), 2 mM Mg^{2+}, and the indicated amounts of purified protein and substrate. The mixture was incubated at 30°C for 10 min, and then stopped by adding a 25 μl color reagent containing acidic dye solution, ammonium molybdate, and Tween 20. The mixture was incubated at room temperature for 10 min, and then the absorbance at 630 nm was measured using an Ultrospec 3300 pro UV spectrophotometer (Biochrom, Great Britain). The blank (control) only included substrate and Mg^{2+}. For determination of K_m, pH optimum, temperature optimum, and Mg^{2+}-dependent enzyme profile, the corresponding factors were changed.

Functional complementation and halotolerance test. To complement yeast mutant *hal2*, the coding region of cotton *GhHL1* was PCR-amplified with the following primers: 5′-CCCTCGAGATGTCTTATGATAAAGAACTG-3′ (the *Xho*I site is underlined) and 5′-CCCTCGAG**TCA**CAAAGATGAAGCTTTCTC-3′ (the *Xho*I site is underlined and the stop codon is in bold). PCR products were digested with *Xho*I enzymes and cloned into yeast expression vector pRS699 at *Xho*I sites to generate construct pRS699-GhHL1. The yeast mutant cells JMR4 (hal2::LEU2) were transformed by electroporation with corresponding vectors and spread on uracil selection SD medium. The colonies were selected and grown on SD medium without uracil for two days. Before the halotolerance tests, cells transformed with corresponding vector were grown for two days to absorbance ~2.7 at 660 nm. For testing in solid medium (2% agar) the cultures were diluted 50-fold with water and 3 μl were dropped on uracil selection solid minimal medium supplemented with 100 μg/ml methionine (SDM) and on the same medium supplemented with 0.25 M LiCl, 0.5 M LiCl, 1 M NaCl, 1.5 M NaCl, or 1.5 M KCl, respectively. Growth was recorded after seven days. For testing in liquid medium, the cultures were diluted 10,000-fold in uracil selection SDM medium and in the same medium containing 0.25 M LiCl, 1 M NaCl, or 1.5 M KCl. Growth curves were recorded by their absorbance at 660 nm.

RESULTS

Sequence characterization of the cotton *GhHL1* gene and its expression patterns. The 1434 bp of full-length cDNA (GenBank Accession No. AJ310755) was obtained using RACE-PCR on the basis of its 990 bp fragment isolated previously [21]. The sequence contains an open reading frame of 1041 bp encoding a protein of 347 amino acids with a predicted molecular mass of 37 kDa and a calculated p*I* of 5.44 (Fig. 1). Database searches indicated that the protein shares high sequence identity with yeast HAL2 (X72847, 37.5%), as well as many plant homologs such as RHL (U33283, 69.33%) and RHL2 (Os12g0183200, 64.81%) both from rice, ZmDP1 from *Zea mays* (AF288075, 68.40%), and SAL1 (At5g63980, 78.95%), SAL2 (At5G64000, 64.18%), and SAL4 (At5g09290, 62.86%) from *Arabidopsis*. Sequence aligning revealed that it contains a conserved motif of D-*Xn*-EE-*Xn*-DP(i/l)D(s/g/a)T-*Xn*-WD-*X*11-GG that is present in all HAL2 homologs [6, 11, 14, 15, 26]. Based on the significant degree of identity with almost all HAL2 homologs, we designated this protein as GhHL1 (*Gossypium hirsutum* HAL2-Like protein 1). Additionally, computer homology-based modeling showed that the three-dimensional structure of GhHL1 is similar to the crystal structure of yeast HAL2 [27], showing a common core fold of both (α + β) and α/β domains (data not

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1     ATCTCTGTCACTTGCCTCCTCCTCCTCCTTCTTCTTCTGCTGTGATTGTGGCAATGTCTTATGATAAAGAACTGGCTGCTGCAAAGA
                                                                  M  S  Y  D  K  E  L  A  A  A  K
88    AAGCAGCCTCTCTCGCTGCTCGTCTCTGTCAGAAAGTACAAAAGGCTTTGCTGCAATCCGATGTTCAATCAAAGAATGATAAAAGTC
      K  A  A  S  L  A  A  R  L  C  Q  K  V  Q  K  A  L  L  Q  S  D  V  Q  S  K  N  D  K  S
175   CTGTAACTGTTGCTGATTATGGCTCACAAGCACTGGTTAGTTTTGTGCTGCAGCAGGAATTTCCTGATAACTTCTCATTAGTTGCTG
      P  V  T  V  A  D  Y  G  S  Q  A  L  V  S  F  V  L  Q  Q  E  F  P  D  N  F  S  L  V  A
262   AGGAGGATTCTAAAGATCTTCGCAAGGATGGTGGCCAGGAAATAGTAGAGCGCATTACAAAACTTGTGAACGATTCTCTAACTATTG
      E  E  D  S  K  D  L  R  K  D  G  G  Q  E  I  V  E  R  I  T  K  L  V  N  D  S  L  T  I
349   ATGGATCATACAATGTTACTTTATCCACAGAAGATGTTCTCAAGGCCATTGATAATGGCAGATCTGAGGGTGGTTCCCAAGGTCGAC
      D  G  S  Y  N  V  T  L  S  T  E  D  V  L  K  A  I  D  N  G  R  S  E  G  G  S  Q  G  R
436   ACTGGGTTTTGGATCCTATAGATGGTACTAAAGGTTTTCTGAGAGGAGATCAATATGCAACAGCATTGGCTTTGCTAGATGGAGGAA
      H  W  V  L  D  P  I  D  G  T  K  G  F  L  R  G  D  Q  Y  A  T  A  L  A  L  L  D  G  G
523   AAGTTGTCCTGGGTGTGCTGGCTTGTCCAAATCTTCCACTAACTTCCCTCAGTGATGCTGGTCAGCATTCTCCAAATAATAAAGTTG
      K  V  V  L  G  V  L  A  C  P  N  L  P  L  T  S  L  S  D  A  G  Q  H  S  P  N  N  K  V
610   GCTGCCTTTTCTTTGCTGTAGTAGATGGTGGAACTTATATGCAGCCACTTGATGGTTCTTCGGCAGTAAAGGTGCAAGTAAGTGCTG
      G  C  L  F  F  A  V  V  D  G  G  T  Y  M  Q  P  L  D  G  S  S  A  V  K  V  Q  V  S  A
697   TTGAAAATCCTGAAGAAGCATCATTCTTTGAGTCTTACGAAGCAGCACACTCCATGCATGATTTATCTAGCTTGATTGCCCAAAAAC
      V  E  N  P  E  E  A  S  F  F  E  S  Y  E  A  A  H  S  M  H  D  L  S  S  L  I  A  Q  K
784   TCGGCGTCAAAGCACCACCGGTTAGAATTGATAGCCAGGCAAAGTATGGTGCTCTATCCAGAGGAGATGGAGCCATATATCTGCGTC
      L  G  V  K  A  P  P  V  R  I  D  S  Q  A  K  Y  G  A  L  S  R  G  D  G  A  I  Y  L  R
871   TTCCACACAAAGGGTATCGAGAAAAAATATGGGATCATGCTGCTGGGTGTATTGTTGTGAGTGAAGCTGGGGGTGTGGTCACAGATG
      L  P  H  K  G  Y  R  E  K  I  W  D  H  A  A  G  C  I  V  V  S  E  A  G  G  V  V  T  D
958   CTGCAGGGCAGCCATTGGATTTTTCAAAGGGAAAGTATCTTGATCTGGACACAGGCATCATTGTCACCAACCAGAAGTTGATGCCAT
      A  A  G  Q  P  L  D  F  S  K  G  K  Y  L  D  L  D  T  G  I  I  V  T  N  Q  K  L  M  P
1045  TACTGTTTAATGCAGTTAGAAAATCTATCCAGGAGAAAGCTTCATCTTTGTGATTCATTTTAGAGGCAGGCTTCATCCTTCCTCATA
      L  L  F  N  A  V  R  K  S  I  Q  E  K  A  S  S  L  *
1132  ACTTGCTCTGTTAAGCTGGTTGAAACTTGATTATTTTTGCCTTCAATGCTTGAACTTTTATCATTCTTCTCCCCAATGCTTATAGGA
1219  AGATTTTCATTTAGCATGCATGAACAAGAATGGGACCAATAAATCTCATCTCTACAATTAAGACAATGTAATTGTAGACTAGAACTT
1306  GAAAGCATGGTCCTAGGCCTTGAGCCTTTCTCTTTGTCCTACCTGGCAGTTGGGTTTAGGTGTAATTAATTAGATAAAGGAACTGTG
1393  TTGTTGAATACAAACATATTACATTATTACGAAAAAAAAAAA
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Fig. 1. Nucleotide sequences and deduced amino acids of cotton *GhHL1* gene. Upper lines, nucleotide sequence; lower lines, amino acid sequence of the putative coding region. The stop codon is marked by an asterisk. The three conserved regions are underlined.

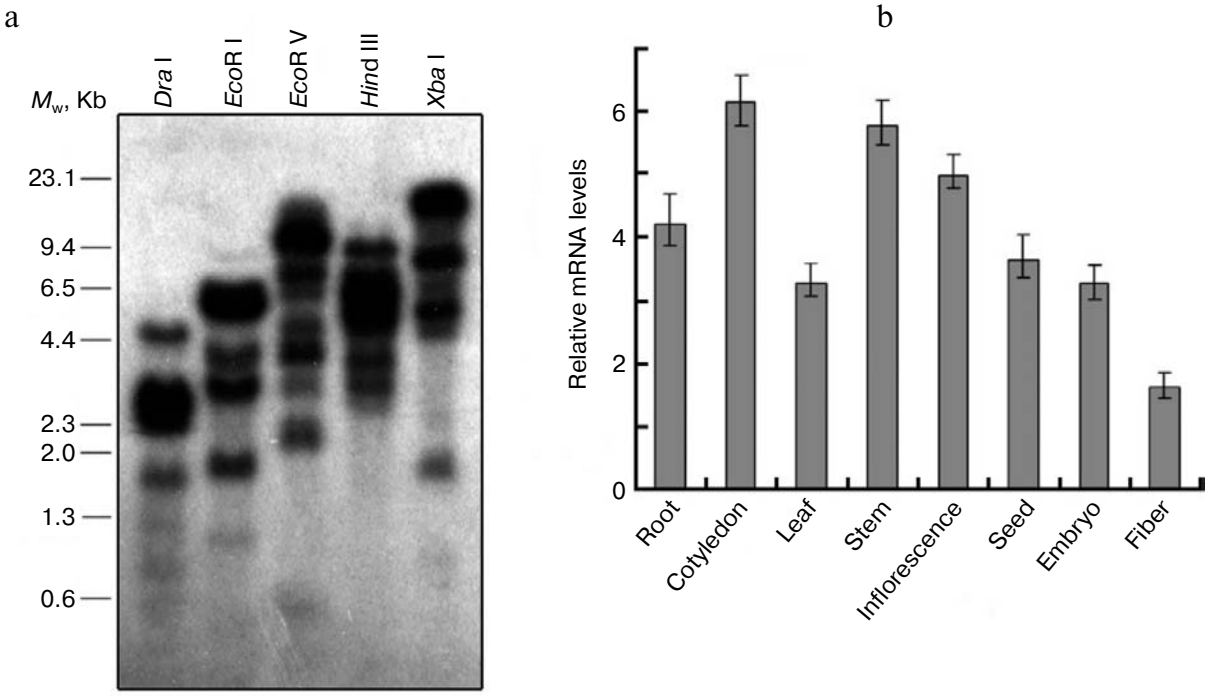

Fig. 2. Southern blot analysis and tissue-specific expression of the cotton *GhHL1* gene. a) The copy number of *GhHL1* genes in cotton was analyzed by Southern blot. b) The tissue-specific expression of *GhHL1* in cotton was determined by real-time PCR analysis.

shown). These findings provide further evidence that the newly cloned cDNA could be a cotton gene encoding a yeast HAL2 homolog.

Southern blot analysis showed that several hybridization bands ranging from 1 to 23 kb were found in each lane, suggesting that *GhHL1* belongs to a multi-gene family in cotton (Fig. 2a), just as in *Arabidopsis* and rice. The transcript level of the *GhHL1* gene in different tissues was also determined using real time PCR. The data indicated that *GhHL1* was constitutively expressed in all tested organs of cotton, but was most highly expressed in cotyledons, stems, and inflorescences (Fig. 2b). The transcriptional levels of SAL1 from *Arabidopsis* and RHL from rice were also reported with much higher expression in shoots than that in roots [13, 15]. However, the expression level in leaves was lower than that in roots.

GhHL1 with catalytic activities of PAPase and IPP1ase. Previous studies showed that yeast HAL2 and its homologs from all other organisms possess phosphatase activity towards both PAP and IP2 substrates [6, 11, 14, 15, 28]. To examine whether the cotton GhHL1 protein has similar enzymatic activity as does HAL2 from yeast, the recombinant protein of *GhHL1* was produced in *E. coli* BL21 and the assay was performed by the modified malachite green procedure. The size of purified protein was shown to be approximately 37 kDa on SDS-PAGE (Fig. 3). The optimum Mg^{2+} concentration, pH,

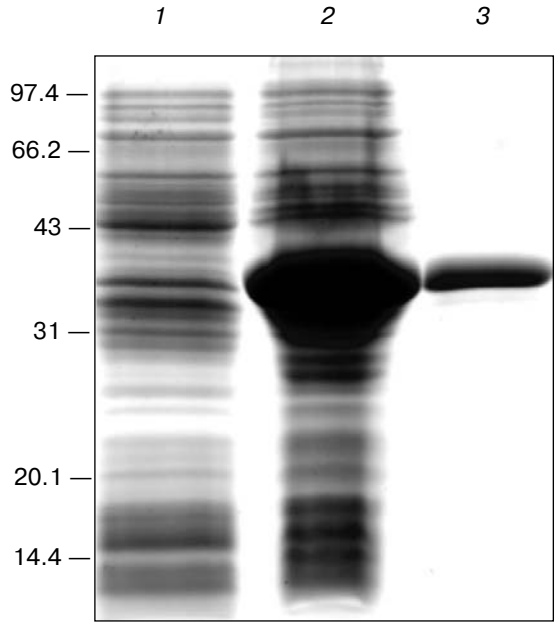

Fig. 3. SDS-PAGE analysis of the *GhHL1* gene product. Lanes *1* and *2* contain protein extracts from *E. coli* cells transformed with plasmid pT7-GhHL1, which were uninduced or induced by 0.5 mM IPTG at 20°C for 4 h, respectively. Lane *3*, purified GhHL1 protein. The numbers on the left indicate the molecular masses of markers in kDa.

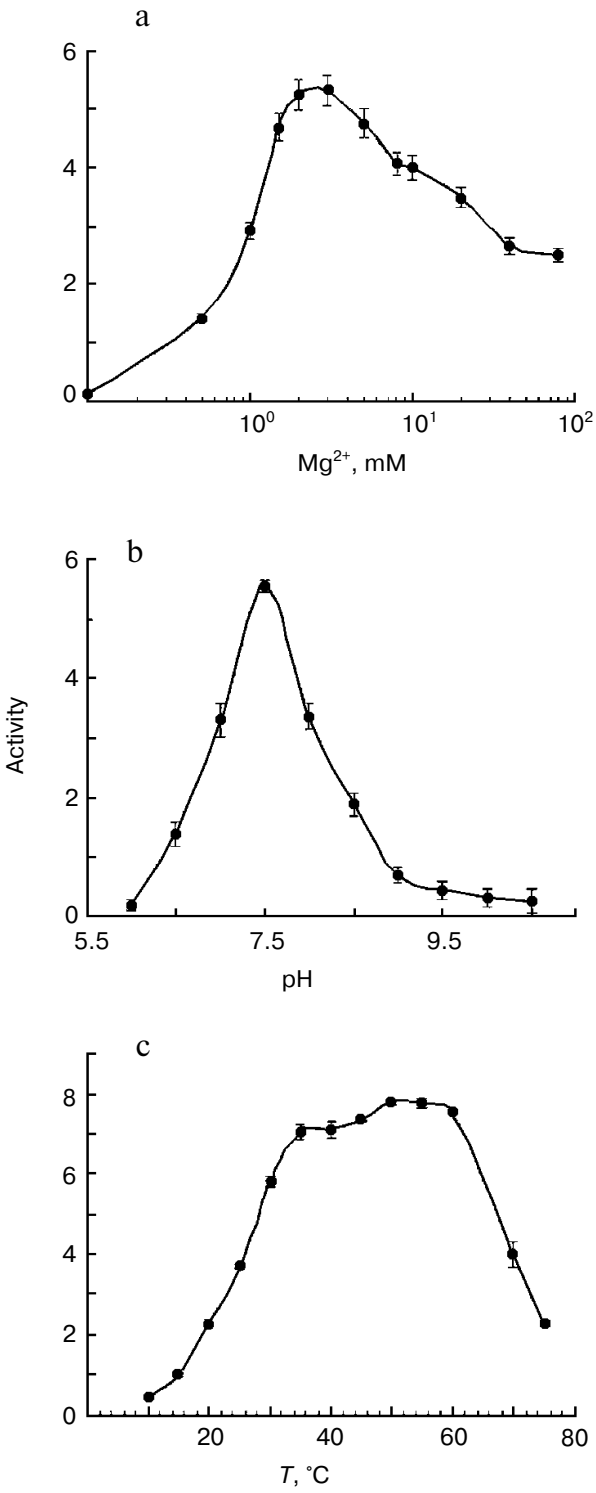

Fig. 4. Mg^{2+}-, pH-, and temperature-dependent profiles of GhHL1 nucleotidase (PAPase) activity. The reactions were performed under standard conditions as described above. a) Mg^{2+}-dependence reactions were checked over the concentration range of 0-80 mM Mg^{2+}. b) pH-dependence reactions were performed over the range pH 5.0-10.5. c) Temperature-dependence reactions were performed over the range 25-80°C. The activities are expressed as μmol P_i/min per mg protein. Reactions were performed three times independently with standard errors.

and temperature for phosphatase activity of cotton GhHL1 was first determined using 3′-PAP as substrate with an optimal Mg^{2+} concentration of 2 mM, which was similar to conditions reported in the cases of all other characterized HAL2 homologs [6, 11, 14, 15, 28]. Under Mg^{2+} concentrations from 1 to 80 mM, the activity maintained higher level (>50%) (Fig. 4a). Optimal pH was around 7.5 at 30°C and 2 mM Mg^{2+}, the enzyme was sensitive to both low pH (<6.5) and high pH value (>9.0), and showed highest activity in the pH range from 7.0-8.5 (Fig. 4b). The temperature optimum of GhHL1, assayed at pH 7.5 and 2 mM Mg^{2+}, is between 30 and 60°C (Fig. 4c). However, its activity was limited at low temperature (<10°C), and relatively sensitive to high temperature showing only 28.9% activity after incubation at 75°C (Fig. 3c). Thus, the enzyme was able to hydrolyze PAP over a wide range of temperatures and Mg^{2+} concentrations.

To further investigate the substrate specificity of GhHL1, 3′-PAP, PAPS, IP2, IP3, 3′-AMP, 5′-AMP, or ADP was used as substrate. All reactions were carried out at 30°C, pH 7.5, and 2 mM Mg^{2+} unless stated otherwise. As shown in the table, the GhHL1 protein efficiently hydrolyzed PAP and PAPS (at 128% of PAP levels) but had low or no activity with other tested compounds including 3′-AMP, 5′-AMP, or ADP. The efficiency of GhHL1 for catalyzing IP2 and IP3 was only 35 and 4.5% of that which occurred using the PAP substrate (table). The enzyme characteristics indicate that GhHL1 was similar to the *Arabidopsis* SAL1 but with higher PAPase activity and relatively lower IPP1ase activity. The K_m value for PAPS hydrolysis was estimated to be lower than 10 μM.

Cation sensitivity of GhHL1. Previous works reported that the HAL2 homologs were strongly inhibited by Ca^{2+}, Li^+, Na^+, and K^+ [11, 15, 16]. To test the cation sensitivity of cotton GhHL1, assay was performed under the conditions of 30°C, pH 7.5, 2 mM Mg^{2+}, 0.2 μg purified His-GhHL1 protein, and 0.2 mM PAP. As expected, GhHL1 activity was inhibited by submillimolar concentrations of Li^+ (IC_{50}, 0.2 mM), by submolar concentrations of Na^+ (IC_{50}, 100 mM), and by submolar concentrations of K^+ (IC_{50}, 200 mM) (Fig. 5a). The GhHL1 enzyme was also strongly inhibited by submicromolar concentrations of Ca^{2+} (IC_{50}, 50 μM) (Fig. 5b). The inhibition of GhHL1 enzyme activity by Ca^{2+} could be rescued by high concentration of Mg^{2+}. At Mg^{2+} concentrations of 5, 2, and 0.5 mM, the IC_{50} for Ca^{2+} was 0.8, 0.05, and 0.01 mM, respectively (Fig. 5b). As such, the inhibition of GhHL1 activity by 1 mM Ca^{2+} gradually decreased following an increase in Mg^{2+} concentration, indicating that the inhibition by Ca^{2+} depends on the ratio of Ca^{2+}/Mg^{2+}, and that the relationship of Ca^{2+} and Mg^{2+} was competitive. Suppression by Mg^{2+} of the high GhHL1 inhibition by Ca^{2+} suggests that intracellular concentrations of Ca^{2+} can play an important role in regulating the activity of cotton GhHL1.

Complementation of yeast *hal2* mutant by cotton *GhHL1*. The functionality of *GhHL1* was assessed by complementation of the yeast mutant strain JMR4 lacking HAL2, a strain that exhibits methionine auxotrophy [6]. The yeast JMR4 cells were transformed with pRS699-GhHL1, pRS699 (as negative control), and pRS699-ScHAL2 (as positive control) plasmids, respectively, and were separately grown on SD medium. As shown in Fig. 6a, ectopic expression of *GhHL1* indeed complemented the methionine auxotrophy at similar levels as did yeast *HAL2*. This suggested that *GhHL1* can encode an active HAL2-like protein being capable of reducing PAPS to sulfite *in vivo*.

To further assess the function of *GhHL1* in response to salt, the growth of transformed yeast cells was tested under different salt stresses. Cells transformed with pRS699 and RS699-ScHAL2 vectors were used as the negative and positive controls, respectively. In uracil selection SD medium without cation supplement the growth rate of the three transformed cells showed no difference (Figs. 6b and 6c). The yeast cells transformed with pRS699 vector grew slightly slower than cultures without cation supplement in uracil selection solid SD medium containing methionine supplemented with 0.25 M LiCl. The growth of the yeast cells containing RS699-ScHAL2 vector was not inhibited until the concentration of LiCl reached 0.5 M, and the growth of the yeast cells transformed with pRS699-GhHL1 were similar to those cells transformed with RS699-ScHAL2 vector (Fig. 6b). In solid SD medium containing methionine supplemented with 1.0 or 1.5 M NaCl, yeast containing *HAL2* or *GhHL1* grew slightly faster than yeast transformed with the negative control RS699 (Fig. 6b). In liquid SD medium containing methionine supplemented with 0.25 M LiCl and 1.0 M NaCl, yeast transformed RS699-GhHL1 and RS699-ScHAL2 likewise grew faster than the negative control strain (Figs. 6d and 6e). However, the three transformed yeast cells showed similarly slow growth rate with a longer lag phase under conditions of SD medium containing methionine supplemented with 1.5 M KCl compared with growth under normal conditions (Figs. 6b and 6f). These results suggest that the expression of *GhHL1* conferred more tolerance to LiCl and NaCl, but not to KCl, which is similar to *HAL2* from yeast as previously reported by Murguia et al. [9].

Substrate specificity of GhHL1 enzyme

Substrate	Activity, %
3'-PAP	100
PAPS	128
IP2	35
IP3	4.58
3'-AMP	<1
5'-AMP	<1
ADP	<1

Note: Activity was determined using 0.2 mM of each substrate. Enzyme activity is expressed as the percentage activity observed with PAP as 100%. To correct for lithium inhibition (as PAPS is supplied with 4 moles of lithium per mole PAPS), we assayed the reference substrate PAP with the same quantity of lithium as supplement. Results are the means of two independent experiments, each performed in triplicate.

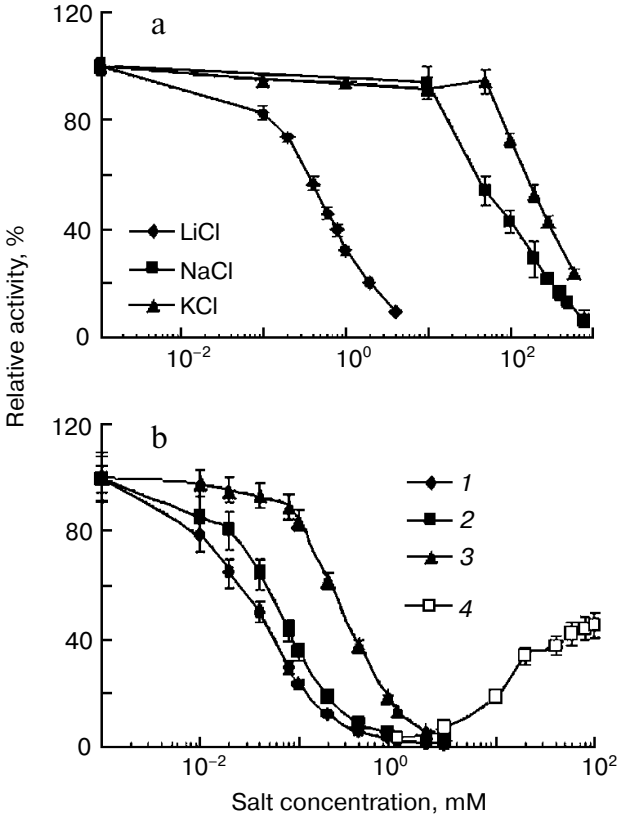

Fig. 5. Effects of various cations on the enzyme activity of GhHL1 with 0.2 mM PAP as substrate. Activity was measured in buffer containing 50 mM Tris-MES, pH 7.5, and 2 mM $MgCl_2$ at 30°C for 10 min except that cations were as indicated. a) Inhibition of GhHL1 PAPase activity by monovalent cations including Li^+, Na^+, and K^+. Activity obtained under standard conditions was considered 100%, and that obtained in the presence of ions was normalized to this activity. b) Effect of Ca^{2+} and Mg^{2+} on GhHL1 PAPase activity. Activities are expressed as percentages of values determined in the absence of Ca^{2+}. *1*) Inhibition by Ca^{2+} with 0.5 mM Mg^{2+}; *2*) inhibition by Ca^{2+} with 2 mM Mg^{2+}; *3*) inhibition by Ca^{2+} with 5 mM Mg^{2+}; *4*) restoration of GhHL1 nucleotidase activity by Mg^{2+} with Ca^{2+} at 1 mM. Results are means of three independent experiments.

DISCUSSION

Enzymes with activity similar to yeast HAL2 have been isolated and characterized from various organisms

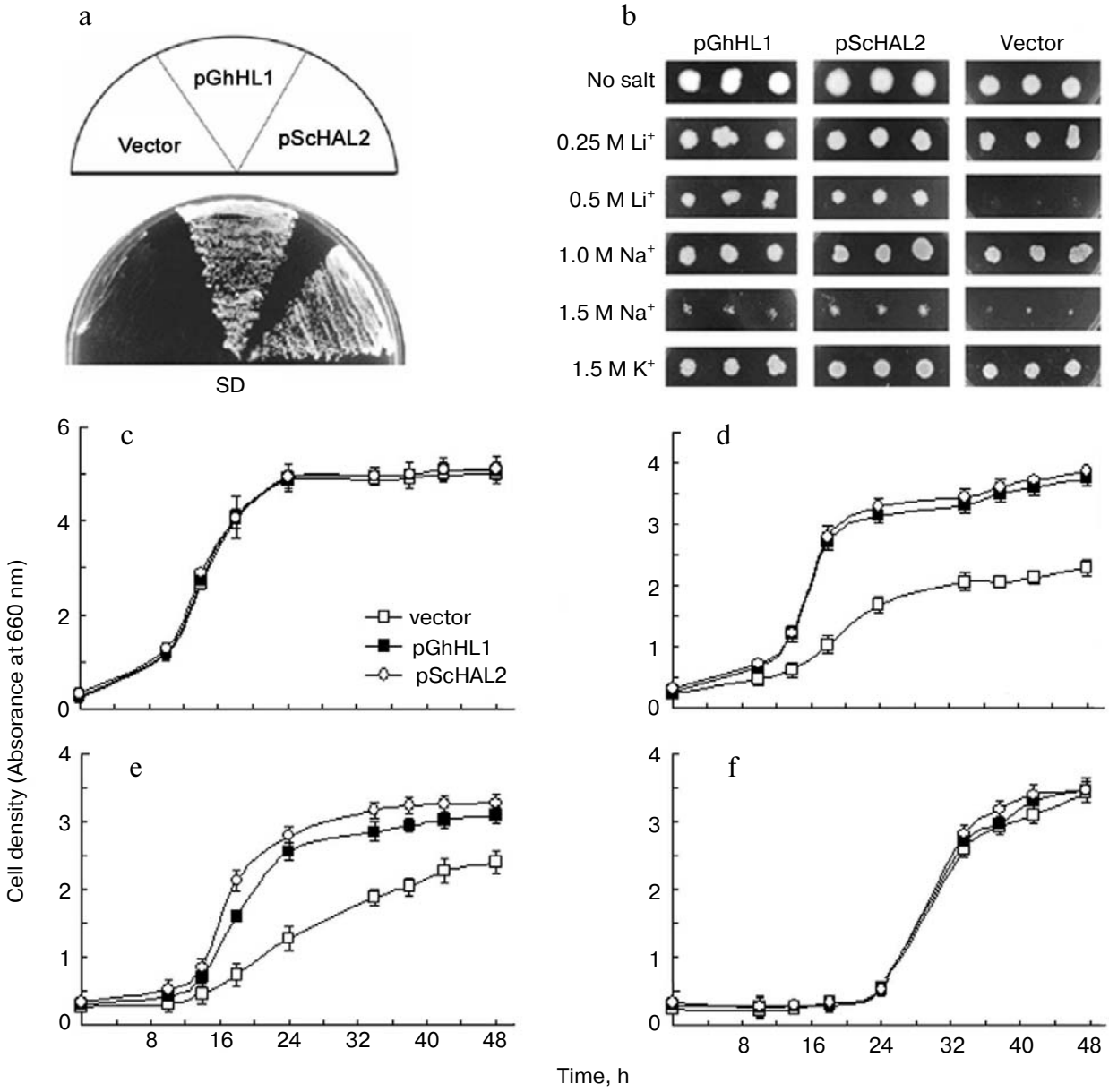

Fig. 6. Complementation analysis of *S. cerevisiae hal2* mutant by *GhHL1* and effects of *GhHL1* overexpression on salt tolerance in yeast. a) Complementation analysis of the *S. cerevisiae hal2* mutant by *GhHL1*. Strains JRM4 transformed with plasmid pRS699 (vector), pRS699-GhHL1 (pGhHL1), and pRS699-ScHAL2 (pScHAL2) were separately streaked onto SD plates. b-f) Effects of *GhHL1* overexpression on salt tolerance in yeast. b) Serial dilutions of saturated cultures were spotted onto uracil selection solid SDM medium without salts and on the same medium supplemented with different cation concentrations as indicated on the left, respectively. Identical results were obtained in three different transformants for the same treatments. c-f) Saturated cultures were diluted 10,000-fold in uracil selection SDM medium without salts (c) and in the same medium containing 0.25 M LiCl (d), 1 M NaCl (e), or 1.5 M KCl (f). Growth curves were recorded by their absorbance at 660 nm at times indicated. Error bars show the variation of three independent experiments.

including bacteria, yeast, plants, and mammals [6, 11, 14, 15, 28]. This suggests that HAL2 and its homologs were conserved from prokaryotes to eukaryotes. To better understand the evolutionary relationships of lineages within *HAL2*-like genes originating from different species, BLAST searches were performed based on *HAL2*-like genes from several organisms, especially from species whose genomes have been completely sequenced by using the inquiry sequence of *CysQ* from *E. coli* [14], yeast *HAL2* [6], *Arabidopsis SAL1* [16], and *RnPIP* from *Rattus norvegicus* rat [28], respectively. Only a single *HAL2*-like gene was found in bacterial species, yeast, and animals [6, 14, 28].

Interestingly, six *HAL2*-like genes exist in the *Arabidopsis* genome and five genes are present in the rice genome (data not shown). Southern blot analysis revealed

that *GhHL1* also belonged to a multi-gene family in cotton. These results indicated that *HAL2*-like genes encode a multi-gene family in plants. Biochemical analysis of cotton GhHL1 showed similar enzyme activity to SAL1 of *Arabidopsis*. However, another homologous AHL (At5g54390) from *Arabidopsis* showed sensitivity to physiological concentrations of Na^+, which was completely different from SAL1 and GhHL1. Moreover, the former only specifically hydrolyze PAP while the latter could also function as IPP1ase. Interestingly, it was the membrane-bound form, not the cytoplasmic form of yeast DHAL2 that plays an important physiological role in yeast under high salt stress conditions [12]. Thus, it can be speculated that this target peptide might play important roles for the normal function of plant *HAL2*-like genes in one of the organelles. The localization differences of plant *HAL2*-like genes were consistent with the different subcellular accumulation of PAP generated by different subcellular localization of sulfotransferase [29].

Our results showed that PAP is the major substrate of the PAPase activity of cotton GhHL1, which is similar to other HAL2 homologs [6, 11, 14, 15, 28]. PAP is an inhibitor of the 5′-3′ exoribonucleases (Xrn1p) in yeast [30], while Xrn1p is the main enzyme catalyzing mRNA degradation of decapped mRNAs in deadenylation-dependent decapping, as well as the nonsense-mediated decay pathways [31]. Orn is a 3′-5′exonuclease that is essential for *E. coli* mRNA degradation, and it is inhibited by PAP in both *E. coli* and humans (via the Orn-homolog in humans Sfn) [32]. It was reported that no Xrn1p ortholog was found in *Arabidopsis* and other plant species [33, 34]. AtXRN4 of *Arabidopsis*, a functional ortholog of Xrn2p/Rat1p, displayed similar roles in plants as Xrn1p did in yeast [35, 36]. Thus, the function of Xrn1p-homologs might be conserved in plants. In addition, BLAST was carried out using the Orn and Sfn as query sequences and an Orn-like gene *AtOrn* (At2g26970) or *OsOrn* (Os02g0465500) was found in *Arabidopsis* and rice, respectively. The identity between *AtOrn* and *OsOrn* was 63%. Both showed approximately 40% identity with Orn and Sfn, respectively (data not shown). Potentially then, yeast HAL2 homologs from plants such as cotton GhHL1 and *Arabidopsis* SAL1 might indirectly affect RNA metabolism by regulating the activity of Xrn1p- or Orn-homologs through hydrolyzing PAP [33, 34]. Further studies are needed to determine whether cotton GhHL1 has a role in PAP-dependent regulation of mRNA levels as a salt stress response in cotton.

It is well known that IP3 is a key secondary messenger in phosphoinositide signaling pathways [37, 38]. As mentioned above, yeast HAL2 and its homologs from various organisms possess phosphatase activity towards both PAP and inositol polyphosphate substrates including IP2 and IP3. However, a homologous SAL1 protein from *Arabidopsis* has been shown to have low activity against IP3, which is ~4% of the activity against PAP [13]. Analysis of the loss of function mutants of *SAL1* (*FIERY1/HOS2*) suggested that the associated IPP1ase activity terminates the IP3 signaling process and functions as a negative regulator in abscisic acid and salt stress signaling pathways [13, 20]. Consistent with this, the *fiery1* mutants null for SAL1 were hypersensitive to both salt and abscisic acid treatment in both seed germination and seedling development [13]. Cotton GhHL1 also showed low activity (4.58%) to IP3, which is similar to SAL1 of *Arabidopsis* [13]. The sequence similarity and the biochemistry characteristics indicated that cotton GhHL1 is most closely related to SAL1 of *Arabidopsis*. If GhHL1 does in fact function like SAL1 [13], it might likewise serve as a negative regulator of salt stress response and abscisic acid signaling in cotton.

Taken together, the results presented here identify the new cotton gene *GhHL1*, a homolog of yeast *HAL2* and *Arabidopsis SAL1*. Our biochemical analysis revealed that GhHL1 has the same dual catalytic activities of IPP1ase and PAPase (i.e. modifies both IP3 and PAP derivatives) as do the other HAL2 homologs previously examined [6, 11, 14, 15, 28]. Overexpression of cotton *GhHL1* in yeast showed a role in salt tolerance associated with ionic stress response. Findings presented here suggest that cotton *GhHL1* is a functional phosphatase gene and might play an important role in salt response in cotton. Further work is needed to analyze this gene determinant of cotton salt stress tolerance as a means to illuminate mechanisms underlying salt response in cotton.

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