Characterization of genes for two-component phosphorelay mediators with a single HPt domain in *Arabidopsis thaliana*

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Abstract Three cDNAs that encode two-component phosphorelay-mediator-like proteins were cloned from *Arabidopsis thaliana*. Putative proteins (ATHP1–3) contain an HPt (Histidine-containing Phospho transfer)-like domain with a conserved histidine and some invariant residues that are involved in phosphorelay. Growth retardation of *YPD1*-disrupted yeast cells was reversed with ATHPs, which indicates that ATHPs function as phosphorelay mediators in yeast cells. The ATHP genes are expressed more in roots than in other tissues, similar to the expression of genes for a sensor histidine kinase, ATHK1, and response regulators ATRR1–4. These results suggest that ATHPs function as two-component phosphorelay mediators between sensor histidine kinase and response regulators in *Arabidopsis*.

Key words: Two-component system; HPt domain, tissue-specific expression; *Arabidopsis thaliana*

1. Introduction

Protein phosphorylation is commonly used for intracellular signal transduction. In prokaryotes, one of these mechanisms is the so-called 'two-component system', a histidine-to-aspartate phosphotransfer mechanism [1–8]. Typically, a two-component system is composed of only two types of signal transducers, a sensor histidine kinase and a response regulator. The sensor domain of the histidine kinase receives an environmental signal, which activates the autophosphorylation of histidine in the transmitter domain. This histidine residue transfers the phosphate group to an aspartate residue in the receiver domain of a response regulator. The activity of the output domain of the response regulator is altered by phosphorylation to control the transcription of signal-responsive genes.

Similar two-component systems have been shown to be involved in osmoregulation in yeast [9], hyphal development in fungi [10], and hormone responses in plants [11–13]. It is thus apparent that the two-component system is not confined to prokaryotes. As information on these two-component systems has been accumulated, two interesting structural features have been clarified. First, eukaryotic two-component proteins such as yeast Sln1p [9] and plant ETR1 [11] were found to be hybrid. These contain not only a sensor histidine kinase domain but also a response regulator domain and are called hybrid sensor histidine kinase. Second, a new protein module was identified from *Escherichia coli* ArcB [14] and yeast Ypd1p [15]. This small module contains an active histidine residue that mediates phosphotransfer, and hence is called the HPt (Histidine-containing Phospho transfer) domain. In the yeast osmoregulation system, Sln1p, Ypd1p, and response regulator Ssk1p form a multistep phosphorelay cascade [15]. A phosphate group is transferred through His in the transmitter domain of Sln1p, Asp in the receiver domain of Sln1p, His in the HPt domain of Ypd1p, and Asp in the receiver domain of Ssk1p. This multistep phosphorelay is also found in prokaryotes [16].

In *Arabidopsis thaliana*, hybrid histidine kinases such as ETR1, CKI1 [13], and ATHK1 [17] and response regulators such as ARR3-7 [18] and ATRR1-4 [19] have been identified. Hybrid histidine kinases and response regulators have also been identified in other plants [20,21]. Thus there may be multistep phosphorelay cascades in the higher plant two-component system. However, no phosphorelay mediator having an HPt domain has been reported so far in plants. In this report, we describe molecular cloning of three cDNAs from *Arabidopsis* that encode novel two-component phosphorelay mediators having an HPt domain, and characterize their function in yeast and tissue-specific expression in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials

Arabidopsis thaliana (Columbia ecotype) plants were grown on GM agar plates under continuous illumination of approximately 2000 lux at 22°C for 3–4 weeks. The plants were used for the construction of a cDNA library and in stress treatments. For the analysis of tissue-specific expression, plants were grown in 15-cm pots filled with a 1:1 mixture of perlite and vermiculite and were watered with 0.1% Hyponex (Hyponex, Tokyo, Japan).

2.2. Stress treatments

For the salt treatment, plants were transferred to and grown hydroponically in a solution of 250 mM NaCl under dim light. For heat and cold treatments, plants were grown under dim light at temperatures of 42°C and 4°C, respectively. For the dehydration treatment, plants were removed from the agar and desiccated in plastic dishes at 22°C and 60% humidity under dim light. In each case, plants were subjected to the treatments for 5 h, frozen in liquid nitrogen, and then stored at −80°C.

2.3. cDNA library screening, cloning and sequencing

A cDNA library was constructed in a HybriZAP vector (Stratagene, La Jolla, CA, USA) with poly(A)$^+$ RNA prepared from unstressed *Arabidopsis* rosette plants. Screening was done by the plaque hybridization method under high-stringency conditions, as described by Mizoguchi et al. [22]. The cDNA inserts were isolated from the positive phage clones and were excised in vivo as pAD-GAL4 phagemid clones. The sequences were determined by the dye-terminator cycle sequencing method (PE Applied Biosystems, Foster City, CA, USA).

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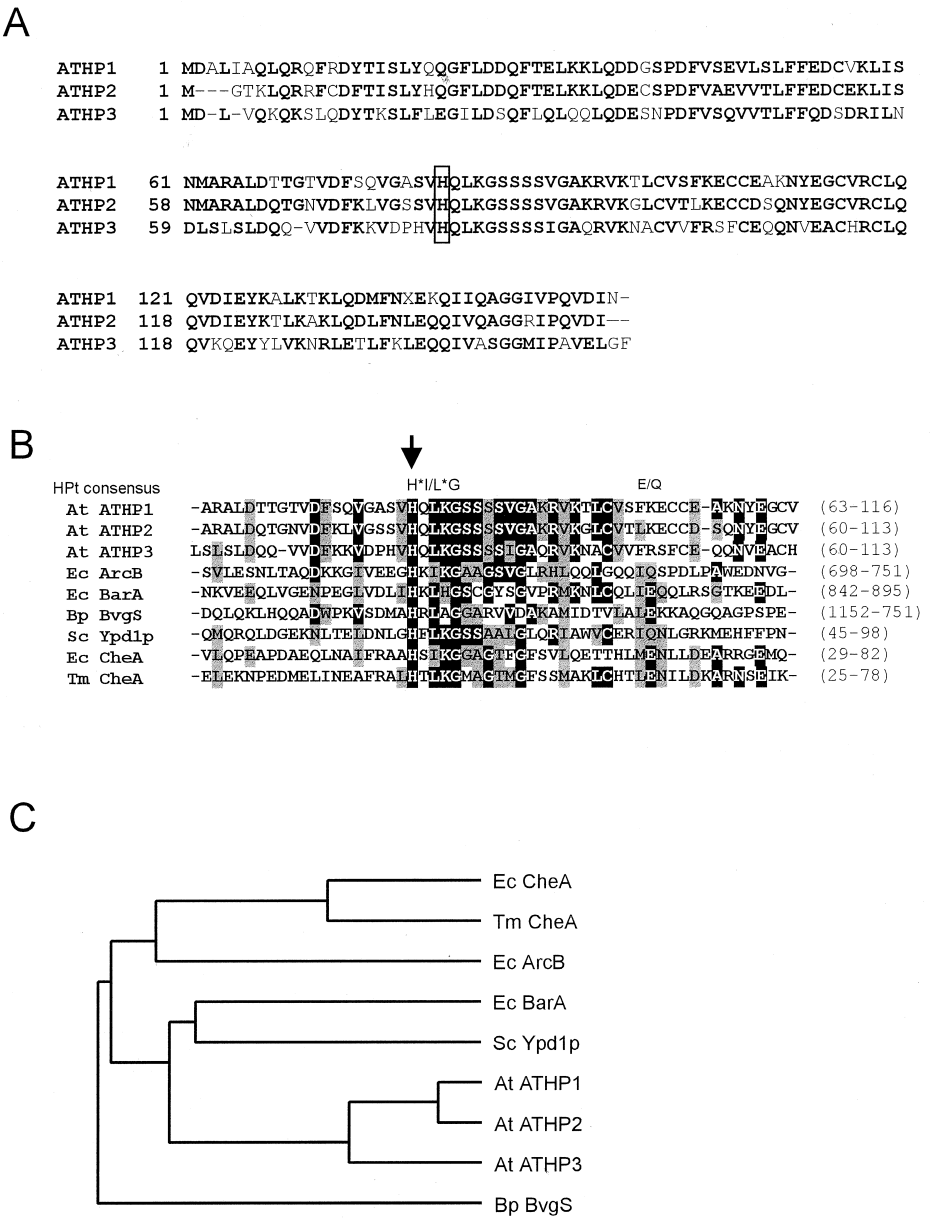

Fig. 1. A: Alignments of amino acid sequences deduced from cDNA sequences of *Arabidopsis* ATHP genes. Similar or identical residues are shown in bold. The open box indicates the histidine that seems to be important for phosphorelay. Accession numbers of *ATHP1, 2* and *3* cDNA sequences are AB012568, AB012569, and AB012570, respectively. B: Alignments of HPt domains of *A. thaliana* (At) ATHPs and the known two-component phosphorelay mediators of *Bordetella pertussis* (Bp) BvgS [27], *Escherichia coli* (Ec) ArcB [28], *E. coli* BarA [29], and *Saccharomyces cerevisiae* (Sc) Ypd1p [15], and the P1 domains of *E. coli* CheA [30] and *Thermotoga maritima* (Tm) CheA [31]. In the case of Ec and Tm CheA, the P1 domain was used for queries. The alignments were calculated with Clustal W [32]. Identical residues are shown on a black background and similar residues are shaded in gray. The histidine residue, which is invariant in all HPt domains, is indicated by an arrow. Conserved residues of HPt domains are indicated as 'HPt consensus'. C: This phylogenetic tree was constructed from the matrix of sequence similarities of the HPt domains shown in panel B by the UPGMA program [33].

2.4. Southern blot analysis

For the preparation of total DNA from *Arabidopsis* rosettes, we used Nucleon Phytopure plant and fungal DNA extraction kits (Amersham, UK) and followed the manufacturer's protocol. Total DNA was digested by appropriate restriction enzymes and was electrophoresed into 0.8% agarose gel and blotted onto a nylon membrane filter [23]. The membrane was hybridized with ^{32}P-labeled cDNA inserts in 50% formamide, 6× SSC, 5× Denhardt's solution, 1% SDS, and 250 μg/ml denatured salmon sperm DNA at 42°C. The filter was washed twice under low- (2× SSC, 0.5% SDS, 37°C) and high-stringency (0.1× SSC, 0.1% SDS, 60°C) conditions.

2.5. Northern blot analysis

Total RNA was prepared by the method described by Ausubel [24]. Thirty micrograms of total RNA were fractionated on a 1% agarose gel containing formaldehyde and blotted onto a nylon membrane filter [23]. The filter was hybridized with the same probes as used in the Southern blotting in 50% formamide, 5× SSC, 25 mM sodium phosphate buffer (pH 6.5), 10× Denhardt's solution, and 250 μg/ml denatured salmon sperm DNA at 42°C. The filter was washed twice with 0.1× SSC, 0.1% SDS, at 60°C for 15 min.

2.6. Yeast complementation test

The plasmid containing P_{GAL1}-*PTP2* was transformed to yeast

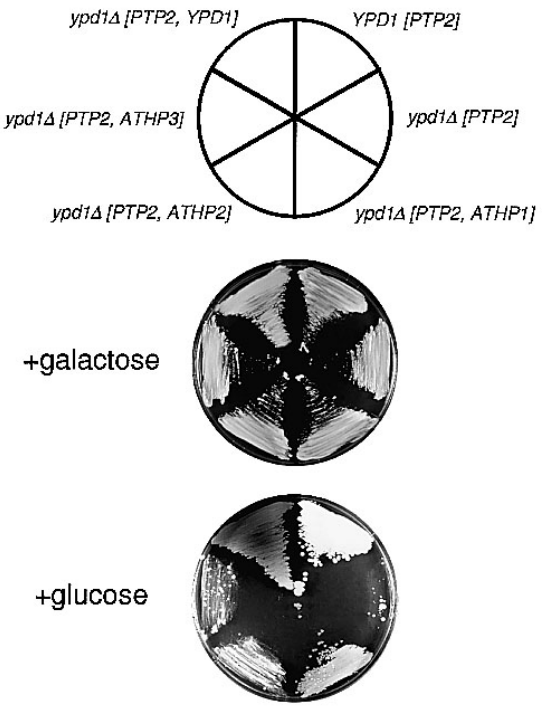

Fig. 2. Functional analysis of *ATHP* genes in yeast cells. In each sector, only relevant genotypes are indicated. *PTP2*: P_{GAL1}-*PTP2*; *YPD1*: wild-type *YPD1*; *ATHP1-3*: ORF region fragments of *ATHP1–3* cDNAs. P_{GAL1}-*PTP2* is a modified *PTP2* gene that is under control of the inducible *GAL1* promoter. On glucose medium plates, *PTP2* expression is repressed, but on galactose plates, *PTP2* expression is induced. The growth of the *ypd1*Δ strain in this study is reduced, but overexpression of *PTP2* on galactose medium can suppress this.

strain TM141 (*MATa ura3 leu2 trp1 his3*) [15], and then the *ypd1*Δ strain was constructed using an insert DNA of the pDPD221 plasmid (kindly provided by T. Maeda). This *YPD1* disruptant was maintained on galactose medium. Then the *ATHP1-3* cDNA fragments were cloned into a YEpGAP112 vector (modified from YEplac112 [25]) and transformed into the *ypd1*Δ strain. As a positive control, the wild-type *YPD1* gene in the pRS315 plasmid was transformed into the *ypd1*Δ strain. Each transformant was screened and maintained on SD medium plates lacking the appropriate amino acids.

3. Results and discussion

3.1. Cloning and sequencing of ATHP cDNA clones

To isolate cDNAs that encode phosphorelay mediators, we first searched the *Arabidopsis* expression sequence tag (EST) database with the amino acid sequence of the yeast *YPD1* gene as a query. We found several EST clones that share sequence similarity with *YPD1*, and categorized these EST clones into three groups. Then we screened an *Arabidopsis* cDNA library using EST clones 187A12T7, 245I14T7, and E1E11T7 as probes under high-stringency hybridization conditions (GenBank accession numbers are R90427, N97149, and AA041089, respectively). Several cDNA clones were isolated and were excised in vivo to the pAD-GAL4 phagemid. After determination of their nucleotide sequences, we identified three independent cDNA groups, each corresponding to one probe, and then selected the longest insert DNA from each group. We found that the length of these cDNA clones was consistent with the mRNA molecular sizes. It is suggested that these are full-length cDNAs.

Fig. 1A shows a sequence comparison of deduced amino acids from the ATHP1–3 (*Arabidopsis thaliana* phosphorelay mediator having HPt domain 1–3) cDNA nucleotide sequences. The putative ATHP1 and 2 proteins share 79% homology, whereas ATHP1 and 3 share only 49% homology. All have a highly conserved region around His-82 of ATHP1. Thus we compared ATHPs with known hybrid HPt kinases in the region around the HPt domain (Fig. 1B). A phylogenetic tree based on the comparison of the HPt domains indicates the evolutional relationships among the two-component factors (Fig. 1C). Evolutional relationships were also observed in two-component response regulators (ATRRs) [19].

The sequence comparison in Fig. 1B suggests that ATHP1–3 contain important conserved amino acid residues for the functional HPt domains. The conserved residues are His-82, Leu-84 (hydrophobic), and Gly-86 of ATHP1, and one of two strictly conserved basic residues, indicated by asterisks (Fig. 1B). But the residues corresponding to Gln-739 of *E. coli* ArcB (Glu/Gln) were not conserved in ATHP1–3. It was suggested from structural analysis that this Glu/Gln residue

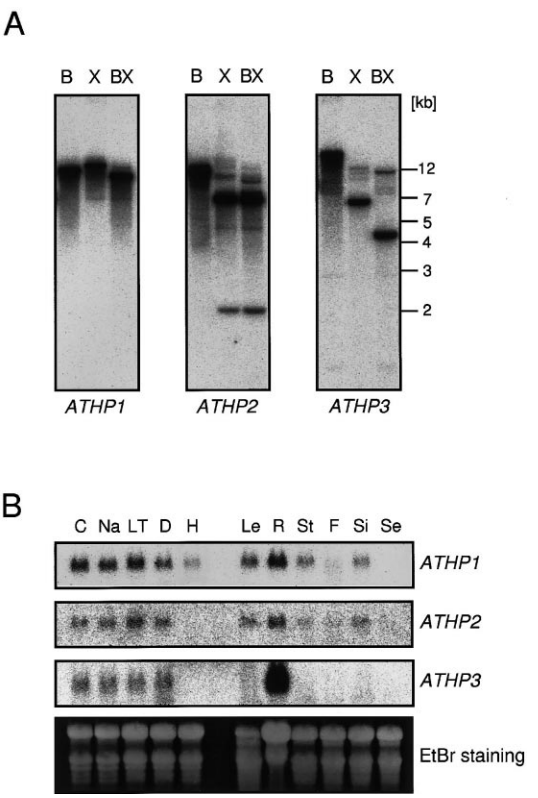

Fig. 3. A: Southern blot hybridization to *Arabidopsis* total DNA with probes of full-length cDNA inserts of ATHP1, 2 and 3 under high-stringency conditions. DNAs were treated with *Bam*HI (B), *Xba*I (X), or both (BX). Molecular sizes are indicated on the right of the panel. B: Expression of *ATHP* genes under various stress conditions and in various tissues. Each lane was loaded with 30 μg of total RNA prepared from whole rosette plants that had been subjected to treatments for 5 h with 250 mM NaCl (Na), 4°C (LT), dehydration (D), or 42°C (H), or untreated (C), and from leaves (Le), roots (R), stems (St), flowers (F), siliques (Si), and developing seeds (S). The blots were hybridized with the probes used in the genomic Southern blots in panel A. Ethidium-bromide-stained RNAs before blotting are shown as controls.

seems to play a crucial role in the activity of phosphorelay mediator [26]. However, ATHP1–3 may take different conformations as phosphorelay mediators and interact with sensor histidine kinase and response regulators in plant cells.

3.2. Complementation of YPD1-disrupted yeast cells with ATHP1–3 genes

For the functional analysis of the ATHP proteins as phosphorelay mediators, we performed a complementation experiment using a yeast *YPD1*-disrupted mutant. In *Saccharomyces cerevisiae*, it had been reported that the *ypd1*Δ mutant is lethal at low osmotic conditions and can be suppressed with *PTP2* overexpression under the control of the GAL1 promoter (*ypd1*Δ [*PTP2*]) [15]. We constructed a *ypd1*Δ [*PTP2*] yeast strain and transformed the mutant with ATHP cDNAs under the control of a yeast constitutive promoter (Fig. 2). Slight viability remained in the *ypd1*Δ mutant, and this strain grew only very slowly on the glucose medium plate. As shown in Fig. 2, this severe growth retardation of the *ypd1*Δ cells was suppressed by the transformation of the wild-type *YPD1* gene on the glucose plate. Transformation of each *ATHP* also suppressed growth retardation of the *ypd1*Δ cells. However, the *ATHP* transformants did not grow as fast as the *YPD1* transformant. These results suggest that ATHPs function as two-component phosphorelay mediators, like Ypd1p in yeast cells.

3.3. Genomic construction and tissue-specific expression of ATHPs

To examine ATHP-related genes on the *Arabidopsis* genome, we used Southern blot analysis. Nuclear DNA was digested with *Bam*HI, *Xba*I, or both, and hybridized under both high- and low-stringency conditions. As shown in Fig. 3A, an intense signal was observed with ATHP1 and the 3 cDNAs as probes under high-stringency conditions. Two intense signals were found with ATHP2 because there is an *Xba*I digestion site in the ATHP2 cDNA. Several weak signals of high molecular weight were detected with ATHP2 and 3. No difference was observed between low- and high-stringency conditions (data not shown). These results suggest that there may be several ATHP-related genes in the *Arabidopsis* genome.

We investigated the expression of the ATHP genes under environmental stress conditions and their tissue-specific expression by Northern blot hybridization (Fig. 3B). Sizes of the ATHP transcripts were about 1.0 kb in length. All ATHPs were expressed under salt, cold, and drought stress, but their expression was reduced under heat stress. However, the ATHP mRNAs were not strongly accumulated under stress conditions. All ATHPs were expressed strongly in roots. ATHP1 and 2 mRNAs were detected in leaves, stems, siliques and strongly in roots, but ATHP3 mRNA was detected only in roots. These results suggest that ATHPs function mainly in roots. Abundant expression in roots was also observed in the ATHK1 and ATRR genes for a sensor histidine kinase and response regulators, respectively [17,19]. The similar expression pattern may suggest that some of them constitute a phosphorelay cascade(s) in roots. Further experiments are now in progress to clarify whether these proteins can physically interact with each other to transfer the phosphate group.

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